

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

PreOmics GmbH and Biognosys AG,
Petitioners

v.

The Brigham and Women's Hospital, Inc.,
Patent Owner

Case No. IPR2024-01473
Patent No. 11,435,360

**DECLARATION OF NANCY E. ADAMS Ed.D.
IN SUPPORT OF PETITION FOR INTER PARTES REVIEW OF
U.S. PATENT NO. 11,435,360**

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I. INTRODUCTION

I, Nancy E. Adams Ed.D., do hereby declare as follows:

1. I have been retained as an independent expert witness on behalf of Petitioners for proceedings before the United States Patent and Trademark Office (“PTO”) regarding the authenticity and public availability of certain prior-art references in relation to U.S. Patent No. 11,435,360.

2. I am being compensated for my work in this matter at my customary hourly rate, which is \$250/hour. I am also reimbursed for any reasonable expenses associated with my work on this matter. My compensation is not contingent on the results of my study, the substance of my opinions, or the outcome of this matter.

3. I understand that this Declaration accompanies a petition for an *Inter Partes* Review (“IPR”) involving U.S. Patent No. 11,435,360, which resulted from U.S. Patent Application No. 17/215,923, filed on March 29, 2021.

A. Qualifications and Professional Experience

4. My complete qualifications and professional experience are described in my curriculum vitae (**EX1013**). The following is a brief summary of my relevant qualifications and professional experience.

5. I am the Librarian for Scholarly Communication and Education at Harrell Health Sciences Library, a Penn State University Library. I have held this

position since July of 2024. Prior to that, I held the position of Assistant Dean of Foundational Sciences at Penn State College of Medicine and Librarian for Education and Instruction at Harrell Health Sciences Library, a combined position that I held between July of 2017 to July of 2024. Prior to that, I held the position of Associate Director of the Harrell Health Sciences Library, which began in 2010. Prior to that, I held numerous Librarian positions at the University level, such as Harrisburg University of Science and Technology, The University of Maine, and Wayne State University. I have accumulated a total of 27 years of experience working in an academic library and medical library. I hold an Ed.D. in Adult Education from Pennsylvania State University and a Masters in Library and Information Science from Wayne State University.

6. I am an expert on library cataloging, classification, and searching. I have published contributed chapters to editions on this subject, *Curriculum Mapping: A Match Made for Librarian Skill* (2020) and *Making it better: library and student services collaboration at a new university* (2012). I have taught workshops on literature searching to a myriad of undergraduate students, graduate students, and advanced-degree professionals for decades. I have given presentations, such as “Searching PubMed like an Expert: The Evolution, a PubMed Searching Discussion Forum.” I have served as the search specialist on a systematic

review team in which my role was to formulate, execute, and document an exhaustive search of the literature answering a specific research question.

7. I am familiar with metadata schematic design and implementation. As a librarian, I routinely use the metadata associated with publications to answer reference questions or track down items that researchers rely on for their work. I have used online cataloging systems to create new catalog records for materials archived by libraries.

8. I am personally familiar with many online catalogs, databases, and search engines, including all of the particular catalogs, databases, and search engines that I relied upon in forming my opinions herein.

B. Scope of This Declaration

9. I have been asked to offer an opinion on the authenticity and public-availability date of the following documents:

- i. Zaccaria, A., Roux-Dalvai, F., Bouamrani, A., Mombrun, A., Mossuz, P., Monsarrat, B., and Berger, F., *Accessing to the Minor Proteome of Red Blood Cells Through the Influence of the Nanoparticle Surface Properties on the Corona Composition*, INTERNATIONAL JOURNAL OF NANOMEDICINE (2015): 1869-1883. (EX A, “Zaccaria”).

- ii. Hu, Z., Hongyan, Z., Zhang, Y., Wu, R., and Zou, H, *Nanoparticle Size Matters in the Formation of Plasma Protein Coronas on Fe₃O₄ Nanoparticles*, COLLOIDS AND SURFACES B: BIOINTERFACES 121 (2014), 354-361. (EX B, “Hu”).
- iii. Cai, X., Ramalingam, R., Wong, H.S., Cheng, J., Ajuh, P., Cheng, S.H., and Lam, Y.W., *Characterization of Carbon Nanotube Protein Corona By Using Quantitative Proteomics*, NANOMEDICINE: NANOTECHNOLOGY, BIOLOGY AND MEDICINE 9 (2013), 583-593. (EX C, “Cai”).
- iv. Bryk *et al.*, *Quantitative Analysis of Human Red Blood Cell Proteome*, JOURNAL OF PROTEOME RESEARCH (2017) 2752-2761. (EX D, “Bryk”).
- v. Nanjappa *et al.*, *Plasma Proteome Database as a Resource for Proteomics Research: 2014 Update*, NUCLEIC ACIDS RESEARCH 42 (2014) D959-D965. (EX E, “Nanjappa”).
- vi. Ahmed, *Sample Preparation and fractionation for proteome analysis and cancer biomarker discovery by mass spectrometry*, JOURNAL OF SEPARATION SCIENCE 32 (2009) 771-798. (EX F, “Ahmed”).

- vii. Cao *et al.*, *Latent Transforming Growth Factor-Beta Binding Protein-1 in Circulating Plasma as a Novel Biomarker for Early Detection of Hepatocellular Carcinoma*, INTERNATIONAL JOURNAL OF CLINICAL AND EXPERIMENTAL PATHOLOGY 8(12) (2015) 16046-16054. (EX G, “Cao”).

10. I have provided additional exhibits attached to my Declaration to support my opinions as to the authenticity and public-availability of these publications, including their supporting materials.

C. Evidence Considered in Forming My Opinions

11. In preparing this Declaration, I have reviewed the documents I reference herein, and each of these is a type of material that experts in my field of library cataloging and reference classification would reasonably rely upon when forming their opinions.

II. AUTHENTICITY AND PUBLIC AVAILABILITY OF PUBLICATIONS

A. Zaccaria (EX A)

12. EX A is a true and correct copy of Zaccaria, A., Roux-Dalvai, F., Bouamrani, A., Mombrun, A., Mossuz, P., Monsarrat, B., and Berger, F., *Accessing to the Minor Proteome of Red Blood Cells Through the Influence of the Nanoparticle Surface Properties on the Corona Composition*, INTERNATIONAL

JOURNAL OF NANOMEDICINE (2015): 1869-1883 (“*Zaccaria*”) that was obtained from the website of the publisher, Taylor and Francis, parent to the Dove Press imprimatur that published *Zaccaria*. I began my research by searching the Google Scholar website by the title of *Zaccaria* for records, and a retrieved record (**EX H**) led me to the full text of *Zaccaria* at the Taylor and Francis publisher’s website at <https://www.tandfonline.com/doi/full/10.2147/IJN.S70503>. **EX H** shows that *Zaccaria* was published in the *International Journal of Nanomedicine*, and that it was first published on March 9, 2015. I then followed the PDF link to download the full text of *Zaccaria*. This published copy is presented at **EX A** in this Declaration. **EX A** shows that *Zaccaria* has a total of 15 pages, including 50 references.

13. **EX A** is in a condition that does not create suspicion about its authenticity. Specifically, this copy is not missing any intermediate pages, the text on each page appears to flow seamlessly from one page to the next, and there are no visible alterations to the document. Accordingly, I see no reason to question the authenticity of this copy of *Zaccaria*.

14. My knowledge of library cataloging informs my opinion that *Zaccaria* was published in 2015 by Dove Press. For example, the face page to *Zaccaria* notes that it was published on March 9, 2015. Furthermore, the Directory of Open Access Journals (DOAJ), a publicly available website providing

information about freely available electronic journals, contains a record for the *International Journal of Nanomedicine* available at <<https://doaj.org/toc/1178-2013>>. The record states that the journal became an open access (freely available online) journal in 2006 (EX I), before the date of publication of *Zaccaria*.

15. Based on the above, it is my professional opinion that *Zaccaria* would have become publicly accessible when it was published in 2015 (EX A).

B. Hu (EX B)

16. EX B is a true and correct copy of Hu, Z., Hongyan, Z., Zhang, Y., Wu, R., and Zou, H., *Nanoparticle Size Matters in the Formation of Plasma Protein Coronas on Fe₃O₄ Nanoparticles*, COLLOIDS AND SURFACES B: BIOINTERFACES 121 (2014), 354-361 (“Hu”), that I obtained from ScienceDirect. I began my research by searching Google Scholar by the title of *Hu* for records, and a retrieved record led me to ScienceDirect at <<https://www.sciencedirect.com/science/article/abs/pii/S0927776514003014>>. The ScienceDirect database record (EX J) shows that *Hu* was published in *Colloids and Surfaces B: Biointerfaces*, and that it was first published in September 2014. I then followed the PDF link to download the full text of *Hu*. This published copy is presented at EX B in this Declaration.

17. In EX B, page 1 shows the title, authors, abstract, key words, and the date of publication. Moreover, page 1 shows that the article was received on

February 19, 2014, and accepted on June 5, 2014. Furthermore, it was available online on June 12, 2014. **EX B** shows that *Hu* has a total of 8 pages, including 49 references.

18. I am familiar with ScienceDirect, which is an online library with digital copies of various printed publications, including journals, as maintained by Elsevier, and in my experience ScienceDirect is a reliable source of the publications contained in its catalog.

19. **EX B** is in a condition that does not create suspicion about its authenticity. Specifically, this copy is not missing any intermediate pages, the text on each page appears to flow seamlessly from one page to the next, and there are no visible alterations to the document. Accordingly, I see no reason to question the authenticity of this copy of *Hu*.

20. My knowledge of library cataloging informs my opinion that *Hu* was published in 2014 in *Colloids and Surfaces B: Biointerfaces*. For example, the catalog records for the Penn State University Libraries (**EX K**) provides that *Colloids and Surfaces B: Biointerfaces* was published sixteen times a year in Amsterdam, Netherlands, by Elsevier, beginning in 1993.

21. Based on the above, it is my professional opinion that *Hu* would have become publicly accessible when it was first published in June 2014.

22. *Hu* identifies “Table S1” on pages 357 and 358.

23. *Hu* states on page 357, with respect to Table S1:

In total, there were 117, 133 and 100 plasma proteins identified in the protein coronas of the Fe₃O₄_30, Fe₃O₄_200 and Fe₃O₄_400 NPs, respectively (the detailed identification results were listed in Table S1). A total of 71 human plasma proteins were detected in the protein coronas of Fe₃O₄ NPs of the three different sizes, with 22, 31 and 11 distinct proteins for the Fe₃O₄_30, Fe₃O₄_200 and Fe₃O₄_400 NPs, respectively (shown in Fig. 4). For example, serum albumin, apolipoprotein A-I and Platelet factor, among others, were observed to bind on the surfaces of all three types of Fe₃O₄_NPs, while ceruloplasmin and serum amyloid P-component, among others, were found to be uniquely bound on the surface of only the Fe₃O₄_30 NPs. Fibronectin, serotransferrin and serum paraoxonase/arylesterase 1 were observed to only be adsorbed on the Fe₃O₄_200 NPs, and coagulation factor XIII A chain and inter-alpha (Globulin) inhibitor H2 were only bound on the Fe₃O₄_400 NPs (Table S1).

24. *Hu* states on pages 357 and 358, again with respect to Table S1:

By using this equation, the relative protein abundances for the plasma proteins bound on the Fe₃O₄ NPs were calculated and listed in Table S1. It was found that although there were 71 common plasma proteins bound on the different sizes of Fe₃O₄ NPs, the relative protein abundances for these proteins differed. For instance, serum albumin was observed in the protein corona of the Fe₃O₄_200 NPs with an average MWSpC of 9.01, while the average MWSpC for serum albumin in the protein coronas of the Fe₃O₄_30 and Fe₃O₄_200 NPs were 1.27 and 2.50, respectively, indicating the selective binding of plasma proteins on different sizes of Fe₃O₄ NPs.

25. *Hu* identifies “Appendix A. Supplementary data” and states that data “Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.colsurfb.2014.06.016>.” This link also results

in

<https://www.sciencedirect.com/science/article/pii/S0927776514003014?via%3Dihub#sec0075>, at which I obtained the Supplementary data, a Word file that is titled “Supporting Information: Nanoparticle Size Matters in the Formation of Plasma Protein Coronas on Fe₃O₄ nanoparticles” on its first page (which is the title of *Hu*), where Table S1 starts on page 2. Also on page 2, it identifies that Table S1 corresponds to: “Total identified plasma proteins in the protein coronas of the Fe₃O₄ NPs of different sizes and their isoelectric points (pI), molecular weights (MW) and relative protein abundance (blank spaces indicate that the corresponding proteins were not identified in the analysis),” which corresponds to the description in *Hu* at pages 357 and 358 that I quoted above. I have included this file as **EX L** to my declaration. **EX L** is in a condition that does not create suspicion about its authenticity that it corresponds to the data that was identified in and published along with *Hu*.

C. *Cai* (EX C)

26. **EX C** is a true and correct copy of Cai, X., Ramalingam, R., Wong, H.S., Cheng, J., Ajuh, P., Cheng, S.H., and Lam, Y.W., *Characterization of Carbon Nanotube Protein Corona By Using Quantitative Proteomics*, NANOMEDICINE: NANOTECHNOLOGY, BIOLOGY AND MEDICINE 9 (2013), 583-593 (“*Car*”), that I obtained from ScienceDirect. I began my research

by searching Google Scholar by the title of *Cai* for records, and a retrieved record led me to ScienceDirect at <
<https://www.sciencedirect.com/science/article/abs/pii/S1549963412005977>> The ScienceDirect database record (**EX M**) shows that *Cai* was published in Nanomedicine: Nanotechnology, Biology, and Medicine, and that it was first published in July 2013. I then followed the PDF link to download the full text of *Cai*. This published copy is presented at **EX C** in this Declaration.

27. In **EX C**, page 1 shows the title, authors, abstract, key words, and year of the publication. **EX C** shows that *Cai* has a total of 11 pages, including 39 references.

28. As noted previously, I am familiar with ScienceDirect, which is an online library with digital copies of various printed publications, including journals, as maintained by Elsevier, and in my experience ScienceDirect is a reliable source of the publications contained in its catalog.

29. **EX C** is in a condition that does not create suspicion about its authenticity. Specifically, this copy is not missing any intermediate pages, the text on each page appears to flow seamlessly from one page to the next, and there are no visible alterations to the document. Accordingly, I see no reason to question the authenticity of this copy of *Cai*.

30. My knowledge of library cataloging informs my opinion that *Cai* was published in 2013 in *Nanomedicine: Nanotechnology, Biology, and Medicine*. For example, the catalog record for the Penn State University Libraries (EX N) provides that *Nanomedicine: Nanotechnology, Biology, and Medicine* was a published four times a year in New York, NY, by Elsevier, beginning in 2005, prior to the publication of *Cai*.

31. Based on the above, it is my professional opinion that *Cai* would have become publicly accessible when it was first published in July 2013 (EX C).

32. *Cai* identifies “Table S2” on page 587 that corresponds to “778 proteins [that] were accurately identified.” *Cai* further states on page 589 the following, including that Figure 3(D) shows a “Histogram of the SILAC ratio ... of the 778 quantitated proteins”:

Figure 3. Characterisation of MWCNT- and CB-protein corona by SILAC proteomics. (A) Experimental design. (B) Sample mass spectra of selected peptides that show various degrees of MWCNT- enrichment and CB-enrichment. (C) Western blotting analysis of MWCNT-pull-down by using antibodies against selected proteins. (D) Histogram of the SILAC ratio (“medium” vs “light”, log2) of 778 quantitated proteins. A positive ratio for a protein indicates an enrichment of this protein in the MWCNT pull-down. (E) Histogram of the SILAC ratio (“heavy” vs “light”, log2) of the quantitated proteins, which indicates the relative enrichment in the CB pull-down. (F) Scatter plot that shows the SILAC ratio and the protein abundance index of each quantitated protein (pink: MWCNT pull-down; green: CB pull-down). (G) SILAC ratio of each quantitated protein with respect to the MWCNT- and CB-pull-downs.

33. *Cai* also identifies on page 592, “Appendix A. Supplementary data” and states that “Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.nano.2012.09.004>.” This link also results in <https://www.sciencedirect.com/science/article/pii/S1549963412005977#s0075>, at which I obtained the Supplementary data, an Excel file that is titled “Supplementary Table 2: Data reported in Figure 3” and includes 778 line items. I have included

this file as **EX O** to my declaration. **EX O** is in a condition that does not create suspicion about its authenticity that it corresponds to the data that was identified in and published along with *Cai*.

D. Bryk (EX D)

34. **EX D** is a true and correct copy of Bryk *et al.*, *Quantitative Analysis of Human Red Blood Cell Proteome*, JOURNAL OF PROTEOME RESEARCH (2017) 2752-2761 (“*Bryk*”), that I obtained from ACS Publications website. I began my research by searching Google Scholar by the title of *Bryk* for records, and a retrieved record led me to ACS Publications at < <https://pubs.acs.org/doi/full/10.1021/acs.jproteome.7b00025>>. The ACS Publications database record (**EX P**) shows that *Bryk* was published in *Journal of Proteome Research*, and that it was first published online on July 24, 2017. I then followed the PDF link to download the full text of *Bryk*. This published copy is presented at **EX D** in this Declaration.

35. In **EX D**, page 1 shows the title, authors, abstract, key words, and the date of publication. Moreover, page 1 shows that the article was received on January 13, 2017, and published on July 8, 2017. The database record external to the publication PDF shows that it was first available online on July 24, 2017, as shown in **EX P**. **EX D** shows that *Bryk* has a total of 10 pages, including 49 references.

36. I am familiar with ACS Publications, which is an online library with digital copies of various printed publications, including journals, as maintained by the American Chemical Society, and in my experience ACS Publications is a reliable source of the publications contained in its catalog.

37. **EX D** is in a condition that does not create suspicion about its authenticity. Specifically, this copy is not missing any intermediate pages, the text on each page appears to flow seamlessly from one page to the next, and there are no visible alterations to the document. Accordingly, I see no reason to question the authenticity of this copy of *Bryk*.

38. My knowledge of library cataloging informs my opinion that *Bryk* was published in 2017 in *Journal of Proteome Research*. For example, the catalog record for the WorldCat, a combined online catalog of holdings of thousands of libraries across North America, (**EX Q**) provides that *Journal of Proteome Research* was published by the American Chemical Society in Washington, DC, beginning in 2002, prior to the publication of *Bryk*.

39. Based on the above, it is my professional opinion that *Bryk* would have become publicly accessible when it was first published in July 2017.

40. *Bryk* states, “Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteo-me.7b00025.” *Bryk* further states that the supporting information includes “Figure S1. Western blot

analysis of selected proteins (PDF)” along with three tables: “Table S1. Stable isotope labeled peptide based protein quantification. Table S2. Enrichment of membrane proteins in the white ghost fraction. Table S3. List of identified proteins and their concentrations in the whole red cells and white ghost. (XLSX).”

41. *Bryk* states that:

- “Ratios of heavy to light MS1 intensities of ‘light’ and ‘heavy’ (standard) peptides were manually extracted from the spectra (Xcalibur Qual Browser) and used for calculation of concentrations of targeted proteins (Table S1).” (page 2754).
- “MPE was computed for the 10 most abundant integral membrane proteins (Table S2).” (page 2755).
- “The total number of proteins that were identified per donor sample matching RBCs and WG analyses was about 2500 (Figure 2F, Table S3).” (page 2755).

42. Following this link results in <https://pubs.acs.org/doi/full/10.1021/acs.jproteome.7b00025>, at which I obtained the Supplementary data which are available there in two files: an Excel file that is titled “Bryk Supplementary Tables S1-S3” and with three sheets labeled “Table S1”, “Table S2”, and “Table S3”; and a PDF file that is titled, “Cover Sheet to SI Material: Quantitative analysis of human red blood cell proteome.” I have included “Bryk

Supplementary Tables S1-S3” file as **EX R** to my declaration. I have included “Cover Sheet to SI Material: Quantitative analysis of human red blood cell proteome” as **EX Z** to my declaration. **EX R** and **EX Z** are in a condition that do not create suspicion about their authenticity that they corresponds to the data that was identified in and published along with *Bryk*.

E. *Nanjappa* (EX E)

EX E is a true and correct copy of Nanjappa et al., *Plasma Proteome Database as a resource for proteomics research: 2014 update*, NUCLEIC ACIDS RESEARCH 42 (2014): D959–D965 (“*Nanjappa*”), that I obtained from Oxford Academic Press website. I began my research by searching Google Scholar by the title of *Nanjappa* for records, and a retrieved record led me to the Oxford Academic Press website at <<https://academic.oup.com/nar/article/42/D1/D959/1064084>>

43. The Oxford Academic Press website record (**EX S**) shows that *Nanjappa* was published in *Nucleic Acids Research*, and that it was first received by the publisher on August 16, 2013 and published on December 3, 2013. I then followed the PDF link to download the full text of *Nanjappa*. This published copy is presented at **EX E** in this Declaration.

44. In **EX E**, page 1 shows the title, authors, abstract, key words, and year of the publication. **EX E** shows that *Nanjappa* has a total of 7 pages, including 46 references.

45. I am familiar with Oxford Academic Press website, which is an online library with digital copies of various printed publications, including journals, as maintained by Oxford Academic Press, and in my experience Oxford Academic Press is a reliable source of the publications contained in its catalog.

46. **EX E** is in a condition that does not create suspicion about its authenticity. Specifically, this copy is not missing any intermediate pages, the text on each page appears to flow seamlessly from one page to the next, and there are no visible alterations to the document. Accordingly, I see no reason to question the authenticity of this copy of *Nanjappa*.

47. My knowledge of library cataloging informs my opinion that *Nanjappa* was published in 2013 in *Nucleic Acids Research*. For example, the catalog record for the Penn State University Libraries (**EX T**) provides that *Nucleic Acids Research* was published semimonthly in Oxford, UK by Oxford University Press, beginning in 1974, prior to the publication of *Nanjappa*.

48. Based on the above, it is my professional opinion that *Nanjappa* would have become publicly accessible when it was first published in December, 2013 (**EX E**).

F. Ahmed (EX F)

49. **EX F** is a true and correct copy of Ahmed, F., *Sample Preparation and Fractionation for Proteome Analysis and Cancer Biomarker*

Discovery by Mass Spectrometry, JOURNAL OF SEPARATION SCIENCE 32 (2009), 771-798 (“*Ahmed*”), that I obtained from Wiley Online Journals. I began my research by searching Google Scholar by the title of *Ahmed* for records, and a retrieved record led me to Wiley Online Journals at < <https://analyticalsciencejournals.onlinelibrary.wiley.com/doi/abs/10.1002/jssc.200800622>>. The Wiley Online Journals database record (EX U) shows that *Ahmed* was published in *Journal of Separation Science*, and that it was first published online on March 10, 2009. I then followed the PDF link to download the full text of *Ahmed*. This published copy is presented at EX F in this Declaration.

50. In EX F, page 1 shows the title, authors, abstract, key words, and year of the publication. EX F shows that *Ahmed* has a total of 28 pages, including 235 references.

51. I am familiar with Wiley Online Journals, which is an online library with digital copies of various printed publications, including journals, as maintained by Wiley Press, and in my experience Wiley Online Journals is a reliable source of the publications contained in its catalog.

52. EX F is in a condition that does not create suspicion about its authenticity. Specifically, this copy is not missing any intermediate pages, the text on each page appears to flow seamlessly from one page to the next, and there are no

visible alterations to the document. Accordingly, I see no reason to question the authenticity of this copy of *Ahmed*.

53. My knowledge of library cataloging informs my opinion that *Ahmed* was published in 2009 in *Journal of Separation Science*. For example, the catalog record for the Penn State University Libraries (EX V) provides that *Journal of Separation Science* was a published monthly in Weinheim, Germany by Wiley-VCH, beginning in 2001, prior to the publication of *Ahmed*.

54. Based on the above, it is my professional opinion that *Ahmed* would have become publicly accessible when it was first published in March 2009 (EX F).

G. Cao (EX G)

EX G is a true and correct copy of Cao *et al.*, *Latent Transforming Growth Factor-Beta Binding Protein-1 in Circulating Plasma as a Novel Biomarker for Early Detection of Hepatocellular Carcinoma*, INTERNATIONAL JOURNAL OF CLINICAL AND EXPERIMENTAL PATHOLOGY 8(12) (2015) 16046-16054 (“Cao”), that I obtained from PubMed Central. I began my research by searching Google Scholar by the title of *Cao* for records, and a retrieved record led me to Pubmed Central at < <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4730094/>>. The PubMed Central database record (EX W) shows that *Cao* was published in *International Journal of Clinical and Experimental Pathology*, and that it was first

published online on December 1, 2015. I then followed the PDF link to download the full text of *Cao*. This published copy is presented at **EX G** in this Declaration.

55. In **EX G**, page 1 shows the title, authors, abstract, key words, and year of the publication. **EX G** shows that *Cao* has a total of 11 pages, including 2 pages of supplementary material and 33 references.

56. I am familiar with PubMed Central, which is an online repository with digital copies of journal articles published in various journals representing research that is funded by the National Institutes of Health. PubMed Central is maintained by the National Library of Medicine, and in my experience PubMed Central is a reliable source of the publications contained in its catalog.

57. **EX G** is in a condition that does not create suspicion about its authenticity. Specifically, this copy is not missing any intermediate pages, the text on each page appears to flow seamlessly from one page to the next, and there are no visible alterations to the document. Accordingly, I see no reason to question the authenticity of this copy of *Cao*.

58. My knowledge of library cataloging informs my opinion that *Cao* was published in 2015 in *International Journal of Clinical and Experimental Pathology*. For example, the PubMed Central journals list shows the archives for this journal at <<https://www.ncbi.nlm.nih.gov/pmc/journals/633/>> (**EX X**), which provides that *International Journal of Clinical and Experimental Pathology* was a

published between 6 to 12 times a year by e-Century Publishing, beginning in 2008, prior to the publication of *Cao*. The website of the *International Journal of Clinical and Experimental Pathology* lists the place of publication as Madison, Wisconsin (EX Y).

59. Based on the above, it is my professional opinion that *Cao* would have become publicly accessible when it was first published in December 2015 (EX G).

60. List of Documents Considered

61. The documents I considered in formulating my opinions are as follows:

Exhibit No.	Description
A	Zaccaria, A., Roux-Dalvai, F., Bouamrani, A., Mombrun, A., Mossuz, P., Monsarrat, B., and Berger, F., Accessing to the Minor Proteome of Red Blood Cells Through the Influence of the Nanoparticle Surface Properties on the Corona Composition, INT. J. NANOMEDICINE. (2015) (“Zaccaria”)
B	Hu, Z., Hongyan, Z., Zhang, Y., Wu, R., and Zou, H, Nanoparticle Size Matters in the Formation of Plasma Protein Coronas on Fe ₃ O ₄ Nanoparticles, COLLOIDS AND SURFACES B: BIOINTERFACES 121 (2014) (“Hu”)
C	Cai, X., Ramalingam, R., Wong, H.S., Cheng, J., Ajuh, P., Cheng, S.H., and Lam, Y.W., Characterization of Carbon Nanotube Protein Corona By Using Quantitative Proteomics, NANOMEDICINE: NANOTECHNOLOGY, BIOL. AND MED. 9 (2013) (“Cai”)

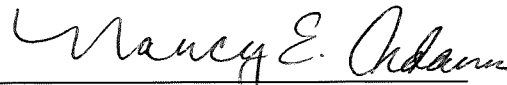
Exhibit No.	Description
D	Bryk <i>et al.</i> , Quantitative Analysis of Human Red Blood Cell Proteome, J. PROTEOME RES. (2017), (“ <i>Bryk</i> ”)
E	Nanjappa <i>et al.</i> , Plasma Proteome Database as a Resource for Proteomics Research: 2014 Update, NUCLEIC ACIDS RES. 42 (2014), (“ <i>Nanjappa</i> ”)
F	Ahmed, F., Sample Preparation and fractionation for proteome analysis and cancer biomarker discovery by mass spectrometry, J. OF SEPARATION SCI. 32 (2009), (“ <i>Ahmed</i> ”)
G	Cao <i>et al.</i> , Latent Transforming Growth Factor-Beta Binding Protein-1 in Circulating Plasma as a Novel Biomarker for Early Detection of Hepatocellular Carcinoma, INT. J. CLIN. EXP. PATHOL. 8 (2015), (“ <i>Cao</i> ”)
H	<i>Zaccaria</i> , Publisher’s database record
I	<i>Zaccaria</i> , Website of journal of publication
J	<i>Hu</i> , Publisher’s database record
K	<i>Hu</i> , University library’s cataloging record for journal
L	<i>Hu</i> , Supplementary materials
M	<i>Cai</i> , Publisher’s database record
N	<i>Cai</i> , University library’s cataloging record for journal
O	<i>Cai</i> , Supplementary materials
P	<i>Bryk</i> , Publisher’s database record
Q	<i>Bryk</i> , WorldCat cataloging record for journal
R	<i>Bryk</i> , Supplementary materials
S	<i>Nanjappa</i> , Publisher’s database record

Exhibit No.	Description
T	<i>Nanjappa</i> , University library's cataloging record for journal
U	<i>Ahmed</i> , Publisher's database record
V	<i>Ahmed</i> , University library's cataloging record for journal
W	<i>Cao</i> , PubMed Central database record
X	<i>Cao</i> , PubMed Central's archive record for publishing journal
Y	<i>Cao</i> , Website of journal of publication
Z	<i>Bryk</i> , Additional supplementary materials

III. CONCLUSION

62. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code.

Respectfully submitted,

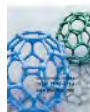


Nancy E. Adams, Ed.D.

Date: 10/1/24

Appendix

Exhibit A



Accessing to the minor proteome of red blood cells through the influence of the nanoparticle surface properties on the corona composition

Affif Zaccaria, Florence Roux-Dalvai, Ali Bouamrani, Adrien Mombrun, Pascal Mossuz, Bernard Monsarrat & François Berger

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Accessing to the minor proteome of red blood cells through the influence of the nanoparticle surface properties on the corona composition

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Abstract: Nanoparticle (NP)–protein interactions in complex samples have not yet been clearly understood. Nevertheless, several studies demonstrated that NP’s physicochemical features significantly impact on the protein corona composition. Taking advantage of the NP potential to harvest different subsets of proteins, we assessed for the first time the capacity of three kinds of superparamagnetic NPs to highlight the erythrocyte minor proteome. Using both qualitative and quantitative proteomics approaches, nano-liquid chromatography–tandem mass spectrometry allowed the identification of 893 different proteins, confirming the reproducible capacity of NPs to increase the number of identified proteins, through a reduction of the sample concentration range and the capture of specific proteins on the three different surfaces. These NP-specific protein signatures revealed significant differences in their isoelectric point and molecular weight. Moreover, this NP strategy offered a deeper access to the erythrocyte proteome highlighting several signaling pathways implicated in important erythrocyte functions. The automated potentiality, the reproducibility, and the low-consuming sample demonstrate the strong compatibility of our strategy for large-scale clinical studies and may become a standardized sample preparation in future erythrocyte-associated proteomics studies.

Keywords: nanoparticles, red blood cells, mass spectrometry, quantitative proteomics, protein corona, minor proteome

Introduction

With the emergence of nanotechnology, the engineering of “smart” nanoparticles (NPs) has opened up a host of possibilities in medicine and biology.^{1–3} Nevertheless, once injected in blood circulation, the fate of these NPs must be anticipated to prevent an NP-associated toxicity.^{4,5} When an NP is introduced into a biological fluid, it is well recognized that proteins immediately cover its surface, resulting in the formation of a protein “corona”, which will rapidly evolve according to the concentration and the affinity of proteins for the NP.^{6,7} Since the last decade, several studies used different analytical methods⁸ and focused on the performance of NPs in the presence of complex protein mixtures^{9–11} to demonstrate the complexity of these NP–protein interactions.^{12,13} First, the “nano-dimension” of these particles endows them with properties completely different from those of bulk materials with the same compositions. Moreover, NPs have a very large surface-to-volume ratio, so that even small amount of particles presents large surface areas available for protein binding. Finally, the physicochemical properties of NPs such as their size, their surface charge, or their chemical functionalization would determine their protein corona composition,^{11,14–17} which may more influence the biological response of the body.^{18,19} This capacity of NPs to concentrate

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at their surface different subsets of proteins according to their physicochemical properties may be useful in proteomics to improve protein detection and identification, and detect potential disease-associated species.^{20–22} In fact, despite the constant technological advances in mass spectrometry (MS) and sample fractionation methods, the limited dynamic range of current mass spectrometers hinders the entire proteome coverage of biological fluids in which the protein concentration range spans more than ten orders of magnitude.^{23,24} Thus, the low-abundant protein compartment where disease-associated biomarkers are thought to reside is still unattainable. Various nanomaterial-associated strategies^{25–28} have been integrated in MS in order to detect disease-associated biomarkers, and have been found extremely useful to improve protein enrichment and sample preparation, particularly NP-associated technology.^{29–31}

Finding biomarker for disease detection, stratification, as well as therapeutic monitoring is a biomedical priority. Erythrocytes or red blood cells (RBCs) are a paradigmatic situation for clinical proteomics and also a major circulating compartment to individualize biomarkers.^{32,33} RBCs circulate all over the body and deliver oxygen to most organs. They are crucial micro-organisms able to capture and provide remote biomolecular information at the periphery. They are also involved in hematological malignancies^{34,35} for which relevant biomarkers are still missing. Because of the presence of hemoglobin (Hb), which represents 98% of the total RBC soluble protein content and masks the 2% of biologically interesting low-abundant proteins, it is difficult to investigate the deep RBC proteome in clinical and large-scale studies. Although technical advances in RBC sample preparation and fractionation have greatly contributed to reduce the protein concentration range, allowing to increase the number of identified proteins by tandem MS (MS/MS),^{36,37} these approaches are time-consuming and require intensive fractionation on gel and large volumes of sample to be really efficient, thus limiting the clinical translation of such technologies for high-throughput large-scale studies. Until now, the capacity of NPs for protein enrichment in complex protein mixture has never been assessed with RBC proteins.

In the present work, the potential capacity of three kinds of NPs to capture RBC proteins was determined by a qualitative and quantitative proteomics analysis. Based on a fast experimental protocol that required low volume of RBC lysates, and without any fractionation step, nano-liquid chromatography (LC)–MS/MS analysis of the different NP-treated samples revealed 893 different proteins, demonstrating the interest of our nanoproteomics approach to analyze

the RBC proteome in a large-scale context. We used label-free quantification³⁸ to confirm the reproducibility and the influence of the NP surface on protein capture, highlighting subsets of proteins specific to each kind of NPs. Through this work, we demonstrated for the first time the interest of chemically modified NPs in sample preparation for RBC proteomics studies.

Methods

Magnetic NPs

Superparamagnetic NPs of 100 nm in aqueous suspension were obtained from Chemicell GmbH (Berlin, Germany). Three different kinds of NPs grafted with different matrices and functional groups were used in our study, fluidMAG-OS (OS-NP), fluidMAG-PAA (PAA-NP), and fluidMAG-Q (Q-NP) (Table 1). They have a multi-domain core allowing a fast and easy magnetic isolation by an external magnet (MagnetoPURE, Chemicell GmbH). More detailed features are available at <http://www.chemicell.com/>.

The size and zeta potential of the magnetic NPs were determined with a Malvern Zetasizer NanoZS. NPs were diluted in phosphate-buffered saline (PBS; pH 7.3) before measurement, and the measurement was conducted at 25°C using 0.5 mg/mL NP concentrations.

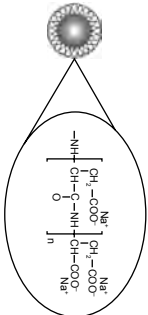
RBC preparation and lysis

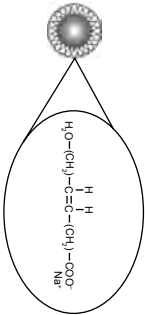
Blood samples were collected from five healthy consenting donors, three men and two women aged 44–62 years (mean 55.4 ± 7.7 years) by venipuncture in ethylenediaminetetraacetic acid vacutainer tubes. RBC lysis was performed according to the protocol previously published by Roux-Dalvai et al.³⁷ Briefly, samples were firstly centrifuged at $1,000 \times g$ at +4°C for 10 minutes to eliminate plasma and buffy coat. To further eliminate leukocytes, a ficoll gradient separation was performed. After removal of the monolayer containing mononuclear cells, erythrocytes were carefully collected, avoiding top RBC layer and polynuclear cells collected in the pellet. Erythrocytes were then washed three times with PBS + PMSF (154 mM NaCl, 10 mM phosphate buffer, pH 7.4 containing 0.1 mM PMSF). At each step, along with supernatant, the upper RBC layer was removed.

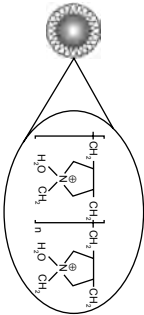
The lysis of RBC was operated by hypotonic shock. Red cells were diluted to a 1:3 ratio with lysis buffer (5 mM phosphate buffer, pH 7.4 containing 1 mM ethylenediaminetetraacetic acid, and 0.5 mM PMSF) containing protease inhibitor and left in ice for 30 minutes. After freezing, red cells were thawed at 37°C. The procedure of freezing/

Table 1 NP properties

	FluidMAG-PAA	FluidMAG-OS	FluidMAG-Q
Matrix	Aspartic acid	Oleic acid	PolyDADMAC
Functional group	Sodium carboxylate	Sodium carboxylate	Quaternary amine group
Diameter (nm)	112.4	107.7	106.9
PDI	0.11	0.12	0.19
Zeta potential (mV)	-37	-26	+41.0







Notes: The manufacturer provides information about the matrix and the functional group. The mean diameter, the PDI, and the zeta potential have been determined with a Malvern Zetasizer NanoZS. NPs were diluted in PBS (pH 7.3) before measurement, and the measurement was conducted at 25 °C using 0.5 mg/mL NP concentrations.

Abbreviations: NP, nanoparticle; PDI, polydispersity index; PBS, phosphate-buffered saline; PolyDADMAC, polydiallyldimethylammonium chloride.

thawing was repeated twice for each sample. At the end of the lysis step, after a centrifugation at $36,000\times g$ for 10 minutes at $+4^{\circ}\text{C}$, the clear supernatants were collected, pooled, and stored at -80°C until use.

RBC proteins–NPs incubation

Five hundred micrograms of each kind of NPs were individually incubated in two replicates for 45 minutes with 200 μ L of hemolysate containing 1 mg of total protein. Then, NPs were attracted to one side of the vial by an external magnet, and supernatant was removed. NPs were washed three times in 500 μ L PBS. Finally, NP-associated proteins were desorbed using 40 μ L of 8 M urea–2% CHAPS, and the protein concentrations were determined by a Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

Analysis of RBC lysate fractions by SDS-PAGE

Each sample was mixed with Laemmli buffer (4% sodium dodecyl sulfate [SDS], 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris-HCl). The mixture was heated in boiling water for 5 minutes and loaded on a precast 4%–15% polyacrylamide gel. Staining and destaining were performed with Coomassie blue and 30% ethanol/7% acetic acid in water, respectively.

LC-MS/MS analysis of RBC proteins

The [Supplementary materials](#) provide a detailed description of LC-MS/MS identification.

Briefly, 10 μg of each duplicated eluate and crude RBC lysate were separately diluted in Laemmli buffer and stacked in one band after a short and low-voltage electrophoretic migration on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel. The stacking bands were cut, proteins were in-gel digested by trypsin, and resulting peptides were extracted from the gel and analyzed by nano-LC-MS/MS using an ultimate 3000 system (Dionex, Amsterdam, the Netherlands) coupled to an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific, Bremen, Germany). The LTQ-Orbitrap was operated in data-dependent acquisition mode with the Xcalibur software. Survey scan MS spectra were acquired in the Orbitrap in the 300–2,000 m/z range with the resolution set to a value of 60,000. The five most intense ions per survey were selected for collision-induced dissociation fragmentation, and the resulting fragments were analyzed in the linear trap quadrupole (LTQ).

Database search and data analysis

The Mascot Daemon software (version 2.3.0, Matrix Science, London, UK) was used to perform database searches against *Homo sapiens* entries in Uniprot protein database. The mass tolerances in MS and MS/MS were set to 5 ppm and 0.6 Da, respectively, and the instrument setting was specified as “ESI-Trap”. Mascot results were parsed with the in-house-developed software Mascot File parsing and Quantification (MFPaQ) v4.0.0 software (<http://mfpaq.sourceforge.net/>), and protein hits were automatically validated if they satisfied one of the following criteria: identification with at least one top-ranking peptide with a Mascot score of more than

39 (P -value <0.001) or at least two top-ranking peptides each with a Mascot score of more than 22 (P -value <0.05). When several proteins matched exactly the same set of peptides, only one member of the protein group was reported in the final list. To evaluate the false-positive rate in these experiments, all the initial database searches were performed using the “decoy” option of Mascot. Mascot results were parsed with the in-house-developed software MFPaQ v4.0.0 (<http://mfpaq.sourceforge.net/>), and protein hits were automatically validated.

Label-free quantification

Quantification of proteins was performed using the label-free module implemented in the MFPaQ v4.0.0 software (<http://mfpaq.sourceforge.net/>). For each sample, the software uses the validated identification results and extracted ion chromatogram (XIC) of the identified peptides in the corresponding raw nano-LC–MS files based on their experimentally measured retention time and monoisotopic m/z values. Quantification of peptide ions was performed based on calculated XIC area values. To compare the abundance profile of one protein in different samples, a protein abundance index (PAI) was calculated for each protein in the different NP samples. PAI was defined as the average of XIC area values for three intense reference tryptic peptides identified for this protein. If only one or two peptides were used to identify the protein, the protein-related PAI was calculated based on the XIC area value of these peptides. As the three different kinds of NPs were experimentally duplicated, six PAI values were obtained for each protein; the highest PAI value was normalized to 1, and the five other PAI values were thus comprised between 0 and 1. Normalized PAIs were submitted to software R for protein hierarchical clustering.³⁹

Results

Magnetic NPs

In this study, we investigated the capacity of three different kinds of 100 nm NPs for protein capture in RBC lysates through the protein identification and quantification of their respective eluted corona. This size of NPs offers a high surface-to-volume ratio and facilitates the NP collection by an external magnet. The main difference between these NPs concerns their surface charge and/or functional group. First, according to dynamic light scattering and zeta potential measurements, the respective NPs were homogeneous in size and monodispersed. Q-NPs displayed a positive charge, and PAA-NPs and OS-NPs both displayed a negative charge (Table 1).

SDS-PAGE analysis

As particles fulfilled critical quality criteria, 500 μg of each kind of NPs, developing 280 cm^2 surface area, were separately incubated in duplicates for 45 minutes with 200 μL of RBC lysates (ie, 1 mg of proteins), followed by extensive washing to remove all unbound proteins. First, the Bradford assays revealed $19 \pm 1 \mu\text{g}$ of proteins eluted from the surface of each kind of NPs, which represented 2% of the initial protein input. Prior to deeper characterization of each NP corona, the same quantity of proteins was loaded on an SDS-PAGE gel (Figure 1) to obtain a protein profile of each condition. For the three types of NPs, SDS-PAGE analysis of the eluted protein corona revealed a pattern obviously different from nontreated sample. Interestingly, the high-abundant Hb was considerably reduced, and the protein profiles of NP-treated samples revealed a great enrichment between 20 kDa and

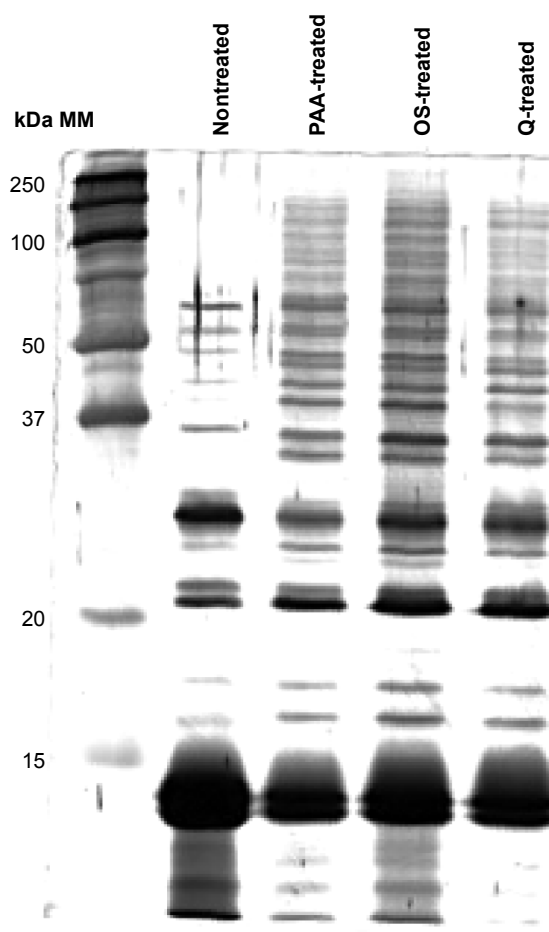


Figure 1 SDS-PAGE of nontreated and NP-treated RBC lysates.

Notes: The same quantity of proteins was loaded on gel for the different tracks. We can see the diminution of hemoglobin proportion in NP-treated samples for which we can distinct two bands corresponding to alpha and beta hemoglobin subunits (around 14 kDa).

Abbreviations: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; NP, nanoparticle; RBC, red blood cell; MM, molecular markers.

250 kDa with the appearance of numerous bands, which were not detected in the control condition.

Protein identification by nano-LC–MS/MS

To better estimate the protein enrichment offered by the NP treatment, a deeper characterization of each NP corona was performed by nano-LC–MS/MS protein identification. Figure 2A sums up the results obtained for each duplicated sample and gives an insight of the harvesting capacity for each condition. The different protein lists are detailed in the [Supplementary materials](#). The two replicates of the crude RBC lysate revealed 108 different proteins, whereas two of the NPs-eluted coronae significantly highlighted more species ($P=0.009$, Mann–Whitney test), from 525 to 576 different species according to the type of NPs (Figure 2A).

Altogether, 909 different proteins were identified from our RBC samples of which 893 were present in the list of NP-treated samples (Figure 2B). Among these 893 proteins, 801 were exclusively identified from NP-eluted coronae, and 92 were shared with crude RBC lysate. A focus on the three NP samples revealed a relatively similar number of identified proteins varying from 525 for OS- and Q-NPs to 576 for PAA-NPs, the three kinds of NPs only sharing 193 proteins (Figure 2C).

Effect of NP treatment on nano-LC–MS/MS analytical coverage

The nano-LC–MS/MS analysis showed that the NP treatment of the sample greatly enhanced the number of identified proteins; this must result from a better MS/MS sampling of

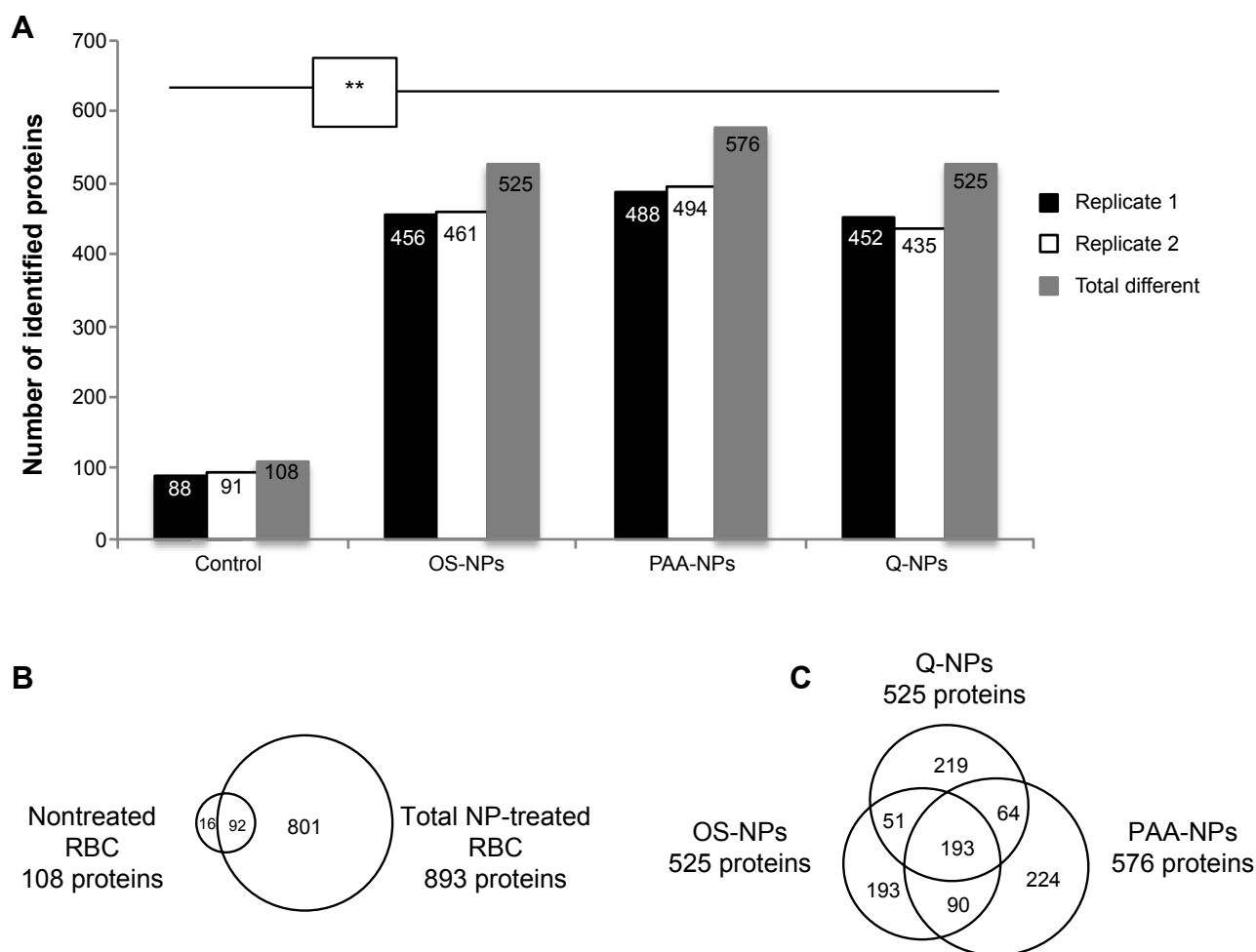


Figure 2 Protein identification results and NP comparison.

Notes: (A) Graphical representation of the number of identified proteins by nano-LC–MS/MS in each replicate, in nontreated and NP-treated RBC lysates. “Total different” columns represent the total number of identified proteins in each condition after merging both replicates. (B and C) Venn diagrams representing the extent of overlap of proteins identified by LC–MS/MS analysis. (B) Comparison made between the nontreated RBC extract (108 proteins) and all NP-treated samples (894 proteins). (C) Comparison among the three NP-treated samples (PAA-NP, 576 proteins; OS-NP, 525 proteins; Q-NP, 525 proteins). The number of identified proteins in NP samples is significantly higher than in nontreated samples (Mann–Whitney test $P=0.009$).

Abbreviations: LC–MS/MS, liquid chromatography–tandem mass spectrometry; NP, nanoparticle; RBC, red blood cell.

the detected ions. To illustrate that effect, we compared the 20 most abundant identified proteins (ie, the 20 proteins with the higher MS/MS queries) in nontreated and NP-treated RBCs (Table 2 and [Supplementary materials](#)). Interestingly, according to the sample treatment, the 20 most abundant proteins were not strictly the same. In fact, only Hb-related subunits and spectrin chains, the two major constituents of RBCs, were shared by all conditions. Nevertheless, the rank and thus the relative abundance of these proteins were different. For example, Hb subunit beta and spectrin alpha chain that were respectively the first and the 20th most abundant protein in nontreated sample occupied the 5th and the 1st rank, respectively, in OS-NP sample. And carbonic anhydrase 1 that appeared just after the Hb-related subunits, as the 12th most abundant protein in nontreated RBC, only occupied the 70th rank in OS-NP sample. These results demonstrate that NP treatment modified the relative protein concentration in RBC sample. This modification of the protein abundance rank was also different according to the kind of NPs, suggesting the differential affinity of the NP surface for RBC proteins. Moreover, we also observed that the number of MS/MS queries assigned to these 20 most abundant proteins was greatly lower in NP-treated condition than in control. In control, 94% of the total MS/MS queries that were generated during the run were assigned to these 20 proteins, of which 91% to the Hb-related subunits. In the NP-treated sample, the 20 most abundant proteins only generated 42%–45% of the total MS/MS queries, of which 20%–29% were assigned to Hb-related subunits, according to the kind of NPs. This observation revealed that NP treatment greatly decreased the proportion of abundant proteins. Finally, for each kind of NPs, we showed that 90%–95% of the 20 most abundant proteins are the same between replicates. We also observed that the 20 most abundant proteins as well as Hb-related subunits generated the same part of MS/MS queries between replicates ([Supplementary materials](#)). These results confirmed the reproducibility of capture of each kind of NPs for these 20 most abundant proteins.

To determine whether NP treatment increased the proportion of low-abundant proteins in RBC samples, the MS/MS queries, the peptide number, and the sequence coverage of ten low-abundant proteins in nontreated sample (ie, proteins with low MS/MS queries) were determined in OS-NPs sample (Table 3). In nontreated sample, these ten low-abundant proteins generated from one to three MS/MS queries, with a low coverage of each protein sequence (from 1.4% to 17.1%). They represented 0.1% of the total MS/MS queries generated by the sample. In OS-NP samples, the same proteins

generated from five to 63 MS/MS queries, allowing a greater coverage of the protein sequence (from 11.9% to 79.6%) through the detection and identification of different peptides for each protein. These ten proteins represented 2% of the total MS/MS queries generated by the NP-treated sample. This enhanced detection of low-abundant proteins was also observed in Q- and PAA-NPs ([Supplementary materials](#)). These observations clearly demonstrated that NP treatment also increased the proportion of low-abundant proteins.

Qualitative and quantitative reproducibility in protein harvesting

Although NP-treated samples allowed a great increase in protein identification, providing access to low-abundant proteins, it was important to confirm the reproducibility of NPs for RBC protein harvesting. First, in a qualitative approach, we compared the MS spectra of each replicate and observed that the MS signal of 90%–94% of identified peptides was present in both replicates per type of NPs, thus confirming the qualitative reproducibility of NPs for protein harvesting (Figure 3A). Then, to assess the quantitative reproducibility of NP-strategy, we used the MFPaQ software,⁴⁰ which was designed to parse and validate protein identifications from Mascot result files and quantify the identified proteins. The quantification module of MFPaQ v4.0.0 was upgraded so that it could handle label-free quantification as described by Mouton-Barbosa et al.⁴¹ More information is available in the [Supplementary materials](#). Briefly, a PAI was calculated for all identified proteins. To assess the quantitative reproducibility of each type of NPs for protein harvesting, the PAI ratio of all identified proteins was determined in duplicates ([Supplementary materials](#)). As shown in Figure 3B, quantitative reproducibility of NPs was found to be good between the duplicates. In fact, the majority of quantified proteins on each kind of NPs revealed a PAI ratio close to the expected value of 1 ([Supplementary materials](#)).

Differential capture of proteins by the NP surface

As the protein capture by NPs was qualitatively and quantitatively reproducible, we aimed to determine if there was an NP-dependent protein harvesting. Based on these quantification results, we normalized the PAIs obtained for each protein in each sample and submitted them to hierarchical clustering with the software R. According to the PAI values of each protein in each condition, the clustering revealed ten differentially quantified protein groups ([Supplementary materials](#)). Among them, groups 4, 5, and 8 revealed 68, 100,

Table 2 List of the 20 most abundant proteins (decreasing from 1 to 20) identified from NPs corona and crude RBC extract

Total MS/MS number	MW	pI	Description	#total MS/MS	%total MS/MS	Rank	%total MS/MS	#total MS/MS	Description	pI	MW	Total MS/MS number
Control RBC												
18,796	16,102	6.75	Hemoglobin subunit beta	2,654	14.1	1	4.4	795	Hemoglobin subunit beta OS	6.75	16,102	18,096
	16,101	7.86	Beta-globin	2,424	12.9	2	4.4	788	Spectrin, beta, erythrocytic	5.15	247,271	
	11,534	5.9	Beta globin (fragment)	1,921	10.2	3	4.3	787	Spectrin beta chain, erythrocyte	5.15	247,171	
	11,496	6.17	Hemoglobin beta (fragment)	1,731	9.2	4	4.2	759	Spectrin alpha chain, erythrocyte	4.95	281,039	
	15,305	8.72	Hemoglobin subunit alpha	1,563	8.3	5	3.3	590	Hemoglobin beta (fragment)	6.17	11,496	
	11,532	5.46	Hemoglobin beta (fragment)	1,499	8.0	6	3.1	568	Hemoglobin subunit alpha	8.72	15,305	
	10,979	6.17	Hbbm fused globin protein (fragment)	1,480	7.9	7	3.1	555	Hemoglobin beta (fragment)	5.46	11,532	
	16,159	7.85	Delta globin	1,429	7.6	8	2.7	495	Hbbm fused globin protein (fragment)	6.17	10,979	
	8,386	6.63	Hemoglobin alpha 2 (fragment)	1,242	6.6	9	2.4	443	Delta globin	7.85	16,159	
	4,263	4.96	Truncated beta globin	968	5.2	10	2.0	358	Solute carrier family 4, anion exchanger, member 1	5.1	102,150	
	16,187	6.64	Hemoglobin subunit gamma-I	242	1.3	11	1.8	330	Transitional endoplasmic reticulum ATPase	5.14	89,950	
	28,909	6.59	Carbonic anhydrase I	113	0.6	12	1.5	272	Proteasome subunit alpha type-6	6.34	27,838	
	59,947	6.9	Catalase	103	0.5	13	1.4	245	Acylamino-acid-releasing enzyme	5.29	82,142	
	247,171	5.15	Spectrin beta chain, erythrocyte	84	0.4	14	1.3	239	Clathrin heavy chain I	5.48	193,260	
	102,150	5.1	Solute carrier family 4, anion exchanger, member 1	72	0.4	15	1.3	236	Hemoglobin subunit gamma-I	6.64	16,187	
	32,325	6.45	Purine nucleoside phosphorylase	59	0.3	16	1.3	228	Myosin-9	5.5	227,646	
	22,049	5.66	Peroxiredoxin-2	57	0.3	17	0.9	171	Delta-aminolevulinic acid dehydratase	6.32	36,728	
	25,133	6	Peroxiredoxin-6	54	0.3	18	0.8	152	Ankyrin-1	5.65	207,334	
	29,285	6.87	Carbonic anhydrase 2	50	0.3	19	0.8	140	Importin-5	4.83	125,032	
	281,039	4.95	Spectrin alpha chain, erythrocyte	49	0.3	20	0.8	136	E3 ubiquitin-protein ligase UBR4	5.7	580,547	
			The 20 most abundant proteins	94.7			45.8		The 20 most abundant proteins			
			Related to hemoglobin (among the 20 most abundant)	91.3			20.3		Related to hemoglobin (among the 20 most abundant)			
OS-NPs												
13,101	281,039	4.95	Spectrin alpha chain erythrocyte	659	5.0	1	4.3	779	Hemoglobin subunit beta	6.75	16,102	18,073
	247,171	5.15	Spectrin beta chain erythrocyte	468	3.6	2	3.8	688	Beta globin (fragment)	5.9	11,534	
	247,271	5.15	Spectrin beta, erythrocyte	465	3.5	3	3.6	656	Beta-globin	7.86	16,101	
	102,150	5.1	Solute carrier family 4, anion exchanger, member 1	459	3.5	4	3.3	596	Hemoglobin beta (fragment)	6.17	11,496	
	16,102	6.75	Hemoglobin subunit beta	428	3.3	5	3.1	565	Hemoglobin beta (fragment)	5.46	11,532	
	11,532	5.46	Hemoglobin beta (fragment)	351	2.7	6	2.7	484	Hemoglobin subunit alpha	8.72	15,305	

(Continued)

Table 2 (Continued)

Total MS/MS number	MW	pI	Description	#total MS/MS	%/total MS/MS	Rank	%/total MS/MS	#total MS/MS	Description	pI	MW	Total MS/MS number
	14,069	6.46	Hemoglobin beta subunit variant (fragment)!	333	2.5	7	2.6	475	Hb β m fused globin protein (fragment)	6.17	10,979	
	11,496	6.17	Hemoglobin beta (fragment)!	331	2.5	8	2.4	442	Delta globin	7.85	16,159	
	16,159	7.85	Delta globin	283	2.2	9	2.0	363	Spectrin beta chain, erythrocyte	5.15	247,171	
	15,305	8.72	Hemoglobin subunit alpha	272	2.1	10	2.0	360	Spectrin, beta, erythrocytic	5.15	247,271	
	16,187	6.64	Hemoglobin subunit gamma-1	212	1.6	11	1.6	286	Ankyrin-1	5.65	207,334	
	16,173	6.64	Hemoglobin subunit gamma-2	212	1.6	12	1.5	263	Spectrin alpha chain, erythrocyte	4.95	281,039	
	8,386	6.63	Hemoglobin alpha 2 (fragment)	199	1.5	13	1.3	230	Truncated beta globin	4.96	4,263	
	207,334	5.65	Ankyrin-1	196	1.5	14	1.2	217	Uncharacterized protein	7.04	98,190	
	580,547	5.7	E3 ubiquitin-protein ligase UBR4	133	1.0	15	1.2	212	Hemoglobin subunit gamma-1	6.64	16,187	
	271,766	5.77	Talin-1	126	1.0	16	1.2	210	Hemoglobin subunit gamma-2	6.64	16,173	
	57,794	6.01	T-complex protein 1 subunit beta	100	0.8	17	1.1	200	E3 ubiquitin-protein ligase UBR4	5.7	580,547	
	82,465	5.49	Calpain-1 catalytic subunit	86	0.7	18	1.1	191	Solute carrier family 4, anion exchanger, member 1	5.1	102,150	
	100,877	5.08	26S proteasome non-ATPase regulatory subunit 2	84	0.6	19	1.0	178	T-complex protein 1 subunit gamma	6.1	61,066	
	51,296	5.49	RuvB-like 2	84	0.6	20	0.9	162	T-complex protein 1 subunit beta	6.01	57,794	
			The 20 most abundant proteins	41.8	41.8				The 20 most abundant proteins			
			Related to hemoglobin (among the 20 most abundant)	20.0	29.5				Related to hemoglobin (among the 20 most abundant)			

Notes: Information about protein description, MW, pI, number, and proportion of MS/MS queries are provided. The % MS/MS has been normalized to the total MS/MS queries per run. Bold proteins are hemoglobin-related subunits or fragments.

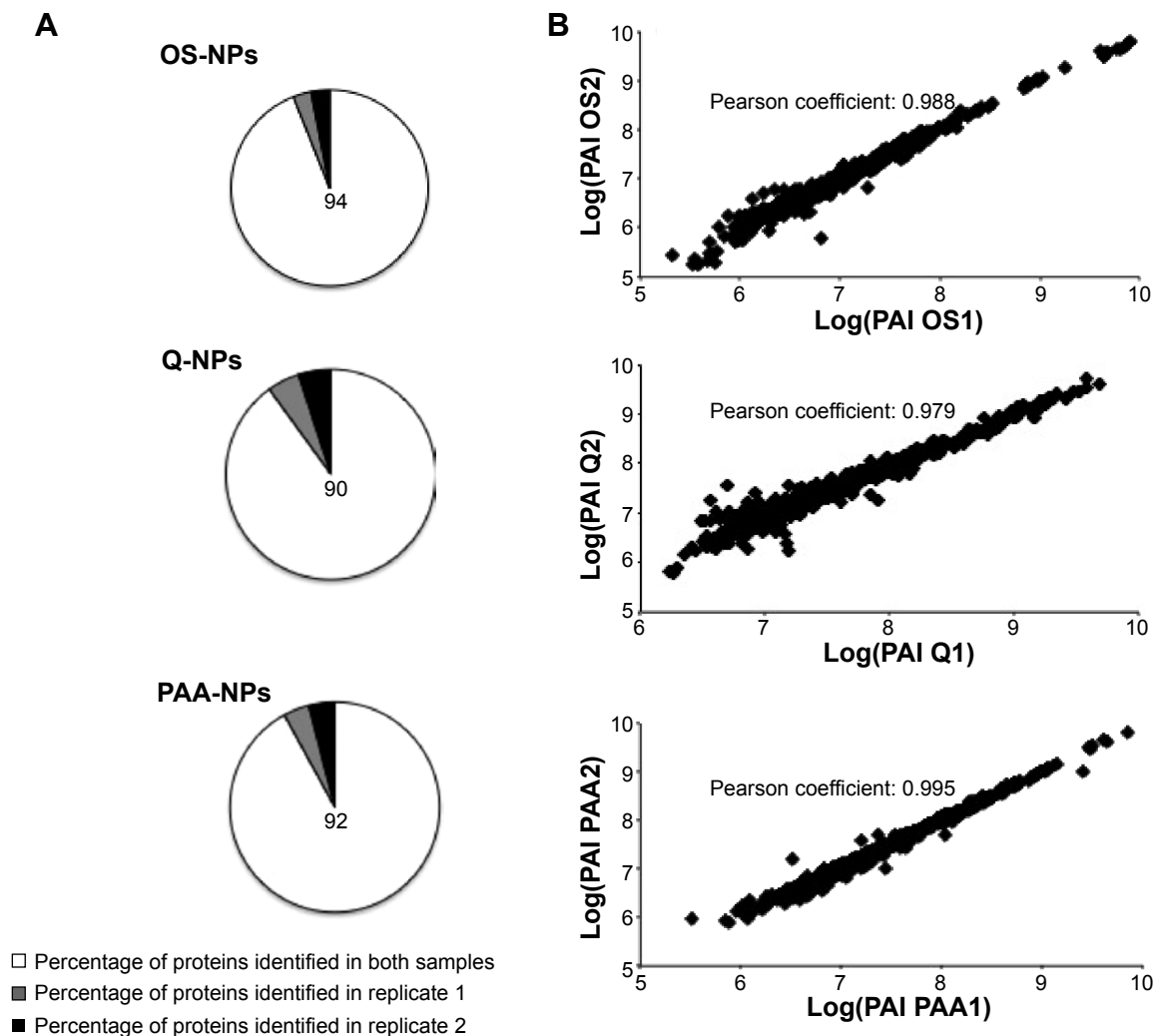
Abbreviations: NPs, nanoparticles; RBC, red blood cell; MS/MS, tandem mass spectrometry; MW, molecular weight; pI, isoelectric point.

Table 3 Comparison between nontreated and OS-NPs-treated RBC, of the ten less abundant proteins identified in nontreated RBC

Control RBC			Description	OS-NPs		
MS/MS queries	Peptide number	Sequence coverage (%)		Sequence coverage (%)	Peptide number	MS/MS queries
1	1	3.4	Calpain small subunit I	60.8	12	63
2	2	3.4	Heat shock protein HSP 90-alpha	26	17	30
2	2	2	T-complex protein I subunit theta	48.5	28	63
2	1	5.2	Proteasome subunit alpha type-7	42.9	8	13
2	2	5	Transkelolase	11.9	5	11
2	2	17.1	Ubiquitin-conjugating enzyme E2 N	79.6	10	28
2	1	1.4	Plastin-2	18	9	19
2	2	14.1	Hemoglobin subunit theta-I	17.6	2	5
3	2	13.2	Proteasome subunit alpha type-2	41.5	6	11
3	2	9.5	Proteasome subunit beta type-5	27.4	7	12

Notes: The total number of MS/MS queries, the number of different identified peptides, and the protein sequence coverage inform about the enrichment of these ten proteins in NP sample.

Abbreviations: RBC, red blood cell; NPs, nanoparticles; MS/MS, tandem mass spectrometry.

**Figure 3** Qualitative and quantitative reproducibility for NPs.

Notes: (A) Qualitative and (B) Quantitative reproducibility for protein harvesting in each kind of NPs over replicates. (A) Pie chart representing the part of MS signals detected in both replicate (white), in replicate 1 (gray), or in replicate 2 (black). (B) Correlation of log(PAI) values for all quantified proteins between replicates in NP-treated samples.

Abbreviations: NPs, nanoparticles; MS, mass spectrometry; PAI, protein abundance index.

and 136 different proteins that specifically constituted the respective corona of Q-, OS-, and PAA-NPs (Figure 4A). To deeply characterize this protein capture specificity, we compared the molecular weight (MW) and the isoelectric point (pI) of proteins in these three groups. PAA-specific proteins revealed significantly higher MWs than OS-NPs (Figure 4B and [Supplementary materials](#)). In fact, the median protein MW in PAA-group was 50,547 Da; it was only 33,667 Da in OS-group. Regarding the pI, the three protein groups showed significant differences (Figure 4C and [Supplementary materials](#)). The positively charged Q-NPs revealed 90% of specific proteins that were negatively charged at pH 7.4 (ie, with a pI <7.4), whereas negatively charged OS- and PAA-NPs harvested less than 70% of specific protein with pI <7.4, confirming previous reports that suggested NP surface charge would influence the protein corona.

Functional analysis of RBC proteins

As NPs considerably increased the number of identified proteins in RBC samples and as the different surfaces harvested different subproteomes, we were interested in determining the functional pathways that were significantly represented by NP-harvested proteins in comparison with nontreated sample. In this aim, we submitted our data set to Ingenuity Pathway Analysis software⁴² (Ingenuity Systems, Redwood City, CA, USA) to perform the functional characterization of the RBC proteome and identify the main molecular pathways and networks. This automatic annotation tool uses a knowledge database and assigns proteins to functional classes or specific canonical pathways (CPs) related to various biological processes.

According to the functional analysis, the 893 proteins identified from the three different kinds of NPs had been associated to 140 different and significantly represented CPs and revealed 98 species related to hematological diseases; the 109 proteins from the crude extract only highlighted 40 different CPs and 31 proteins previously described in hematological diseases.

The ten more significant CPs for crude RBC lysate (Figure 5A) were related to the cellular origin and known functions of RBC. Most of them involved protein and nucleic acid degradation, anaerobic glycolysis, and response to oxidative stress. Although these ten CPs were not the most significant in NP-treated lysates, they were more represented in these samples than crude extract. Figure 5A shows the ratio of proteins identified in these ten CPs for crude and NP-treated RBC extracts. This ratio matches to the number of proteins from our data set that maps to the pathway divided by the total number of proteins referenced in this pathway

by the software. We noticed in all CPs that these ratios were higher for the NP-treated samples, intending that NP increased the identification of proteins related to these CPs. On the other hand, when we considered NPs as the reference sample (Figure 5B), the ten more significant CPs were related to cellular assembly and organization of cytoskeleton, which play an important role in RBC. These pathways that are related to important RBC functions were almost absent from the set of proteins identified in the crude RBC sample, illustrating the impact of the enrichment following protein harvesting using NP technology.

Discussion

Although the behavior of NPs in the presence of complex protein mixture is still far from being completely understood, several studies have demonstrated that the physicochemical features of NPs greatly influenced the composition of their protein corona. In 2008, Lundqvist et al¹⁴ demonstrated, in plasma, qualitative differences in the protein corona of different types of NPs, according to the surface properties and the size of NPs. In 2012, Capriotti et al¹¹ demonstrated that charge density on cationic liposomes influenced both qualitative and quantitative plasma protein capture. Recently, Tenzer et al¹⁵ performed both qualitative and quantitative proteomics to demonstrate that the NP size significantly determined the protein corona. Interestingly, they also demonstrated that the relative proportion of plasma proteins was modified on NP surface compared to their proportion in control plasma. These results suggest that different kinds of NPs may harvest different subproteomes and modify the range of protein concentration in complex samples. As these two properties may be helpful in proteomics approaches to increase the proteome coverage of a biological sample, we assessed for the first time the ability of chemically modified NPs to increase protein identification in RBC samples. The challenge with RBC sample is to decrease the concentration of Hb, which represents 98% of the total protein concentration. NPs provide great advantages for ex vivo experiments; their functionalized surfaces, their nanometer size, and their great surface-to-volume ratio endow them with unique physicochemical properties making them very attractive for protein harvesting.

In this study, we used three kinds of 100 nm superparamagnetic NPs that strongly differ in their surface chemistry. The magnetic properties of these NPs considerably facilitated their handling during washing and elution steps through the use of an external magnet. We selected a strong anion exchanger (Q-NP), a strong cation exchanger (PAA-NP), and a hydrophobic (OS-NP) surface to increase

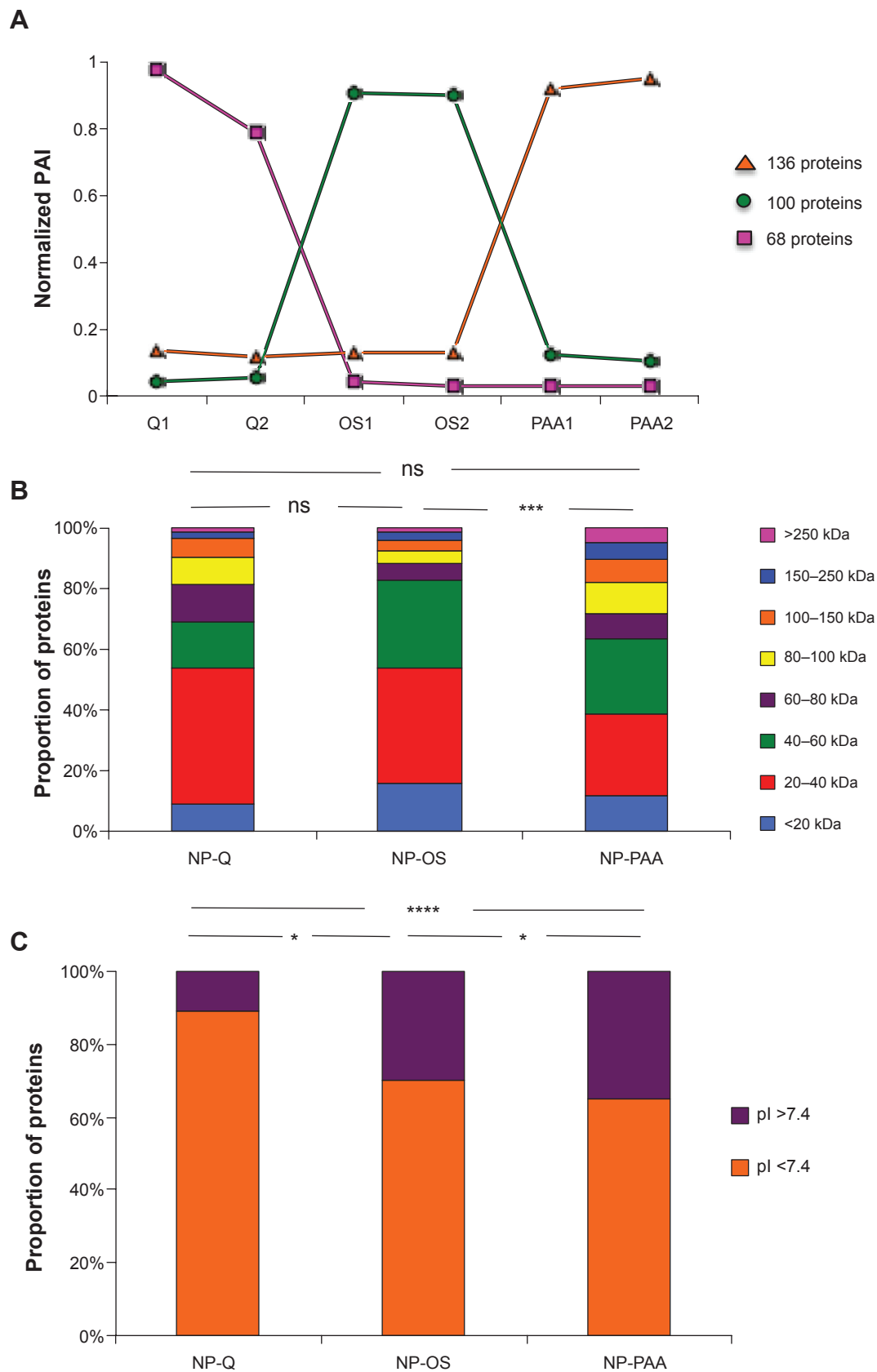


Figure 4 Characterization and comparison of protein groups specific to NP surface by quantitative mass spectrometry.

Notes: (A) Graphical representation of the mean normalized PAI value of three protein groups (obtained by hierarchical clustering) specific to NP surface. Here, 136, 100, and 68 proteins are preferentially captured by PAA-NPs, OS-NPs, and Q-NPs, respectively. Histogram representing (B) the MW and (C) the pI of these specific proteins for each NP surface. * $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$.

Abbreviations: NP, nanoparticle; PAI, protein abundance index; MW, molecular weight; pI, isoelectric point; ns, non significant.

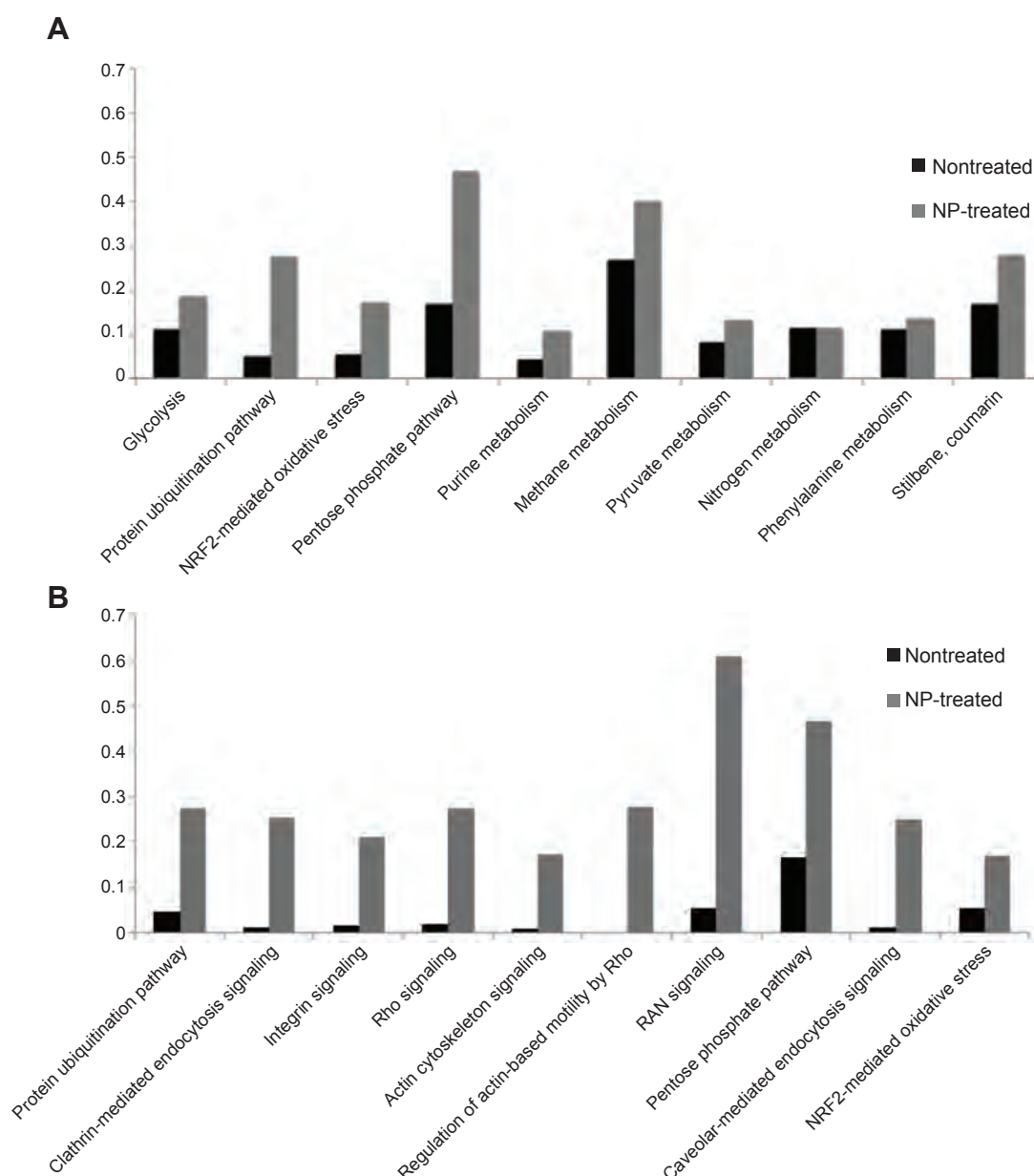


Figure 5 Functional annotation of RBC proteins identified by nano-LC-MS/MS.

Notes: Top ten of significant CPs for (A) untreated and (B) NP-treated RBC extract. For each CP, the bars show the ratio between the number of identified proteins classified in that pathway and the total number of proteins referenced in this pathway by the software for untreated (black bar) and treated RBC extract (gray bar).

Abbreviations: RBC, red blood cell; LC-MS/MS, liquid chromatography–tandem mass spectrometry; CPs, canonical pathways; NP, nanoparticle.

the probability to harvest different subsets of proteins. After the incubation of NPs with RBC protein extract, the nano-LC-MS/MS analysis of each eluted corona enabled the identification of more than 500 proteins, increasing by 400% the number of identified species compared to a control extract of RBC (108 proteins). Actually, synergic effects occurring at the NP surface can explain this improvement in protein identification. First, the proportion of high-abundant proteins is decreased on NP surface. Moreover, as observed by Tenzer et al³⁵ the abundance rank of proteins (estimated

by the number of MS/MS queries) was different between NP-treated and untreated sample. These results confirm that NPs modify the range of protein concentration at their surface. Interestingly, the 20 most abundant proteins harvested by NPs were also different according to the NP chemical functionalization ([Supplementary materials](#)). This observation confirms that the affinity of each protein may be different according to the NP properties, influencing the protein concentration on the NP surface. The second effect concerns the increase of low-abundant proteins on NPs.

In fact, we observed that low-abundant proteins identified in nontreated sample were present in higher abundance on NPs. Here also, the higher affinity of several low-abundant proteins for the different kinds of chemical functionalization allowed their accumulation on the NP surfaces. Thus, through the respective decrease of high-abundant proteins and increase of low-abundant proteins, NP treatment greatly reduces the range of concentration in the sample, making detectable more peptides by the MS instruments. Moreover, because RBC proteins have different affinities for the three kinds of NPs, distinct subproteomes were harvested on each surface, and we finally identified a total of 893 different proteins from these three types of NPs. Among these 893 identified proteins, label-free quantification allowed us to determine which were NP-specific protein signatures. Focusing on the MW and the pI, we found significant differences between these three NP-specific protein groups. While the positively charged Q-NP revealed 90% specific proteins with $pI < 7.4$ (ie, negatively charged at pH 7.4), the negatively charged OS and PAA-NPs bound less than 35% of specific proteins with $pI > 7.4$. We also observed that PAA-NPs harvested specific proteins with higher MW than the ones harvested by OS-NPs. According to these results, we confirmed that properties such as surface charge or chemical functionalization impact on NP–protein interaction.

Although the complexity of the NP–protein interactions is far to be understood, an NP-associated sample preparation of RBC samples in clinical proteomics appears obvious. During its 4-month life span, RBC travels along the entire blood stream to deliver oxygen to most organs. As a major circulating compartment, it is considered as a crucial micro-organite able to contain biomarkers associated both to hematological and non-hematological disorders.³³ In the aim to detect protein modifications in pathological context, the access to the RBC proteome is a major issue to identify potential therapeutic targets. Although technical advances in RBC sample preparation and fractionation have greatly contributed to a better proteome coverage in RBC samples, they remain both time- and sample-consuming and thus incompatible with large-scale clinical studies. We demonstrated that our NP treatment is a very reproducible strategy that specifically allowed the access to the RBC minor proteome. Thus, signaling pathways highlighted in nontreated sample and implicated in important RBC functions such as oxygen transport, glycolysis, and protection against oxidative stress were more represented by NP treatment, offering the possibility to better understand mechanisms responsible for dysfunctions of these key

functional pathways. Moreover, NP treatment specifically highlighted RhoA, Rac, and Cdc42 signaling that have essential functions in morphology and deformability regulations of the erythrocyte cytoskeleton.^{43,44} The maintenance of normal deformability is crucial to permit RBC to enter into narrow capillaries, and dysfunctions of these pathways have been associated with hemolytic anemia. Access to such mandatory pathways for erythrocyte functions would greatly improve investigations on erythrocyte disorders. This access to the minor RBC proteome could also provide precious information for non-hematological disorders. For instance, the specific identification by NP treatment of alpha-synuclein and DJ-1,⁴⁵ two major proteins associated to Parkinson's disease,^{46,47} may open the way to new proteomics analysis of RBCs in this disease.^{48,49}

Finally, one great advantage of our approach, in comparison with immuno-depletion³⁶ or ProteoMiner,³⁷ concerns its efficiency from low volume of sample (200 μ L of RBC containing 1 mg of protein). In their study, Ringrose et al³⁶ and Roux-Dalvai et al³⁷ both performed on-gel fractionation after immuno-depletion or ProteoMiner, and identified 700 and 1,578 different proteins from 200 mg and 5.7 g of starting material, respectively. We would certainly identify more proteins if we had combined such fractionation to NP treatment. Although getting high quantity of RBC proteins is not a limiting factor, as 50 mL of total blood could be easily obtained, the possibility to work on low volumes is more compatible with clinical-automated facilities. And the magnetic feature of NPs allowed us to easily automate all the experimental protocol. Villanueva et al⁵⁰ have already described this kind of platform and demonstrated the reproducibility of NPs for protein harvesting, suggesting the ability to simultaneously process hundreds of samples in reproducible and clinically adapted conditions.

Conclusion

For the first time, we took advantage of the complexity of the NP–protein interactions to increase the proteome coverage in RBC lysates, via the capture of complementary subproteomes on different kinds of NPs. Through a qualitative and quantitative proteomics approach, we confirmed the impact of the NP surface functionalization and charge on protein capture, both demonstrating the reduction of the sample concentration range on the NPs and the specificity of capture for each surface. These synergic effects opened a window on the RBC minor proteome, which is a major issue to identify biomarkers associated to hematological and/or non-hematological disorders.

Disclosure

The authors report no conflicts of interest in this work.

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Exhibit B



Nanoparticle size matters in the formation of plasma protein coronas on Fe₃O₄ nanoparticles



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ABSTRACT

When nanoparticles (NPs) enter into biological systems, proteins would interact with NPs to form the protein corona that can critically impact the biological identity of the nanomaterial. Owing to their fundamental scientific interest and potential applications, Fe₃O₄ NPs of different sizes have been developed for applications in cell separation and protein separation and as contrast agents in magnetic resonance imaging (MRI), etc. Here, we investigated whether nanoparticle size affects the formation of protein coronas around Fe₃O₄ NPs. Both the identification and quantification results demonstrated that particle size does play an important role in the formation of plasma protein coronas on Fe₃O₄ NPs; it not only influenced the protein composition of the formed plasma protein corona but also affected the abundances of the plasma proteins within the coronas. Understanding the different binding profiles of human plasma proteins on Fe₃O₄ NPs of different sizes would facilitate the exploration of the bio-distributions and biological fates of Fe₃O₄ NPs in biological systems.

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1. Introduction

Nanoparticles (NPs) have been widely applied in drug/gene delivery, disease diagnosis and biosensing due to their unique properties compared with their corresponding bulk counterparts [1–5]. When NPs enter biological systems, proteins can bind onto their surfaces, leading to the formation of so-called “protein coronas”, which represents the first interfacial physicochemical properties of the NPs [6,7]. It has been observed that protein coronas bound on the surfaces of NPs critically affect the biological identity of the nanomaterial [6–9] and/or result in physiological and pathological changes, including macrophage uptake, blood coagulation, complement activation and cellular toxicity [10–17].

Studies of protein coronas for different types of NPs, such as copolymers [18,19], silica [15,20,21], polystyrene [21–23] and metallic NPs [24,25], have been performed extensively. It was found that protein coronas on these NPs could be affected by several factors, such as the chemical composition [26], cell culture medium [27], serum inactivation [28], plasma concentration [21,29], surface

properties [24,30] and particle size [20,23] of the NPs. For instance, differences in plasma proteins bound on the surfaces of SiO₂, TiO₂ and ZnO NPs have been observed due to differences in the chemical compositions of the materials [26]. Additionally, it has been reported that the formation of a protein corona on cationic liposomes is strongly related to the membrane charge density [30]. Moreover, it has been illustrated that particle size could critically influence human plasma proteins bound on the surfaces of silica NPs [20].

Fe₃O₄ NPs have been extensively developed for applications in cell separation, protein separation, targeted drug delivery and as contrast agents in magnetic resonance imaging (MRI) [31–36]. Although many types of Fe₃O₄ NPs have been synthesized with different sizes and functionalities to meet the needs of various applications, studies of the interactions between Fe₃O₄ NPs and human plasma proteins are relatively rare. Recently, Mahmoudi et al. found that superparamagnetic iron oxide NPs induced irreversible changes in the conformation of human transferrin protein [37]. Additionally, it was found that the plasma proteins adsorbed onto iron oxide NPs vary depending on the initial NP surface coating and the concentrations of plasma, while the formation of protein corona would further influence the internalization of the iron oxide NPs into macrophages [38]. However, the above-mentioned studies only focused on protein coronas formed on superparamagnetic

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iron oxide NPs less than 10 nm in size and with coatings of polymers, the protein coronas on the pristine Fe₃O₄ NPs in larger sizes were not investigated. Furthermore, whether the particle size of Fe₃O₄ NPs influences the formation of the plasma protein corona is unknown. Although pristine Fe₃O₄ NPs are not directly applied in biomedical fields, they are precursors of the magnetic NPs used in biological and biomedical applications. During the fabrication process, pristine Fe₃O₄ NPs can be inhaled by humans, pass through membranes and barriers, and finally enter the blood [39]. Here, we quantified the plasma protein coronas bound on three different sizes of pristine Fe₃O₄ NPs (~30 nm, 200 nm and 400 nm) by means of label-free quantitative proteomic strategy. Our results demonstrated that the particle size was an important factor that not only influenced the protein composition of the human plasma protein coronas on Fe₃O₄ NPs but also affected the abundance of plasma proteins in the protein coronas.

2. Materials and methods

2.1. Chemicals and materials

Fe₃O₄-30 (~30 nm), dithiothreitol (DTT), iodoacetamide (IAA), TPKC-treated trypsin, bovine serum albumin (BSA), formic acid (FA), tetraethyl orthosilicate (TEOS) and (3-aminopropyl) triethoxysilane (APTES) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (ACN, HPLC grade) and ammonium solution (25%) were from Merck (Darmstadt, Germany). Iron (III) chloride hexahydrate (FeCl₃·6H₂O), anhydrous sodium acetate (NaAc), ethylene diamine, isopropanol, ethylene glycol (EG) and ethanol were bought from Tianjin Kemiu Chemical Reagent Co., Ltd. (Tianjin, China). SDS-PAGE marker and loading buffer were obtained from Thermo Scientific (San Jose, CA). The water used in the experiments was doubly distilled and purified by a Mill-Q system (Millipore, Bedford, MA, USA).

2.2. Human plasma

Human blood was taken from 20 different healthy donors and collected in tubes containing EDTA to prevent blood clotting. The tubes were centrifuged for 5 min at 1000 RCF to pellet the blood cells. The supernatant (the plasma) was transferred to new tubes and mixed together in order to eliminate individual differences. Then, the plasma was divided into portions and stored at –80 °C until use. Upon thawing, the plasma was centrifuged again for 2 min at 16,000 RCF to further reduce the presence of red and white blood cells. The plasma was used immediately upon thawing and was never refrozen.

2.3. Synthesis of Fe₃O₄ NPs of different sizes

Fe₃O₄ NPs with sizes of ~200 and ~400 nm were synthesized according to previous literature [40]. Briefly, FeCl₃·6H₂O (1.35 g, 5 mmol) was dissolved in EG (40 mL) to form a clear solution, followed by the addition of NaAc (3.6 g) and polyethylene glycol (1.0 g). The mixture was stirred vigorously for 30 min and then sealed in a Teflon-lined, stainless-steel autoclave. The autoclave was heated and maintained at 200 °C for 8 h and 48 h for the preparation of the ~200 nm and ~400 nm Fe₃O₄ NPs, respectively. Finally, the mixture was allowed to cool to room temperature. The black products were washed several times with ethanol and dried under vacuum at 60 °C overnight.

2.4. The formation of human plasma protein corona on Fe₃O₄ NPs

To ensure comparability between the identification results for the protein coronas on the Fe₃O₄ NPs of different sizes, the ratio

of the total particle surface area to the plasma concentration was kept the same for the three different particle sizes. For this purpose, the mass of Fe₃O₄ NPs of different sizes (suspended in PBS with a concentration of 0.2%, w/w) possessing a total surface area ($A = 4\pi R^2$) of 0.01 m² was calculated and incubated in a constant amount of human plasma at 37 °C with shaking at 1000 rpm for 1 h. The ratio of the plasma volume to the particle surface area was adjusted to a constant value of 5.56 mL/m², as reported previously (PBS was added to obtain a total volume of 1 mL) [23,24]. After that, the nanoparticle–protein complexes were collected by a magnet and washed at least 3 times to remove the loosely bound and unbound proteins. The amount of plasma proteins absorbed onto the Fe₃O₄ NPs was roughly calculated by subtraction of the amount of plasma proteins before and after incubation. In addition, the microcentrifuge tubes were changed after each washing step in order to eliminate the potential influences of proteins adsorbed on the surfaces of the tubes. Then, the nanoparticle–protein complexes were resuspended in 500 µL of 100 mM NH₄HCO₃ solution (pH ~8.1) and divided into two parts. One portion was pelleted, resuspended in SDS-PAGE loading buffer, then boiled at 95 °C for 5 min and finally separated with 1-D SDS-PAGE to illustrate the binding patterns of the plasma proteins on the Fe₃O₄ NPs. The other portion was analyzed with the on nanoparticle digestion shotgun proteomics method [41]. Briefly, the nanoparticle–protein complexes were resuspended in a solution containing 8 M urea and 100 mM NH₄HCO₃ (pH ~8.1) to denature the proteins, then reduced and alkylated with DTT (10 mM, 2 h at 37 °C) and IAA (20 mM, 30 min in the dark at room temperature), respectively [42]. Next, the solution was diluted with 100 mM NH₄HCO₃ to 1 M urea, and the proteins were digested with trypsin at 37 °C overnight with shaking (the amount of trypsin applied to digest the plasma proteins was set to an enzyme:protein ratio of 1:40 according to the amount of adsorbed proteins). Finally, the digestion solution was adjusted to pH 2–3 with a 10% TFA solution and desalted with homemade C18 SPE columns. Briefly, after the C18 SPE columns were sequentially activated and equilibrated with methanol and 0.1% TFA solution, respectively, the digestion solutions were loaded onto the SPE columns, washed extensively with 0.1% TFA solution and then eluted with 0.1% TFA in 80% ACN. Finally, the elutions were lyophilized in a vacuum freeze dryer. The samples were stored at –40 °C until RPLC-MS/MS analysis.

Furthermore, to investigate the plasma protein adsorption dynamics of the Fe₃O₄ NPs, Fe₃O₄ NPs of different sizes and with a total surface area of 0.005 m² were also incubated with the same amount of human plasma at 37 °C with shaking at 1000 rpm for 1 min and 30 min. After that, the nanoparticle–protein complexes were collected and washed at least 3 times to obtain the hard protein coronas. Finally, the nanoparticle–protein complexes were resuspended in 1× SDS-PAGE loading buffer, boiled at 95 °C for 5 min and separated with 1-D SDS-PAGE to illustrate the binding dynamics of the Fe₃O₄ NPs of different sizes.

2.5. RPLC-MS/MS analysis

The digested peptides from the human plasma protein corona were dissolved in 100 µL of 0.1% FA solution. The analysis was carried out on a 1-D nano LC-MS/MS system with 5 µL of the resuspended peptides injected each time. The C18 trap column (7 cm × 200 µm i.d.) was connected to a reverse phase (RP) C18 column (12 cm × 75 µm i.d.) packed with C18 AQ beads (5 µm, 120 Å pore size) from Microm BioResources in tandem by a union. For the RPLC separation, a Waters Nano-ACQUITY UPLC system was used to deliver the mobile phases of 0.1% FA in water (mobile phase A) and ACN (mobile phase B) with a total flow rate of ~350 nL/min; the binary separation gradient of mobile phase B was increased from 2% to 30% in 60 min. Tandem mass spectrometry was performed on

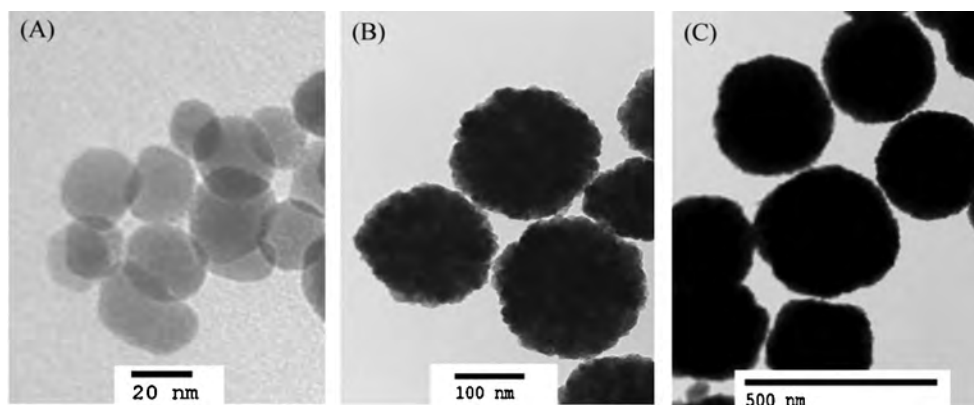


Fig. 1. TEM images of the Fe_3O_4 NPs of different sizes (Fe_3O_4 -30 (A), Fe_3O_4 -200 (B) and Fe_3O_4 -400 (C)).

a triple TOF 5600 mass spectrometer (AB SCIEX, USA) operated in positive ion data dependent (IDA) mode with one MS survey scan followed by 30 MS/MS scans using a 120 s exclusion window. The scan range of the full MS was set from m/z 350 to m/z 1250, while the MS/MS was from m/z 100 to m/z 1500.

2.6. Database searching

The MS/MS spectral data acquired by the triple TOF 5600 mass spectrometer were searched by the “Paragon Algorithm” of the Protein Pilot software (AB SCIEX, USA) with trypsin specificity in “Thorough ID” mode [43]. The peptide false discovery rate (FDR) was controlled <1% by setting the criterion for the corresponding confidence of global FDR of hit. The results from the human plasma protein coronas were searched against the International Protein Index (ipi.human.3.80.fasta). To confirm the identification results for the plasma proteins bound on Fe_3O_4 NPs, each sample was analyzed in at least three replicates, and only proteins that were both detected at least 2 times in triplicate analysis and identified by at least 2 unique peptides in each run were considered as positively identified.

3. Results and discussion

3.1. Characterization of Fe_3O_4 NPs

The Fe_3O_4 NPs of different sizes were characterized by transmission electron microscopy (TEM, Fig. 1). The size distributions and zeta potentials of the Fe_3O_4 NPs with different particle sizes of 30, 200 and 400 nm (denoted as Fe_3O_4 -30, Fe_3O_4 -200 and Fe_3O_4 -400) are listed in Table 1. The Fe_3O_4 NPs exhibited approximate zeta potential values of -28.9 mV, -23.4 mV and -24.3 mV for Fe_3O_4 -30, Fe_3O_4 -200 and Fe_3O_4 -400, respectively. The particle size of NPs influences their surface curvature and free energy, which may result in different aggregation states of the NPs in suspensions. This could explain why the Fe_3O_4 NPs of different particle sizes were found to be aggregated in PBS buffer to different degrees (shown in Table 1).

Table 1
The sizes and zeta potentials of the Fe_3O_4 NPs.

Fe_3O_4 nanoparticle	Size (nm)	Zeta potential (mV)	Z-Ave diameter (nm)	PDI
Fe_3O_4 -30	30 ± 10.3	-28.9 ± 0.87	822.4 ± 73.9	0.41 ± 0.02
Fe_3O_4 -200	225 ± 11.5	-23.4 ± 0.35	643.1 ± 34.6	0.38 ± 0.03
Fe_3O_4 -400	375 ± 16.3	-24.3 ± 1.34	571.1 ± 10.8	0.44 ± 0.06

The error bars represent the standard deviation from the mean ($n=3$).

3.2. Plasma protein binding patterns for Fe_3O_4 NPs with different sizes

Once introduced into biological systems, proteins would compete for binding sites on the surfaces of NPs. After incubation in human plasma for 1 min, 30 min and 1 h, the obtained nanoparticle–protein complexes were washed at least 3 times with PBS to obtain the hard protein corona of Fe_3O_4 NPs. The nanoparticle–protein complexes were resuspended in SDS loading buffer and separated using 1-D SDS-PAGE. The adsorption dynamics of the human plasma protein coronas on the Fe_3O_4 NPs are shown in Fig. 2. It was observed that the adsorption of plasma proteins on the three different sizes of Fe_3O_4 NPs evolved as the incubation time went along and reached a relative equilibrium state after 1 h of incubation in human plasma. On the other hand, the plasma proteins displayed different binding patterns depending on the size of the Fe_3O_4 NPs, illustrating that nanoparticle size impacted the formation of the protein corona. Additionally, the total band intensities for the plasma protein coronas exhibited obvious differences depending on NP size (shown in Fig. 2D); i.e., there were much more plasma proteins bound on the Fe_3O_4 -200 and Fe_3O_4 -400 NPs than on the Fe_3O_4 -30 NPs, which may be due to the obvious aggregation of the Fe_3O_4 -30 NPs, indicating that NP aggregation also affects the formation of protein coronas. Moreover, the optical intensity distributions of the plasma protein coronas formed on Fe_3O_4 NPs of different particle sizes were further analyzed with the Image Lab program (shown in Fig. 3), which also confirmed the different adsorption patterns on the Fe_3O_4 NPs and the equilibrium state achieved after 1 h of incubation in human plasma.

3.3. Identification and quantification of plasma proteins bound on Fe_3O_4 NPs with different sizes

In our study, to guarantee identification accuracy, each sample was analyzed in at least three replicates, and only proteins detected in at least two runs and identified by at least two unique peptides in each analysis were considered as constituents of the protein coronas formed on the Fe_3O_4 NPs. The protein coronas bound on the Fe_3O_4 NPs after 1 h of incubation with human plasma were

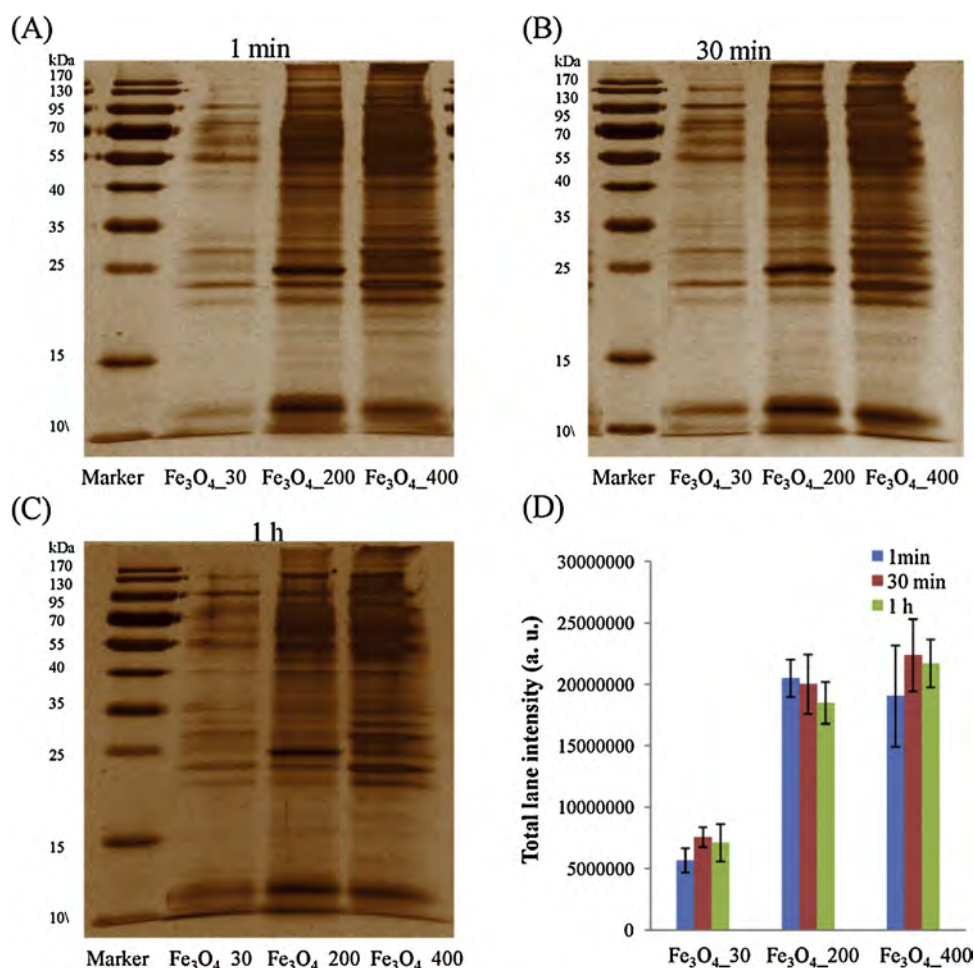


Fig. 2. Silver stained SDS-PAGE gel lanes showing the plasma protein binding profiles on different sizes of Fe₃O₄ NPs after incubation in human plasma for 1 min (A), 30 min (B) and 1 h (C) and the corresponding optical intensities of each gel lanes for the plasma proteins adsorbed on the Fe₃O₄ NPs. The error bars represent the standard deviation from the mean ($n = 3$).

used to investigate the size effects on the formation of protein coronas on the Fe₃O₄ NPs. In total, there were 117, 133 and 100 plasma proteins identified in the protein coronas of the Fe₃O₄_30, Fe₃O₄_200 and Fe₃O₄_400 NPs, respectively (the detailed identification results were listed in Table S1). A total of 71 human plasma proteins were detected in the protein coronas of Fe₃O₄ NPs of the three different sizes, with 22, 31 and 11 distinct proteins for the Fe₃O₄_30, Fe₃O₄_200 and Fe₃O₄_400 NPs, respectively (shown in Fig. 4). For example, serum albumin, apolipoprotein A-I and Platelet factor, among others, were observed to bind on the surfaces of all three types of Fe₃O₄ NPs, while ceruloplasmin and serum amyloid P-component, among others, were found to be uniquely bound on the surface of only the Fe₃O₄_30 NPs. Fibronectin, serotransferrin and serum paraoxonase/arylesterase 1 were observed to only be adsorbed on the Fe₃O₄_200 NPs, and coagulation factor XIII A chain and inter-alpha (Globulin) inhibitor H2 were only bound on the Fe₃O₄_400 NPs (Table S1). In addition, with the analysis of GoMiner and Panther databases [44,45], it was found that most of the 71 common proteins bound on the Fe₃O₄ NPs of all sizes were related to cellular process, metabolic process, response to stimulus, immune system process and other processes (shown in Fig. 5A). By contrast, the plasma proteins uniquely bound on the surfaces of the Fe₃O₄ NPs of only one specific size (Fe₃O₄_30/Fe₃O₄_200/Fe₃O₄_400) were observed to participate in different biological processes (shown in Fig. 5B). For instance, plasma proteins that related to metabolic processes were

found to comprise 33.3% of the total distinct proteins bound on the Fe₃O₄_30 NPs, which were observed to take up 9.5% and 16.7% of the total distinct proteins bound on the Fe₃O₄_200 and Fe₃O₄_400 NPs, respectively. Additionally, the plasma proteins uniquely bound on the Fe₃O₄_30 NPs were observed to be related to the cell cycle (6.7%), while it was not found for plasma proteins that uniquely bound on the Fe₃O₄_200 and Fe₃O₄_400 NPs. Thus, the size of the Fe₃O₄ NPs seemed to be an important factor regulating the composition of the plasma protein corona on the Fe₃O₄ NPs, which may pose great impacts on the biological fates of NPs.

On the other hand, based on the spectral counting (SpC) method [46], we applied the label-free quantitative proteomic strategy to quantitatively analyze the plasma proteins bound on the surfaces of the Fe₃O₄ NPs of different sizes. Here, the SpC of each protein identity was normalized to the protein mass and described as the relative protein quantity by applying the following equation:

$$MWSpC_k = \left(\frac{(\text{SpC}/\text{Mw})_k}{\sum_{i=1}^n (\text{SpC}/\text{Mw})_i} \right) \times 100$$

where MWSpC is the percentage normalized spectral count for protein k , SpC is the spectral count identified and Mw is the molecular weight in kDa for protein k . This correction takes into account the protein size and evaluates the real contribution of each protein to the hard corona composition [20,21]. By using this equation, the relative protein abundances for the plasma proteins bound on the

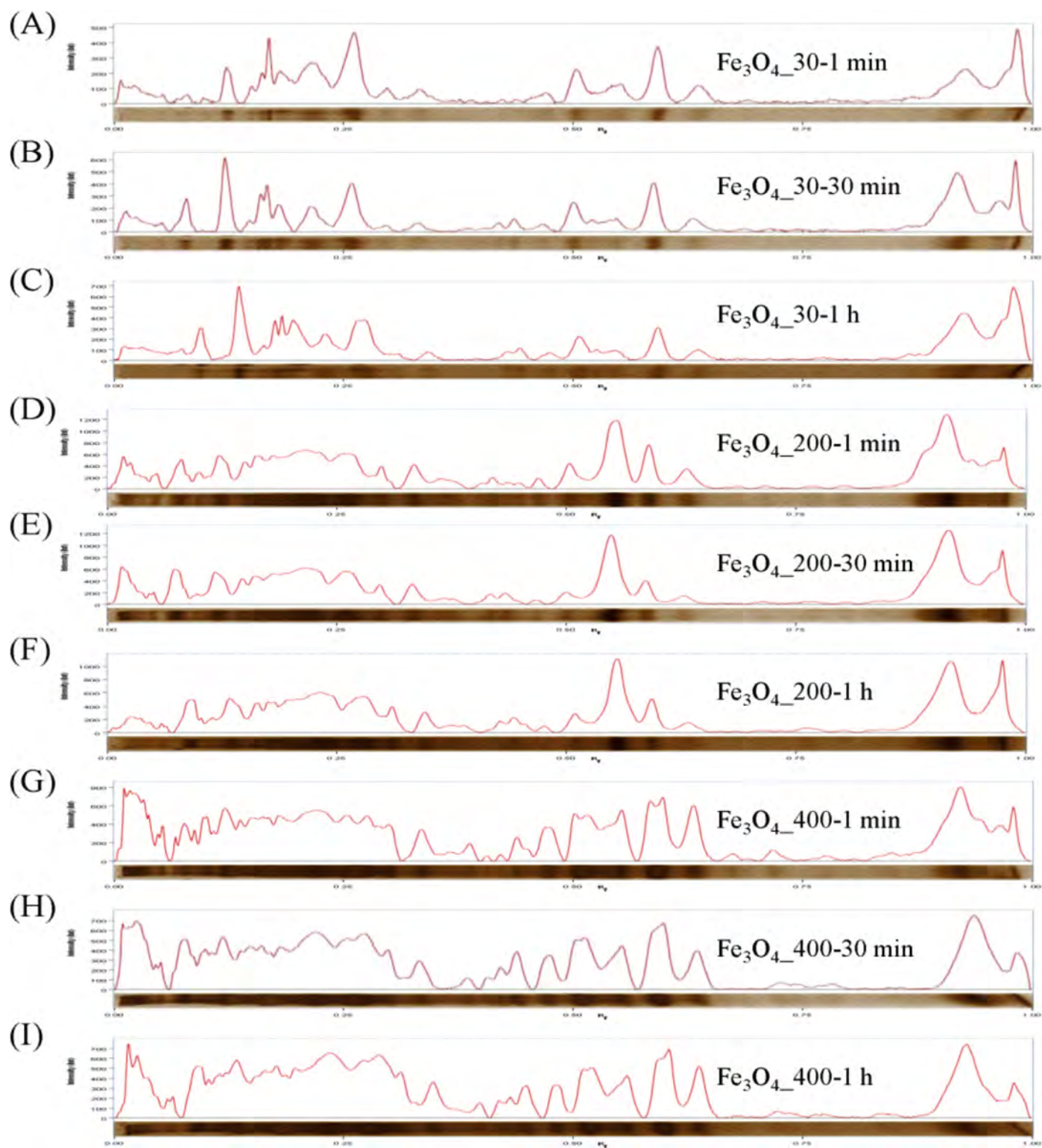


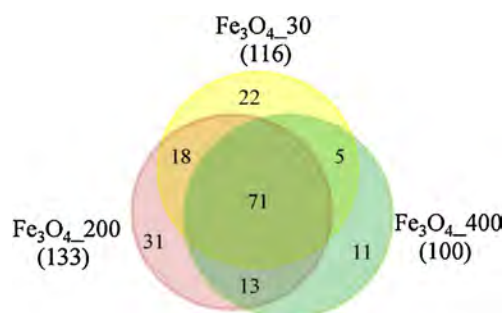
Fig. 3. The optical intensities across the SDS-PAGE gel lanes for the plasma protein binding profiles on different sizes of Fe_3O_4 NPs (Fe_3O_4 _30 (A–C)/ Fe_3O_4 _200 (D–F)/ Fe_3O_4 _400 (G–I)) after incubation in human plasma for 1 min (A, D and G), 30 min (B, E and H) and 1 h (C, F and I).

Fe_3O_4 NPs were calculated and listed in Table S1. It was found that although there were 71 common plasma proteins bound on the different sizes of Fe_3O_4 NPs, the relative protein abundances for these proteins differed. For instance, serum albumin was observed in the protein corona of the Fe_3O_4 _200 NPs with an average MWSpC of 9.01, while the average MWSpC for serum albumin in the

protein coronas of the Fe_3O_4 _30 and Fe_3O_4 _200 NPs were 1.27 and 2.50, respectively, indicating the selective binding of plasma proteins on different sizes of Fe_3O_4 NPs. Additionally, platelet factor 4 was observed in the corona of Fe_3O_4 _30 with an average MWSpC of 8.57, which accounted for 3.51% and 4.01% in the protein coronas of the Fe_3O_4 _200 and Fe_3O_4 _400 NPs, respectively. Furthermore, the

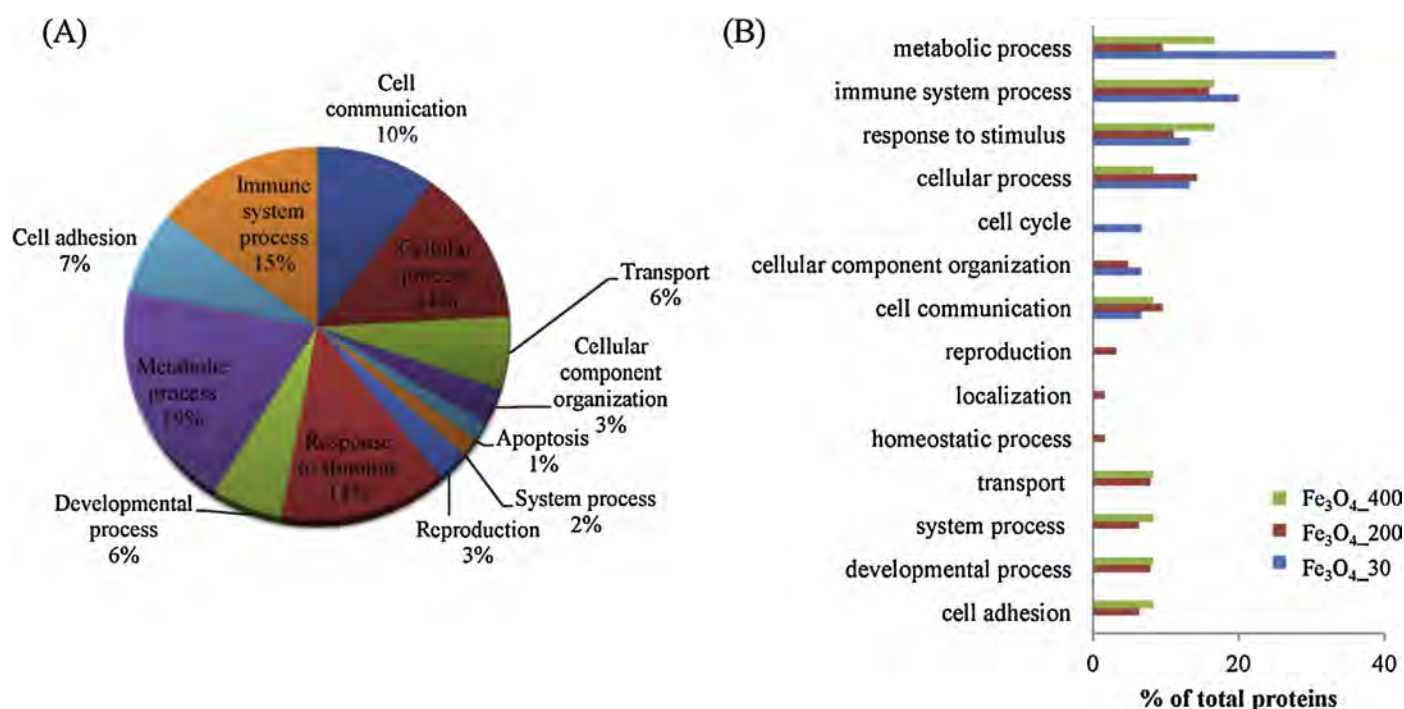
Table 2The top 20 most abundant plasma proteins bound on the Fe₃O₄ NPs of different sizes.

Rank	Fe ₃ O ₄ _30	Fe ₃ O ₄ _200	Fe ₃ O ₄ _400
1	Platelet factor 4	Apolipoprotein A-I	Apolipoprotein E
2	Apolipoprotein E	Serum albumin	Vitronectin
3	Vitronectin	Apolipoprotein C-III variant 1	Histidine-rich glycoprotein
4	Platelet basic protein	Apolipoprotein C-I	Platelet basic protein
5	IGK@ IGK@ protein	Apolipoprotein E	Platelet factor 4
6	IGKC Anti-RhD monoclonal T125 kappa light chain	Isoform LMW of Kininogen-1	Complement C1q subcomponent subunit C
7	Putative uncharacterized protein	Vitronectin	Complement C1q subcomponent subunit A
8	Complement C1q subcomponent subunit C	Platelet factor 4	Isoform 1 of Gelsolin
9	IGHG1	Histidine-rich glycoprotein	APOC1 Apolipoprotein C-I
10	Apolipoprotein C-III variant 1	Platelet basic protein	Complement C3 (Fragment)
11	IGHG1	Isoform HMW of Kininogen-1	Prothrombin (Fragment)
12	IGHG1	Isoform 1 of Gelsolin	Serum albumin
13	Prothrombin (Fragment)	Apolipoprotein A-II	C1QB Uncharacterized protein
14	Complement C1q subcomponent subunit A	Complement C3 (Fragment)	IGHG1
15	CLEC3B Putative uncharacterized protein DKFZp686H17246	Isoform 1 of Inter-alpha-trypsin inhibitor heavy chain H4	IGHG1
16	Apolipoprotein A-I	clusterin isoform 3	IGHG1
17	Isoform LMW of Kininogen-1	ITIH4 protein	APOH Beta-2-glycoprotein 1
18	Complement C3 (Fragment)	inter-alpha (globulin) inhibitor H4 isoform 2 precursor	IGKC Putative uncharacterized protein
19	Beta-2-glycoprotein 1	Prothrombin (Fragment)	Complement component 4B
20	IGHM Putative uncharacterized protein DKFZp686I15212	Apolipoprotein C-II	IGKC Anti-RhD monoclonal T125 kappa light chain

**Fig. 4.** Venn diagram displaying the number of proteins identified in the protein coronas of the different sizes of Fe₃O₄ NPs.

plasma proteins with an average MWSpC ≥ 1 in the protein corona on the Fe₃O₄ NPs are illustrated in Fig. 6, which also reveals the distinctive properties of the plasma protein coronas formed on the different sizes of Fe₃O₄ NPs.

Moreover, it has been reported that adsorbed protein layer on NPs would affect the cellular uptake and subsequent trafficking in cells, while in vivo, the specific binding of proteins would influence bio-distribution and blood clearance [47]. For example, the adsorption of opsonins such as IgG and complement factors is supposed to promote rapid clearance from the bloodstream, while the binding of dysopsonins, such as serum albumin and apolipoproteins, would promote prolonged circulation time in blood [21,47,48]. Although proteins with higher relative

**Fig. 5.** Biological processes related to the common proteins identified on all three sizes of Fe₃O₄ NPs (A) and those related to the plasma proteins uniquely bound on only one specific NP size (Fe₃O₄_30/Fe₃O₄_200/Fe₃O₄_400).

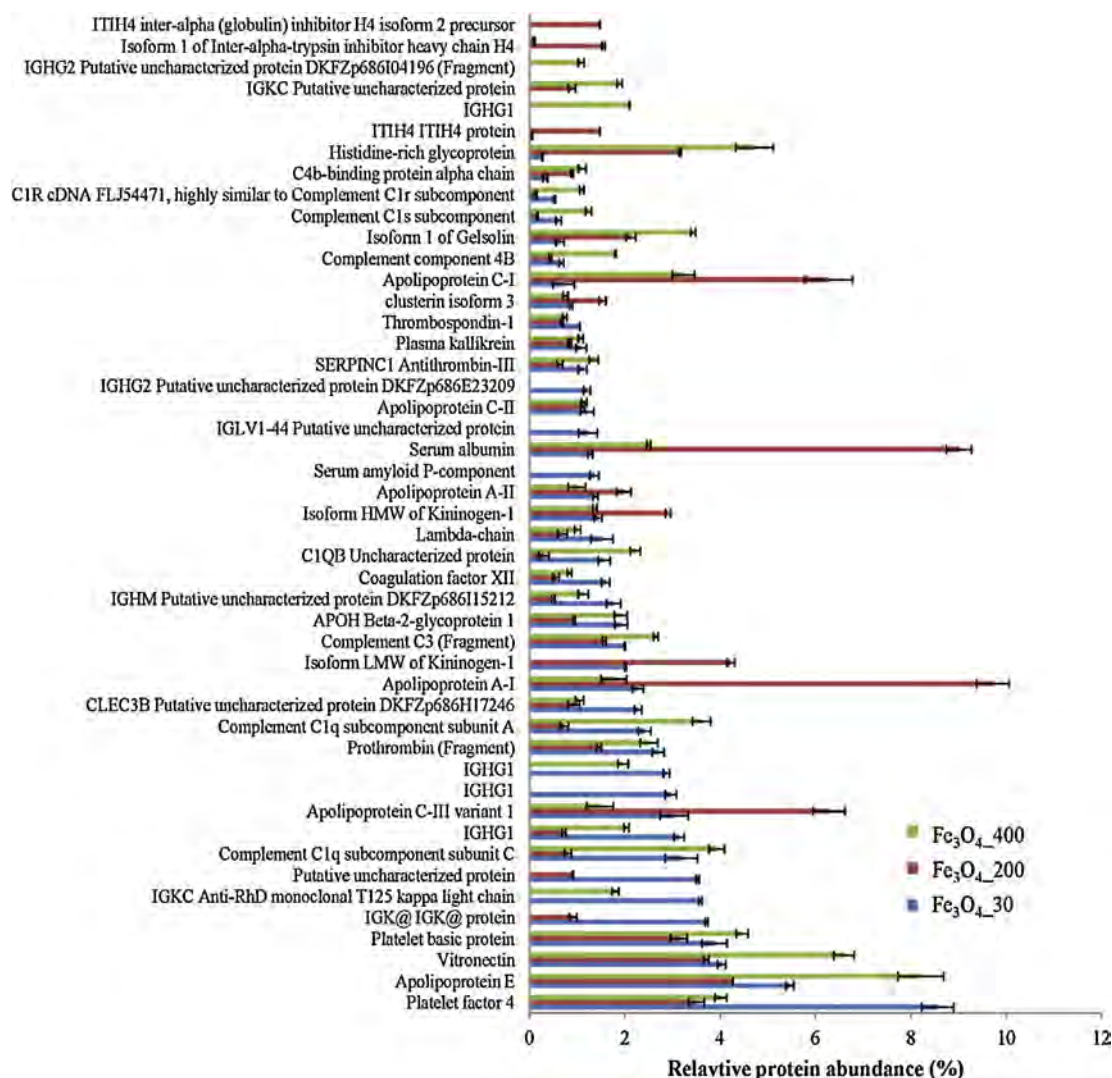


Fig. 6. Relative protein abundance of proteins (normalized to molecular weight, MWSpC) satisfying the criterion that MWSpC ≥ 1 in the coronas of the different sizes of Fe_3O_4 NPs. As the proteins were detected in more than 2 replicates, the error bars represent the standard deviation from the mean ($n = 2$ or $n = 3$).

abundances do not directly reflect biological impacts, proteins with extremely low relative abundances are not expected to be critical factors that determine the bio-distributions and biological fates of NPs [48,49]. Here, we list the top 20 most abundant plasma proteins comprising the protein coronas formed on the Fe_3O_4 NPs in Table 2 to further illustrate the different binding patterns of the plasma proteins and understand the following potential biological impacts. It was found that as the most abundant protein in human plasma, serum albumin was the 2nd most abundant protein in the protein corona around the Fe_3O_4 -200 NPs, while it was the 12th most abundant protein in the protein corona on the Fe_3O_4 -400 NPs and was not among the top 20 most abundant proteins for the Fe_3O_4 -30 NPs (27th). Thus, the quantitative results confirmed the selective binding of plasma proteins to different sizes of Fe_3O_4 NPs. Additionally, platelet factor 4, which is supposed to promote rapid clearance from the bloodstream, was found to bind on the aggregated Fe_3O_4 -30 NPs with the highest protein abundance. Apolipoprotein A-I and apolipoprotein E, which are supposed to promote prolonged circulation times, were the most abundant proteins bound on the Fe_3O_4 -200 and Fe_3O_4 -400 NPs, respectively. Therefore, the different binding profiles of plasma proteins on the different sizes of Fe_3O_4 NPs may have different impacts in human organisms.

4. Conclusions

In this study, the plasma protein coronas formed on pristine Fe_3O_4 NPs of three different particle sizes were investigated for the first time. According to the results, it could be concluded that the particle size induced different surface curvature and aggregation state of the Fe_3O_4 NPs, both of which may have played important roles in the formation of plasma protein coronas on these Fe_3O_4 NPs; the protein compositions as well as the abundances of the plasma proteins in the protein coronas of Fe_3O_4 NPs were found to be critically affected by particle size. Moreover, the proteins bound on the Fe_3O_4 NPs of different particle sizes may pose great influences on the biological distribution and fates of the Fe_3O_4 NPs.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.colsurfb.2014.06.016>.

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Exhibit C



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Characterization of carbon nanotube protein corona by using quantitative proteomics

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Abstract

The protein corona of a nanomaterial is a complex layer of proteins spontaneously and stably formed when the material is exposed to body fluids or intracellular environments. In this study, we utilised stable isotope labeling by amino acids in cell culture (SILAC)-based quantitative proteomics to characterise the binding of human cellular proteins to two forms of carbon nanoparticles: namely multi-walled carbon nanotubes (MWCNTs) and carbon black (CB). The relative binding efficiency of over 750 proteins to these materials is measured. The data indicate that MWCNTs and CB bind to vastly different sets of proteins. The molecular basis of selectivity in protein binding is investigated. This study is the first large-scale characterisation of protein corona on CNT, providing the biochemical basis for the assessment of the suitability of CNTs as biomedical tools, and as an emerging pollutant.

From the Clinical Editor: This team of investigators performed the first large-scale characterization of protein corona on carbon nanotubes, studying 750 proteins and assessing the suitability of CNTs as biomedical tools and as an emerging pollutant.

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Key words: Nanomaterials; Quantitative-proteomics; Carbon-black; Carbon-nanotubes

Abbreviations: ATCC, American type culture collection; CB, Carbon black; CID, Collision induced dissociation; CNTs, Carbon nanotubes; CVD, Chemical vaporization deposition; DMEM, Dulbecco's modified eagle's medium; ECL, Enhanced chemiluminescence; emPAI, empirical peptide abundance index; FBS, Fetal bovine serum; HSP, Heat shock protein; LC-MS/MS, Liquid chromatography-Mass spectrometry/Mass spectrometry; MS, Mass Spectrometry; MWCNTs, Multi-walled carbon nanotubes; NaCl, Sodium chloride; PMSF, Phenylmethanesulfonyl fluoride; PVDF, Polyvinylidene fluoride; PBS, Phosphate buffer saline; PDB, Protein data bank; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; SILAC, Stable isotope labelling by amino acids in cell culture; SPIO, Superparamagnetic iron oxide nanoparticles; TEM, Transmission electron microscope.

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Carbon nanotubes (CNTs) are cylindrical graphene structures closed at either end with caps that contain pentagonal rings.¹ A plethora of research over the past 20 years has revealed many chemical and physical properties of this nanomaterial that are both academically fascinating and commercially appealing. Many applications of CNTs involve direct contact of these materials with biological systems, either in the form of therapeutic treatments² or as a result of unintentional exposure.³ Biological consequences of CNT exposure have received increasing attention over the past decade. For instances, many in vitro studies have indicated that various forms of CNTs can penetrate the plasma membrane of mammalian cells and induce cytotoxicity.^{4,5} In addition, in vivo studies have revealed the involvement of CNT exposure in inflammation, epithelioid granulomas, fibrosis, and biochemical/toxicological changes in the lungs.⁶

Like other nanoparticles, CNTs are known to absorb a mixture of proteins to form a 'protein corona'.^{7–14} The binding of cellular proteins to CNTs and the subsequent alterations of protein structure and function may provide a molecular explanation of the physiological responses to CNT administration. Existing data

indicate that the binding efficiency of proteins is not merely related to their abundance in the given sample, which may be due to the protein size or charge.¹⁵ The interactions of bacteriorhodopsin¹² and bovine serum albumin¹³ with CNTs are accompanied by changes in molecular conformation, and the CNT binding of ribonuclease A,¹⁴ R-chymotrypsin and soybean peroxidase¹⁶ leads to a reduction in the enzymatic activity of these proteins. These examples suggest that, at least in vitro, CNT binding can affect protein structures and functions. Furthermore, the cytotoxicity of CNTs is attenuated after the binding of proteins.¹⁷ When a mixture of proteins is exposed to CNTs, some proteins show preferential CNT binding.¹⁸ For instance, lipoproteins and coagulation-related proteins like antithrombin and coagulation factor V have a high affinity to CNTs, whereas immunoglobulin and acute response proteins like fibronectin and haptoglobin related protein demonstrate a lower affinity.¹⁵ The CNT affinity of a protein may be influenced by the abundance of certain amino acid residues in the protein that have physiochemical properties conducive to CNT interaction. Peptides rich in amino acids histidine, tryptophan and phenylalanine have been shown to bind to CNTs preferably.^{19,20}

Mass spectrometry (MS)-based proteomics is beginning to unravel the formation and composition of protein corona on nanomaterials. Plasma proteins that stably bind to different types of polystyrene nanoparticles have been analysed by MS²¹ with the identification of 36 to 58 proteins bound to each type of nanoparticle. Similarly, MS proteomics has identified 23 plasma proteins that adhere to superparamagnetic iron oxide (SPIO) nanoparticles.²² These studies demonstrate the feasibility of using MS-based proteomics to characterise the protein corona of nanomaterial. However, none of the studies are characterizing the protein absorption of the CNT has not been quantitatively documented, despite the popularity of this class of nanomaterial. To fill this knowledge gap, we employed a comprehensive approach to investigate the biochemical properties of the protein corona of CNTs and carbon black (CB) by using stable isotope labelling by amino acids in cell culture (SILAC) proteomics. This technique not only allows the identification of proteins in the corona, but also the measurement of the enrichment level of each protein by the material.²³ To our knowledge this is the first comprehensive study in adopting a SILAC based MS in documenting the interaction of human cellular proteins with the carbon nanoparticles.

Methods

Carbon nanomaterials

Different types of CNTs with various diameters and lengths made by a chemical vaporization deposition (CVD) method were purchased from Nanotech Port Co Ltd (Shenzhen, China). Properties of the CNTs used in this study are summarised in SI Table 1. The CNTs and CBs were put onto a copper grid which was covered with carbon film and observed under a transmission electron microscope (TEM), as described.²⁴

Protein lysate preparation

HeLa cells were from the ATCC collection and were maintained in DMEM medium (high glucose) supplemented

with 10% fetal bovine serum and 5% Anti-Anti (Gibco, Invitrogen). Cells were harvested by trypsinization, washed with phosphate buffered saline (PBS), and incubated in lysis buffer (100 mM NaCl; 25 mM Tris pH 7.3; 1 mM EDTA; 0.1% Triton-X-100, complete protease inhibitor cocktail (Roche)) was added. Typically, 2 ml of lysis buffer was used for 5×10^7 cells. The cells were sonicated for 1 min (in 5 s pulses) on ice with a 3 mm probe sonicator (Ultrasonic processor, 70–100 W). Then, the lysates were centrifuged at 21,000 g, 15 min, 4 °C and collected the clear supernatant for further proteomics analysis.

Zebrafish embryos were collected and cultured after hatching and the embryos were examined every day and the abnormal ones were discarded. The 50 fishes were lysed 72 hrs after hatching by homogenization in 500 µl of lysis buffer that contained a complete protease inhibitor cocktail (1:25 diluted, Roche Diagnostic) and 0.5 M phenylmethanesulfonylfluoride (PMSF, Sigma) on ice for 15 min. Then, the lysates were clarified by centrifugation (21,000 g, 15 min, 4 °C). Mouse liver was freshly dissected from a four-month old C57WT female mouse, weight 1.2 g, after homogenizing with 4.5 ml of lysis buffer for 5 min on ice, 21,000 g for 15 min at 4 °C. The supernatant was transferred to a new tube and the protein concentration was detected with a Bradford assay.

Three liters of *Thalassiosira pseudonana* were spun down and harvested for cell lysis, 8.5 ml of cell lysis buffer and were added into the collected algae. The extracted algae were sonicated for 1 min, 4 times (20 amplitudes, 9 pulses), cells were then vortexed by 0.5 cm diameter glass beads for 1 min each time for 4 times. The lysates were clarified by centrifugation, (21,000 g, 15 min, 4 °C). The supernatant (protein solution) was transferred to a new tube. Fifty adult *Drosophila* were anaesthetized by carbon dioxide for 1 min, 2 ml of cell lysis buffer and 80 µl of 25× protease inhibitor cocktail were added. The *Drosophila* and buffer were homogenized by a homogenizer for 3 min, and the cells were lysed for 2 hrs at 4 °C. Then, the lysates were centrifuged (21,000 g, 15 min, 4 °C). The supernatant was transferred to a new tube.

Protein corona isolation

Cell lysates were incubated with 5 mg of CNTs or CB for 3 hrs at 4 °C. After centrifugation at 800 g (3 min, 4 °C), the pellets were thoroughly washed with washing buffers (100 mM NaCl; 25 mM Tris-HCl pH 7.3; 1 mM EDTA, complete protease inhibitors) until the OD280 of the supernatant was less than 0.1. The bound proteins were eluted by heating the pellets in 50 µl of LDS sample buffer (Invitrogen) to 70 °C for 10 min. In some experiments, 5 mg of 7 CNTs of different sizes were incubated with the same amount of FBS (4 mg) or cell lysate proteins (5 mg), and then followed a standard pull-down experiment. In some experiments, lysates were adjusted to pH 7.2 and pH 11, and then incubated with CNTs, and washed with a washing buffer of pHs 7.2 and 11, respectively. Three-way SILAC encoding of HeLa cells was done as described.²³ Equal protein amounts (as measured by a Bradford assay) of the control cell lysate (light) and MWCNT (diameter 20–40 nm)-binding proteins (medium) were mixed together, CB-binding proteins (heavy) were then added into the mixture with the same

volume as CNT-binding proteins. The mixed protein samples were electrophoresed, gel-sliced and digested as described previously.^{23,25}

Mass spectrometry

Mass spectrometry was performed on an LTQ-orbitrap mass spectrometer system (ThermoElectron, Bremen, Germany) coupled to a Dionex 3000 nano-LC system (Camberley, UK) A 100×0.075 mm Agilent C18 column (3.5 µm particle diameter) with an A mobile phase of 0.1% formic acid in water and B mobile phase of 0.1% formic acid in acetonitrile were used. The peptides were eluted using a 13.5-cm-long, 75-µm-inner diameter tip column (YMC-Gel, Liquid Chromatography). The pump flow rate was set at 0.3 µl/min, and peptide elution was achieved using a linear gradient of 5% to 40% B in A for the first 40 min followed by a rapid increase to 95% B for the next 20 min. Conventional MS spectra (a survey scan) were acquired at high resolution ($M/\Delta M$, 60,000 full width half maximum) over the acquisition range m/z 200–2000 and a series of precursor ions were selected for the MS/MS scan. The former examined the accurate mass and charge state of the selected precursor ions and the latter acquired the spectrum (CID spectrum or MS/MS spectrum) of the fragment ions generated by collision-induced dissociation as described.²³

The MS/MS spectra were searched against the UniProt human database with the Mascot search engine (Matrix Science) setting with the following criteria: peptide tolerance=10 ppm; trypsin as the enzyme; carboxyamidomethylation of cysteine as a fixed modification and oxidation of methionine as variable modifications; medium SILAC labels were: label 13C(6) (R), and label 2 H(4) K; and heavy SILAC labels were: label 13C(6) 15 N(4)(R), and label 13C(4) 15 N (4) K. The MS-Quant programme^{23,26} was used for quantitation; the peptide ratio calculation carried on for each arginine- and/or lysine- containing peptide, and the peak area of labelled arginine/lysine divided by the peak area of non-labelled arginine/lysine were calculated for each single scan mass spectrum. The ratio of each protein was the average of the peptide ratios for all arginine- and lysine-containing peptides sequenced from this protein. The criteria of protein validation were as follows: only individual peptide with Mascot score of at least 25, and total protein score of at least 60 were used. Only proteins with two unique peptides were considered. This filter results in a false-positive rate of below 0.7%.²⁷

Western blotting

Following an SDS-PAGE, the proteins were transferred to a hydrated polyvinylidene fluoride (PVDF) membrane at 100 V for 1 hr by using the Western blot system (Bio-rad). The membrane was blocked with 5% skim milk powder in a washing buffer (0.1%, v/v Tween 20 in PBS, pH 7.2) for 40 min. The blot was then incubated with mouse anti-β-Actin antibody (Sigma Inc.Cat.No.A5441), 1:10000 dilution in blocking buffer (5% milk, 0.1%, v/v Tween 20 in PBS, pH 7.2) for 60 min at R.T., washed four times in the washing buffer for 25 min and incubated with secondary antibody goat anti-mouse IgG (H+L) (Zymed, Cat. No. 62 -6520), 1:10000 dilution in blocking buffer

for 1 hr at R.T., and then, washed 3 times in a washing buffer for 10 min each. The membrane was developed by using an ECL Western blotting kit (Amersham Biosciences). Mouse anti-β-tubulin antibody (1:5000, Sigma Inc. Cat.No.T4026), HSP60 (1:500, sigma Inc. Cat.No.H3524), and HSP70 (1:5000, Sigma Inc. Cat.No.H5147) were used to detect the specific proteins by following the same steps.

Data analysis

Protein Center (Thermo), a proteomics data mining and management software, was used to eliminate redundancy and compare datasets, and to convert protein IDs to gene symbols and obtain gene ontology and sequence length information. Theoretical pI, the Kyte-Doolittle hydrophobicity and amino acid usages of the identified proteins were analysed by using PROMPT software.²⁸ Protein secondary structure information was obtained from the PDB.²⁹

Results

Protein corona of carbon nanoparticles

To test the binding of a complex mixture of proteins to CNTs, we incubated pristine multi-wall CNTs (MWCNTs) with a mean diameter of 20–40 nm with a cell lysate prepared from the human cell line HeLa. Proteins from HeLa cells were chosen because they provide a representative source of the human proteome. In parallel, CB, a pure elemental carbon in the form of colloidal nanoparticles, was also tested for the binding capacity for the same protein mixture. The same amount of HeLa cell lysate proteins was used in the binding assay (Figure 1, A). Both MWCNT and CB formed loose agglomerates that could be collected by low-speed centrifugations after protein binding. The proteins that bound stably to these carbon matrices were eluted after extensive washing and then analyzed by SDS-PAGE. Remarkably, MWCNTs bound to a significant amount of HeLa cell proteins, whereas much less proteins were pulled down by the same amount of CB (Figure 1, B). By comparing the coomassie-blue staining intensities of the eluted proteins with that of the input, we estimated that about 1.5% of the entire pool of HeLa cell proteins in the incubation reaction are able to bind to the MWCNTs after 3 hrs of incubation, but only less than 0.1% of these proteins are able to bind to the same amount of CB. The efficient interaction of proteins with CNTs is not restricted to proteins from cultured human carcinoma cells, protein extracts prepared from zebrafish, mouse, drosophila and algae also demonstrated a substantial affinity to CNTs (Figure S1).

We examined the MWCNT and CB used in the protein binding experiments by using transmission electron microscopy (Figure 1, C and D). The diameters of CBs appear significantly smaller than MWCNTs. This suggests the dimensions of carbon nanomaterials may affect the formation of protein corona. We further characterized the possible influence of CNT length and dimension on its protein binding capacity. Fetal bovine serum (FBS) proteins were incubated with CNTs of various types, diameters and lengths (Table S1 and Figure 2, A) and the

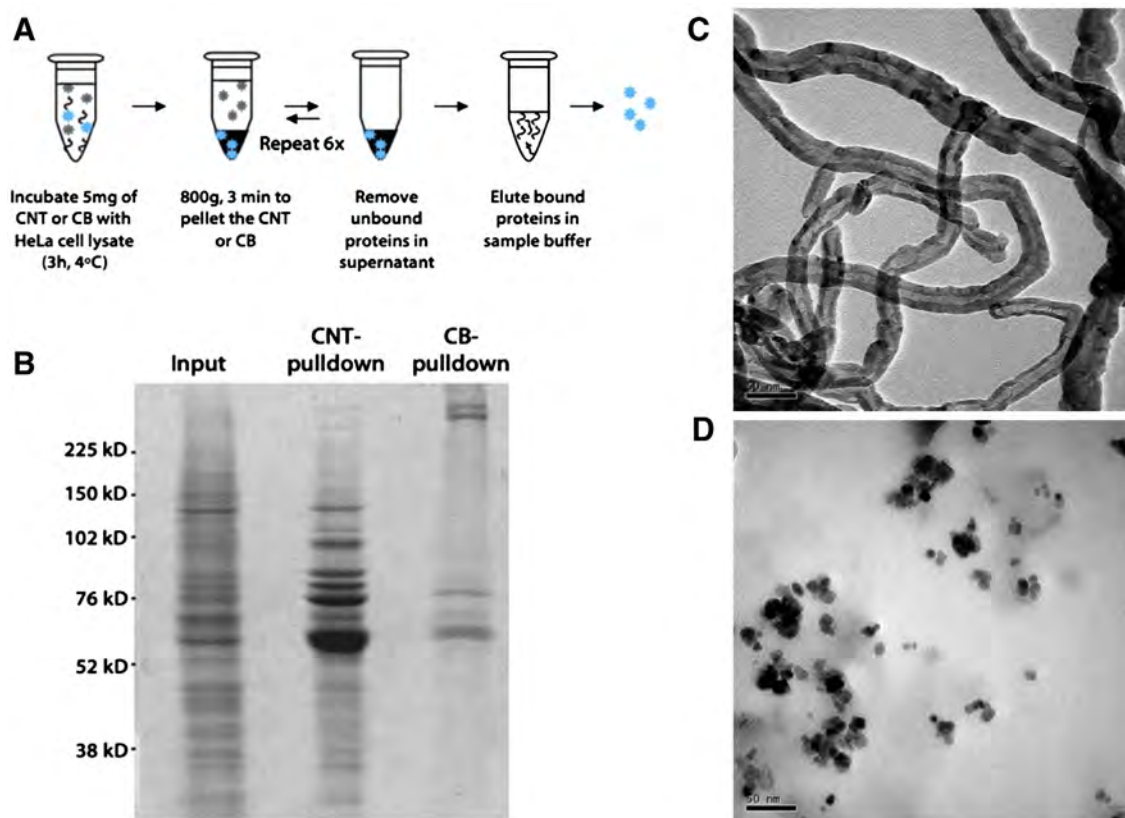


Figure 1. Protein binding assay to study MWCNT- and CB-protein corona. **(A)** Scheme of carbon nanoparticle pull-down experiment. **(B)** Proteins from HeLa cell lysate pulled down by MWCNTs and CB were separated by SDS-PAGE and stained by coomassie blue. **(C-D)** Transmission electron micrographs of the MWCNTs **(C)** and CNTs **(D)** used in this study (Bars: 50 nm).

recovery of proteins in the CNT pull-downs was studied. MWCNTs of diameters of 20–40 nm or above could pull-down a significant amount of proteins (Figure 2, B, lanes 5–8), whereas those with diameters less than 10 nm bound to virtually no proteins (Figure 2, B, lanes 3–4). Single walled CNTs, typically with diameters less than 2 nm, also show no significant protein binding (Figure 2, B, Lane 2). For MWCNTs that are wider than 20 nm, an increase in nanotube diameter did not further increase the protein binding capacity, as the amount of proteins pulled down by CNTs with diameters from 20–100 nm is essentially the same (Figure 2, B, Lanes 6, 8). This result confirms the roles of the size of carbon nanomaterials in protein binding, and is consistent with previous studies where protein binding was more efficient with larger CNTs¹³ or nanoparticles²¹ than smaller ones.

Next, we asked whether the observed protein binding reflects a specific interaction of proteins with CNTs, or if it is merely a result of non-specific trapping of proteins by the CNT and CB agglomerates. We compared the protein binding capacity of MWCNTs that had similar diameters, but different averaged lengths (Figure 2, C), as short MWCNTs do not form agglomerates as efficiently as long MWCNTs. MWCNTs of different lengths bind to a similar amount of proteins (Figure 2, B, Lanes 5, 6 and 7, 8). This further supports the notion that protein interactions with CNTs is likely to be determined by

factors such as surface curvature and area, or lumen dimensions, but not by the level of agglomeration.

Systematic characterization of carbon nanoparticle-protein corona

A comparison of the gel electrophoretic profiles of total HeLa cell lysate and the proteins pulled down by MWCNTs indicated that some proteins were selectively enriched into the CNT-protein corona (Figure 1, B). This suggests that, in a complex protein mixture, some proteins are able to bind more efficiently and/or more stably with MWCNTs. It is important to identify the proteins that bind to MWCNTs specifically. In order to accomplish this, we performed proteomics analysis to identifying the proteins that selectively bind to MWCNT of diameters of 20–40 nm. We also aimed to compare the types of proteins that bound to this preparation of MWCNT with those that bound to CB. Cell lysates were prepared from three populations of HeLa cells that had been metabolically encoded using three different SILAC media (“light”, “medium” and “heavy”, Figure 3, A and Methods). A CNT pull-down was performed on the “medium” lysate, and a CB pull-down was performed on the “heavy” lysate. Equal volumes of proteins eluted from these pull-down experiments were mixed. The mixture was also spiked with an equal amount of unfractionated “light” lysate. The mixtures were then separated by SDS-PAGE, and

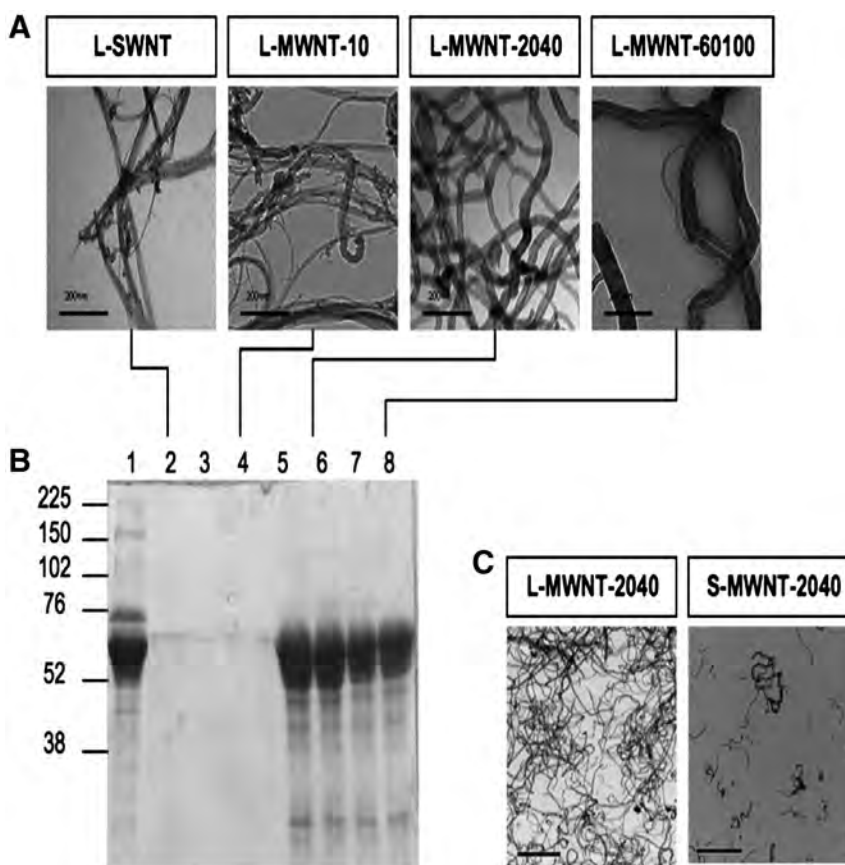


Figure 2. CNTs of different sizes exhibit different protein-binding capacities with. **(A)** TEM images of CNTs (length: 5–15 μm) with different diameters (Magnification 87000 $\times$). From left to right: L-SWNT with diameter <2 nm; L-MWNT-10 with diameter <10 nm; L-MWNT-2040 with diameter in range of 20–40 nm; L-MWNT-60100 with diameter in range of 60–100 nm. **(B)** FBS pulled down by CNTs of different sizes. Lane1: FBS; Lane2: SWNT; Lane3: S-MWNT-10; Lane4: L-MWNT-10; Lane5: S-MWNT-2040; Lane6: L-MWNT-2040; Lane7: S-MWNT-60100; Lane8: L-MWNT-60100. **(C)** MWNTs-2040 of different length. L-MWNT-2040, length: 5–15 μm ; S-MWNT-2040, length: 1–2 μm .

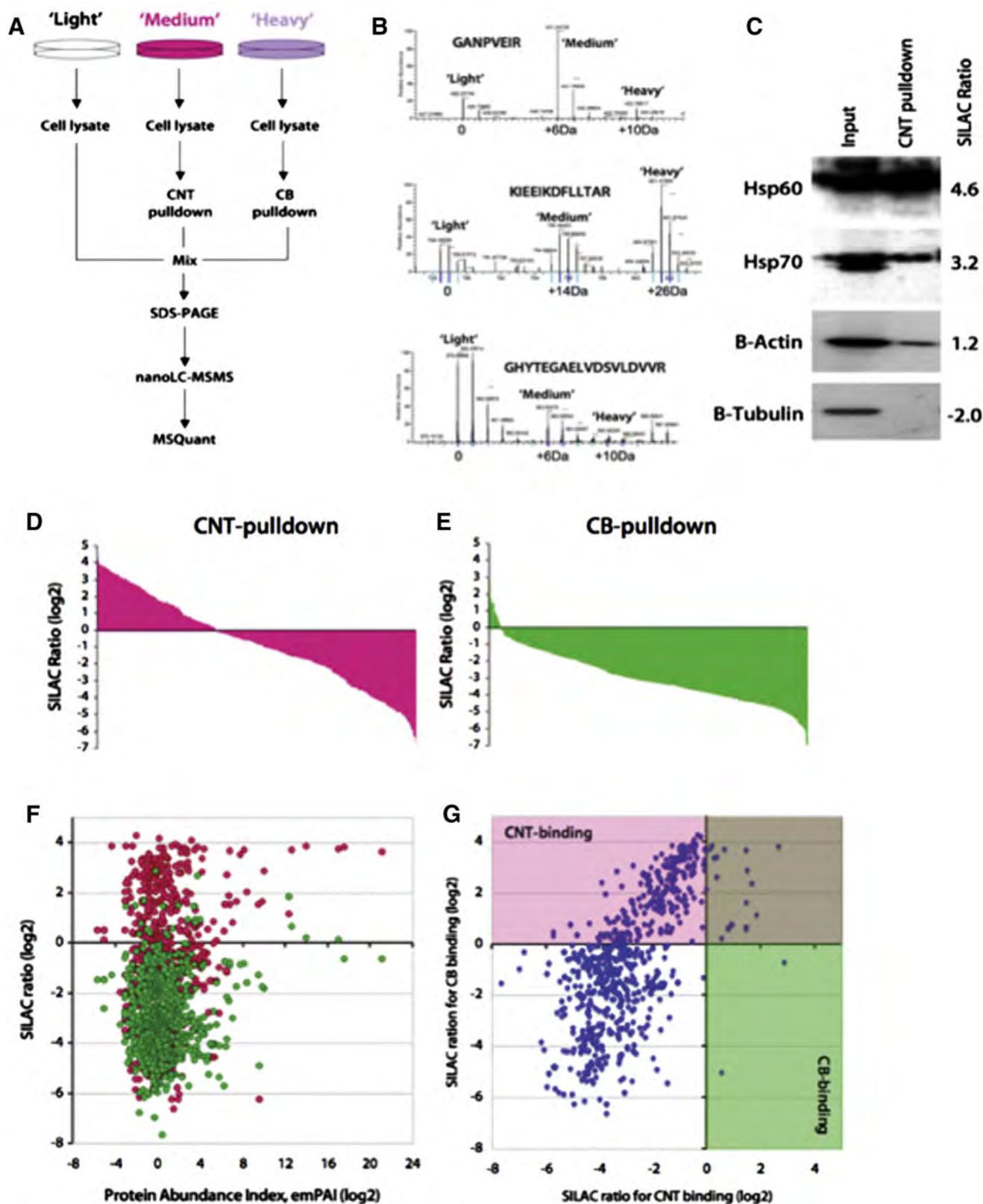
analyzed with the “gel-LC-MS/MS” approach. More than 9,000 unique peptides were unambiguously mapped to more than 1,750 human proteins. The abundance distribution of each identified peptide among the “light”, “medium” and “heavy” categories was quantified by the MSQuant programme.²⁴ In total, 778 proteins were accurately quantified (Table S2). This three-way SILAC protocol allows a comparison of the binding efficiencies of each protein to MWCNTs (medium to light ratios), and to CB (heavy to light ratios). The mass spectra of representative peptides are shown in Figure 3, B. Furthermore, to validate the MS data, we performed Western blotting analyses on HSP60, HSP70, β -actin and tubulin. Quantitative proteomic data show that HSP60, HSP70 and β -actin, but not tubulin, were enriched in the MWCNT pull-down. Western blotting results also showed an enrichment of HSP60, HSP70, and actin in the pull-down, while tubulin was only detectable in cell lysate input, but not in the pull-down (Figure 3, C).

Our MS analysis confirmed that the number of proteins enriched by MWCNT-binding is much higher than that enriched by CB (Figures 3, D and E). An examination of the SILAC ratio of each quantified protein and its empirical peptide abundance index (emPAI), a semi-quantitative indication of the overall abundance of protein in the mixture,³⁰ reveals no strong correlation between the MWCNT- and CB-binding efficiencies

of a protein and its abundance in the lysate (Figure 3, F). The relative abundance of the MWCNT-binding proteins spread over 8 orders of magnitude. Some of the proteins with the highest binding efficiencies, as indicated by the SILAC ratios, are low abundance proteins. This suggests that protein binding to MWCNTs is unlikely to be a non-specific aggregation of the highly abundant proteins with MWCNTs. Instead, the binding of proteins to MWCNT and CB appears to be protein-selective. We compared the MWCNT- and CB-binding efficiencies of all the quantitated proteins (Figure 3, G), and observed that while a small number of CB-binding proteins also bind to MWCNTs, there is no apparent correlation between the binding efficiencies of proteins to these two materials. In fact, most of the proteins with high CB-binding efficiencies bind to MWCNTs only marginally or not at all. This indicates that MWCNTs and CB not only have different protein binding capacities, but also different protein binding specificities.

Functional classification of the carbon nanotube-protein corona

To correlate the MWCNT-binding property of proteins with their functions, we grouped the quantitated proteins into 15 bins; each contained 50 proteins (except for the last bin which



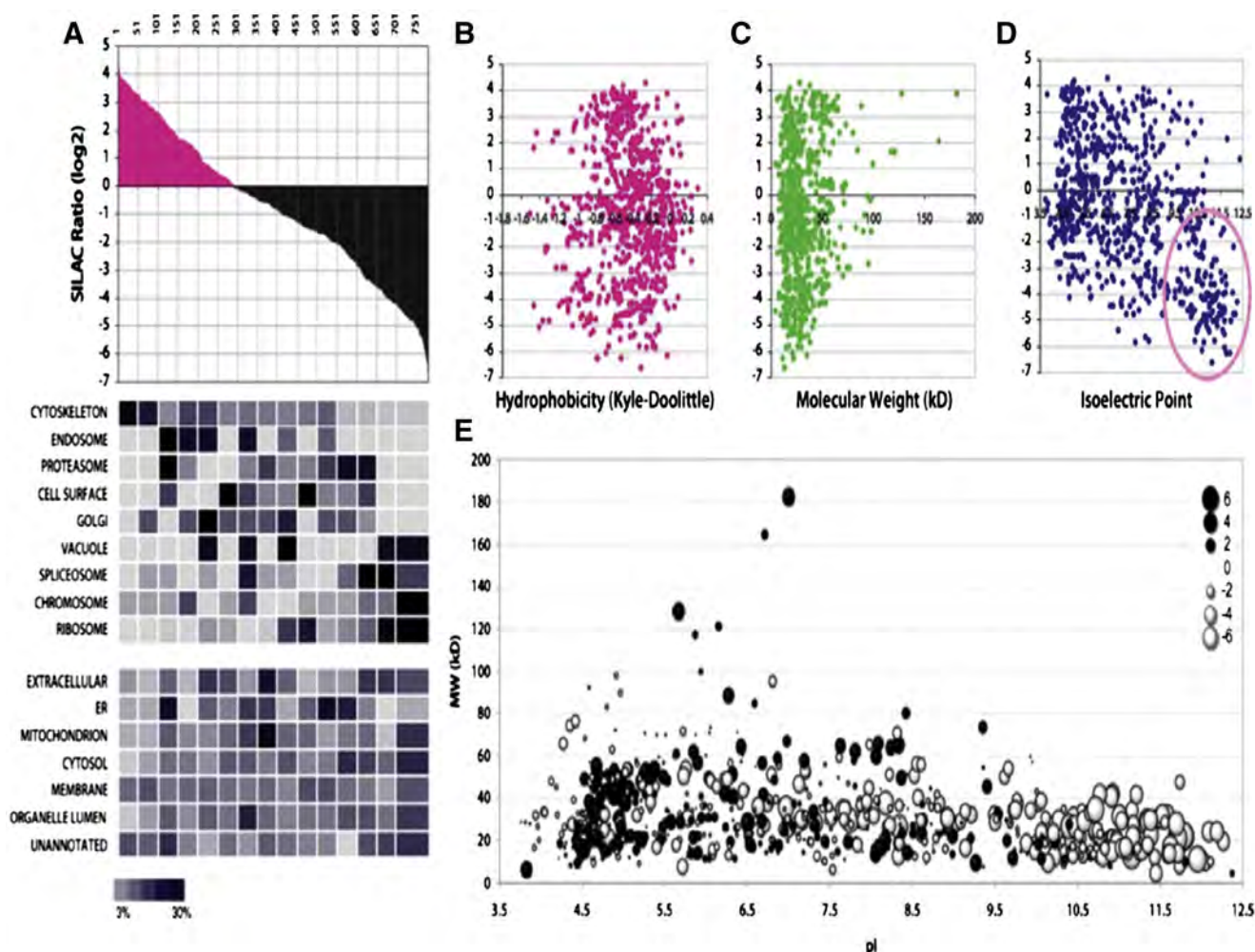


Figure 4. Correlation of MWCNT-binding with functional and physical properties. (A) Proteins are grouped according to their SILAC ratios and the percentage of proteins in each functional category is indicated. (B–D) Scatter plots of the SILAC ratio and hydrophobicity, molecular weight and isoelectric point of each quantitated protein. (E) The molecular weight and isoelectric point of each quantitated protein are shown. The SILAC ratios are indicated by the size and colour of the dots.

contained 78 proteins). The distribution of gene functions, as defined by slimmed-down gene ontology categories (Table S4), in each bin was then compared (Figure 4, A). Cytoskeletal proteins were dramatically over-represented among the MWCNT-binding proteins, as Bins 1 and 2 (proteins with the highest binding efficiency with MWCNTs) contain more than 50% of all the cytoskeletal proteins identified and quantified in the entire experiment. Endosomal proteins were also significantly enriched by MWCNTs. By contrast, nuclear proteins, such as chromatin, ribosomal and spliceosomal proteins, were over-represented in proteins with very low SILAC ratios, *i.e.*, proteins that do not bind effectively to MWCNTs. Proteins in other gene ontology categories, *e.g.*,

mitochondrial and membrane proteins, exhibit no preference in terms of CNT-binding. The strong preference of cytoskeletal and endosomal proteins to CNT interactions suggests a possible link between MWCNTs to these intracellular structures. A recent study has revealed the striking ability of pristine CNT to interfere with actin organization.³¹ The preferential binding of cytoskeletal proteins to CNTs may be a biochemical basis for CNT cytotoxicity.

Physiochemical properties of CNT-binding proteins

We next studied the possible correlation of physiochemical properties of proteins with their MWCNT-binding efficiencies.

Figure 3. Characterisation of MWCNT- and CB-protein corona by SILAC proteomics. (A) Experimental design. (B) Sample mass spectra of selected peptides that show various degrees of MWCNT- enrichment and CB-enrichment. (C) Western blotting analysis of MWCNT-pull-down by using antibodies against selected proteins. (D) Histogram of the SILAC ratio ("medium" vs "light", log2) of 778 quantitated proteins. A positive ratio for a protein indicates an enrichment of this protein in the MWCNT pull-down. (E) Histogram of the SILAC ratio ("heavy" vs "light", log2) of the quantitated proteins, which indicates the relative enrichment in the CB pull-down. (F) Scatter plot that shows the SILAC ratio and the protein abundance index of each quantitated protein (pink: MWCNT pull-down; green: CB pull-down). (G) SILAC ratio of each quantitated protein with respect to the MWCNT- and CB-pull-downs.

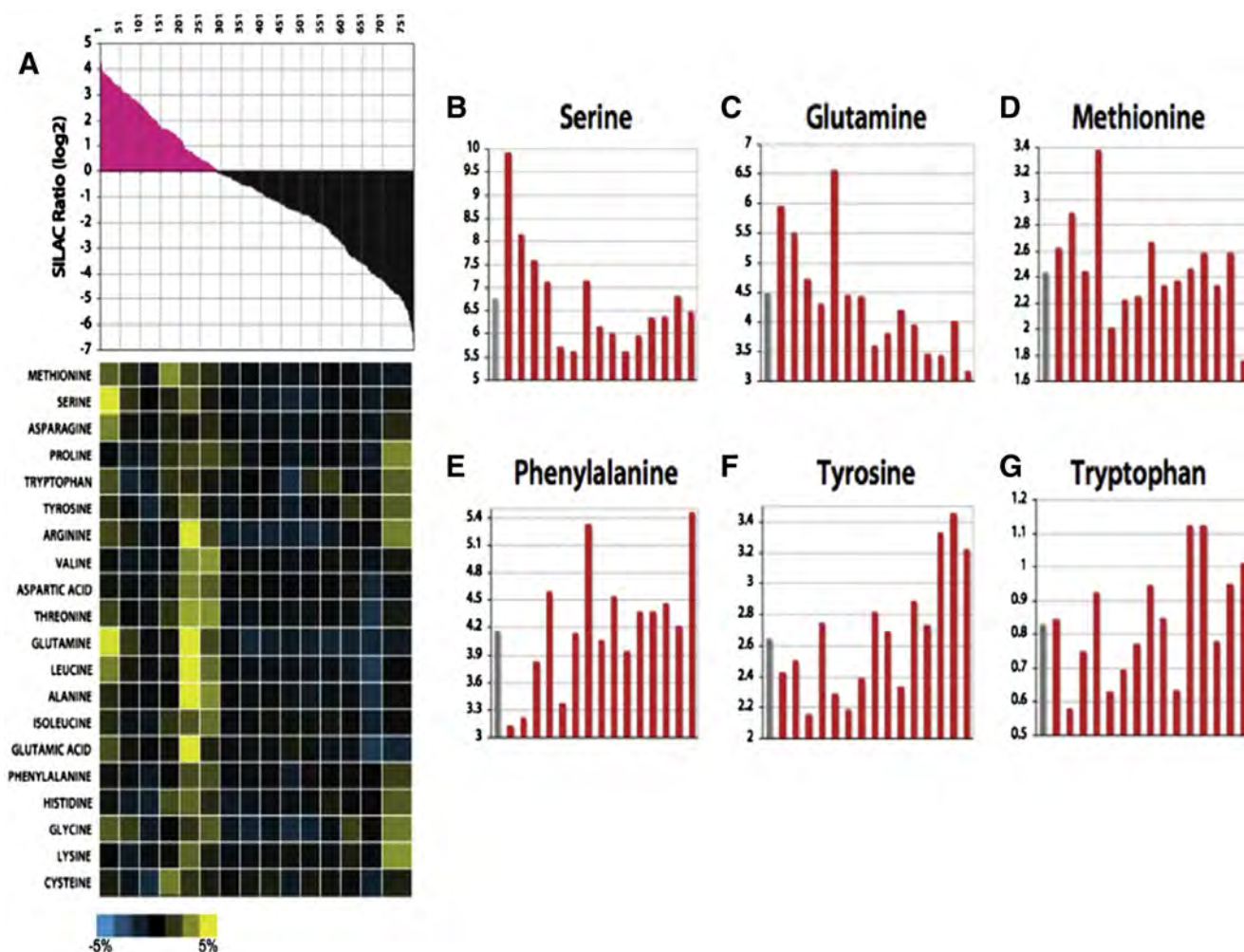


Figure 5. Amino acid usage within the proteins pulled down by MWCNTs. (A) Proteins are grouped according to their SILAC ratios and the amino acid usage within the proteins in each group is calculated. The colours of the boxes indicate the derivation of the measured amino acid usage from the average usage of each amino acid in the human proteome. (B–G) Usage of selected amino acids in each SILAC ratio group (same grouping as in 4A). Grey bars: average usage of amino acid in human proteins.

First, the overall Kyle-Doolittle hydrophobicity of proteins was plotted against the SILAC ratios (Figure 4, B and Table S4). The scatter plot indicated that there is no obvious correlation between the hydrophobicity of a protein and its CNT affinity. We did not observe a preference of MWCNTs to bind hydrophobic proteins, despite previous studies indicating that hydrophobic interactions are an important factor of peptide binding to CNTs.^{19,20} A survey of the molecular weights of quantitated proteins (Figure 4, C and Table S4) also indicated a lack of correlation between molecular sizes and MWCNT-binding, except for the under-representation of proteins over 50 kDa among the ones with very low SILAC ratios. An investigation of the distribution of isoelectric points (pI, Figure 4, D and Table S4) indicated that the majority of non-CNT-binding proteins were very basic (circled). Taken together, a theoretical 2D gel (Figure 4, E) shows a strong bias in CNT-binding efficiencies against small, basic proteins. This supports the hypothesis that interactions between MWCNTs and proteins might be modulated by electrostatic forces.¹³ Remarkably, the protein binding of

MWCNTs also appears to depend on the pH in the incubation buffer (Figure S2).

Previous studies have suggested that the peptides rich in aromatic amino acids have a higher affinity to CNTs.^{19,20} We tested this hypothesis by examining the amino acid usage of proteins with different MWCNT affinities (Figure 5, A). CNT-binding efficiency correlates well with the frequencies of serine, and to a lesser extent, methionine, glutamine and asparagine. For example, the frequency of serine in proteins with the highest affinity to MWCNTs is more than 1.5 folds higher than the serine usage in all the quantitated proteins (Figure 5, B). Unexpectedly, all three aromatic amino acids are over-represented in proteins found not to bind to MWCNTs (Figures 5, E–G), which suggests that proteins rich in these amino acids are less prone to CNT binding.

To study the potential correlation between MWCNT-binding affinity and the secondary structure of proteins, we cross-referenced the quantitated proteins in this study with the Protein Data Bank (PDB). Out of 778 proteins, 269 are found to have

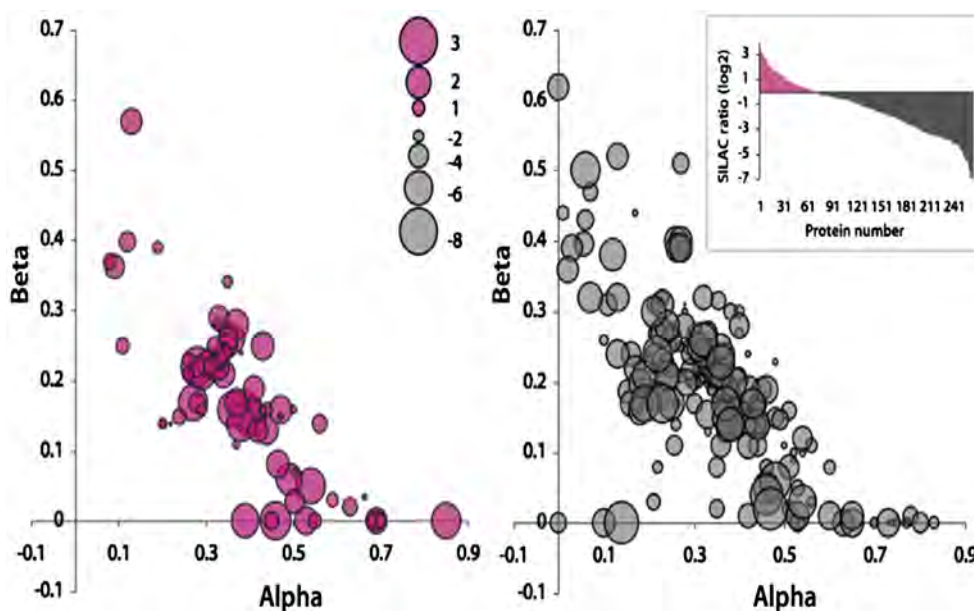


Fig. 6. Correlation of secondary structures and MWCNT-binding. The relative proportions of alpha helical and beta strand contents of a subset of proteins identified in this study were obtained from the Protein Data Bank. The SILAC ratios of the selected subset are shown in the inset. The alpha helical and beta strand content of proteins with positive SILAC ratios (MWCNT-binding, pink data) and negative SILAC ratios (non-CNT-binding, grey data) are shown as bubble plots, with the values of the SILAC ratios indicated by the bubble sizes.

information on their secondary structure (Figure 6 inset and Table S4). The theoretical proportions of alpha helical and beta strand content of each protein was calculated. Proteins found to bind MWCNTs (Figure 6, pink data) appeared to show a slight bias towards having high alpha helix content, while those with beta strand content was under-represented among the CNT-binding proteins. In contrast, among the proteins that did not bind to MWCNTs (grey data), such a bias toward alpha helical structure was not observed.

Discussion

Until recently, studies on the nanomaterial interactions with biomolecules have been limited to the use of purified proteins or peptides. In the present study we exposed CNTs to a complex protein mixture, and report the large-scale identification of the resulting corona proteins. A complete protein lysate of HeLa cells was used as an archetypal human proteome, which provided the representative inventory of proteins for the binding assay. Our quantitative approach not only allowed the identification of proteins associated with nanoparticles, as have been done previously,^{21,22,32,33} but also measured each protein's level of enrichment in the binding assay. Though not a direct measurement of binding coefficients, our data provides an unprecedented assessment of the efficiency and specificity of the protein binding to two nano materials, namely MWCNT and CB.

We observed that MWCNTs bind to a large amount of proteins. Some proteins apparently bound to CNT highly efficiently, with 44 proteins found to be enriched by over 10 fold in MWCNT after the binding assay. In comparison, much less proteins can bind to sepharose and magnetic bead

matrices.²³ Interestingly, both the protein binding capacity and specificity of MWCNTs are highly distinct from those of CB. This suggests that the protein-CNT interaction depends on the 3-dimensional arrangement of carbon atoms in CNTs, and not on the chemical properties of carbon itself. A recent study of mice treated with CNT, asbestos and CB³⁴ showed that CNTs inflict a much more serious effect on protein expression in the lungs than CB. Our results seem to provide an explanation for this difference in molecular perturbation.

The physical nature of protein-CNT interaction is not well understood. Recently, a study by Zhang et al. demonstrated that the protein-binding patterns onto the nanoparticle correspond closely to the nanoparticle surface properties, size as well as properties of proteins.³⁵ Our data show that effective protein binding was possible only if the CNT exceeds a certain size threshold. Our findings commensurate with the several findings that the particle size can play a sizeable role in determining the protein binding and the formation of corona.^{21,36–39} However, we do not see a correlation between protein sizes and binding efficiencies, implying that proteins might not be trapped in the CNT lumen. More likely, protein binding may require a suitable surface curvature, as have been previously suggested.¹³ By measuring the protein selectivity of MWCNT, we were able to test various hypotheses on the nature of protein-CNT interactions, and this yielded surprising results. Our results disagree with the reported preference towards hydrophobic proteins, or peptides containing aromatic amino acids during MWCNT binding.²⁰ The use of purified proteins or peptides, which were often applied at high concentrations, with nanoparticles may not reflect the physiological situation where proteins interact with CNTs in a complex mixture. The peptide motifs found by these studies to efficiently bind to CNTs may be not

accessible for binding or may be differently folded when present in a complex mixture of proteins in vivo.

Taken together, we have demonstrated that CNTs are able to selectively enrich some specific proteins from protein mixtures, and specific CNT protein binding capacities may be a general phenomenon that should provide new insights into the study and application of CNTs. This study helps to broaden future CNT studies in at least two aspects. Firstly, proteins with a high affinity to bind with CNTs are potential targets after the latter enters the cell. The conformation and functions of these proteins may be affected, resulting in disruptions to cellular structure and functioning. A recent cytotoxicity study showed that MWCNTs with larger diameters exhibit a higher level of toxicity to cells.³⁹ As we observed a higher level of protein binding in larger MWCNTs, the apparent link between CNT protein binding and toxicity will be an important topic for future studies. Interestingly, the toxicity of CNT was found to decrease after the binding of serum proteins,¹⁷ probably because these proteins have saturated the protein binding sites of the CNT and prevented further interactions of cellular proteins. Secondly, the identification of CNT-binding proteins may have implications on the future applications of CNTs. The binding of these proteins to CNTs could complicate the efficacy of CNTs as biosensing probes or delivery tools for therapeutic cargos. Our study has provided an inventory of proteins with high affinity to CNT. This inventory will offer future directions for the surface modification and the blocking procedures that will minimise these non-specific protein interactions. The proteomic approach developed in this study can also provide a convenient technical platform for the evaluation and optimisation of these procedures.

Acknowledgment

We thank the members of the Lam and Cheng labs for fruitful discussion.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.nano.2012.09.004>.

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Exhibit D

Quantitative Analysis of Human Red Blood Cell Proteome

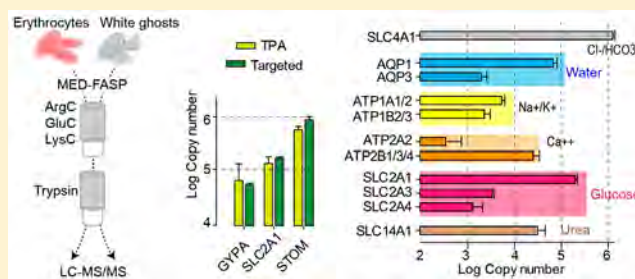
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S Supporting Information

ABSTRACT: Red blood cells (RBCs) are the most abundant cell type in the human body. RBCs and, in particular, their plasma membrane composition have been extensively studied for many years. During the past decade proteomics studies have extended our knowledge on RBC composition; however, these studies did not provide quantitative insights. Here we report a large-scale proteomics investigation of RBCs and their “white ghost” membrane fraction. Samples were processed using the multienzyme digestion filter-aided sample preparation (MED-FASP) and analyzed using Q-Exactive mass spectrometer. Protein abundances were computed using the total protein approach (TPA). The validation of the data with stable isotope-labeled peptide-based protein quantification followed. Our in-depth analysis resulted in the identification of 2650 proteins, of which 1890 occurred at more than 100 copies per cell. We quantified 41 membrane transporter proteins spanning an abundance range of five orders of magnitude. Some of these, including the drug transporter ABCA7 and choline transporters SLC44A1 and SLC44A2, have not previously been identified in RBC membranes. Comparison of protein copy numbers assessed by proteomics showed a good correlation with literature data; however, abundances of several proteins were not consistent with the classical references. Because we validated our findings by a targeted analysis using labeled standards, our data suggest that some older reference data from a variety of biochemical approaches are inaccurate. Our study provides the first “in-depth” quantitative analysis of the RBC proteome and will promote future studies of erythrocyte structure, functions, and disease.

KEYWORDS: erythrocyte, red blood cell, white ghosts, membrane proteomics, FASP, MED FASP, copy number, total protein approach, TPA



INTRODUCTION

Red blood cells (RBCs) are the most abundant human blood cells. Apart from their pivotal role in oxygen and carbon dioxide transport, RBCs are involved in the transfer of glycoposphatidylinositol-linked protein immune complexes.¹ The plasma membrane of the RBCs is mechanically supported by spectrin tetramers connected by actin junctional complexes, forming a 2D six-fold triangular network anchored to the lipid bilayer.² To date, the membrane protein composition and structure has been investigated by chemical staining,^{3,4} immunoassay methods,^{5–7} crystallography,⁸ scanning electron microscopy, and atomic force microscopy.⁹

In contrast with the conventional biochemical approaches targeting single or few proteins, large-scale proteomics enables insights into thousands of proteins in a single experiment, including their abundances. However, all early proteomics analyses of RBCs failed to provide comprehensive data sets. The reasons were the presence of hemoglobin interfering with identification of lower abundant proteins and the limitations of proteomics technology to analyze membrane proteins. Further progress in analysis of cytosolic fraction of RBCs was possible using extensive fractionation of proteins^{10,11} and by reduction of hemoglobin content using peptide libraries.¹² Depletion of hemoglobin by metal affinity chromatography¹³ allowed

reproducible measuring and relative quantification of over 1000 proteins across cytosolic preparations from a series of RBCs donors.¹⁴ Using the N-terminomics procedure, Overall et al. identified 1369 human erythrocyte proteins.¹⁵ Analysis of the membrane preparation of RBCs from Diamond–Blackfan anemia patients, using extensive gel fractionation, allowed the identification of >1100 mainly nonmembrane proteins.¹⁶ Applying diverse depletion strategies to a triton insoluble membrane skeleton fraction enabled semiquantitative profiling of 77 proteins.¹⁷

Despite its potential, so far proteomics has not provided a comprehensive and quantitative picture of the erythrocyte. We aimed to (1) perform an in-depth proteomics analysis of whole RBCs and their “white ghost” (WG) preparations and to (2) quantify proteins in whole cells and their membrane fraction, including low abundance membrane transporters.

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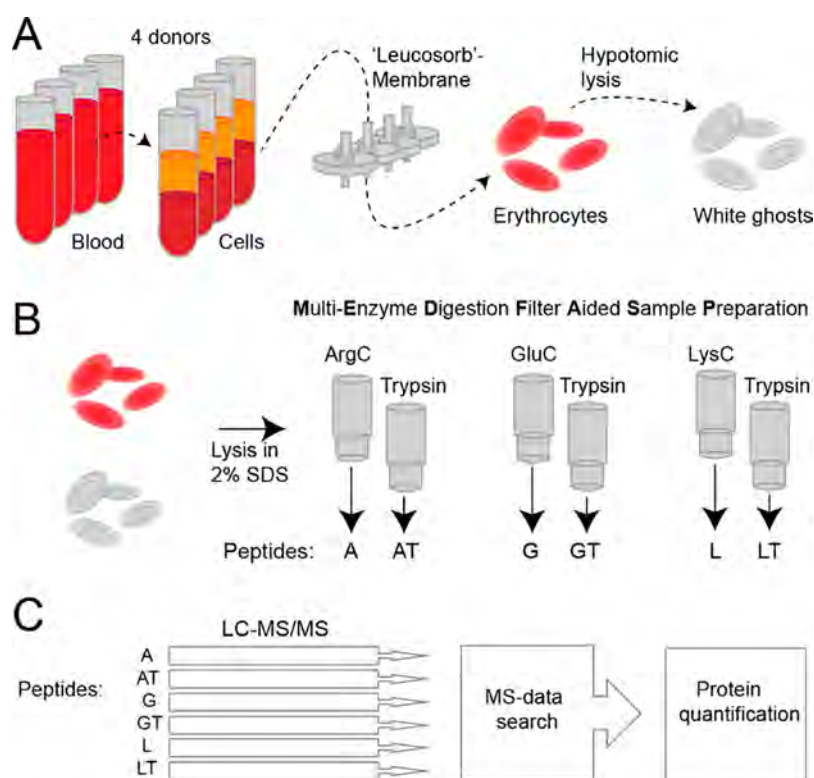


Figure 1. Overview of the analytical workflow. (A) Venous blood was collected from four healthy donors. Erythrocytes were collected by centrifugation and remaining leucocytes were depleted by filtration. We used purified erythrocytes for isolation of membrane fraction, so called the “White Ghosts”. (B) The purified cells and the membrane fractions were lysed in buffer containing 2% SDS, and the protein extracts were processed using the MED FASP method. Each sample was successively digested using endoprotease ArgC and trypsin, endoprotease GluC and trypsin, and endoprotease LysC and trypsin. (C) The isolated peptides were chromatographed on a C₁₈ column coupled to mass spectrometer. The mass spectra were searched using MaxQuant software. Proteins were quantified using the “Total Protein Approach”.

MATERIALS AND METHODS

Erythrocytes

Blood samples were provided by the authors and further laboratory employees who gave their informed consent. Nine mL of fasting venous blood was collected with minimal stasis into EDTA tubes. The procedure was modified from previously published methods¹⁶ (Figure 1). To minimize proteolysis, all samples and buffers were kept at 0–4 °C during all steps. RBCs were collected from the plasma by centrifugation for 5 min at 150g and were suspended in PBS containing 1 mM EDTA. Then, the slurry was passed through a leucocyte depletion filter (Acrodisc, PSF Syringe Filters, Pall, Ann Arbor, MI). RBCs were washed four times with PBS and stored overnight on ice at 4 °C in PBS containing 10 mM glucose and EDTA-free protease inhibitor cocktail tablets (Roche, Mannheim, Germany).

Preparation of “White Ghosts”

WGs were isolated according to Pesciotta et al.¹⁴ In brief, the purified RBCs were lysed using 10 volumes of hypotonic lysis buffer composed of 5 mM sodium phosphate, 1 mM EDTA, complete, mini EDTA-free protease inhibitor cocktail, pH 8.0; the WGs were then isolated by centrifugation at 21 000g for 40 min. WG were purified by four to five additional washes and centrifugations.

Lysis of the Cells and Membrane Preparations

Whole RBCs and WGs were lysed in 0.1 M Tris-HCl containing 2% SDS and 0.05 M DTT at 100 °C for 5 min.

Protein and Peptide Determination

Total protein concentration in lysates and the peptide content in the digests (see below) were assayed using a tryptophan fluorescence-based WF assay in microtiter plate format.¹⁸ For measurements, Corning Costar 96-well black flat-bottomed polystyrene plates (Sigma-Aldrich, Taufkirchen) were used.

Multienzyme Digestion Filter-Aided Sample Preparation (MED FASP)

Sample aliquots containing 100 µg total protein were processed using the MED FASP method¹⁹ with modifications described recently.²⁰ Proteins were first cleaved overnight using endoprotease ArgC, GluC, or LysC, and in the second step they were digested with trypsin for 2 h. The enzyme-to-protein ratio was 1:50. Digestions with LysC and trypsin were carried out in 50 mM Tris-HCl, pH 8.5. For the cleavage with ArgC the buffer was supplemented with 10 mM MgCl₂. The digestion with GluC was carried out in 25 mM ammonium bicarbonate. All reactions were conducted at 37 °C. Aliquots containing 10 µg total peptide were concentrated to a volume of ~5 µL and were stored frozen at –20 °C until mass spectrometric analysis. Ten µg aliquots of the peptides were deglycosylated with 4 units PNGase F at room temperature for 2 h.

Liquid Chromatography–Tandem Mass Spectrometry

Analysis of peptide mixtures was performed using a QExactive HF mass spectrometer (Thermo-Fisher Scientific, Palo Alto). Aliquots containing 5 µg total peptide were chromatographed on a 50 cm column with 75 µm inner diameter packed C₁₈.

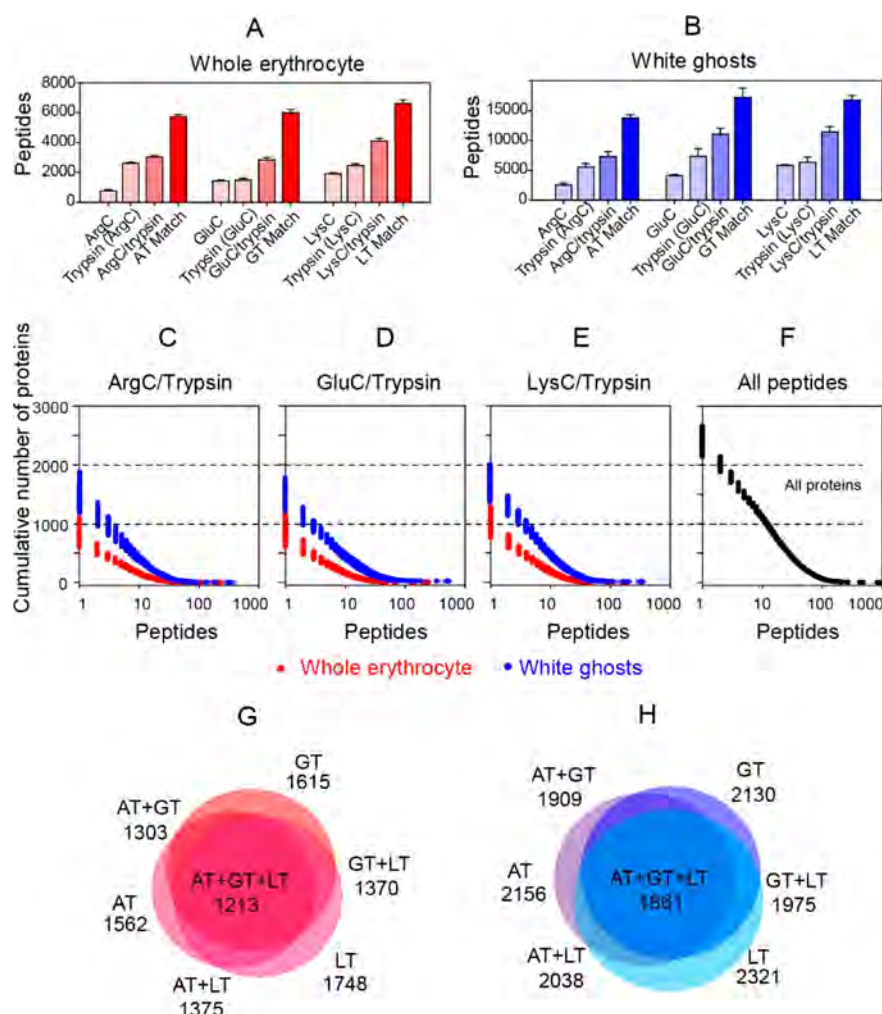


Figure 2. Peptide and protein identification and quantification in lysates of whole erythrocytes (red–pink colors) and their white ghosts (blue–purple colors). (A,B) Identification of peptides obtained by three different cleavage approaches using either ArgC followed by trypsin, GluC followed by trypsin, LysC followed by trypsin. In each group the first, second, third, and fourth bars indicate number of peptides identified from the digest with the first enzyme and the second enzyme (in all trypsin), the composite number of peptides from both digestions, and the total number of peptides from both digestions using the “matching between runs” algorithm of the MaxQuant software. (C–E) Cumulative number of proteins sorted by the number of matching peptides from individual digestion approaches. (F) Cumulative number of proteins sorted by the number of matching peptides from all approaches. (G,H) Venn plots with the number of identified proteins using various digestion approaches.

material (100 Å pore size; Dr. Maisch, Ammerbuch-Entringen, Germany). Peptide separation was carried out at 300 nL/min for 75 min using a two-step acetonitrile gradient 5–40% over the first 60 min and 40–95% for the following 15 min. The backpressure varied between 450 and 650 bar. The temperature of the column oven was 55 °C.

The mass spectrometer operated in data-dependent mode with survey scans acquired at a resolution of 50 000 at m/z 400 (transient time 256 ms). Up to the top 15 most abundant isotope patterns with charge $\geq +2$ from the survey scan (300–1650 m/z) were selected with an isolation window of 1.6 m/z and fragmented by HCD with normalized collision energies of 25. The maximum ion injection times for the survey scan and the MS/MS scans were 20 and 60 ms, respectively. The ion target value for MS1 and MS2 scan modes was set to 3×10^6 and 3×10^5 , respectively. The dynamic exclusion was 25 s and 10 ppm. The mass spectrometry data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository²¹ with the data set identifier: PXD005200.

Stable Isotope-Labeled Peptide-Based Protein Quantification

Peptides with isotopically labeled lysine ($^{13}\text{C}_6$, $^{15}\text{N}_2$) and arginine ($^{13}\text{C}_6$, $^{15}\text{N}_4$) residues were purchased from (Sigma-Aldrich). The peptide matching sequences of erythrocyte band 7 integral membrane protein/stomatin (SLC4A1/STOM) (TISFDIPPQEILTK, YLQTLTIAAEK), glycophorin A (GYPA) (TVYPPEETGER), and glucose transporter 1/solute carrier family 2 member A (GLUT1/SLC2A1) (TFDEIASGFR) were selected on the basis of the proteomics analysis using the LysC and trypsin digestion. The selected peptides were repeatedly identified in the analyses of the WG preparations. Ten μg aliquots of the digest of the WG lysate were spiked with 0.5, 1, 2, or 5 pmol of the labeled peptides and were LC–MS/MS analyzed as described above. Ratios of heavy to light MS1 intensities of “light” and “heavy” (standard) peptides were manually extracted from the spectra (Xcalibur Qual Browser) and used for calculation of concentrations of targeted proteins (Table S1).

Data Analysis

The spectra were searched using MaxQuant software. All raw files were searched together in single MaxQuant run using separate parameter options for MS files from analyses of Arg C, LysC, GluC, and tryptic peptides. For the tryptic peptides generated from the GluC-precleaved material a mixed GluC/tryptic specificity was set. A maximum of two missed cleavages was allowed. Carbamidomethylation was set as fixed modification. The “matching between the runs” option was used. The maximum false peptide and protein discovery rate was specified as 0.01. Protein abundances were calculated using the “total protein approach” (TPA) method.²² The calculations were performed in Microsoft Excel.

The copy number of protein was calculated using the relationship

$$\text{Copy number} = \text{RBC volume} \times N_A \times \text{total protein content per cell}$$

where

$$c(i) = \frac{MS - \text{signal}(i)}{\text{total MS} - \text{signal}} \times \left[\frac{\text{mol}}{g \text{ total protein}} \right]$$

and N_A is the Avogadro number.

We assumed the mean volume of RBCs of 90 fL²³ and the average total protein content per cell as 20% of RBCs mass.²⁴ The values obtained using different proteinase were averaged.

Calculation of copy numbers using data from the analysis of the WG fractions ($\text{Copy number}_{\text{WG}}$) was performed using the membrane protein enrichment (MPE) factor. This factor reflected the ratio of protein concentration in the whole RBCs sample and the respective protein concentration in the subsequent WG sample. MPE was computed for the 10 most abundant integral membrane proteins (Table S2).

$$\text{Copy number} = \text{Copy number}_{\text{WG}}(i) / \text{MPE}$$

Membrane proteins are often glycosylated with predominant N-glycosylation. To assess whether the N-glycosylation has an effect on the label free quantitation, we compared the proteins abundances assessed from analysis of the deglycosylated and untreated samples.

RESULTS

Proteomics Analysis

In this study we analyzed RBC samples originating from four donors (Figure 1). We analyzed whole cell lysates and membrane fractions prepared by hypotonic lysis of RBCs, resulting in WGs. Proteins were extracted with 2% SDS and subjected to the filter-aided sample preparation (FASP) approach, which allows unbiased analysis of soluble and membrane proteins.^{20,25} To cover the highest number of RBCs proteins and to achieve the height sequence coverage, each protein extract was consecutively digested using two enzymes. In the first cleavage step, endoproteinase Arg C, GluC, or LysC was used. After elution and collection of peptides, in the second step, the material remaining in the filter was treated with trypsin (Figure 1B). Protein digestions were carried out in the absence of any denaturant, a condition ensuring high digestions yields with very low rates of peptides with missed cleavage sites.²⁰ Each peptide fraction was analyzed separately by LC-MS/MS, and all recorded spectra were

searched together (Figure 1C). Each of the protein digestion protocols resulted in a similar number of identified peptides per analyzed sample of 2500–3000 in the analysis of the whole RBCs and 6000–7000 peptides in the WG analysis (Figure 2A,B). The cumulative numbers of identified peptides per sample were about 6000 and 15 000 for the whole cell and WG, respectively. Comparing the three digestion strategies used, the combination of LysC and trypsin enabled identification of the highest number proteins; however, the two other enzyme combinations significantly extended the total number of identified proteins (Figure 2C–E). The total number of proteins that were identified per donor sample matching RBCs and WG analyses was about 2500 (Figure 2F, Table S3). The Venn plots show that 30% of proteins were identified using only one or two cleavage approaches (Figure 2G,H). This result emphasizes the importance of multicleaveage strategies for comprehensive characterization of proteomes.²⁶

RBC Purity

Purity of the RBCs samples is prerequisite for their unbiased proteome analysis. In this study, we followed the efficient, reproducible RBC processing protocol by Pesciotta et al.¹⁶ RBCs were stored for 24 h to reduce the number of reticulocytes in 4 °C.

Next, RBCs were depleted of small cells, including platelets and microparticles, by repeated sample washing and low-speed centrifugation. Finally, leucocytes, neutrophils, and other cells larger than RBCs were removed by filtration (Figure 1). Because our analysis provided absolute abundance values of proteins, we could estimate the extent of contamination of the RBCs by platelets and leucocytes. In contrast with RBCs, leucocytes and neutrophils are nucleated cells. Each nucleus of a 2n human cell contains $\sim 2.7 \times 10^8$ copies of all core and linker histones. We identified $(1.5 \text{ to } 7.0) \times 10^4$ histone molecules across the samples (Table S3). This value accounts for one leucocyte or neutrophil per 10^4 RBCs. Out of the platelet-specific antigens such as CD36, CD41, CD42, and CD61 occurring at 20–80 000 copies per platelet,²⁷ we identified only CD36 at a copy number of 64 ± 17 . This suggests a maximal platelet contamination below 1 platelet per 1000 erythrocytes. In fact, the contamination of our preparations RBC and WG by platelets could be even lower. Notably, we did not identify some of the top ten most abundant platelet proteins such as platelet factor 4 or platelet basic protein, which occur in these cells at 530 000 and 355 000 copies, respectively.²⁷ Thus quantitative proteomics data confirmed the expected depletion of leucocytes and platelets from the RBC samples. Because a minute contamination of the erythrocyte preparations by other blood cells cannot be avoided we consider proteins above a threshold of 100 copies as those occurring in erythrocytes. However, we do not rule out the possibility that some proteins with abundances below this limit may also originate from RBCs. In addition we cannot exclude contamination of the RBC and their membrane preparations by plasma proteins. For example, the most abundant plasma protein, albumin, was present at several thousand copies.

To estimate contamination of the RBC preparation we compared the abundances of ribosomal subunits that were calculated in this study with abundances abundance of these proteins in reticulocytes.²⁸ In our preparation the median abundance of these subunits was 200 copies per cell, whereas in reticulocytes the corresponding value was 220 000. This

suggests that RBC preparations used in our analyses were contaminated by <0.1% reticulocytes

Protein Quantification

Across the preparations the MPE was in the range of 8–12 (Figure 3A). 1890 identified proteins occurred at >100 copies

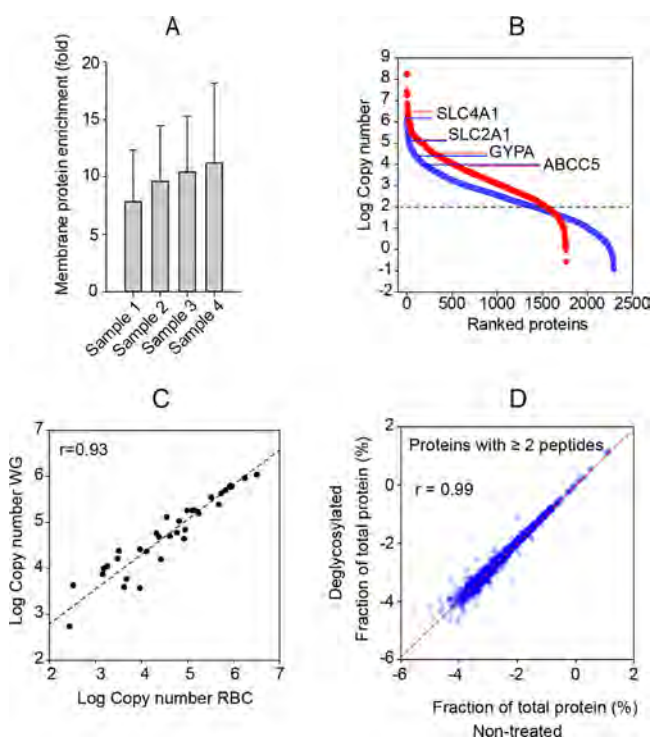


Figure 3. Quantitative analysis. (A) Average enrichment of top 10 most abundant membrane proteins in the WG fractions relative to whole RBCs. (B) Distribution of protein copy numbers across all proteins in whole cells (red circles) and WG fractions (blue circles). The dashed line shows the 100 copy per cell sample purity threshold. (C) Correlation of the copy numbers for the known plasma membrane proteins calculated using data from analysis of whole cells and WG fractions. (D) Correlation of assessed protein abundance with and without protein deglycosylation.

per cell; 1536 identified in the whole cell lysate and 1406 found in the WG fraction (Figure 3B). For the known RBC membrane proteins we observed an excellent correlation [$r(\text{Pearson}) = 0.93$] between copy numbers of proteins calculated using data from both whole cells and WGs data sets (Figure 3C). The analysis revealed that N-glycosylation has practically no effect on protein quantification (Figure 3D).

Analysis of the Whole RBCs

Analysis of whole RBCs lysates allowed the identification of 2090 proteins. Of those, 450 were quantified across all samples (Figure 3B). Hemoglobin is the dominant protein of the RBCs. Its subunits alpha (HbA) and beta (HbB) exist at about 200 million copies, whereas the less abundant subunits delta (HbD) and gamma (HbG) occur at about 30 and 4 million copies, respectively (Figure 4A). Surprisingly, we also found significant amounts of the subunits theta (HbQ) (400 000 copies), zeta (HbZ) (900 000 copies), and mu (HbM) (100 000 copies); these are generally considered to be embryonic forms of hemoglobin. Identification of these isoforms in the presence of >100-fold excess of HbA and HbB stresses the power of mass spectrometry compared with classical biochemical methods.

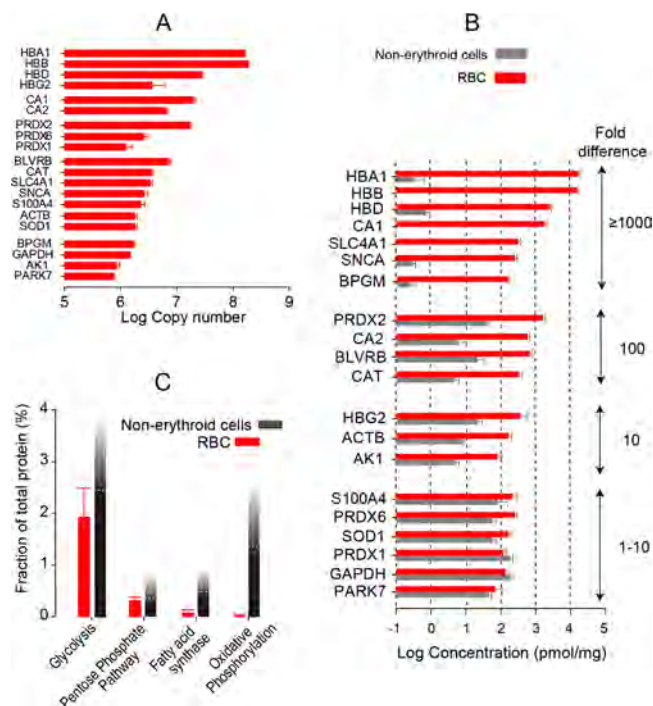


Figure 4. Proteomics analysis of the whole RBCs. (A) Copy numbers of the 20 most abundant proteins in RBCs. (B) Comparison of the concentrations of the most abundant proteins in RBCs compared with nonerythroid human cells. The values for the nonerythroid cells are based on proteomics analyses of human hepatocytes³¹ and cultured cells.^{31,32} (C) Total protein content of basic metabolic processes in RBCs compared with the nonerythroid cells. The black bars show the average value across different human cells. The gradient area of the bars shows standard deviation.

Carbonic anhydrase is the second most abundant protein. Its isoform 1 (CA1) is present at about 20 million copies. Peroxiredoxin-1, -2, and -6 (PRDX1, PRDX12, and PRDX16) occur with similar copy numbers. The next group of highly abundant proteins consists of biliverdin reductase B (BLVRB), which catalyzes reduction of methemoglobin, and catalase (CAT) and superoxide desmutase (SOD1), which protect from toxic effects of oxygen peroxide and radicals, respectively. Bisphosphoglycerate mutase (BPGM), the central enzyme of the Rapoport–Luebering cycle, is present at 1.6 million copies. BPGM converts 1,3-bisphosphoglycerate, which is formed by glycolysis, to 2,3-bisphosphoglycerate (2,3-BPG). The main role of 2,3-BPG is to shift the equilibrium of hemoglobin toward the deoxy state. Housekeeping enzymes glyceraldehyde-P dehydrogenase (GAPDH) and deglycase (PARK7)²⁹ are present at 1.4 and 0.7 million copies, respectively. Calcium binder S100A4 is present at about 2 million copies. Another enzyme found to be present at 2–3 million copies was α -synuclein. Notably, it has recently been reported that RBCs as the major source of α -synuclein in blood.³⁰

To compare abundance of proteins between RBCs and nonerythroid cells, such as hepatocytes³¹ and cultured cells,^{31,32} we used protein concentrations, which are not affected by cell size (Figure 4B). Hemoglobin subunits, CA1, SLC4A1, α -synuclein, and BPGM exist in RBCs at concentration higher by a least 1000 times compared with common cells. The concentrations of proteins protecting from exposure to oxygen, PRDX2, BLVRB, and CAT, are 100 times higher compared with nonerythroid cells. Proteins with “housekeeping” functions

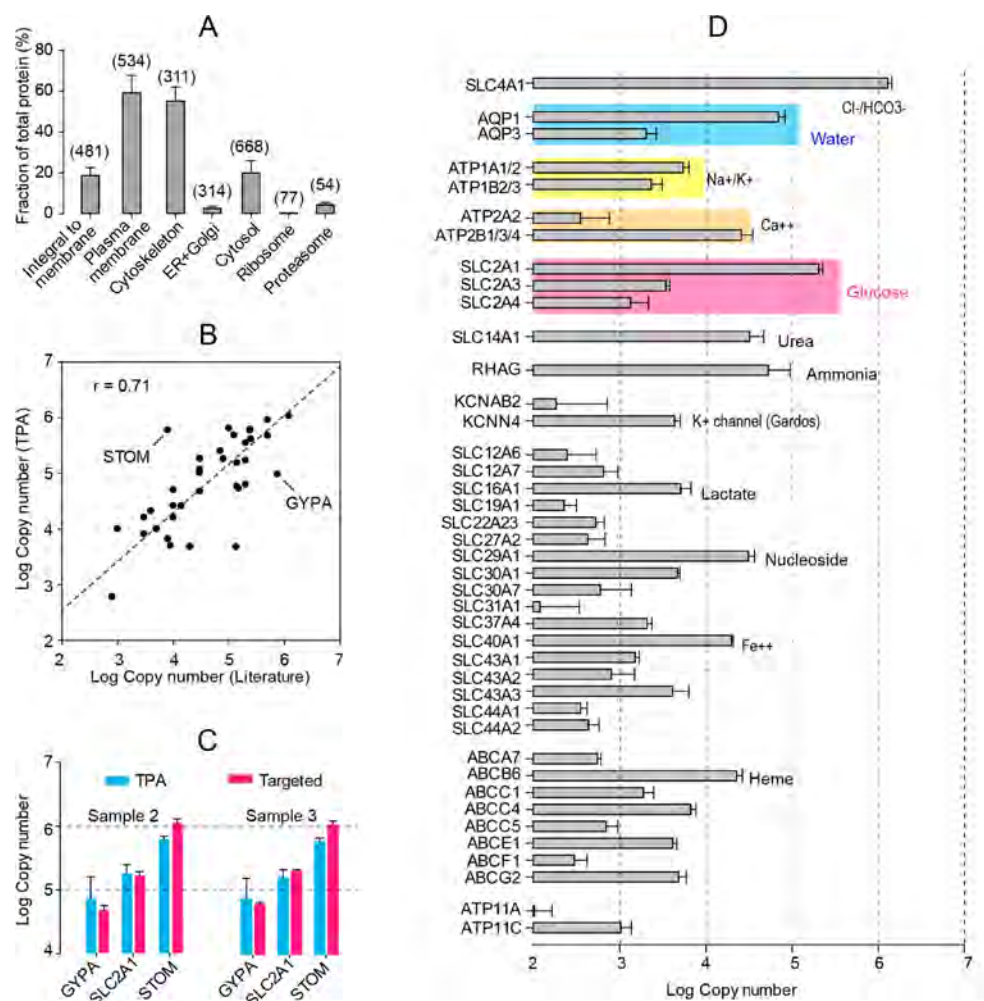


Figure 5. Composition of the WG preparation. (A) Global composition of WGs based on Gene Ontology (GO) annotations. In parentheses is the total number of proteins per GO term. (B) Comparison of the protein copy numbers determined by total protein approach (TPA) with literature values (Table 1). r , Spearman's correlation coefficient. (C) Comparison of the copy numbers of Glycophorin A (GYPA), Glut1 (SLC2A1), and Band 7-Stomatin (STOM) determined by TPA and targeted analysis. Two donor samples were analyzed. (D) Copy numbers membrane transporter proteins identified with >100 copies.

have similar concentrations in RBCs and nonerythroid cells. Enzymes involved in the glycolytic and pentose phosphate pathways constitute a similar portion of total protein of RBCs compared with nonerythroid cells. In contrast fatty acid synthase and enzymes of oxidative phosphorylation are present at significantly lower amounts in RBCs. Notably, not all key enzymes of the oxidative phosphorylation were identified, indicating interruption of this process. In addition, we were unsuccessful in identification of some of these proteins by Western blotting (Figure S1). This suggests their occurrence in a partially degraded state and most probably only in “young” RBCs. Proteins originating from nucleus, mitochondria, and other organelles identified in our and previous proteomics RBCs studies^{10,15} were suggested by us and the previous authors to represent nonfunctional remnants of erythrocyte maturation.

Analysis of the WG Fraction: Identification of Novel Membrane Proteins

Analysis of WG fractions resulted in the identification of 2400 proteins. The plasma membrane and cytoskeletal proteins constitute the most abundant portion of total protein (Figure 5A). Approximately 20% of the total WG protein is predicted

to be integral to membranes. The WG is a crude membrane preparation and hence contains a substantial proportion of nonplasma membrane proteins. Cytosolic proteins contribute in 20% of total protein to WG preparation, and more than 300 proteins have ER or Golgi “Gene Ontology” annotation. An almost complete set of ribosomal and proteosomal proteins was identified in this fraction.

For about 50 proteins of the WG fraction the copy numbers have been reported in the past. We found that copy number values calculated by the TPA approach were similar to the literature data for most proteins (Table 1, Figure 5B). A comparison of the data sets provides a Spearman correlation coefficient r of 0.71. However, obvious discrepancies were found for some proteins. The TPA-based copy number values for STOM were about 2 orders of magnitude higher compared with the literature data. In contrast, GYPA appeared to be 5–10 times less abundant compared with previous studies. Across references the copy numbers of SLC2A1 vary between 200 000 and 700 000.³³ To clarify these differences we have quantified these proteins by means of a so-called “targeted approach”. Two donor WG samples were spiked with stable isotope-labeled peptide standards and were analyzed by LC–MS/MS. The results confirmed the accuracy of the TPA quantification of

Table 1. Comparison of Protein Copy Numbers Obtained by the Proteomics Analysis and the Literature Data^a

protein	gene	copies ($\times 10^{-3}$) literature	copies $\pm$ st. dev ($\times 10^{-3}$) proteomics
AE1 (band 3)	SLC4A1	1200	1074 $\pm$ 114
b-Actin	ACTB	500	908 $\pm$ 65
Stomatin (band 7)	STOM	10	643 $\pm$ 79 (1100) ^b
b-Spectrin	SPTB	242	612 $\pm$ 69
a-Spectrin	SPTA1	242	590 $\pm$ 67
Glyceraldehyde-P DH	GAPDH	500	475 $\pm$ 120
Ankyrin	ANK1	124	479 $\pm$ 32
Protein 4.2	EPB42	250	411 $\pm$ 20
Protein 4.1	EPB41	200	350 $\pm$ 35
Tropomyosin	TPM3	70	253 $\pm$ 66
b-Adducin	ADD2	30	181 $\pm$ 14
p55	MPP1	80	181 $\pm$ 9.6
Tropomodulin	TMOD1	30	184 $\pm$ 26
Glut1	SLC2A1	200–700	170 $\pm$ 18 (200)
Dematin (band 4.9)	EPB49	140	153 $\pm$ 20
CD59	CD59	20–40	116 $\pm$ 39
a-Adducin	ADD1	30	103 $\pm$ 5.1
Rh polypeptide	RHCE, RHD	200 (D and CE)	63 $\pm$ 17
Glycophorin A	GYPA	500–1000	96 $\pm$ 67 (52)
Aquaporin 1	AQP1	120–160	58 $\pm$ 10
Kell glycoprotein	KEL	3–18	50 $\pm$ 5.4
CD47	CD47	10–50	47 $\pm$ 5.4
RhAG	RHAG	100–200	53 $\pm$ 25
Nucleoside transporter	SLC29A1	10	26 $\pm$ 4.2
LW glycoprotein	ICAM4	3–5	21 $\pm$ 10
Urea transporter	SLC14A1	14	26 $\pm$ 8.2
AChE	ACHE	10	16 $\pm$ 2.9
Lutheran glycoprotein	BCAM	1.5–4	16 $\pm$ 5.7
CD99	CD99	1	10 $\pm$ 2.6
CD58 (LFA-3)	CD58	3–7	10 $\pm$ 0.9
KX	XK	1.5–4	8.1 $\pm$ 3.2
CD44	CD44	6–10	6.6 $\pm$ 2.2
DAF (CD55)	CD55	20	4.8 $\pm$ 2.6
Duffy	FY	6–13	5.0 $\pm$ 2.5
CR1	CR1	0.02–1.5	0.6 $\pm$ 0.3

^aFrom data compilation by Burton and Bruce.³³ ^bIn parentheses are values that were assessed by targeted proteomics analysis.

STOM, GYPA, and SLC2A1 (Figure 5C), suggesting that some older quantification data based on biochemical approaches needs to be revised.

Membrane channels and transporters are essential for the exchange of metabolites and ions between the cytoplasm and the environment of the cell. Our analysis provides a comprehensive picture of the occurrence of these proteins in the RBCs membrane. 41 proteins of this class were identified at more than 100 copies per cell (Figure 5D). The most abundant one is the solute carrier family 4, member 1/band 3 anion exchange protein (SLC4A1/AE1) present in >1 000 000 copies per RBCs, whereas the glucose transporter SLC2A1 occurs at about 200 000 copies. Proteins involved in transport of water, urea, ammonia, nucleosides, calcium, and iron exist at 20 000–60 000 copies. The Na⁺/K⁺ ATPase and the K⁺ channel *Gardos* are present at 3000–5000 copies. Other transporter proteins, often without known specificity, exist at fewer than 1000 copies. Some of these transporters, including the drug transporter, ATP-binding-cassette (ABC) subfamily A, member 7 (ABCA7) and the choline transporters, solute carrier family 44, members 1 and 2 (SLC44A1 and SLC44A2), have not previously been reported for RBCs.

In addition to the well known structural and transport proteins of the plasma membrane, our analyses mapped many other proteins with predicted transmembrane domains (Table S3). For example, 16 proteins with names “Transmembrane protein *number*” (TMEM*number*) and 7 “Transmembrane emp24 domain-containing” proteins were found at 100–8000 copies. Most of these have not been functionally characterized.

DISCUSSION

Proteomics Analysis

To our knowledge, this study provides the most comprehensive proteome analysis of the human erythrocyte so far. We provide absolute protein abundance estimations in RBCs and their WG fractions. To increase the depth of analysis and quantitation accuracy we applied three different protein cleavage strategies using the MED FASP format. We identified 2650 proteins, among them 1890 proteins occurring at more than 100 copies per cell. Notably, this study allowed identification of low abundant membrane transporters and other membrane proteins with unknown functions. Because our data are quantitative, the created database can serve to the community

as a useful tool for further studies on RBCs diseases and erythrocyte aging during storage in blood banks.³⁴

Protein Copy Numbers

The composition of the plasma membrane of human RBCs has been studied over the past several decades. Previously, copy numbers of membrane proteins were determined by classical and biochemical methods, such as radioimmunoassay^{6,7} and densitometry of Coomassie-stained polyacrylamide gels.⁴ Because all of these methodologies allowed only indirect measurements, the accuracy of the data appears to be moderate. For instance, the copy numbers reported by Anstee et al. for CD44 varied from 1888 to 10,450 between individual assays and antibodies used.⁶ Relatively high copy numbers for GYPA were derived from Scatchard plots,⁷ which are known to be prone to generating large errors.³⁵ Copy numbers assessed by Coomassie staining⁴ are biased by uneven binding of the stain to proteins varying in their amino acid composition. This phenomenon was not taken into consideration. In addition, many of these calculations assumed a standard protein content of 0.6 pg per single WG, an estimate provided by Dodge et al.,³⁶ but overlooked the fact that the total membrane protein content can vary between methods used for WG preparation.

Because quantitative analyses using only single enzymes for protein digestion may be heavily biased against proteins lacking specific cleavage sites, in this study we applied three different protein digestion strategies. The protein abundance data reported here were calculated by averaging the TPA values obtained from the three different protein digests. In the present study copy number values were computed using the TPA approach. The copy numbers calculated by TPA showed excellent correlation with the literature data.²² Accuracy of the approach was validated by comparing with data from targeted approaches³⁷ and demonstrated that on average, the deviation between values obtained by TPA and targeted proteomics was 1.5 fold. We provided a global proteome quantification; for many proteins the abundance values reported here are only estimates. Nonetheless the copy number values attained for the RBCs membrane correlate well with the literature (Figure S5B). Although for a few proteins, such as GYPA and STOM, the TPA and previous reports were quite different, targeted analysis showed that the values were close to the TPA copy number estimates. Similarly, the TPA values for GLUT1 copy number agreed well with the labeled standard-based quantification.

Membrane Transporters

Membrane transporters are of pivotal importance for cell homeostasis and play a crucial role in diseases. This study is the first to provide comprehensive and quantitative data set of membrane transporter proteins in the RBCs membrane.

We identified eight members of the ABC transporters family (ABCA7, ABCB6, ABCC1, ABCC4, ABCC5, ABCE1, ABCF1, ABCG2). Some of them had already been identified in previous nonproteomics studies.³⁸ ABCB6 and ABCB10 were assigned to mitochondria, although it has been recently shown that ABCB6 is secreted in exosomes, and resides on the erythrocyte cell surface.³⁸ ABCB6 is functionally relevant in the plasma membrane, where its expression prevents the accumulation of specific porphyrins in the cell.³⁹ We found ABCB6 at 10 000–20 000 copies in RBCs, whereas in human hepatocytes and cultured hepatoma cells this protein occurs at 50 000–75 000 copies.³¹ This three- to five-fold difference may be attributed to the fact that due to their function the RBCs require an efficient porphyrin clearance system.

The presence of human multidrug resistance protein 1 (MRP1/ABCC1) in the human erythrocyte membrane is well established.⁴⁰ Recently, the indirect evidence suggesting that ABCC4 (MRP4) and ABCC5 (MRP5) contribute to cGMP transport in RBCs was confirmed with a new inhibitor of ABCC4 transport.⁴¹ In another study, immunoprecipitation of ABCC5 from CHAPS-solubilized extracts reduced active transport by ~45%, showing that MRP5 is an important cGMP-transporting protein in human erythrocytes.⁴² We provided the first evidence of the presence of the ABCA7 transporter on the red blood cell membrane. The potential roles of this protein, such as binding of APOA1 and phospholipid efflux from cells,⁴³ have to be further investigated in the context of the red blood cell membrane.

Loss of some membrane surface results in hereditary spherocytosis syndrome manifesting as hemolytic anemia,⁴⁴ which may be attributed not only to the qualitative but also quantitative changes of the RBC membrane proteins. For example, absence of band 3 causes severe hereditary spherocytosis,⁴⁵ whereas a decrease in human erythroid protein 4.1R may result in hereditary elliptocytosis.⁴⁶ SDS–polyacrylamide gel electrophoresis (PAGE) is the technique recommended by the International Council for Standardization in Haematology for the quantitation of relative membrane protein contents in diseases such as hereditary elliptocytosis.⁴⁷ Currently, there is an increasing need for a precise tool for the detection of copy numbers of red blood cell membrane proteins, also in relation to such diseases as Alzheimer's disease or cancer. Data obtained by flow cytometry indicate that in late onset Alzheimer disease, the GLUT1, ABCA1, and the ABCG2 transporters are significantly increased in comparison with age-matched control subjects.⁴⁸ Because ABCG2 polymorphisms are known to modify essential pharmacokinetic parameters, uric acid metabolism, and cancer drug resistance, a direct determination of the erythrocyte membrane ABCG2 protein expression may provide valuable information for assessing these conditions or for devising drug treatments.⁴⁹

■ ASSOCIATED CONTENT

§ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.7b00025.

Figure S1. Western blot analysis of selected proteins. (PDF)

Table S1. Stable isotope labeled peptide based protein quantification. Table S2. Enrichment of membrane proteins in the white ghost fraction. Table S3. List of identified proteins and their concentrations in the whole red cells and white ghosts. (XLSX)

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Author Contributions

A.B. and J.R.W. designed the research, performed experiments, analyzed results, and wrote the paper.

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Notes

The authors declare no competing financial interest.

The mass spectrometry data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository²¹ with the data set identifier: PXD005200.

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Exhibit E

Plasma Proteome Database as a resource for proteomics research: 2014 update

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ABSTRACT

Plasma Proteome Database (PPD; <http://www.plasmaproteomedatabase.org/>) was initially described in the year 2005 as a part of Human Proteome Organization's (HUPO's) pilot initiative on Human Plasma Proteome Project. Since then, improvements in proteomic technologies and increased throughput have led to identification of a large number of novel plasma proteins. To keep up with this increase in data, we have significantly enriched the proteomic information in PPD. This

database currently contains information on 10546 proteins detected in serum/plasma of which 3784 have been reported in two or more studies. The latest version of the database also incorporates mass spectrometry-derived data including experimentally verified proteotypic peptides used for multiple reaction monitoring assays. Other novel features include published plasma/serum concentrations for 1278 proteins along with a separate category of plasma-derived extracellular vesicle proteins. As plasma proteins have become a major

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thrust in the field of biomarkers, we have enabled a batch-based query designated Plasma Proteome Explorer, which will permit the users in screening a list of proteins or peptides against known plasma proteins to assess novelty of their data set. We believe that PPD will facilitate both clinical and basic research by serving as a comprehensive reference of plasma proteins in humans and accelerate biomarker discovery and translation efforts.

INTRODUCTION

Plasma proteome represents an important subproteome, as it harbors proteins secreted from almost all tissues (1,2). In addition to classical blood proteins, plasma contains proteins secreted by various cells, glands and tissues along with proteins derived from commensal and infectious organisms and parasites residing inside the body (1). The plasma proteome comprises 22 highly abundant proteins including albumin, immunoglobulins, transferrin and haptoglobin, which make up 99% of total protein abundance in plasma. The remaining fraction is composed of proteins of much lower abundance including proteolytically cleaved protein fragments (3). This wide dynamic range of protein abundance, greater than 10 orders of magnitude, makes plasma proteome a challenging proteome to analyze. Plasma proteins also undergo various types of post-translational modifications such as glycosylation, which add to its complexity (4,5). An additional level of heterogeneity is brought about by inter- and intra-individual variation due to age, gender and genetic factors. All these factors make plasma the most complex and diverse body fluid, and its analysis poses challenge for the proteomic community.

Despite the analysis of plasma not being straightforward from an analytical standpoint, it is the most investigated body fluid in clinical diagnostics. Components of plasma including circulating tumor cells, cell-free RNA and DNA, metabolites, electrolytes and proteins are all considered as molecular markers for early detection of diseases, disease monitoring and prognosis (6–9). When compared with other body fluids such as cerebrospinal fluid, gastric juice, bile and synovial fluid, plasma is more readily accessible and requires a simple collection procedure (1,10,11). Thus, detection and quantitation of endogenous or foreign antigens or antibodies directed against these antigens in plasma could be used to determine the physiological and pathological states of an individual (4). Several plasma or serum proteins have already been identified as potential biomarkers of diseases including cardiovascular diseases, autoimmune diseases, infectious diseases and neurological disorders (12–19).

Owing to the importance of plasma proteins, several proteomic efforts have been carried out to explore human plasma proteins. In 2002, Anderson and Anderson compiled immunoassay- and 2D gel electrophoresis-based investigations of plasma proteome and reported the presence of 289 proteins (1). Adkins *et al.* used two different separation techniques followed by mass spectrometry (MS) for characterization of proteins

from depleted serum and reported 490 proteins (20). Human Proteome Organization (HUPRO) initiated a pilot phase of a major community initiative—the Human Plasma Proteome Project (HPPP)—in 2002 to determine the human plasma or serum protein constituents (21). With the involvement of 35 laboratories across the globe, this led to the identification of 3020 plasma proteins (22). As a part of this initiative, we developed a web-based resource called the Plasma Proteome Database (PPD) (23). Recent incorporation of depletion strategies to remove high-abundance proteins and multiple fractionation strategies coupled to LC-MS/MS approaches and high-resolution MS have resulted in a substantial increase in the number of proteins identified from plasma. For example, a study by Liu *et al.* coupled two different fractionation methods to MS and identified 9087 proteins in plasma, which is the largest data set on plasma proteins reported thus far (24). Farrah *et al.* have analyzed raw MS/MS data from plasma/serum, submitted to the Proteomics Identifications Database (PRIDE) and Human Plasma PeptideAtlas using Trans-Proteomic Pipeline and reported a set of high-confidence 1929 proteins (25–27). We systematically documented these newly described human plasma proteins and made them available for the biomedical community through the updated version of PPD.

RESULTS

Documentation of plasma proteins in PPD

Plasma proteome occupies an important niche at the interface of proteomics, diagnostics and medicine. To enable sharing of data across laboratories involved in HPPP, we developed the PPD as a web-based resource for plasma proteins (23). To keep up with exponential increase in plasma proteome data published in recent times, we systematically documented newly described human plasma proteins and curated information pertaining to them in PPD. For this, as a first step, we carried out a PubMed-based literature by searching for groups that have extensively contributed to plasma or serum proteomics. Additional PubMed searches were carried out to fetch scientific articles by using keywords pertaining to plasma proteins. The information from articles was annotated in PPD by experienced researchers at the Institute of Bioinformatics (IOB) along with clinical investigators from collaborating clinical centers in India. Currently, PPD contains 10 546 proteins linked to 509 scientific articles. However, as experimental data were obtained from diverse experimental platforms and published in the form of peer-reviewed research articles, we did not attempt any *post hoc* determination of any false positives in the database. However, as an alternative, to increase the confidence of identification of proteins in plasma or serum, we have included the number of articles reporting the detection of each protein in plasma or serum. This is listed as ‘Total number of studies’ under ‘Experimental evidence’ in the molecule page of respective protein as shown in Figure 1. In this database, the detection of 12 proteins in plasma is supported by >50 publications and

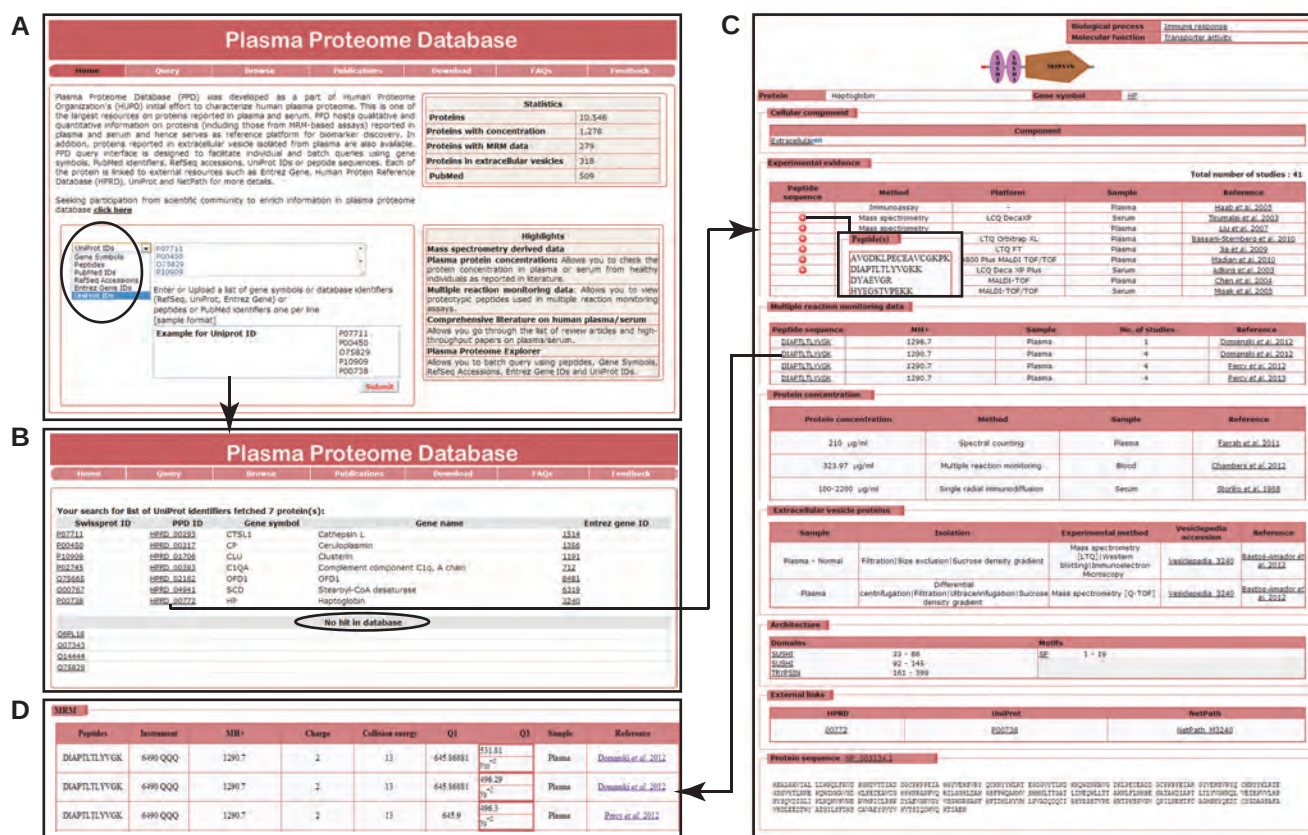


Figure 1. Data analysis workflow using Plasma Proteome Explorer and a screenshot of a 'Molecule Page' for Haptoglobin in PPD. When queried using UniProt IDs (as shown in A), the plasma proteome explorer displays two different type of results (shown in B). These correspond to (i) proteins present in PPD, which are hyperlinked to their corresponding molecule pages and (ii) proteins not present in PPD, which are linked to an external database, UniProt. Clicking the molecule leads the user to the respective molecule page (shown in C). The graphical representation shows domains and motifs found in the protein. The molecule page also displays the alternate localization of protein, the associated biological process and molecular function of the protein. In addition, the plasma concentration reported in healthy individuals along with corresponding PubMed identifiers and any other information regarding presence in plasma EVs is displayed. Further, MRM data is provided with information on proteotypic peptides, peptide m/z values, charge, collision energy, transitions determined, type of fragment ions and the mass spectrometer used along with a link to the corresponding publication (shown in D).

that of 167 proteins is supported by >10 publications. Of the remaining 10 367 proteins, 2199 are supported by two publications, whereas 6762 proteins are supported by a single publication. A histogram depicting the distribution of proteins with corresponding number of publications is shown Figure 2. In the current update, we have included peptide data for proteins identified from MS-based studies, which is one of the newly added features of PPD. A comparison of previous and current versions of PPD in Table 1 shows a 3-fold increase of protein information in this 2014 update. From our analysis, we observed that 4668 and 1972 proteins were unique to PPD when compared with data from other publicly available data resources on plasma or serum proteins—the Sys-BodyFluid and Healthy Human Individual's Integrated Plasma Proteome (HIP2), respectively (28,29). The data present in the Sys-BodyFluid database were annotated from 15 articles, whereas the HIP2 database contained plasma proteome data cataloged from 4 articles. In contrast to other databases that provide plasma/serum protein catalogs generated from a limited number of studies, PPD includes data annotated from 509 peer-reviewed publications.

In addition to documentation of plasma proteins and peptide information, for each protein in PPD, we have provided external links to Human Protein Reference Database (HPRD) (30), NetPath (31), Entrez Gene (32) and UniProt (33) (Figure 1). For the benefit of users, a collection of important published research articles and reviews describing high-throughput investigations on plasma/serum proteome has been provided. We are seeking participation of the scientific community by inviting investigators to submit relevant articles for inclusion in PPD and have created a new portal in PPD to submit such articles. To facilitate engagement with the community, there is a 'feedback' option, which allows users to submit their comments or suggestions. Finally, we have provided an option to download PPD data in XML and Microsoft Excel formats (<http://www.plasmaproteomedatabase.org/download>).

Plasma concentration of proteins

An important step in plasma biomarker discovery is the relative quantification of proteins in healthy and disease conditions. Determination of relative and absolute

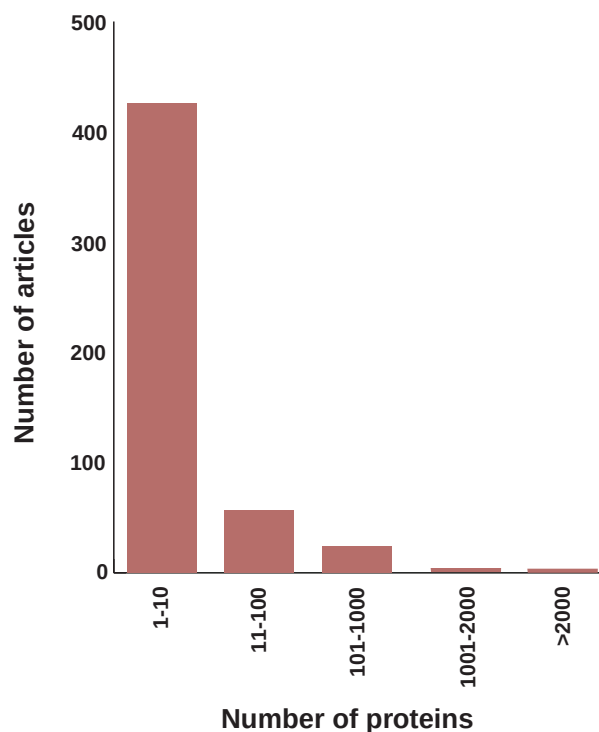


Figure 2. Distribution of proteins annotated in PPD according to number of corresponding publications. The histogram shows that a high number of proteins (8793 proteins) in plasma have been reported only in a single study. Of the 509 articles annotated, 426 were found to support the presence of ≤ 10 proteins in plasma or serum.

Table 1. A comparison of data in the current version of PPD with the initial database in 2005

Data Features	PPD-2005	PPD-2014	Number of articles annotated in the current update
Total number of proteins	3778	10 546	509
Total number of proteins with plasma levels	Not annotated	1278	276
Total number of proteins derived from plasma extracellular vesicles	Not annotated	318	9
Total number of proteins with MRM data	Not included	279	24

concentration of plasma proteins is helpful for monitoring disease predisposition, onset, diagnosis and progression. Several studies have been carried out in this direction. An early effort was reported by Anderson and Anderson in 2002, where they cataloged plasma concentration range for 70 proteins from published literature (1). Subsequently in 2005 and 2008, their group documented plasma levels for 177 and 211 proteins, respectively, which were reported to be associated with cardiovascular disease, stroke and cancer based on literature evidence (34,35). Recently, Farrah *et al.* provided an estimate for plasma levels for 1200 proteins based on spectral counting (26).

We have incorporated plasma protein levels in this update of PPD and documented plasma protein concentrations for 1278 proteins from 276 articles. Availability of this information will be useful for both clinicians and researchers alike. To document plasma protein levels, we searched PubMed using Gene symbols OR Protein name OR alternate name(s) of the protein AND Plasma level* OR Serum level* as queries. We engaged four medical students (S.K., B.N.J., K.V.S. and R.K.K., who are also co-authors) to document the plasma protein levels in normal individuals from the literature. The plasma protein concentration data in PPD can be browsed as shown in Figure 1 along with the method used for the measurement in each case. We also developed a web-based submission portal (http://www.plasmaproteomedatabase.org/protein_concentration) for submission of data pertaining to protein concentration in plasma/serum by the clinical/analytical community. The researchers can submit the information obtained from their study through this portal, and the information will be processed in PPD along with the source of the information. The 'Plasma protein concentration data' file contains a list of plasma proteins sorted based on their concentration in plasma/serum. This file can be downloaded at <http://www.plasmaproteomedatabase.org/download>.

Incorporation of data from multiple reaction monitoring experiments

Traditionally, enzyme-linked immunosorbent assay and western blotting have been used for protein identification and quantitation in plasma. The success of these conventional methods relies heavily on the availability of specific antibodies against each protein. These methods also provide limited throughput, as each assay can monitor one protein at a time. Recent advances in MS have enabled simultaneous identification and quantitation of hundreds of proteins in plasma in a single experiment (36–38). Targeted approaches like multiple reaction monitoring (MRM) have enabled quantitation of plasma proteins with high specificity as well as increased throughput (36,38).

MRM assays use multiple parameters to identify and quantify specific protein/s in complex samples. Proteotypic peptides that uniquely represent a protein are first selected, and assays are developed, which take into account the retention time of the peptides, their m/z values and measurement of specific fragment ions (39). This multi-step process provides superior specificity and allows for accurate quantification of protein levels over a wide dynamic range of abundance (36,40). MRM analysis has thus revolutionized biomarker research and is being widely used to determine altered protein levels across various disease phenotypes. A compilation of proteotypic peptides of plasma proteins that generate good signals in MS experiments along with their transitions will be immensely beneficial in developing MRM assays. In PPD, we have annotated 591 reported peptides corresponding to 279 proteins for MRM analysis. This includes details of precursor m/z, fragment ion m/z, collision energy, charge

of the precursor ion and instrument used for the analysis (Figure 1).

Proteins identified from extracellular vesicles in plasma

Extracellular vesicles (EVs) are membranous sacs released by both normal and diseased cell types and are also reported in body fluids such as blood, urine, saliva and synovial fluid (41–44). EVs include ectosomes, exosomes and apoptotic bodies. They facilitate communication between cells by transfer of membrane and cytosolic proteins and are also shown to play role in mediating immune response and antigen presentation (44). EVs are shown to be present in plasma from healthy and certain cancer patients including glioblastoma and colon cancer (45,46). Therefore, identification and characterization of EVs in plasma should aid in disease diagnosis and discovery of biomarkers. Considering the importance of EVs, we have curated and included plasma EV proteins in PPD. There are 318 EV proteins annotated in PPD and each is linked to Exocarta and Vesiclepedia, both manually curated resources for proteins, messenger RNA, micro RNA and lipids reported from EVs including exosomes (42,43).

Plasma Proteome Explorer—a batch query interface in PPD

We have enabled a batch query utility called ‘Plasma Proteome Explorer’, which allows users to query the plasma proteome by providing a list of peptides, gene symbols, Entrez Gene IDs, RefSeq accessions or UniProt IDs. Data analysis workflow of plasma proteome explorer is represented in Figure 1. The results obtained are displayed in a web page with following features—PPD ID, which are hyperlinked to PPD ‘molecule page’, gene symbol and Entrez Gene ID.

Plasma proteome explorer will enable biologists with limited bioinformatics skills to compare their own experimental data set with that of plasma proteome. In a single step, users can differentiate between novel plasma proteins, novel MS-derived peptides detected from plasma, known plasma proteins and peptides. This will enable researchers in biomarker development to screen candidate molecules that can be detected in plasma, from a larger set of candidate protein biomarkers identified in the discovery phase to pursue further for validation. We believe that the plasma proteome explorer will occupy the intermediate niche between ‘biomarker discovery’ and ‘biomarker validation’ phases. We have enabled a separate query tab, which allows querying of proteins based on protein name, gene symbol, Entrez Gene ID, UniProt ID, sample type, experimental method and MS-based platform. In addition, search by protein sequence can be carried out in PPD. The ‘Browse’ option allows the user to pursue proteins categorized on the basis of their protein name, biological process, molecular function, proteins with information on MRM data and plasma exosomal proteins.

CONCLUSIONS

The main goal of the PPD is to foster research in the area of clinical proteomics, especially the discovery of plasma biomarkers, to diagnose and monitor human diseases. We have introduced a number of new features to expand the utility of this database, particularly in the field of biomarker discovery and validation. Plasma proteome explorer will prove useful for selecting secreted candidate biomarkers from a larger set of differentially expressed genes/proteins in any disease or perturbed biological conditions. As the database develops, we foresee the need for the incorporation of information on disease association and availability of reagents such as enzyme-linked immunosorbent assay kits. We anticipate that PPD will provide a platform to bring together the communities of biomedical researchers engaged in biomarker discovery and validation along with proteomic researchers developing analytical solutions to overcome problems in plasma proteome research. We foresee that PPD will serve as a centralized reference repository for such a community approach.

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Exhibit F

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Review

Sample preparation and fractionation for proteome analysis and cancer biomarker discovery by mass spectrometry

Sample preparation and fractionation technologies are one of the most crucial processes in proteomic analysis and biomarker discovery in solubilized samples. Chromatographic or electrophoretic proteomic technologies are also available for separation of cellular protein components. There are, however, considerable limitations in currently available proteomic technologies as none of them allows for the analysis of the entire proteome in a simple step because of the large number of peptides, and because of the wide concentration dynamic range of the proteome in clinical blood samples. The results of any undertaken experiment depend on the condition of the starting material. Therefore, proper experimental design and pertinent sample preparation is essential to obtain meaningful results, particularly in comparative clinical proteomics in which one is looking for minor differences between experimental (diseased) and control (nondiseased) samples. This review discusses problems associated with general and specialized strategies of sample preparation and fractionation, dealing with samples that are solution or suspension, in a frozen tissue state, or formalin-preserved tissue archival samples, and illustrates how sample processing might influence detection with mass spectrometric techniques. Strategies that dramatically improve the potential for cancer biomarker discovery in minimally invasive, blood-collected human samples are also presented.

Keywords: Antibodies / Diagnoses / Plasma / Serum / Urine

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1 Introduction

Proteomics incorporates the study of expression patterns, molecular interactions and functional states of proteins present in a cell, organ, or an organism under consideration. Although DNA may provide the blueprint for cellular functions and development, it is proteins that are the bricks and mortar from which life is built, and they are the molecules that perform the functions and dynamic processes that ultimately determine the phenotype of the cell. Cellular messenger ribonucleic acid (mRNA) expression levels may not necessarily be cor-

related directly to protein expression levels; therefore, monitoring the protein changes of cells is a more meaningful measurement parameter of cellular homeostasis. Minor changes in protein expression may be responsible for altering protein cascades, which may eventually lead to transformation and uncontrolled growth of cells. Furthermore, mutations may lead to processes that allow the cell to become tumorigenic and ultimately metastatic. Since proteins are the agents that control these processes, it is important to understand the changes in global protein expression patterns in order to be able to identify proteomic biomarkers that are involved in various cellular functions, differentiation, regulation, proliferation, and cancer progression [1].

The availability of global profiling strategies combining genomic sequencing with powerful high-throughput (HT) screening methods that provide high resolution has facilitated the comprehensive quantitative identification, relative abundance levels, and post-translational modification (PTM) states of proteins across various tissues and cellular fractions in a systemic manner [2]. However, the understanding of the diverse structural charac-

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Abbreviations: Abs, antibodies; FFPE, formalin-fixed and paraffin-embedded; HT, high-throughput; LAP, low abundant protein; mRNA, messenger ribonucleic acid; PCT, pressure cycling technology; PM, plasma membranes; PTM, post-translational modification; SELDI, surface-enhanced laser desorption ionization

teristics and interactions of the dynamic proteins represent a significantly greater analytical challenge than that posed by the static nucleic acid analysis required for deciphering the genome sequence. Elucidation of large-scale perturbations resulting from pathological processes to proteomic patterns provides insight as to the reason of such changes, as well as offering the potential of discovery of clinically relevant proteomic biomarkers of disease states that have substantial diagnostic, prognostic and/or therapeutic potential or disease recurrence, as well as facilitating risk assessment modalities [3].

The best strategy for sample preparation would be no sample preparation at all; however, the complexity of the dynamic proteome far exceeds the capacity of the currently available analytical systems. In higher animals, and plants, proteomic methods employing mass spectrometers do not presently – nor expected in the immediate future – define more than a small portion of a proteome on a routine HT basis. This limitation precludes analysis in a simple one-step process of the whole proteome of any complicated eukaryotic organism. Therefore, all proteomic methods are *a priori* targeted for simplification by fractionation and separation steps in some way either intentionally, or by the limitation of the analytical method employed to explore specific structural functions of interest using either chromatography, or other methods. Lacking a better alternative, therefore, this current prevailing approach simplifies the proteome while preserving most of the vital information essential for a meaningful analysis by today's methods [4].

It is generally assumed that in order to provide adequate analysis, the analytical method needs to include the following steps: (a) sampling, in which the sample is a good statistical representation of the investigated population; (b) specimen preservation, during which the sample is expected to be kept stable until the analysis is completed; (c) appropriate sample preparation; and (d) statistical analysis and bioinformatics data treatment. A major bottleneck of this analytical process lies in the sample preparation step, as it is often a time-consuming, and a laborious step. The purpose of any sample preparation technique is the cleanup of the sample and/or the extraction, enrichment or preconcentration of the analytes in order to improve the quality of the analysis. However, it is important to consider that any sample treatment depends on both the nature of the sample as well as the analytical technique that will follow, which in case of current proteomics will always require a case-by-case development, as no universal preparation technique is currently applicable to all proteomic samples [5].

Ideally, sample preparation should be as simple as possible in order to reduce time and avoid introduction of steps that could lead to sample loss, while eliminating interferences from the matrix (tissue). Moreover, it

should preferably include, if needed, a dilution or a concentration step in order to obtain an analyte at a concentration that is optimal for further MS analysis. Furthermore, it may be necessary, sometimes, to transform the analyte into a different chemical form in order to facilitate either the separation or detection of the protein under consideration [5].

2 Strategies for sample preparation

2.1 Approaches relying on physicochemical characteristics

Approaches relying on physicochemical characteristics for sample purification and enrichment prior to protein analysis are widespread as discussed below.

2.1.1 Tissue disruption and cell lysis by homogenization

Homogenization is one of the preparation steps employed for preparation of biological samples for proteomic analysis, and includes such processes as mixing, stirring, dispersing, or emulsifying in order to change sample's physical, but not chemical properties. Homogenization for proteomics incorporates five main categories: mechanical, ultrasonic, pressure, freeze-thaw, and osmotic/detergent lyses as detailed below.

Mechanical homogenization for tissues and cells can be accomplished by devices such as rotor–stator, and open blade mills (e.g., Waring blender and Polytron), or pressure cycling technology (PCT) such as French presses [6, 7]. Rotor–stator homogenizers can homogenize samples in volumes from 0.01 mL to ~20 L depending on the tip and motor used. For optimum results, the tissue should be cut into slices, the size of which is slightly smaller than the diameter of the applied stator, as larger samples may clog generator's inlet, making it impossible to achieve effective homogenization [8]. Depending on the chemical resistance of a cutting tool, it is possible to homogenize samples under acidic or basic conditions in order to prevent degradation by endogenous enzymes [9]. Heat transfer to processed mixture is low to moderate and the process usually requires external cooling. Sample loss is minimal compared to PCT, where by means of a pressure-generating instrument (Pressure Bioscience, West Bridgewater, MA) alternating cycles of high and low pressure are applied to induce cell lysis [8]. Pressure homogenizer is effective for homogenizing eukaryotic cells as well as microorganisms in suspension. The PCT method was assessed in *Escherichia coli* cell suspensions with improved protein solubilization and electrophilic resolution [10]. It was also employed for fast purification of proteins from cells in culture [11], although molecules such as mRNA were lost [12]. The PCT is ineffective toward tissues or organs without previous preparation

in any other type of homogenizer [8]. Liquid homogenization using Dounce homogenizer and Potter–Elvehjem homogenizer at low temperatures has been successfully used [13]. Blenders can be used to homogenize in either dry, or liquefied samples [8].

Mechanical homogenization may result in loss of activity of some proteins that are sensitive to heating and cooling during the process as was observed when dispersing human breast cancer tissue and calf uterus, resulting in rapid denaturation of the estrogen, and progesterone receptors; in such a case, use of detergent lyses at low temperature did not result in a significant loss of biological activity [14]. In case of nonsatisfactory results, another type of homogenizers (*i.e.*, ultrasonic) could be employed to achieve an optimal homogeneity [15].

Ultrasonic homogenizers (also known as disintegrators or sonicators) are based on the piezoelectric effect that generates high-energy ultrasonic wave resolved after explosion/implosion of gas microbubbles, while interacting with the sample, to effectively destroy solid particles as well as cells. Although, this type of homogenization did not affect enzymatic activity of 13 investigated enzymes in leukocytes [16], the procedure may, however, lead to the disruption of noncovalently bound molecular clusters such as multienzyme complexes [17]. Ultrasonic devices are mostly effective to homogenize small pieces of soft tissues, as tough and dense tissues are not suitably homogenized by this method [8].

Freeze-thaw homogenization uses the destructive effect of ice crystal formation during the freezing process. The method is relatively fast, effective, and does not introduce external impurities into the sample because no frozen solution comes from an external environment. This method is effective toward most of the bacteria, plant and animal cells in water solution, and may be used as an additional or a final step after mechanical or ultrasound homogenization [8], although some microbial cells preconditioned in starvation media, such as *Vibrio parahaemolyticus*, are resistant to this method [18]. Moreover, the possibility of inducing changes in activity or properties of bioactive molecules (*e.g.*, enzymes and membranes) after few freeze-thaw cycles was noticed in case of G-protein coupled receptor kinases, β -arrestins [19].

Osmotic and detergent disruption of cells' walls and membranes of erythrocytes as well as homogenization of nuclear and mitochondrial membranes in cell extracts was reported. It was found useful for RNA extraction from the bacterium *Brucella abortus* internalized in macrophages [20], or for determining survival of *Staphylococcus aureus* after phagocytosis by human granulocytes [21]. Osmotic lyses were reported for microbial cell disruption, as in *Staphylococci*, after addition of lysostaphin to a hypertonic solution [22], or addition of lysozyme to the buffer in case of *Pseudomonas* species and other bacteria

[23]. The most commonly used detergents are Triton X-100, Tween 80, NP 40, and Saponin [8].

2.1.2 Protein solubilization

Proteins in biological samples are generally found in their native state associated with other proteins and often integrated as a part of large complexes, or into membranes. Once isolated, proteins in their native state are often insoluble. Breaking interactions involved in protein aggregation (*e.g.*, disulfide hydrogen bonds, van der Waals forces, ionic and hydrophobic interactions) enables disruption of proteins into a solution of individual polypeptides, thereby promoting their solubilization [24]. However, because of the great heterogeneity of proteins and sample-source related interfering contaminants in biological extracts, simultaneous solubilization of all proteins remains a challenge. Integration of proteins into membranes, and their association and complex formation with other proteins and/or nucleic acids hamper the process significantly. No single solubilization approach is suitable for every purpose, and each sample and condition requires a unique treatment. Sample solubilization can be improved by agitation or ultrasonification, but an increase in temperature must be avoided. The selection of the appropriate solubilization protocol and buffers has especially been facilitated by the availability of commercial kits [13], although it is somewhat more expensive than routine reagent methods.

Detergents within a 1–4% concentration range, to prevent hydrophobic interactions, are used to solubilize proteins. In sample preparation for 2-DE, only neutral (octylglucoside, dodecyl maltoside, Triton X-100), or zwitterionic (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate (CHAPS), CHAPSO, SB 3-10, SB 3-12, and ABS-14) detergents are used due to their compatibility with the separation mechanisms. The anionic detergent SDS improves solubilization but interferes with the first dimension separation, and must be removed if present in the preparation; commercial kits are available for this purpose (*e.g.*, ETTan™ 2D Clean Up kit, GE Health Care; ProteoSpin™ Detergent Clean UP micro and maxi kits, Norgen Biotek Corporation) [25].

To avoid protein modifications, aggregation, or precipitation resulting in occurrence of artifacts and subsequent protein loss, sample solubilization process necessitates the use in sample buffer of: (a) chaotropes (urea, thiourea, charged guanidine hydrochloride, GdnHCl) that disrupt hydrogen bonds and hydrophilic interactions enabling proteins to unfold with ionizable groups exposed to solution; (b) ionic, nonionic and zwitterionic detergents (SDS, CHAPS, or Triton X-100); (c) reducing agents that disrupt bonds between cysteine residues and thus promote unfolding of proteins (DTT/dithioerythritol (DTT/DTE) or tributylphosphine (TBP) or tris-carboxy-

ethyl phosphine (TCEP)); and (d) protease inhibitors [26]. In the presence of chaotropes, proteins are denatured and they easily aggregate and precipitate. The presence of urea and thiourea, and detergents helps maintain them in solution. In the past, urea at concentrations of 8–9 M was solely used, but it was found that the number of proteins solubilized increases when thiourea at a concentration of 2 M was added to 5–9 M urea. Heating of urea and thiourea containing solutions must, however, be avoided to prevent their hydrolysis and undesirable side reactions. GdnHCl is not compatible with the IEF process [13]. After disulfide reduction with reducing agents, TBP or TCEP, it was found that in order to prevent further oxidation, the newly produced free sulfhydryl group (SH) needs to be protected by alkylation, such as by addition of iodoacetamide when 2-DE separation is employed, because proteins' electrical charge – and hence their *pI*s – are maintained using this reagent [13].

Although there is no general procedure to select an appropriate detergent, nonionic and zwitterionic detergents such as CHAPS and Triton X series are less denaturing than ionic detergents, and have been used to solubilize proteins for functional studies. On the other hand, ionic detergents are strong solubilizing agents that lead to protein denaturation. However, sodium cholate and deoxycholate are soft detergents compatible with native protein extraction, although variables like buffer composition, pH, salt concentration, temperature, and compatibility of the chosen detergent with the analytical MS procedure, and how to remove it (by dialysis for example) all are crucial factors that need to be considered. Usually, tissue disruption and cell lyses require the combination of detergent and mechanical methodologies [13]. The proper use of above reagents, together with optimized cell disruption method, dissolution, and concentration techniques collectively determines the effectiveness of proteome solubilization methodologies (<http://www.mnhn.fr/mnhn/by/eDMS/2DE.pdf>).

2.1.3 Protection from proteolysis

It is estimated that nearly 7000 proteases and their homologs defined in the human genome may be related to one of the metalloserine-, cysteine-, or aspartyl-protease groups. If not inhibited, liberated/activated endogenous proteases, during cell membrane disruption for example, could lead to enzymatic protein degradation producing artifacts that complicates further analysis [8]. Protein degradation could be minimized by quick and small-scale tissue extraction, boiling sample in SDS buffer with high-pH Tris base, or by lowering the pH and performing ice-cold (20% TCA) precipitation [27]. Alternatively, denaturation in boiling water, focused microwave irradiation, or use of organic solvents may be used [28]. While active in high concentration of urea, proteases may be effectively inhibited by the presence of thiourea

in the lyses solution [29]. Heat shock proteins (sHsps) were found to protect proteins *in vitro* from proteolytic degradation [30]. It has been recommended to add specific protease inhibitors such as PMSF, aminoethyl benzylsulfonyl fluoride (AEBSF), ethylene diamine tetra acetic acid (EDTA), pepstatin, benzamidine, leupeptin, aprotinin, or cocktails thereof with a broader activity spectrum during cell disruption and subsequent preparation [8].

Although most protein electrophoretic separations are carried out under denaturing conditions, sometimes it may be necessary to preserve the native protein conformation in order to obtain detailed information on protein function and their possible interactions. In such a case, separation under native conditions in 2-DE is imperative [25]. When IEF during 2-DE was carried out for protein extraction, degradation was considerably increased, which could be prevented by using cup loading to apply protein to the strip [31], and carefully apply protease inhibitors in the reswelling buffer as they may modify proteins, or introduce charge trains and adducts [32]. As for LC separation, protease inhibitors as GdnHCl have been added both in binding and elution buffers maintained at 0–4°C [33]. Common protease inhibitors include PMFS, AEBSF, EDTA, or ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetra acetic acid (EGTA), tosyl lysine chloromethyl ketone (TLCK), or tosyl phenyl chloromethyl ketone (TPCK) and benzamidine. Commercial preparations are also available such as Ettan Protease inhibitor mix (GE Health Care) and Halt Protease Inhibitor (Pierce) [13].

2.1.4 Removal of contaminants

Salts, buffers, detergents, nucleic acids, polysaccharides, lipids, and particulates frequently present in sample solutions often tend to interfere with protein separation steps, inhibit the digestion process, collide with MS analysis, or complicate statistical analysis; therefore, there is a need to remove these contaminants at a proper time during the analysis [34].

Salt migrates away from proteins during IES contributing to their precipitation and aggregation. Moreover, a high electric current carried by the salt load could interfere with electrophoretic separation of proteins and reduces the efficiency of 2-DE, and produces an elevation of temperature during IEF [13, 35]. Thus, if present in concentrations >100 mM, salts should be removed prior to IEF, although cup loading in 2-DE tolerates a slightly higher salt concentration. It is also possible to dilute sample below the critical concentration and apply larger sample volume on the IPG gel. Sample dilution is also recommended prior to CE provided that proteins of interest are available at detectable concentrations [36]. Salt removal is often accomplished *via* dialysis using either dialysis bags as in the traditional procedure, which is

time consuming, or employing spin microdialysis, ultrafiltration, gel filtration, precipitation with TCA or organic solvents, and SPE [8]. Other alternatives are the use of commercially available clean-up kits such as Ettan™ mini dialysis kits (GE Healthcare), Zeba Sephadex and microdesalt spin columns (Pierce), ProteoSpin™ CBED micro- and mini-kits (Norgen Biotech Corporation) [37].

The efficiency of four desalting procedures (desalting column packed with Sephadex G-100, on-target washing, centrifugal filter devices, and C₁₈ microcolumns) showed that for intact proteins, the best procedure was the application of C₁₈ microcolumns, pipette tips, and centrifugal filter devices [38]. A method for extraction of proteins from human body fluids (plasma, urine, amniotic fluid, and tears) employed the use of a centrifugal filter device and a sample buffer containing CHAPS for efficient lipid and salt removal [39]. Other techniques for salt removal included fast protein LC (FPLC), desalting columns, SPE, ultrafiltration or dialysis [32], or an automated HT nickel and glutathione disks [40] for protein purification.

The rupture of cellular structures after disruption of a cell provokes the liberation of hydrolytic enzymes, mainly proteases, which begin to exert their action with a slow kinetic. These enzymes are usually resistant to denaturation, although solubilization of protein in strong denaturing agents may prevent their action. Nevertheless, the use of protease inhibitors in the solubilization buffers has been essential in most preparations [13].

Most common detergent removal methods include dialysis, gel filtration chromatography, hydrophobic adsorption chromatography and protein precipitation. For detergents with high CMC and/or small aggregation number, dialysis is usually the preferred choice. For a wide spectrum of detergents present in the sample, gel filtration can be applied; however, it results in a considerable sample dilution [8]. Ion exchange chromatography effectively excludes nonionic and zwitterionic detergents, although it was also successfully applied for SDS removal [41]. SDS can also be effectively removed with nanoscale hydrophilic phase chromatography [42] or acetone precipitation, especially if carried out at –20°C. Ceramic hydroxyapatite (HAP) chromatography was developed for the complete removal of SDS bound to soluble or membrane proteins [43]. For zwittergent removal, equal efficacy of gel filtration chromatography and a detergent affinity bead chromatography column was reported, which was slightly better than dialysis. SPE was found efficient in CHAPS removal for dilute protein solutions than the standard dialysis or the gel filtration methods [44]. Commercially available detergent precipitation or gels effective for binding and removal milligram quantities of various detergents from protein solutions were employed (e.g., Extracti-Gel® D Detergent

Removing Gel, and the SDS-Out™ SDS Precipitation Reagent Kit, Pierce; Ettan Out 2D clean-up kit, GE Healthcare; Proteo Spin Out Detergent clean-up micro and mini kits, Norgen BioTek Corporation). Hydrophobic adsorption employing the use of insoluble resin (e.g., CALBIOSORB™, Calbiochem) has also been employed to remove excess detergents [13].

Lipids are widely present in biological fluids such as plasma complexed with proteins and this interaction reduces protein stability and might affect their pI and MW. In addition, these lipids can complex with detergents leading to reduction in protein enrichment and/or separation efficiency. In case of 2-DE separation, buffers – including CHAPS – were reported to remove lipids and salts efficiently [8]. Precipitation in acetone or combination of TCA/acetone also removed lipids efficiently, although lipids' elimination was more effective when they were associated with proteins in membranes [13]. Precipitation employing ACN supplemented with 1% trifluoroacetic acid (TFA) and 1% *n*-nonyl-β-Δ-glucopyranoside was helpful in dissolving membrane proteins and lipids [45].

Delipidation of human serum lipoprotein by use of RP C₁₈ SPE cartridges was reported to produce a higher, more reproducible, and a faster protein yield and desalting than commercial liquid–liquid methanol diethyl ether delipidation of lipoproteins for mass spectrometric analysis of the proteome [46].

The presence of polysaccharides, especially if they are charged, should be avoided because they can aggregate, clog the pores of polyacrylamide (PAC) gels in 2-DE leading to either precipitation or extended focusing time, complexing of proteins and/or horizontal streaking. Moreover, they bind positively charged proteins, and interfere with protein migration during electrophoresis [47]. The disturbances caused are similar to those produced by nucleic acids. Protein precipitation with TCA, acetone, ammonium sulfate, or phenol/ammonium acetate followed by ultracentrifugation was found to be effective in removal of polysaccharides. Commercial precipitation kits for removal of polysaccharides found in protein samples makes the procedure easier and faster [13].

Nucleic acids interfere with carrier ampholites and proteins, leading to poor recovery of DNA- or RNA-binding proteins; therefore, elimination of nucleic acids needs to be carried out by precipitation with TCA ultracentrifugation, or by digestion with protein-free DNase and RNase [26]. However, if DNases and RNases appear on 2-DE patterns, proteins tend to precipitate. Proteins associated with nucleic acids may be lost from the sample unless the nucleic acid fraction is extracted with a detergent cocktail [48]. Ultracentrifugation and addition of basic polyamine (e.g., spermine) was found effective in removal of large nucleic acids, as well as high MW pro-

teins. High ionic strength extraction and high pH extraction seemed to be potent in minimizing interactions between negatively charged nucleic acids and positively charged proteins [49]. A convenient alternative was reported that utilized QIASHredder (QIAGEN) and subsequent centrifugation [50].

To remove other contaminants such as small ionic molecules, nucleotide metabolites, phospholipids, either TCA/acetone precipitation or other salt-excluding techniques were effectively performed [51]. High-speed centrifugation is an alternative method [52]. Particulates must also be removed, as like nucleic acids they may plough the gel pores, which could be conveniently achieved by centrifugation [13].

2.1.5 Protein enrichment

Proteins concentration range in a sample such as blood is beyond the dynamic range of any single analytical sample. Therefore, prior to analysis, it is necessary to reduce sample's complexity by fractionation in order to enrich for proteins of interest (biomarkers in this review's context). During any enrichment process, conditions must be stable to avoid protein interactions among the rest of mixture components (*e.g.*, nonspecific interactions with other proteins). Prefractionation involves isolation of the sample into distinguishable fractions containing restricted numbers of molecules. This can be accomplished by many approaches including precipitation, centrifugation, LC, and electrophoresis-based methods, filtration, or equilibrium sedimentation. The selection of the technology strongly depends on the nature of the sample to be analyzed, the physicochemical properties of the proteins and their subcellular location, and the object of the study. It should be kept in mind, however, that there is no general enrichment protocol that exists for enriching low-abundant proteins (LAPs) [13, 25].

Selective precipitation employs acetone, TCA, ethanol, isopropanol, diethyl ether, chloroform/methanol, ammonium sulfate, PEG, and affinity precipitation [53, 54]. The precipitant ammonium sulfate causes protein destabilization, a phenomenon known as “salting out”. Addition of various organic solvents causes an increased attraction between particles of opposite charge in the sample solution leading to protein precipitation. Precipitate recovery relies on the sample redissolving in a smaller volume, followed by centrifugation or filtration. Immunoprecipitation is a more specialized approach that employs antibodies (Abs) selective for one or a group of proteins with similar epitopes (*e.g.*, phosphor or glycans) [55]. Separation of cell substructures can be accomplished by ultracentrifugation at different centrifugal forces in sucrose/mannitol gradient, which allows for separation of different cellular components (*e.g.*, membrane, mitochondrial, Golgi, nuclear, or other locally

abundant proteins) according to the density characteristics of the structure [56].

Electrophoretic enrichment methods employ gel separation in one- (1-DE) or two-dimensions (2-DE); the latter method enables simultaneous visualization of 100s of protein spots, their PTMs and quantification of protein levels, although separation of hydrophobic and membrane proteins, as well as alkaline and low molecular weight peptides poses severe limitations. Reproducibility of protein patterns between separate laboratories is difficult to standardize because of protocol variations, artifacts and technology limitations, as reviewed earlier [25].

IEF has emerged as a useful approach for protein prefractionation because of its high resolution, relatively short separation times, and its modest cost. Commercial isoelectric fractionation systems are available that perform focusing in solution-phase, off-gel and in-gel formats at the protein or peptide level. The nongel-based IEF methods have the advantage of convenience, as well as being able to accommodate large volumes, and thus are not limited by samples' amount [57].

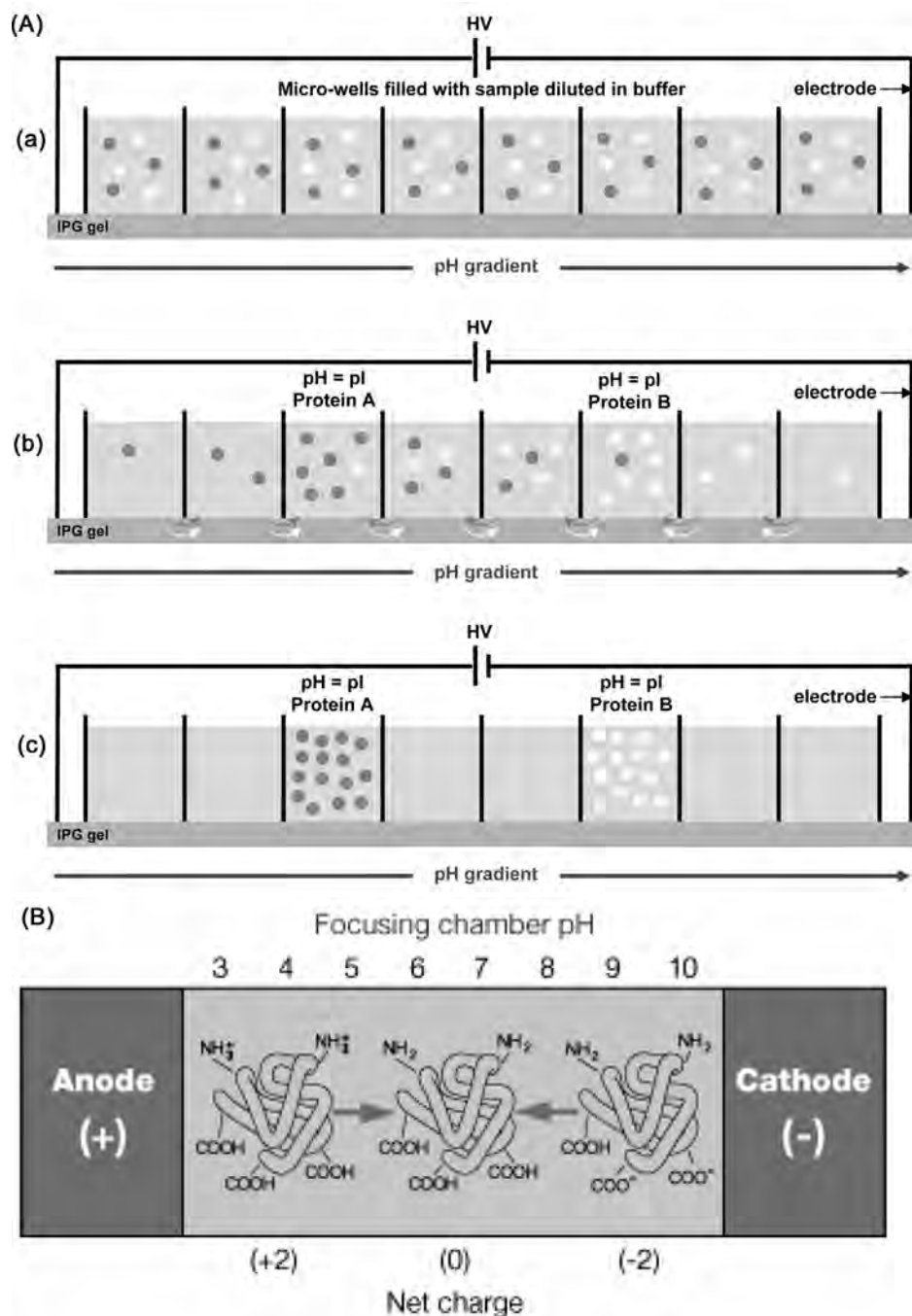
The OGEL fractionator from Agilent Technologies (Santa Clara, CA) uses a novel IEF method, which instead of performing focusing in free solution in the presence of carrier ampholytes, proteins and peptides are focused in an IPG strip, which is sealed against a multichamber frame containing sample and focusing solutions [58, 59] (Fig. 1A). During the separation, sample species migrate through the IPG gel and become focused according to their pIs. At the completion of focusing, proteins diffuse into the well adjacent to the section of the IPG strip within which they have been focused. This IEF approach reduces the risk of protein precipitation during focusing. For complex samples (plasma, serum, and cell or tissue lysates) total sample loads of 50 µg–5 mg are recommended. For simple mixtures, sample capacity is lower. The salt concentration of the sample should not exceed 10 mM. If startup voltage is low (below 100 V), this indicates that sample's salt concentration is too high. Typical focusing times are 12–24 h. At completion of focusing, the focused fractions could be recovered from each well *via* a pipette. This focusing permits harvesting a population of proteins having pI values precisely matching pH gradient of any IPG strip. Finally, due to the fact that only proteins cofocusing in the same IPG interval will be present, much higher sample loads can be accommodated, permitting detection of low-abundance proteins.

The first liquid-phase preparative IEF system (Rotofor) was introduced about 20 years ago by BioRad Laboratories (Hercules, CA) to fractionate proteins according to their pI, based upon the technology developed in Milan Bier Laboratory [60], and over the intervening two decades it has evolved into a family of systems ranging from the standard format to mini- and micro-formats. All three systems are multicompartiment electrolyzers that

share a common design in which the sample is separated into multiple fractions in a cylinder focusing chamber segmented into compartments by parallel monofilament polyester screens. The focusing chamber is connected to anolyte and catholyte chambers by cation and anion exchange membranes, respectively (Fig. 1B). These membranes isolate electrolytes from the sample in the focusing chamber, yet allow the current to flow for fractionation. The standard and mini Rotofor focusing chamber contains 20 compartments with 19 screens, while the microversion contains 10 compartments with 9 screens.

All three systems provide for cooling to dissipate heat during focusing. Run time for all three systems is typically 3 h. At the completion of fractionation, focused samples are harvested by penetration of the collection port seal by an array of needles, with vacuum-assisted transfer to collection tubes in the external harvesting station (standard and mini Rotofors), or to the internal collection tray (micro Rotofor).

A microscale liquid phase IEF fractionator (MicroSol-IEF) or the ZOOM IEF fractionator from Invitrogen Corporation (Carlsbad, CA) is based upon the method of Zou



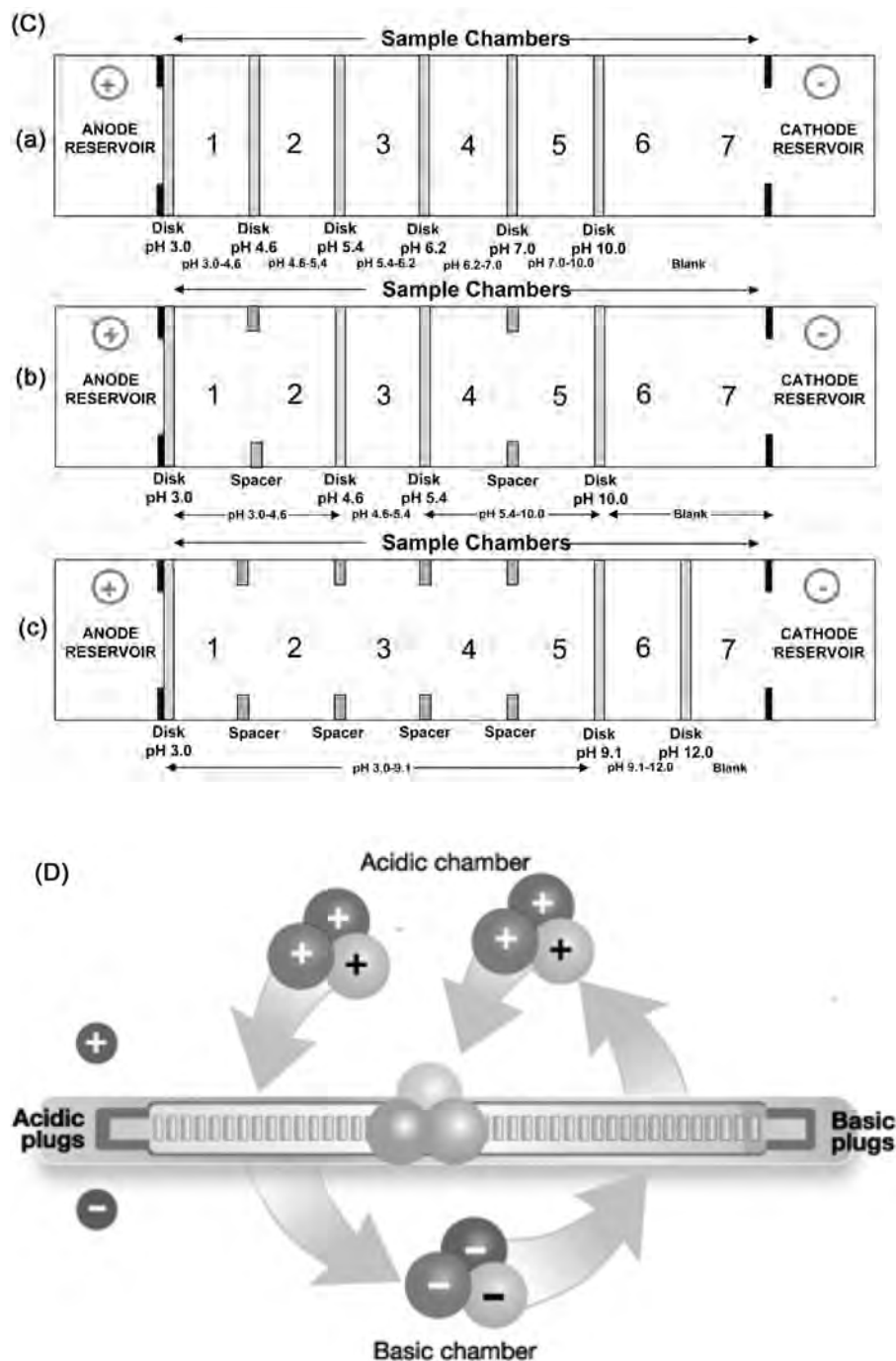


Figure 1. (A) Fractionation principle of the Agilent 3100 OFFGEL fractionator. (B) Separation principle for free-solution IEF in the BioRad RotoFor systems. A protein migrates in response to the electric field through the pH gradient established by the carrier ampholytes until it reaches the compartment where pH equals pI and becomes focused. (C) Formats for the Invitrogen Zoom IEF fractionator (a) standard format where six disks are used to create five fractions from pH 3.0 to pH 10.0, (b) extended format where seven disks are used to create six fractions from pH 3.0 to pH 12.0, and (c) three-disk extended format where three disks are used to create two fractions of pH 3.0–9.21 and pH 9.1–12.0. (D) Fractionation principle of the protein Forest digital Protein-Chip. Charged polypeptides migrate through the chip between the acidic and basic sides until they encounter a gel plug whose pH is at or near their pI. The uncharged polypeptide will no longer migrate and becomes trapped in these plugs. Courtesy of Tim Wehr, BioRad Laboratories (Hercules, CA).

and Spicher [61], in which a multicompartiment apparatus (4.7 mL total volume, 650 μ L/chamber) partitions complex protein samples on the basis of pH into multiple tandem electrode chambers divided by isoelectric membranes (ZOOM disks). Chambers can be set into specific pH ranges by buffers for fractionation of complex proteomes under denaturing conditions using solution-phase IEF in small sample volumes (Fig. 1C). Typical fractionation time is about 3 h, with a stepwise increase in voltage from 100–600 V. Because ~20% of the protein in each sample chamber remains adhered to the chamber walls, it has been recommended to add about 300 μ L of a denaturing solution to each chamber, place the chamber assembly on a rotary shaker for 10 min, and combine the wash solutions with the appropriate fraction, and fractions can then be harvested by a pipette [57].

The Digital Protein Chip (dPC) system recently introduced by Protein Forest (Lexington, MA) is a miniature electrophoretic device that employs parallel IEF for rapid fractionation and enrichment of complex protein mixtures [62]. The chip contains a linear array of 41 gel plugs, with 0.05 pH resolution between adjacent plugs (Fig. 1D). The chips allow placing ~2 μ L of acrylamido buffer into each chip feature, followed by UV polymerization. Three pH ranges are available: 4.20–6.20, 6.00–8.00, and 7.20–9.20. The dCP fractionator can accommodate up to six chips. Run time is 30–45 min, depending upon the fraction range. At the completion of focusing, gel plugs can be extracted from the chip for downstream analytical steps such as Western blotting or in-gel digestion followed by LC-MS.

Following fractionation of intact proteins by IEF, ampholytes and urea need to be removed prior to proteolytic digestion. This can be accomplished by TCA precipitation of the proteins, or by rapid desalting using a size-exclusion spin column. Focusing peptides is much easier than focusing proteins as peptides are natural ampholytes that will self-generate a pH gradient in the focusing chamber under voltage, which eliminates the problems of ampholytes in downstream analysis. Peptides are also soluble under focusing conditions, obviating the need for additives that would interfere with downstream LC-MS or MALDI-MS analysis. From bioinformatics standpoint, focusing at the peptide level complicates the picture because a given protein will exist as its component peptides and be defocused over multiple fractions, and therefore over multiple MS data files [57, 63].

A remedy to the slow migration of proteins in multicompartiment electrolyzers (MCEs) (Proteome Systems, Woburn, MA), in which a solution phase IEF is separated into discrete pH zones using membranes that can be set for specific pH ranges, due to the sieving effect of isoelectric membranes, was the introduction of hydrogel beads instead of membranes as pI barriers sandwiched in between the various chambers [64]. A composite isoelec-

tric beads made up of ionic acrylamide derivative monomers copolymerized with the pores of a central ceramic hard core thus minimizing mass transfer resistance of proteins that are transiently adsorbed onto the beads was employed. This method reduces separation time. Protein mixtures with species covering a large pI spectrum were fractionated by slicing out the extreme pI ranges (below and above predetermined pI defined by the pI of selected beads). The remaining protein mixtures (pI species between the previous pI of selected beads) were prefractionated under the same rules, thus yielding multiple fractions of predetermined pI ranges. Moreover, electrochromic chambers were separated by steric membranes with a cutoff of ~1000 Da, thus preventing the migration of species toward the electrochromic chambers [65].

Another multifunctional electrokinetic 2-D liquid-based, IEF prefractionation technology, the Gradiflow® System BF400 (French Forest, NSW, Australia) [66], comprises recirculating hydraulic flow of protein mixtures through a separation cartridge of two shallow separation compartments consisting of three polyacrylamide membranes located between two restriction membranes with an orthogonal electrophoretic transport of different proteins across a single separation uncharged membrane between the recirculating components. The Gradiflow technology operates on the principle of binary fractionations, by allowing for the electrophoretic separation of proteins based upon both a protein's charge and molecular shape (size) parameters, so that generally only two populations can be collected in each run: the upstream and downstream fractions [67]. Major drawbacks of the Gradiflow are the lack of sample loading flexibility (>8 mL volume), sharply defined molecular size cut-off, and potential protein loss at each membrane-based separation step according to pI and molecular weight [68].

The ProteomeLab® PF2D system (Beckman Coulter, Fullerton, CA) is an automated, 2-D fractionation system expressly designed for high resolution analysis of complex protein mixtures for downstream proteomic analysis that uses IEF in the first dimension followed by non-porous RP-HPLC selectivity. This system has been shown to work effectively and reproducibly separating basic proteins that proved to be a challenge for a 2-DE [69], as well as highly hydrophobic microsomal proteins [70].

Another liquid-based IEF prefractionation technology developed about 50 years ago that works in the absence of either a stationary phase or a solid support gel material is a continuous free-flow electrophoresis (FFE) system for purifying cells and subcellular organelles [71]. FFE separates charged particles ranging in size from molecular to cellular dimensions according to their electrophoretic mobility or pI, where vertical free flowing buffer maintains a pH gradient across a single focusing chamber [72]. It continuously injects samples into a carrier ampholine solution flowing as a thin laminar film (0.3–

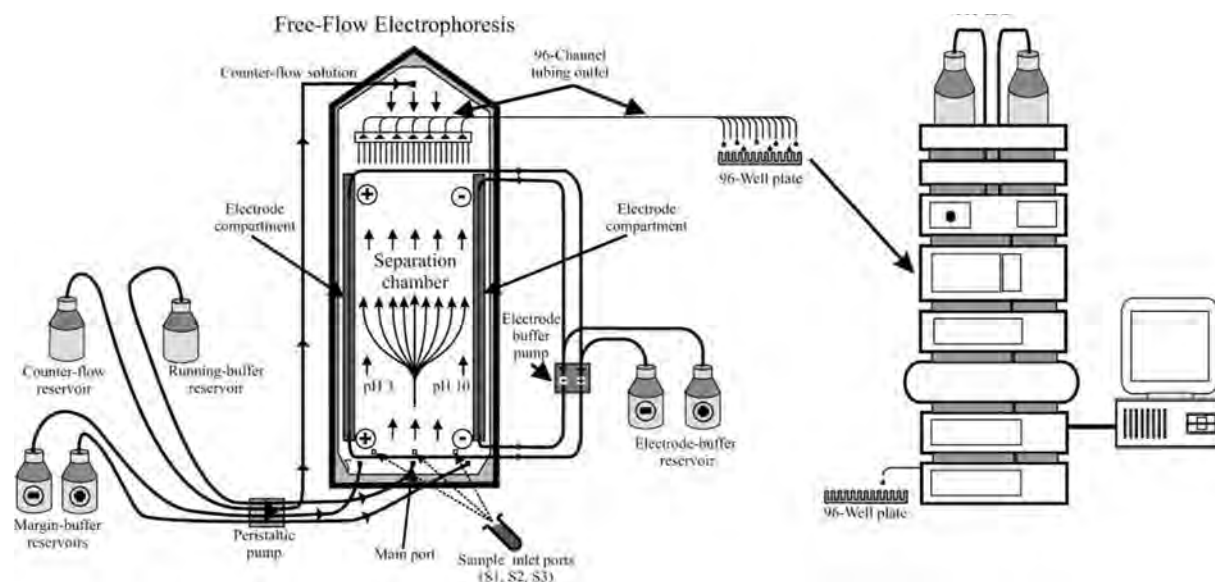


Figure 2. Schematic representation of the continuous FFE apparatus coupled offline to RP-HPLC. For analytical imaging separation, a portion of each first-dimension FFE-IEF fraction ($50\ \mu\text{L}$ /total volume, $2\ \text{mL}$) was injected directly from the 96 deep-well plate using the Agilent 1100 HPLC equipped with a well-plate autosampler. From ref. [74]; with permission.

1.0 mm wide) between plates. By introducing an electric field perpendicular to the direction of flow, proteins can be separated by IEF according to their different pI values [73]. An off-line RP-1100 HPLC system (Agilent Technologies) can be coupled to a FFE device [74], as shown in Fig. 2. A 3-D image of a tryptic digest of a cytosolic extract of human colon cell line LIM1215 separated protein is shown in Fig. 3, whereas Fig. 4 shows a mucoprotein-depleted human urine that was subjected to both non-reducing 2-DE and the 2-D FFE-IEF/RP-HPLC system following a MS/MS detection method for comparison. Advantages of this system are: (a) protein or peptide separation in the first dimension (IEF), where very narrow range pH gradients are generated, are performed in a liquid phase, (b) the RP-HPLC stationary phase extends the resolving power of this 2-D system compared to other 2-D systems based solely on coupled HPLC columns, (c) the system is not sample limited, (d) separation efficiency is maintained by continual flushing of the separated sample, and (e) the system is capable of separating both peptides and proteins over a broad pH range. Early versions of FFE, such as RIEF and Rotofor, have evolved to the TECAN's FFE (now BD Biosciences, San Jose, CA). Regardless of the sample application method (continuous vs. interval), the applied electrical field causes charged sample components to move toward the respective counter electrode on the basis of electrophoretic mobility or pI . The advantages of FFE fractionation include good sample recovery, probably due to absence of gel media or membranous material, and higher sample loading capacity with continuous sample feeding. Additionally, reproducibility and high resolution is provided by throughput fractiona-

tion as samples can be collected in 96 fractions, which can enrich for proteins with specific pH range, and the capacity for native or denaturing electrophoresis [75]. Major drawbacks of FFE are that buffer constituents may interfere with MS measurements, leading to protein loss during buffer exchange [76]. Moreover, removal of buffer additives (e.g., glycerol) before each separation step is very cumbersome and leads to sample dilution [77].

Since 1944 several microfluidic, miniaturized FFE (μFFE) devices (e.g., free-flow zone electrophoresis (FFZE), free-flow IEF (FFIEF), free-flow isotachiphoresis (FFITP), and free-flow field-step electrophoresis (FFFSE)) were developed to allow rapid separation (in seconds) and small volume (in μL) with various modes. Eventually, such microfluidic FFE systems might find application in small portable devices and point of care tools [78]. An overall drawback of IEF prefractionation, in general, is that fractions collected contain high amount of ampholytes, which can, however, be effectively removed by microcolumns filled with C_{18} RP material [8].

The requirements for HT proteomic applications (e.g., hydrophobic interactions, immobilized metal affinity chromatography (IMAC), and affinity-based separations) have fueled the development of high speed separations [79], although they have been mostly used in the separation of peptides for shotgun proteomics, particularly continuous bed monolithic columns [80], which have the advantage of improved mass transfer properties, wide range of pore sizes, and low back pressure at high flow rates. Monolithic disks with ion exchange functionality were used to fractionate proteins from liver and hepatocellular carcinoma followed by analysis with 1-DE and 2-

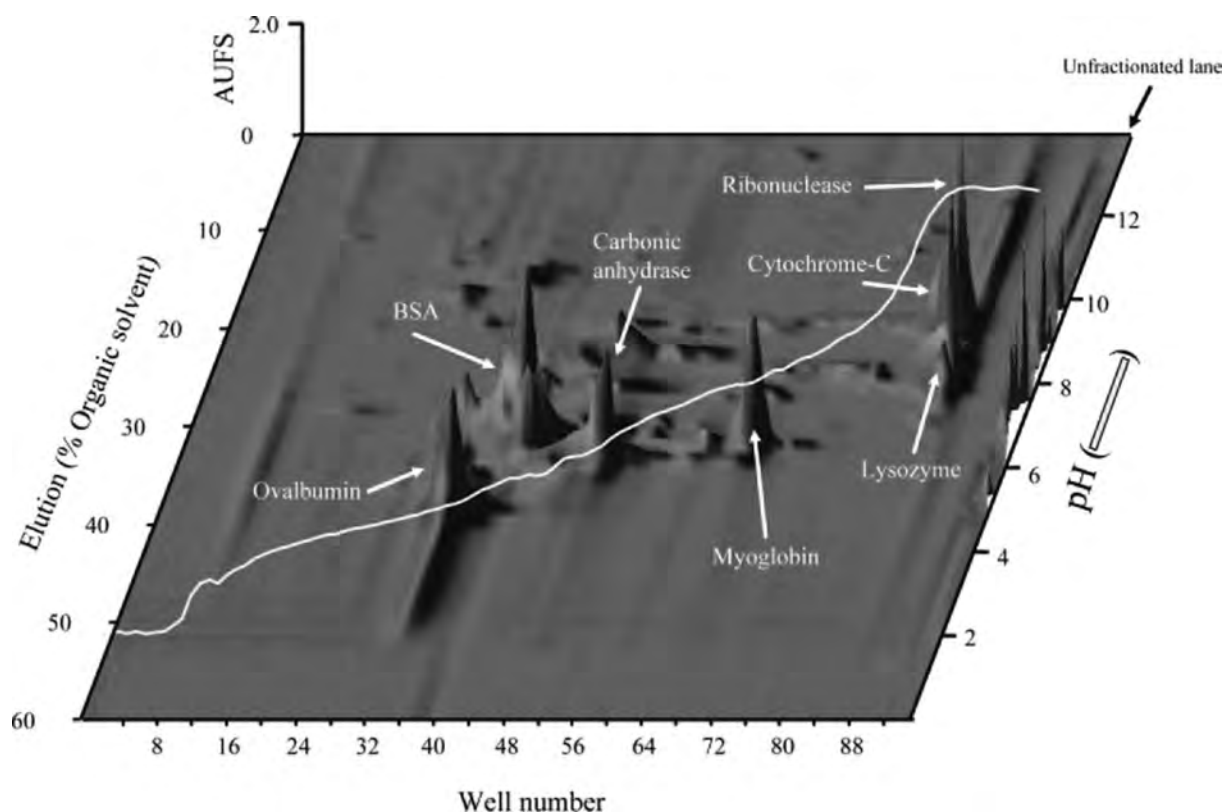


Figure 3. 3-D visualization of a native 2-D FFE-IEF (pH gradient 3–10)/RP-HPLC separation of standard proteins. Peak intensity (z-axis) absorbance plot at 215 nm. From ref. [74]; with permission.

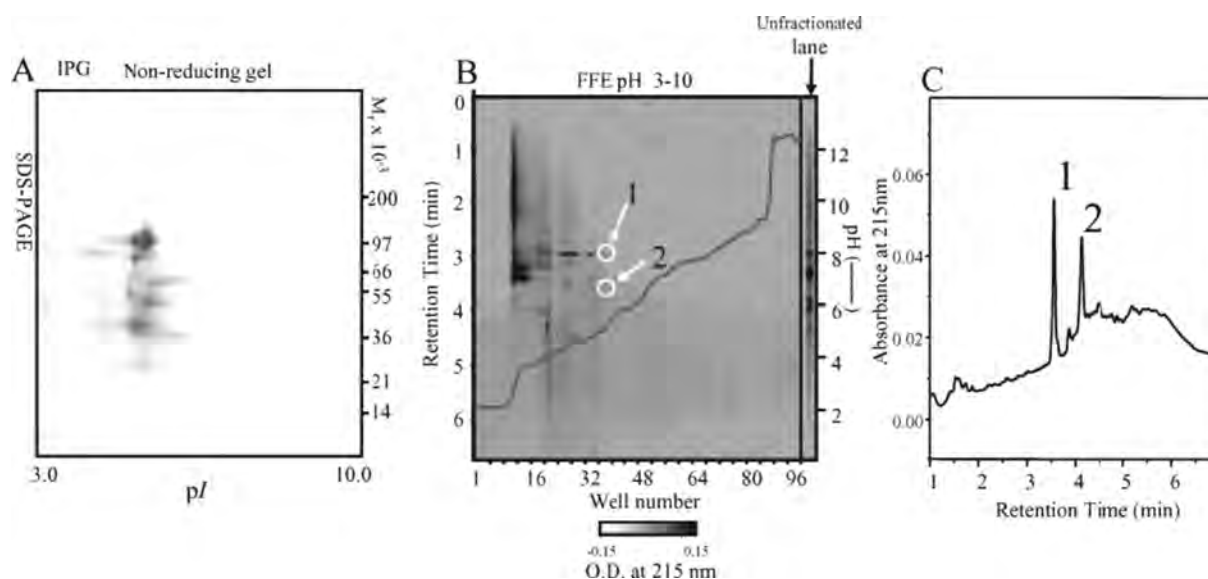


Figure 4. Comparison of human urinary protein separation by nonreducing 2-D FFE-IEF/RP-HPLC separation of 1.25 mg of human urinary proteins dissolved in 5 mL of IEF running buffer containing 0.2% w/v HPMC, 0.4% v/v Servalyte pH 3–10. (A) Nonreducing 2-DE. (B) FFE-IEF/RP-HPLC, pH 3–10. (C) RP-HPLC chromatogram of FFE fraction 42, pH 5.26, from circled peaks 1 and 2 in panel B, identified by N-terminal Edman degradation as CD59 gpi-anchored membrane protein, and spasmodic polypeptide, respectively. From ref. [74]; with permission.

Table 1. Instrumental techniques

Instrument	Principle	Manufacturer
Offgel OGE/IEF electrophoresis	A free-flow protein purification method based on isoelectric electrophoresis without the need of carrier ampholytes, in which protein solutions are flowed under an immobilized pH gradient gel (IPG) where an electric field is applied perpendicular to the direction of flow	Agilent Technologies
Rotofor	Proteins are separated in free solution by liquid phase IEF	BioRad Labs
Gradiflow BF 400	A size/charge IEF method using polyacrylamide membranes and tangential flow allowing binary fractionation to collect up- and down-stream populations	Frenchs Forest
Multichannel electrolyte IEF/Digital Protein Chip	Solution phase IEF separated into discrete pH zones using isoelectric membrane disks, or gel plugs, that can be set for certain pH ranges	MCE, Protein Forest
Microscale (Zoom) IEF	Electrode chamber with narrow range immobilized IPG gradients formed using precast polyacrylamide disks	Invitrogen Corporation
PF 2-D LC	An automated 2-D liquid-phase fractionation system using chromatofocusing and nonporous RP-HPLC selectivities	BioRad, Beckman-Coulter, Lumicyte
Free-flow electrophoresis (FFE)	Sample injected into a separation chamber of two parallel plates, then transported within a 0.5 mm of aqueous film formed between the two plates that are flanked by an electric field perpendicular to the laminar flow. Charged particles are deflected allowing for separation and collection of 96 fractions	BD Biosciences
Biosensor surfaces	Biosensor surfaces functionalized with specific proteins are used to affinity purify binding partners and their complexes, which can then be isolated for downstream analysis	BIAcore, IAsys, Vir, Genoptics

Modified from refs. [57, 66].

DE, surface-enhanced laser desorption ionization (SELDI) and electrospray MS/MS [81].

The development of lab-on-chip technologies, in which channels are etched onto glass or polymer chips [82, 83], or protein arrays that are coated or immobilized in a grid-like pattern on small surfaces [84], as well as nonporous magnetic particles have greatly reduced nonspecific adsorption compared with conventional chromatographic supports [85]. The various instrumental techniques detailed above are presented in Table 1.

2.2 Approaches relying on biological characteristics

Approaches relying on biological characteristics include prefractionation into cellular compartments (e.g., cytoplasm and membranes), subcellular compartments and organelles. They are simpler and more focused than physicochemical ones discussed in the previous sections, and can considerably increase our understanding of the proteome.

2.2.1 Subcellular fractionation (SF)

SF is the first step among enrichment techniques for reduction of sample complexity, which is of importance for analysis of intracellular organelles (e.g., nucleus, mitochondria, Golgi apparatus, lysosomes, exosomes, peroxisomes, and phagosomes) and multiprotein complexes. SF is most efficiently combined with 2-DE-MS analysis as well as with gel-independent techniques. SF allowing fractionation of organelles consists of two main steps: (a) disruption of the cellular organization (homogenization) and (b) fractionation of the homogenate to separate the different populations of organelles. Centrifugation is a traditional method for organelle isolation [86], and is compatible with further steps in protein solubilization and separation. Problems with reproducibility are frequent when protein depletion technologies are used [87]. Cells are collected by a low-speed centrifugation step and mechanically homogenized. After homogenization, the nuclei are removed by a low-speed centrifugation and can be purified from the pellet that contains cell debris and unbroken cells for further analysis. The postnuclear supernatant (PNS) contains the cytosol and

other organelles in free suspension, which can be subsequently separated by differential gradient centrifugation [88]. Although time consuming, labor intensive and resulting in dilute fractions, centrifugation is commonly used. While differences in composition of subcellular components affect relative densities of fractions, the degree of separation also depends on the gradient medium used. Sucrose is the most used medium [64], although other reagents such as Ficoll, Percoll, Nycodenz, or Metrizamide have been employed. Other techniques like FFE or immunoisolation have also been applied to SF of organelles [89]. Sometimes after centrifugation, an additional precipitation step may be required, especially when dealing with plant tissue [13].

Ideally, the purity of isolated organelles is important for the comprehensive analysis of total organelle proteome, although complete purification is often impossible for functional proteomic studies because cross contamination of fractions is a significant problem, which can be monitored by the use of appropriate markers. Therefore, enrichment of some organelles or certain subcellular fractions could be beneficial for the detection of low abundant proteins (LAPs) and tracking of their changes after stimulation of cells [90]. As an example, the stacked Golgi fraction from rat liver was fractionated using two sucrose step gradient centrifugation, followed by trypsin digestion, and the peptides were subsequently separated by multidimensional protein identification (MudPIT) technique. Forty-one proteins were discovered, two with confirmed Golgi location, and an arginine dimethylation species was identified on 18 of these proteins alluding to the role of methylation in Golgi function [91]. Peroxisomes were isolated from rat liver by homogenization followed by using the Nycodenz density gradient centrifugation, and organelles purified by immunosonication with Abs to peroxisomal membrane protein (anti-PM70) bound to magnetic beads. Purified peroxisomal fraction 34 was separated by 1-DE to visualize proteins that were identified after trypsin digestion by LC-MS [92].

Bioinformatics analysis of 3962 proteins of the yeast *Saccharomyces cerevisiae* previously localized by green fluorescent proteins (GFP) tagging and microscopy to 22 subcellular organelles or compartments pointed out that different compartments showed significantly different distributions of protein pI and hydrophobicity, with mitochondrial and endoplasmic reticulum (ER) proteins showing the most differences to other organelles to these two experimental parameters [93]. Therefore, for proteins to be separated and identified efficiently, analytical strategies need to be employed that pay careful attention to the degree of acidic, basic, hydrophobic, and hydrophilic proteins in each compartment [94].

Because of limited resolution of the available fractionation methods, it is often difficult to isolate and thus

profile pure organelles for identification of the protein components of specific fractions containing the target organelles using MS. Moreover, many secretory proteins are often observed to dramatically shuttle between organelles. Therefore, determination of their true cellular localization requires the concurrent analysis of multiple organelles from the same cell lysate. An integrated experimental approach that simultaneously profiles multiple organelles (e.g., ribosome, mitochondria, proteasome, lysosome, ER, and Golgi apparatus) based on the SF of cell lysates by density gradient centrifugation, isobaric tags for relative and absolute quantification (iTRAQ™) labeling and MS analysis of proteins in selected fractions, followed by principle component analysis (PCA) statistics of the resulting quantitative proteomic data, has been carried out using that approach [95].

2.2.2 Purification of protein complexes and microdomains

Proteins rarely function in isolation, but are rather organized in functional units that are different in size, number of interacting partners and stability, ranging from huge stable ribosomes or nuclear pore domains to small and transient signal transduction complexes. Studying of these multiprotein complexes and microdomains provides information about the spatio-temporal organization of signal transduction or metabolic processes within a cell as a major part of this information is lost when cells are lysed and proteins digested before analysis. Because isolated protein complexes have much reduced complexity, this allows for identification of low copy number proteins present in the complex and connecting them to particular function [90]. Multiprotein complexes and associated proteins can be isolated and purified by a variety of techniques such as affinity-based, recombinant pull-downs, LC, blue native gel electrophoresis (PN-PAGE), 2-DE/LC/CE and FFE methods, followed by MS analysis [86, 89, 96–98].

In a rat study aiming at targets for tissue-/organ-specific delivery of therapeutic and imaging agents *in vivo*, tissue subfractionation with subtractive proteomics and bioinformatics analyses reduced tissue complexity by more than five orders of magnitude and unmasked a subset of proteins at the blood tissue interface [90].

2.3 Sequential extraction method

An optimized sequential extraction method for fractionation of proteins in their native state according to their subcellular localization yielded four subproteomes enriched in: (a) cytosolic proteins, (b) membrane and organelle-associated proteins, (c) soluble and DNA-associated nuclear proteins, and (d) cytoskeletal proteins, respectively. Four extraction buffers of defined ionic and osmotic composition containing surfactants enabled

stepwise disintegration of cells and selective extraction of certain subcellular components. This method allowed for the assessment of spatial rearrangements of signaling proteins, as demonstrated on signal-dependent redistribution of phosphorylated mitogen-activated protein kinase (MAK) and nuclear factor kappa B (NF κ B), between cytoplasm and nucleus [99].

2.4 Membrane proteins' extraction

Membrane proteins are important for proteomics as they represent a large population of the proteome in the form of receptors, transporters, channels, and a variety of cellular mechanism, which makes them a major target of pharmacological interest. However, membrane proteins are still under identified and underrepresented during whole cell proteome analysis [8]. Although membrane proteins with up to 12 transmembrane α -helices have been resolved and identified by 2-DE-MS [100], most membrane proteins have been resistant to this approach.

Membrane proteins are often enriched by ultracentrifugation in sucrose gradient, lectin affinity chromatography in combination with centrifugation, silica beads or biotinylation and interaction with immobilized streptavidin [101]. Solubilization of this fraction is accomplished by using detergents, whose choice depends on the nature of the experiment. Combinations of chloroform and methanol were used to extract hydrophobic chloroplast membrane proteins [102]. The aqueous two-phase system, at least one of them containing a water-soluble polymer, which employed detergent DDM, Triton X-114 or PEG for the selective binding of one or more proteins of interest to the one of the incompatible aqueous phases, was used for membrane enrichment [103, 104]. A combination of chloroform and methanol was employed for differential extraction of membrane proteins from chloroplasts [105]. Centrifugal sucrose gradient fractionation was employed for isolation of mitochondrial membranes [106]. Identification of membrane proteins has been further complicated by the lack of tryptic cleavage sites across transmembrane chain fragments. Enzymatic digestion often results in large, hydrophobic species, which hinder identification. To enlarge sequence coverage, a mixture of proteases and cyanogens bromide with addition of detergents was carried out [107, 108].

Analyses of proteins from a number of proteomic studies of cell membranes have demonstrated that a significant component of the identified proteins is not predicted to contain transmembrane regions. The presence of such proteins may arise as a result of contamination of the membrane preparations or through real associations. Identification of integral proteins, as well as those that are intimately associated with the microsomal membranes of K562 cells, first necessitated removing noncovalently associated peripheral proteins and the

residual proteins were then 1-DE separated and analyzed by LC-MS/MS. Tandem lectin affinity was also examined as an approach for the enrichment of membrane glycoproteins. Approximately, 41% of the isolated proteins were assigned as membrane proteins based on the presence of transmembrane regions or covalent PTMs that could account for membrane association. Collectively, these results indicate that there is a significant component of nonintegral proteins that appear to be as closely associated with membranes as integral elements [109].

Plasma membranes (PMs) are important for cells as they form a selectively permeable barrier to the environment, and many essential tasks of PMs are carried out by their proteinaceous components, including molecular transport, cell–cell interactions and signal transduction. Because of their low abundance and immense heterogeneity, they require special treatment in order to identify and characterize them. An effective tool for PM isolation is partitioning in aqueous polymer two-phase systems (e.g., PEG and dextran) in which membranes are separated according to differences in surface properties, rather than size and density. The main advantages of this method are the high yield and purity, together with rapid processing. Different factors such as molecular weight and concentrations of the polymers and salts can be explored to optimize the partition behavior, so that an effective partitioning can be carried out within a few hours without employing specialized equipment [110].

Examination of membrane proteins of enriched and selectively isolated from microdissected ovarian tumor cells within the pellets was carried out by treatment with two different protein extraction methods that employed either SDS detergent or ACN organic solvent. The detergent-based preparation extracted proteins effectively from pellets, and was compatible with subsequent proteome analysis using capillary IEF/nano RP LC separation, coupled with nano ESI MS. Among proteins identified from an amount of pellet equivalent to 20 000 cells, 773 proteins were predicted to contain one or more transmembrane domains, corresponding to 22% membrane proteome coverage within the Swiss-Port Human protein sequence entries [111].

Aqueous polymer two-phase systems have been described for the preparative isolation of PMs [112]. Separation was attained due to differential affinity between two immiscible aqueous polymer phases. Qproteome Cell Compartment Kit[®] from Qiagen was used for separation of cytosolic, membrane and nuclear proteins, and Nuclear Subfractionation Kit for isolation of nucleic acid binding proteins from isolated nuclei [113, 114]. An immunoaffinity purification of PM with Ab superparamagnetic beads, following sucrose gradient separation of mouse liver PM, was described [115].

Two common methods have been used to isolate cholesterol-rich membrane microdomains with distinct

lipid and protein composition termed “lipid rafts” by treatment with either high pH or nonionic detergents followed by treatment with either high pH or nonionic detergents, and subsequent density gradient centrifugation; however, both methods have been plagued by contamination from nonraft proteins, which was overcome by using quantitative methods as stable isotope-labeling with amino acids in cell culture (SILAC) to determine the subset of cholesterol-dependent proteins depleted from rafts by cholesterol-disrupting drugs using a high-resolution MS method [116]. Seven-hundred and three detergent-resistant fractions proteins (of which 392 raft proteins) and 585 carbonate-resistant fractions were identified; among the rafts and raft-associated proteins a significant number of serine/threonine kinases/phosphatases, as well as numerous heterotrimeric G protein subunits, suggesting that rafts may be general signaling coordinators. Comparison of this data with previous published one on lipid rafts showed that only less than half of the 19 proteins in a detergent-resistant fraction from Jurkat T cells identified in a study [117], and approximately two-thirds of 70 proteins identified in another study [118] to be authentic raft proteins; the remaining being false positive. However, carbonate-resistant fractions were less specific for raft isolation and more difficult to interpret than detergent-resistant method [119].

Lipid rafts were originally defined as detergent-resistant membranes (DRMs) due to their relative insolubility in cold nonionic detergents. Recent findings suggest that although DRMs are not equivalent to lipid rafts, the presence of a given protein within DRMs strongly suggests its potential for raft association *in vivo*. A differential detergent extraction method employing 2% Triton X-100 was shown to enable rapid DRM isolation, minimized nuclear contamination and yielded fractions compatible with MS analysis [120].

Caveolin-enriched membranes were isolated by either cationic silica affinity purification or buoyant density methods. Subsequent 2-D separation followed by MALDI-TOF analysis showed improved identification of membrane proteins and their PTMs. On the other hand, cationic silica purification yielded predominantly ER, whereas the cold-detergent method yielded a large number of caveolae residents, including caveolin-1 [121].

An affinity-based isolation method was used to enrich and purify parts of blood vessel endothelial cells that contact the blood in organs such as lung and lung tumors by infusing colloidal silica particles into the blood stream of rats, where these particles attached to the endothelial cells, followed by centrifugation of tissue homogenates to separate endothelial cell membrane and the attached caveolae from the remainder of the cells. For purification, an Ab that recognized caveolin coupled to magnetic beads was used to isolate caveolae and their associated proteins. Purified caveolae displayed a greater

than 20-fold enrichment for specific markers. Analysis by 2-DE produced high-resolution vascular endothelial protein maps of major rat organs and showed 37 proteins to be present only in the endothelial membrane; 11 of which possess an extracellular portion that could be presented to blood cells. Expression profiling and γ -scintigraphic imaging with Abs suggested two of these cell surface proteins (aminopeptidase P and annexin A1) as selective *in vivo* targets for Abs in lungs and solid tumors, respectively. Radio immunotherapy targeted against Annexin A1 selectively decreased tumor size and increased animal survival [122].

A sequential fractionation strategy following homogenization of cells by centrifugation of the postnuclear supernatant at $100\,000 \times g$ that separated total membrane fraction from cytosol allowed extraction of peripheral membrane proteins from membrane pellet in 0.1 M sodium carbonate, pH 11.0; the remaining integral membrane proteins were analyzed [123]. Alternatively, Triton X-114 was applied to enrich for the integral membrane protein fraction [124].

A general MS-based proteomic “shave-and-conquer” sequential extraction strategy was used that targets specifically glycosyl phosphatidyl inositol-anchored proteins (GPI-APS) from *Homo sapiens* and from *Arabidopsis thaliana* that act as enzymes and receptors in cell adhesion, differentiation and host–pathogen interactions and are potential diagnostic and therapeutic targets. Raft-enriched membranes of human HeLa cells were purified by homogenization of cells and ultracentrifugation in sucrose gradients. After extraction of peripheral membrane proteins by sodium carbonate, lipid rafts were obtained from membrane fractions by two-phase separation in the presence of Triton X-114. The isolated membrane fractions were treated with phosphatidylinositol phospholipase C, which hydrolyzes phosphatidylinositol, releasing the soluble GPI protein from membrane/detergent phase and allowing its recovery in the aqueous phase. Proteins were then separated by 1-DE and identified by MS. Six GPI-APs were identified in *H. sapiens* lipid raft-enriched fraction and 44 GPI-APS in an *A. thaliana* membrane preparation [125].

3 Sample preparations from biological fluids

3.1 Body fluids

Many of the diagnostic, prognostic, or monitoring response to therapy biomarkers used in clinical practice are found in biological fluids (the most widely used ones are blood and urine, which are easier to access compared to tissue biopsies, besides being minimally invasive). Body fluids are very complex mixtures of molecules with a wide range of polarity, hydrophobicity, and size over a

range of several orders of magnitude. Ideally, a crude, unprocessed sample should be analyzed, which would avoid all artificial losses or biases arising from sample preparation. However, since all body fluids contain a large amount of different ions, lipids, carbohydrates, *etc.*, these samples generally cannot be analyzed in the native form in a mass spectrometer, and a pre-MS separation is a necessary prerequisite in order to cope with the complexity and dynamic range of these samples [25, 63].

3.1.1 Urine

Among body fluids, urine is especially attractive for biomarker discovery in urological diseases because: (a) it contains fewer proteins than, for example, blood because only a few organs are located directly along the path of urine production and excretion (*i.e.*, kidney, urinary tract, including bladder), (b) it can be obtained in large quantities using noninvasive procedures, (c) repeated sampling from the same individual is achievable, (d) it contains proteins and polypeptides of lower molecular mass (<30 kDa) that are highly soluble, which facilitates analysis of such polypeptides in their natural state without the need for additional manipulation (*e.g.*, tryptic digest) [126], and (e) for proteins >30 kDa, urinary polypeptides are stable and do not generally undergo significant proteolysis within several hours of collection, in contrast to blood where activation of proteases and the generation of an array of proteolytic breakdown products is often associated with its collection [127].

The urinary proteome appeared to be stable when urine was stored up to 3 days at 4°C, or up to 6 h at room temperature, probably because following its storage for several hours in the bladder, proteolytic degradation by endogenous proteases is complete by the time of voiding [126]. Urine, however, has disadvantages as a source for protein markers due to: (a) the wide variation in protein concentration, which is largely due to differences in person's fluid intake. This problem can be mitigated by standardization based on creatinine [128] or peptides present in urine [129], (b) inconsistency of its pH that may alter the activity of proteases in a fraction of the urinary proteome leading to greater variability of the proteome during the day due to factors such as different diets, metabolic or catabolic processes, circadian rhythm, exercise, and circulatory levels of various hormones [130]. However, the basal or housekeeping proteins of urine remains largely unaffected by these changes [129], and (c) clear differences between early stream and mid stream urine samples have been found [131]. Therefore, standardization of urine collection protocols for biomarker discovery is a must [132].

3.1.2 Blood

Plasma is the largest single component of blood, comprising about 55% of total blood volume. Plasma is

obtained from whole blood after addition of an anticoagulant (*e.g.*, citrate, heparin or EDTA) to prevent blood to clot, preferably in the presence of inert catalyst such as glass beads or powder. Although serum is a more convenient specimen, its protein profile is different from plasma [133], as an array of proteases are activated immediately upon clotting, resulting in the generation of many degradation products. Consequently, the Human Proteome Consortium has recommended that blood be examined as plasma rather than as serum and established standardized sample collection protocols [127].

Serum and plasma potentially contain elements of all proteins produced in the body, and studies suggest that the low molecular weight (LMW) protein/peptides in these fluids (*e.g.*, peptide hormones or small secreted proteins) are correlated with pathological conditions and present opportunities for potential clinical utility for diagnostic, prognostic, or predictive response to therapy markers [134]. The technical challenges in the analysis of plasma/serum proteome is that their proteins are present at unequal concentrations as a few are so dominant (*e.g.*, albumin and globulin present at almost 90% of total proteins by weight) that they mask the detection of other proteins, especially low abundant ones, which are considered to be of clinical importance [25]. The 30 most abundant proteins in the plasma of healthy persons, as well as the half-lives of some are presented in Table 2. Ranking these proteins by molar than by mass abundance changes the perspective on the relative abundance of many plasma proteins, particularly the small ones. Most larger proteins have half-lives of several days, while those with M_r of <30 000 Da are cleared by filtration in the kidneys, giving half-lives of a few hours, like retinol-binding protein, and are extended several folds by binding to a carrier protein such as albumin [135]. Without fractionation, the complexity of plasma/serum is overwhelming that important biological information will be in background noise and will not be detected by current available MS technologies [136].

Standardizing sample preparation procedures for plasma/serum profiling, including the type of collection tubes and coagulants, the clotting and incubation time before sample isolation, storage conditions, strategies used for removal of high abundant proteins (HAPs), as well as fractionation techniques employed either to generate several fractions or to selectively obtain a particular subset of peptides/protein, although time-consuming and error prone is critical for obtaining reliable biomarkers and building a biomarker pattern, since slight changes in a given sample preparation could lead to very different protein profiles [137].

3.1.3 Sample collection, handling, and storage

In spite of the impact of this parameter on the sensitivity, selectivity and reproducibility of the analysis, only a few

Table 2. Ranking of plasma proteins in healthy persons in order of molar or mass abundance, with examples of half lives^{a)}

No.	Protein rank	Concentration		Molecular mass (M _r) in Da	Half life
		M · mol/L	mg/L		
1	Albumin	500–800	35 000–52 000	66 438	15–10 days
2	IgG	40–100	7 000–16 000	160 000	
3	Apolipoprotein A-I	36–72	1 000–2000	28 079	5 days
4	Transferrin	25–45	2 000–3600	79 600	7 days
5	Apolipoprotein A-II	22–60	200–550	8 691	5 days
6	α_1 -Proteinase inhibitor	18–40	900–2000	55 000	1–2 days
7	α_1 -Acid glycoprotein	12–30	500–1200	40 000	
8	Transthyretin	15–30	200–400	54 000	
9	Hepatoglobulin	3–20	300–2000	104 000	
10	Hemopexin	15	900	57 000	2.5 days 4 days
11	IgA	4–24	700–4000	170 000	
12	Apolipoprotein C-III	6–20	60–180	8 765	
13	α_2 -Macroglobulin	7–17	1 300–3000	30 000	
14	α_2 -HS glycoprotein	12	600	50 000	
15	Gc globulin	8–14	400–700	51 000	
16	Apolipoprotein C-I	6–12	40–80	6 631	
17	Fibrinogen	6–12	2 000–4000	340 000	
18	α_1 -Antichymotrypsin	7	500	68 000	
19	C3	5–10	900–1800	180 000	
20	b ₂ -Glycoprotein I	4–8	150–300	40 000	10–12 h
21	Vitronectin	4–8	250–450	55 000	
22	α_1 -B Glycoprotein	3–6	150–300	63 000	
23	Apolipoprotein A-IV	3–6	130–250	43 375	
24	Apolipoprotein C-II	2–7	20–60	8 915	
25	β_2 -Glycoprotein II	2–5	12–30	63 000	
26	Antithrombin III	3	200	65 000	
27	Inter α -trypsin inhibitor	2–4	400–700	160 000	
28	Plasminogen	2–4	150–350	81 000	
29	Ceruloplasmin	1.5–5	200–600	135 000	
30	Retinol-binding protein	1.5–3	30–60	21 000	

^{a)} Modified from ref. [135].

studies have been carried out that showed sampling procedures to have the greatest effect on proteome profiling, whereas handling procedures and storage conditions to have relatively minor effects [138]. However, standardized protocols for plasma/serum handling and storage are needed in order to have comparable results between different laboratories [139].

Studies on the effects of blood collection on many types of laboratory analyses [140] have shown that optimization and standardization of collection tubes is important for reliable analysis of plasma/serum proteins. Commercially available blood collection tubes contain multiple components (e.g., silicones commonly used for lubricants for stoppers or coatings for the internal surface of collection tubes; polyvinyl pyrrolidones or PEGs added as surface wettings; clot inhibitors or activators; substances in polymeric gels to separate blood constituents; polymers in rubber stoppers and plastic tubes) [141] that may interfere with MS analysis when they are shed into these body fluids. The kind of tube used can also influence adsorption of plasma/serum proteins to tubes' inner surfaces. Significant differences have been found

when using red-top tubes (glass tubes containing no preservatives or anticoagulants) *versus* tiger-top tubes (also known as serum separator tubes, SST) [138, 142].

The coagulant added to blood for making plasma influences MS protein profiles [143]. For example, although platelets are more stable in citrate anticoagulants, collecting tubes often contain a liquid form that dilutes the plasma. Heparin that binds to and enhances the activity of antithrombin III also binds to a number of other proteins [144]. Because the activity of many proteases requires metals, the chelation of EDTA prevents coagulation [126]. Freshly collected EDTA-treated blood is only slightly stable, but over longer periods of time, marked changes appear as elapsed time before centrifugation increases [126]. Plasma protein profiles obtained by EDTA treatment were most divergent from those obtained by citrate or heparin treatment [138], probably because EDTA leads to platelet clumping and aggregation that could change the protein content of plasma [145].

Clotting or time of incubation before separating blood cells from serum influences protein profiles. Profiles from plasma samples changed as time lag increased

Table 3. Strategies used for HAPs depletion and enrichment

Method	Principle	Advantages	Disadvantages
(A) Depletion			
Centrifugal ultrafiltration	Membrane filtration combined with solvents	Fast, easy to use, inexpensive	Potential loss of components binding to HMW proteins
SPE disk formats	Bases on ion exchange, metal chelating, affinity or dye ligands, bacterial protein A and G, or combination thereof	High selectivity, reproducibility and sensitivity when using series of different columns	High cost of Abs depletion columns, generally short lifetime (L), sample dilution or loss may occur, not all LAPs can be accessed by these columns
Disk plates SPE formats	Same as SPE columns	Highly suitable for HT and automation	Same as SPE columns
Organic solvents, extraction	Solvent precipitation of HAPs with simultaneous extraction of LAPs	Ease of use, convenience	Requires organic solvents, leads often to sample dilution
(B) Enrichment			
Solid phase ligand library (Equalizer technology)		Simple, convenient, potentially useful to discover/predict nonhuman proteins	Technology not mature and needs further development and validation
Hydrazide resins for glycopeptide-capture/thioaffinity resins for cysteinyl peptide capture		Simple, reduced proteome complexity, high sensitivity, allows for PTMs detection	Ab specificity not always quite optimal, little information provided on spatial resolution of complexed proteins, not suitable for all potential proteins in the proteome

Modified from ref. [149].

from 1, 12, or 24 h at either 4°C or room temperature, although profiles obtained from different individuals under the same conditions were consistent, probably due to metabolism of blood cells, alterations of cells' membrane integrity, and/or release of degraded products from the clot [138]. Changes in protein profile (*e.g.*, intensity of peaks; either increase or decrease) from serum samples were also observed for different clotting times at both 4°C and room temperature, probably due to either degradation of plasma peptides or formation/accumulation of new peptides during and after clotting [142, 146].

Storage conditions exert effects on plasma/serum protein profiles. Minimal changes were observed in the samples stored at room temperature within the first 4 h or 6 h, whereas noticeable changes were found after 8 h, especially for peaks in the *m/z* range < 3000 [138, 146], and were more pronounced after 24 h [147]. For plasma/serum stored up to 24 h at 4°C, the profiles were quite similar, but if time was prolonged to 48 or 96 h, significant changes were observed [146]. No major differences were found for samples stored for long term at –20°C, –80°C, or in liquid N₂ [139, 146], although freeze/thaw cycles are believed to change sample composition prob-

ably due to peptide aggregation, precipitation or adsorption to surfaces [142, 148].

3.1.4 Depletion of high abundance proteins (HAPs) and enrichment of diluted ones

Several strategies are available for depletion of HAPs from plasma/serum as illustrated in Table 3 [149]. Centrifugal centrifugation is a simple variation of membrane filtration technology where centrifugation forces a liquid against a semipermeable membrane leading to retaining solids and solutes of high molecular weight, whereas the liquid and LMW solutes pass through the membrane depending on membranes' molecular weight cut-off (MWCO) [149] that ranges from 10 to 50 kDa, and centrifugation speeds from 3000–4000 × *g* [150]. Low speed centrifugation (–4000 × *g*), use of diluted plasma/serum to a final concentration of ~5%, or use of denaturing conditions (*i.e.*, addition of ACN to 25% final concentration) [151] are required to disrupt protein–protein/peptide interactions so that LMW components that may be bound to albumin or other larger species are released and are free to pass through the membrane [152].

Different types of SPE depletion formats, including columns, cartridges, microcolumns or spin columns have

been used based on ion exchange, metal chelation, affinity ligands, dye ligands (e.g., Cibacron blue chlorotriazine ligand dye), bacterial proteins A and G, Abs (polyclonal multiple affinity removal systems, MARS), or combination of these have been marketed by companies such as Agilent, GenWay, Biotech, BioRad, Sigma–Aldrich, Amersham Biosciences, Ball Corporation, Pierce and others [84, 153, 154]. Ideally, a spin column that allows parallel processing of multiple samples in convenient microcentrifuges, and highly selective column formats that could deplete 18–22 of the most abundant proteins that compromise 98–99% of total plasma/serum protein content, but leaving LMW ones would be desirable [25].

A new MARS column from Agilent Technologies (Willmington, DE) for the specific depletion of 14 high abundant proteins from plasma/serum combined with RP-C₁₈ column for postdepleted fractionation performed at 80°C for protein recovery of immunodepleted proteins, followed by HPLC-Chip/MS analysis resulted in identification of the cytokine interleukin (IL)-27 beta chain present in pg/mL concentrations in plasma [155]. The Seppro™ Mixed 12 spin column (GenWay Biotech., San Diego, CA; now licensed to Beckman-Coulter) containing 12 avian polyclonal immunoglobulin yolk (IgY) Abs against the 12 most abundant proteins are covalently coupled to microbeads to pack the column. Recycling of the column up to 135 times did not lead to apparent loss of specificity or capacity [156]. The Sigma ProteomePrep 20 (Top 20) depletion column, which removes the 20 high abundant plasma serum proteins, allows up to 100–200-fold more liquid volume to be loaded into downstream separation and analyses after depletion. Only eight mediums to LAPs that were predominantly found in the unbound (depleted fraction) were also found in the bound fractions in 1-DE [157].

Concerns exist about the use of immunodepletion methods under nondenaturing conditions because cytokines and other low-abundance proteins bound to the target proteins may be simultaneously removed via the “albumin sponge effect”. Consequently, about 210 proteins – including some potential biomarker candidate proteins – were found to be associated with the six most abundant plasma proteins, and removal of albumin using a resin causes a significant loss of several cytokines [158]. However, the use of polyclonal antibody spin columns to deplete abundant plasma proteins has a good potential to reduce this nonspecific binding, or sponge effect [159]. Procedures that enable the recovery of low abundance proteins from immunoaffinity columns, such as use of mild solvent treatment of the bound proteins, salt-out preparation, and molecular sieve filtration [159], nonprotein binding resin [160], or membrane containing small peptides [161] to selectively remove albumin, but not albumin-bound proteins from plasma have promising potential [162]. These depletion techniques

could result in some sample dilution requiring enrichment downstream to enable depletion of LAPs [163], and the abundant proteins can themselves bind either specifically or nonspecifically to other proteins in the sample leading to their loss [164].

A recent study on human serum combining protein fractionation by Off-GEL™ IEF and RP-HPLC run and detection by an IT mass spectrometer showed that serum proteins are equally distributed at overall levels of concentration; thus, depletion columns cannot unfortunately stand their promise of making 1000s of low abundance proteins accessible by even removing the 6–20 most abundant proteins, because the next most abundant ones will have similar ratios compared to the remaining proteins as the depleted most abundant proteins have had [165].

Selective binding and enrichment of LMW peptides and proteins from human plasma using nonporous silica particles have also been described [166]. Alternative selectivity could be obtained by changing the characteristics of the nanoporous silica. Lower molecular weight proteins were best concentrated using smaller pore size silica with a LOD of 15 ng/mL for plasma spiked with insulin in a MALDI-TOF MS detector. A similar enrichment has been shown using nanoporous controlled pore glass beads [167] or nanoporous surfaces [168].

SPE disk plates for high abundance protein removal from plasma/serum samples using different functionalities such as ion exchange, dye ligand, and RP disks have employed the same principles attempted in SPE columns. The main advantage associated with the use of SPE disk plates is the increased ability for HT automation as they are generally used in a 96-well plate format allowing simultaneous processing of large number of samples robotically [169].

Precipitation of HAP from plasma/serum with simultaneous extraction of peptides and LMW proteins using organic solvents (e.g., two volume of CAN containing 0.1% TFA) in the presence of ion-pairing agents dissociates peptides and small proteins from large abundant proteins such as albumin, while small proteins and peptides stay in solution, which facilitates their analysis [149, 170].

A two step fractionation approach for plasma using MARS-6 immunodepletion column with multi-lectin affinity chromatography to deplete the glycosylated proteins allowed for identification of protein biomarkers such as angiotensinogen present at high levels in patients with obesity and its associated complications such as diabetes and hypertension [171].

The selective affinity capture of binding proteins and their complexes with downstream proteomic analysis was initially reported in the mid 1990s [172]. A number of platforms exist, which include direct immunoprecipitation [173], affinity columns, magnetic beads, biosensor

surfaces, and antibody arrays [174]. Affinity chromatography can be used not only to deplete certain types of proteins but also for enrichment of specific classes of proteins such as phosphoproteins, glycoproteins, thiosulfide proteins, ubiquitinated proteins, and characterization of groups of proteins in terms of function and structure (protein complexes) by tandem affinity purification (TAP) steps, in which a TAP tag, which consists of two type G binding domains, fused either N- or C-terminally to the target protein, and the construct is introduced into the host cell organism in order to understand the functions of protein networks. However, it must be remembered that adding tags to proteins can modulate their behavior, and the method relies upon protein over expression, which may influence signaling pathways [175].

Affinity-based techniques are advantageous if suitable reagents are available because of their high selectivity and relatively gentle elution conditions, which minimize protein denaturation [176]. N-linked glycoproteins in plasma or serum in humans or mice, respectively, could be captured using lectin affinity column (Qiagen), hydrazine resin that covalently link the N-linked glycopeptides following oxidation of the sugars [177–179], which are subsequently digested by trypsin, and N-linked tryptic peptides released from the resin by digestion with N-glycosidase, followed by LC-MS/MS [180, 181]. A number of serum proteins, including albumin, are not glycosylated and therefore are not effectively removed using these strategies [178].

Another affinity method targets cystine-containing peptides [182]. Cysteines constitute ~1.7% of amino acids in proteomes, and therefore enriching peptides with cysteines provides a substantial simplification of complex peptide mixtures. In a study on a mammary epithelial cell proteome, a thiol-specific resin was used to enrich cysteine-containing peptides before fractionation by strong cation exchange and identification by LC-MS/MS. A number of low abundance proteins were detected in the cysteinyl-enriched fraction that was not identified in non-enriched fractions [183].

Protein phosphorylation is one of the most common PTMs that regulate protein localization, complex formation and degradation. The most common methods for enrichment of phosphoprotein samples involve either affinity chromatography using immobilized antiphosphopeptide Abs, IMAC with immobilized Fe^{3+} , Ga^{3+} , Al^{3+} , or Zr^{4+} ions [184], or the use of titanium dioxide microcolumns followed by downstream MS analysis. These supports are now available in a range of formats including ZipTips (Millipore, Billerica, MA), SwellGel Gallium disks, and as part of a CD microlaboratory (Gyrolab MALDI IMAC1 (Uppsala, Sweden)). These techniques have been successfully used to identify numerous new phosphoproteins and phosphorylation sites [185–187]. In some cases, initial enrichment using antiphosphotyrosine Abs,

which have better specificity than the antiphosphoserine/threonine Abs, followed by IMAC have been used [188]. Although generally stable, in one study a batch-to-batch variation of C_8 magnetic particles has been observed [142].

Paramagnetic nonporous particles with chromatographic functionality have gained acceptance as platforms for the chromatographic manipulation of samples prior to downstream analysis [189]. Such beads are commercially available (Dyna, CPG, Bruker Daltonics, Miltenyi, Polymer Labs, Promega, QuickPick, BIAcore, IAsys, Vir, BioRad) in a wide range of functionalities including biological affinity, IMAC (Cu and Fe), RP (C_3 , C_8 , and C_{18}), anion- and cation-exchange. They offer a number of advantages, making them ideally suited to sample preparation for downstream proteomic analysis, as the process is simple and gentle, often allowing protein complexes to be recovered intact [189]. The magnetic particles can subsequently be recovered by such devices as the Kingfisher Magnetic Particle Processor (Thermo Scientific, Waltham, MA) or a Tecan Genesis Liquid handling workstation (Tecan, Durham, NC) [190–192]. The magnetic beads technology has allowed for the development of automatic magnetic particle based immunoassay systems (e.g., Beckman-Coulter Access (Fullerton, CA), Bayer ADVIA Centaur (Bayer, Leverkusen, Germany), Abbott ARCHITECT i2000 (Abbott Diagnostics, Abbott Park, IL)) [193].

Biosensor technology (e.g., BIAcore, Uppsala, Sweden; IAsys (Neosensors, Sedgefield, UK)), used as affinity detector combined with micropreparative HPLC, has been used for the identification, purification, and characterization of ligands for orphan receptors during chromatographic identification of the ligands of interest. Biosensors were also used as microaffinity purification platforms (both flow-based and cuvette-based systems) [194–196] for subsequent capture and MS/MS peptide analysis.

Mass spectrometric immunoassay (MSIA) that couples mass identification of proteins with antibody capture results in a unique biomarker signature, which overcomes the inability of traditional ELSA to recognize the specific form of the ligand assayed in spite of its excellent sensitivity [67, 197]. For example, whereas the performance of 115 Ab-antigen pairs was investigated in a study aimed at developing protein microarrays, only 20% of the arrayed Abs provided specific and accurate measurement of their cognate ligands at concentrations at or below 1.6 $\mu\text{g/mL}$ [198]. Immuno-MS has been used to analyze a number of potential biomarkers in urine (e.g., β -2 microglobulin, transthyrein, cystatin C, urine protein 1, retinol binding protein, albumin, transferring, and human neutrophil defensin peptide) using individual microcolumn tips (MSIA Tips, Intrinsic Bioprobes, Tempe, AZ) loaded into a multichannel pipette [199]. Rapid and cheap HT production of mouse-derived mAb using immunization, automated fusion and cell culture,

Table 4. Chromatographic selectivities for multidimensional separation/fractionation methods based on physical or chemical properties

Method	Properties	Elution conditions
Anion/cation exchange Chromatofocusing RP	Charge Charge Hydrophobicity	Above/below pI, increasing salt concentration pH gradient Step wise or gradient elution with increase in concentration of organic solvent
Hydrophobic/hydrophilic Interaction Size exclusion	Hydrophobicity Size (Stoke's radius)	Step wise or gradient elution with increase in organic solvent concentration/decreasing salt concentration Aqueous buffers often with low levels of detergents to minimize nonspecific adsorption
Affinity enrichment/depletion Lectin chromatography Metal affinity	Affinity Affinity Affinity	Competition, conformational change Competition, e.g., glucosamine Competition using increasing imidazole concentration or pH change
Ligand dye HAP	Pseudo affinity Pseudo affinity	Change in pH, salt concentration or use of a displacer Increasing phosphate concentration for this mixed-mode ion exchange

Modified from refs. [67] and [204].

and sensitive screening based on antigen-coated microarrays has been reported [198]. Such Abs need to be characterized for affinity and specificity using a technology as immuno-MS. These developments would ultimately lead to panels of highly specific Abs raised against all proteins predicted in the genome [84].

By optimizing a magnetic bead-based platform (Protein G beads linked with immobilized polyclonal Abs) amenable to HT peptide enrichment, followed by multiple reaction monitoring (MRM) MS using a stable isotope standards with capture by anti-peptides Abs (SISCAPA) and enrichment measured by selection ion monitoring, which employed a linear IT mass spectrometer for quantitative testing of biomarker candidates, an ion signal enhancement of $>10^3$ was achieved, believed sufficient for quantifying biomarkers in plasma at the range of ng/mL, which are applicable to any protein and biological fluid of interest [200].

Use of protein equalizer technology to reduce protein concentration differences by sharply reducing the differences of the most abundant components, while simultaneously enhancing the concentration of the most dilute species (*i.e.*, equalize) body fluid samples such as plasma/serum or urine. The technology uses a diverse solid phase library of combinational peptide affinity ligands coupled to spherical porous beads $\sim 65\ \mu\text{m}$ in diameter (Equalizer™ beads) that carry 50 pmol of hexapeptides supplied by Ciphergen Biosystem, Fremont, CA, which incorporate 20 different amino acids for synthesis of different ligand structure [201]. The progressive increase in detectable species when using larger sample/bead ratio suggests that theoretically 20^6 or 64 million protein species can be enriched [202]. Because the library has equal amounts of each ligand, theoretically the maximum amount of each protein binds the same number of

ligands. Under overloading condition, this has the effect of diluting those proteins present in excess of the ligand concentration, and concurrently those of relatively lower abundance. Unbound components are washed out, and capture species are desorbed [203]. The entire sample treatment takes about half a day and yields a protein solution that could be measured by MS. This fractionation method under the simplest condition (adsorption followed by a single elution), or with sequential desorption, or in association with other fractionation methods, can theoretically be valuable in situations when proteins are predicted by genome or mRNA sequences, but previously undetected, as in biological extracts of nonsequenced organisms, and they may now – at least theoretically – be detected [203].

Analysis of serum passed through a solid phase ligand library and sequentially eluted showed altered patterns compared with 2-DE; however, the new protein spots were not sequenced [202]. Therefore, it is not clear if solid phase ligand libraries substantially increase the detection of low abundance plasma proteins, nor is it clear if such strategies can be used in the near future to discover quantitative changes in different plasma samples [157]. Further, developments of this technique are thus warranted before its utility for biomarker discovery can be fully evaluated.

Chromatographic selectivities for multidimensional separation methods based on physical or chemical properties are presented in Table 4.

4 Sample fractionation to reduce proteome complexity

Due to the high complexity of plasma/serum samples, various fractionation methods have been used either to

Table 5. Common advantages and limitations of separation methods for mass spectrometers

Ionization source	Advantages	Disadvantages
Surface-enhanced SELDI chips	Affinity capture on MALDI chips with chromatographic functionality, ease of use, automation, convenience, low sample volume, raw samples analyzed, various chip surfaces, does not require sophisticated bioinformatics tools, detection with a broad molecular mass region in a single analysis	Loss of important information, problems at preanalytical and postanalytical steps, bias toward high abundant proteins particularly in the low mass range, performance could change over time
Derivatized carrier material (MELDI)	Allows for detection of larger number of peptides than SELDI due to use of particles with higher surface areas, permits raw sample analysis, HT	Carrier material must be carefully chosen, only highly porous, spherical and low micrometer size range particles can be used as carriers
Surface-derivatized magnetic beads	Can be automated, employs wide range of derivatized beads with different functional groups, sensitive, compatible with MS	Lack of reproducibility between commercial batches of the same beads
Glycoprotein/glycopeptide capture	High selectivity, reduced sample complexity	Loss of information on nonglycosylated proteins, increased false positive protein identification
Nonporous substrates (silicon wafers, silica particles and glass beads)	Allows for harvesting of distinct subsets of the proteome	Not a mature technology, needs standardization/validation
2-DE chromatography	Applicable to large molecules, high resolution, allows visualizing changes in molecular mass (M_r), pI or PTMs	Not applicable to peptides Is or M_s poorly represented
CE	Automation, relatively sensitive, low sample volume needed, low cost, MS/MS compatibility	Not well suited for peptides >20 kDa, precipitation of peptides in capillaries when acidic running buffers are used
LC	Automation, highly sensitive, accurate, multidimensional, versatile, HT potential, MS/MS compatibility	Time consuming, sensitive toward interfering compounds, limited mass range, often unsuitable for analysis of intact proteins
Protein microarrays	HT, low sample volume, chips have potential for assaying a wide range of biochemical activity, various platforms and detection methods are available	Abs are not available for all screened proteins, no standardization is available for biomarker discovery, low sensitivity, qualitative

Modified from refs. [25, 84, 129, 135].

generate several fractions (e.g., gel, CE, and LC), or to selectively obtain a particular subset of proteins/peptides with common features based on their similar affinities to a particular solid support (e.g., SELDI, MELDI, surface-captured magnetic beads, nonporous substrates) [149]. These strategies are illustrated in Table 5.

A higher dimensional 4-D separation strategy for an in-depth analysis of plasma/serum proteome that combines three orthogonal protein separation methods (protein depletion by affinity column, microscale solution IEF using ZOOM fractionator, and 1-DE), slicing gels and in-gel tryptic digestion carried out on sliced gels, followed by nano LC fractionation and MS/MS analysis, is believed to allow a detection dynamic range approaching 10^9 (<10 ng/mL), which is the concentration range expected for biomarkers in blood [157].

Heparin chromatography has been used to fractionate proteins from extracts of prokaryotic organisms or eukaryotic cells. Heparins are negatively charged polydispersed linear polysaccharides that have the ability to bind a wide range of biomolecules including enzymes, serine protease inhibitors, growth factors, extracellular matrix proteins, DNA modification enzymes and hormone receptors. In this chromatography, heparin is not only an affinity ligand but also an ion exchanger with high charge density and distribution, in which biomolecules can be specifically and reversibly adsorbed by heparins immobilized on an insoluble support, having the advantage of being able to enrich heparin-binding proteins using its concentration effect, which is particularly advantageous for analysis in 2-D, MS or other proteomics approaches [205].

IMAC, a useful fractionation method used to enrich metal-associated proteins, represents an affinity separation approach based on the interaction between proteins and metal ions immobilized on a solid support. By changing various metal ions and other experimental conditions such as pH and elution composition, IMAC can selectively isolate metal-binding protein fractions for further specific proteomic analysis, as characterizing the metalloproteome and its PTMs [206].

Other nonchromatographic purification have also been developed that can deal with protein suspensions [207]. A three phase partitioning (TPP) was originally described for interfacial precipitation of proteins, as well as for protein refolding. As such, TPP lacks selectivity, but was adequate for preparation with reasonable purity [208]. A recent version known as macroaffinity ligand facilitated TPP (MLFTPP) using eudragit S-100 as the affinity microligand, xylanase enzyme was purified from crude mixture of the fungus *Aspergillus niger* protein [209]. An aqueous two-phase system (ATPS) (e.g., PEG and dextran or phosphate) could partition introduced proteins forming two phases. Separation could be achieved by manipulating the partition coefficient of proteins by varying the average molecular weights of the polymers, the large strength of the salts or introducing an affinity ligand [210]. Expanded bed chromatography (EBC) is a technique that utilizes all the concepts of traditional backed bed technology, but the bed (i.e., calcium alginate or zinc alginate beads) is a fluidized form [211]. It combines filtration/centrifugation, concentration, and purification in a single step. EBC has benefited from using an immobilized affinity medium that can fluidize such as polyhistidine fusion tags [212].

5 Sample preparations from frozen tissue samples

The local concentration of the biomarker is expected to be high in the vicinity of the tumor microenvironment. Therefore, fine-needle aspiration biopsies (FNABs) are one way to obtain these samples [134]. Because many different cell types are typically present in tissue biopsies, laser microdissection (LMD) techniques have been developed to provide a rapid method for separating and processing homogenous subpopulation of cells for biochemical analysis [213]. Use of LMD may subject samples to potential artifactual processing, including changes at two different stages: (a) during the stage of tissue sections that enables selection of the relevant cell types, and (b) during the dissection process itself. These changes could impact the level of protein recovery and the quality of subsequent proteomic studies [214]. Isolated cells and captured minute tissue samples can then be directly analyzed employing, for example, MALDI-MS, or through the use of an automated multidimensional HT separation platform

that combines CIEF, with nano-RP LC [215]. CIEF is a variant of the commonest mode of CE (CZE) that combines the high resolution power of conventional gel IEF with CE instrumentation, and because of its focusing effect, it has often been used as the first step in multidimensional separation of complex mixtures of peptides and proteins [216] based on *pI* and a running buffer that contains ampholytes generating an electrically stable pH gradient, thus providing the highest possible efficiency for protein separation. The high analyte concentrations in small peak volumes as a result of electrokinetic focusing/stacking and the resolving multidimensional separation results in sensitive proteome analysis by enhancing the dynamic response and detection sensitivity of the coupled MS instruments. Instead of performing multi-runs or multidimensional separations, comparable or even better HT proteome results could theoretically be achieved by simply increasing the number of CIEF fractions due to the intrinsic high resolution nature of electrokinetic focusing, a feature that is particularly important for proteome analysis of limited tissue samples [217].

6 Dealing with formalin-fixed, paraffin-embedded (FFPE) tissue

FFPE tissue is the most common clinical specimen available after fixing and paraffin wax embedding for every tissue, from biopsy or surgical origin samples, for application of diagnostic assays on tissue after microscopic examination, and this huge amount of human material becomes a valuable resource for research in molecular medicine and biomarker discovery. It has been shown that protein crosslinking (and also nucleic acid) [218], due to formalin fixation, prevents protein profiling by Western blot and protein microarrays [219, 220]. The only routinely employed method currently available for protein analysis in FFPE tissues is immunohistochemistry, which is a qualitative technique. Although, a wealth of information exists on the expression signature of mRNA expressed by a specific type of cancer [221, 222], little data are currently available about protein expression signature in normal and cancerous cells [223–226] because difficulties of obtaining clinical samples, as well as the complexity of the dynamic proteome.

A shotgun proteomic method called direct tissue proteomics (DTP) was developed to provide extraction procedure that disrupts the crosslinked proteins from FFPE tissue samples, allowing for chemical identity of proteins in cells, tissues and fluids by MS/MS analysis [225]. Different extraction buffers were tested. ACN buffer (30% ACN, 100 mM ammonium carbonate), which is compatible with trypsin digestion and direct LC-MS/MS analysis can extract from 13 to 42% of total extractable proteins of large quantities of available samples. Another buffer (buffer D) was recommended; it contains radio-immunopreci-

pitiation (RIPA) buffer (150 mM NaCl, 10 mM Tris-HCl (pH 7.2), 2% SDS, 1% Triton X-100, 5 mM EDTA) heated at 94°C for 30 min followed by 60°C for 3 h to rehydrate proteins and hydrolyze the formaldehyde crosslink, followed by incubation with 1 µg of trypsin in buffer D (1:20 dilution) for 18 h at 37°C, then sample lyophilization and resuspension in buffer B (5% CN, 0.5% acetic acid, 0.005% heptafluorobutyric acid) and analyzed on a Finnigan LTQ-LIT mass spectrometer coupled to nano electrospray source. A 0.1% RapiGest buffer (Waters, Milford, MA) in 50 mM NH₄HCO₃, which is also compatible with MS, was found to give ~77% higher protein content than buffer D by densitometric quantification of 2-D silver stained gels [225].

Because DTP method is not quantitative, an absolute quantification method termed AQUA that employs an internal peptide standard was used for quantification of pictogram levels of prostate-specific antigen (PSA) in normal and cancerous prostate FFPE tissue. Using minute prostate biopsy sections, 428 prostate-expressed proteins were identified. The DTP strategy is a general method that is believed to provide a roadmap for successful identification of critical molecular targets of multiple cancer types [227].

A commercial multiplexed protein extraction system that is robust, fast, standardized and easy to use as a protein measurement technique for the solubilization of nondegraded, full length, and immunoreactive proteins from FFPE tissue samples was developed. It is specifically designed for the solubilization of high amounts of proteins from 10% neutral formalin-fixed tissue samples (Qproteome FFPE Tissue Kit, Qiagen) from cancer and noncancer tissues like colon carcinoma/non cancerous colon tissue, gastric cancers/nontumor gastric tissue, breast cancer, and nontumorous pancreatic tissue was employed. After deparaffination of cut sections placed on slides, with other unfixed frozen cryostat sections for comparison, it was possible to conveniently detect membrane, cytoplasmic and nuclear proteins, and no differences were found in the protein yield and abundance by Western blot and RP protein microarrays. Extraction of protein membrane (as demonstrated by the analysis of Her2 and E-cadherin proteins immunohistologically) in breast cancer biopsy was employed to test that technique [228]; which was found to be practical.

7 Concluding remarks and future prospects

It is evident from the body of this review that the field of sample preparation for proteomics is still in its infancy. There is no general standardized strategy for overall sample preparation, separation or purification. An ideal strategy, of course, is to entirely avoid sample preparation, but unfortunately today mass spectrometers do not

allow for direct identification/quantification of myriad proteins present in complex biological matrices. Until that goal is realized, a HT preparation strategy, with application of microfluidic devices, microchips (lab-on-chip) and automation, focusing on isolation of protein from organelles, as well as analysis of PTMs such as phosphopeptides, glycosylated proteins and other PTMs that play important roles in signal transduction processes is envisioned, in order to have a better understanding of the global processes occurring within cells from which sample preparation should be mild and suitable for extraction of noncovalent complexes between proteins, peptides, nucleic acids, and metabolic products [8, 13].

Major drawbacks to the general acceptance of multidimensional HPLC purification strategies are their being technically demanding, time consuming, need to be optimized for recovery and reproducibility, not easy to automate for HT analysis and requirement for extensive MS analysis time that could result in data analysis bottlenecks. Alternative platforms such as magnetic beads, multiwell plates or chips could address many of these issues. Simple batch wise elution techniques can lead to effective fractionation that is amenable to a HT automated application. Use of multidimensional techniques for MS analysis can reduce sample complexity and also increase the number of samples analyzed [176]. Improvement in data processing and analysis by integrating these processes into a linear process will increase overall efficiency [229].

The discovery role of potential biomarkers and drug targets far exceed the rate of early validation, and the gap is expected to increase [135]. Therefore, standardization of sample preparation methods to obtain reproducible data between laboratories is a must. Moreover, more investigation is needed focusing on reduction of sample complexity, developing promising sample preparation methods such as improved multiaffinity removal system, multidimensional LC, and use of nonporous solid phases [149]. Subcellular fractionation allows access to intracellular organelles and multiprotein complexes; LAPs and signaling complexes can be enriched, and at the same time, the complexity of the sample can be reduced [90].

Fractionation of protein mixtures to isolate species by their common biological activity is an approach that is not yet well established, not because of lack of interest, but rather because of lack of effective methods for immediate implementation. Selection of protease activities would no doubt have a strong impact on the understanding of specific pathway regulations with direct interest in diagnostic. Most proteases are part of very low abundance species that might stay silent for long periods, but can be detected by their specific peptide signature [65].

Application of SELDI technology and the generated proteomic patterns for the analysis of differences in proteins between healthy and cancer-bearing individuals

has raised many concerns due to various discrepancies, as illustrated in the body of this review, in a recent review on the subject [230] and in Table 5 [231, 232]. MALDI profiling, although has given better results than SELDI, still needs further standardization and evaluation [233]. Although, a recently developed carrier-based approach called MELDI is an improvement on the MALDI approach, and appears to enhance the robustness and throughput of large-scale proteomic studies [234, 235], nevertheless, it still needs confirmation [230].

The development of orthogonal high-dimensional proteomic strategies that include two or more protein separations has been shown to overcome to some extent the complexity of the plasma proteome leading to the detection of a large number of LAPs (<100 ng/mL), where cancer biomarkers are expected to be found, and also the identification of their PTMs when using intact protein fractionation schema together with shotgun LC-MS/MS analysis methods that are associated with the disease; this is a feature that is not readily available with regular protein digest-based fractionation approaches [136].

With an increased use of unique separation platforms, together with an equally expanding repertoire of fractionation technology aiming at precise molecular characterization of chemical modifications contributing to the complexity of protein patterns, coupled with quantitative and differential methods for protein analysis by MS, exceptional target protein biomarker signatures for diagnostic, prognostic, and evaluating response to therapy, which are more relevant than current descriptive or nucleic acids-based biomarker technology, is expected to be attained in the not too distant future.

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Exhibit G

Original Article

Latent transforming growth factor-beta binding protein-1 in circulating plasma as a novel biomarker for early detection of hepatocellular carcinoma

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Abstract: This study aimed to assess the diagnostic value of the latent transforming growth factor-beta binding protein-1 (LTBP-1) in distinguishing hepatocellular carcinoma (HCC) from patients with hepatitis or liver cirrhosis. The protein levels of LTBP-1 or AFP in circulating plasma were measured by enzyme-linked immunosorbent assay (ELISA) or chemiluminescence in four cohorts: HCC ($n = 167$), liver cirrhosis ($n = 50$), chronic hepatitis B (CHB, $n = 50$), and healthy individuals ($n = 104$). Receiver operating characteristics (ROC) curves and area under the curves (AUC) of the proteins were calculated. Results showed that plasma levels of LTBP-1 were significantly higher in HCC patients than those in other three groups. LTBP-1 showed a better diagnostic performance (AUC = 0.74, 95% CI: 0.67-0.80) in distinguishing HCC from the CHB or cirrhosis patients, compared to AFP (AUC = 0.59, 95% CI: 0.52-0.65). In the early-stage HCCs investigated, diagnostic performance of LTBP-1 (AUC = 0.77, 95% CI: 0.70-0.84) remained better than that of AFP (AUC = 0.61, 95% CI: 0.52-0.69). Combination of LTBP-1 and AFP showed increased diagnostic efficiency than any of the two proteins performed alone, for both all HCC (AUC = 0.78, 95% CI: 0.72-0.83) and early-stage HCC (AUC = 0.80, 95% CI: 0.74-0.87). These findings proposed that LTBP-1 may be a promising biomarker for distinguishing HCC from the CHB or liver cirrhosis patients, especially for the early-stage HCC.

Keywords: LTBP-1, biomarker, hepatocellular carcinoma, early detection, AFP

Introduction

Hepatocellular carcinoma (HCC) accounts for 70-80% of liver malignancies, is the third most common cause of cancer-related death worldwide [1, 2]. In China, chronic infection with hepatitis B virus (HBV) is one of the most important risk factor for HCC, and nearly 10% of the country's population is HBV carrier (accounting for two-thirds of all carriers worldwide) [3]. It is estimated that about 263,000 people died from HBV-related liver cancer or cirrhosis each year in China [3]. The poor survival rate of HCC is largely due to diagnosis at an advanced, non-resectable stage, resulting in inestimable indirect economic losses [4]. Hence more effective surveillance strategies should be developed to screen for early-stage HCC among the population at risk.

Alpha-fetoprotein (AFP) is a routinely used diagnostic biomarker for HCC, but its sensitivity is only 39-65% at the frequently-used cut-off 20 ng/ml [4, 5]. The unsatisfactory sensitivity limited the utilization of AFP as a screening biomarker for distinguishing HCC from patients at high risk [6]. Other biomarkers, such as lectin-bound AFP (AFP-L3), α -L-fucosidase, des- γ -carboxyprothrombin (DCP), γ -glutamyl transferase, glypican-3, etc., seemed to improve the diagnosis, but were dissatisfactory for early-stage HCC. Therefore, there is certainly a requirement of identifying novel biomarkers for early detection of HCC [7, 8].

Transforming growth factor-beta 1 (TGF- β 1) controls various important pathophysiological processes including tissue homeostasis, fibrosis, and carcinogenesis. The latent transform-

ing growth factor-beta binding protein 1 (LTBP-1), a secreted protein, is a part of the extracellular matrix (ECM). LTBP-1 targets latent TGF- β 1 and localizes it to ECM by interacting with integrin, fibrillin and fibronectin [9-11]. It has been reported that expression of LTBP-1 increased gradually, from grade II to grade IV, in human malignant gliomas [12]. Immunohistochemistry (IHC) analysis showed that the IHC staining of LTBP-1 was exceptionally strong in the tumor stroma of malignant mesothelioma [13], pancreatic ductal adenocarcinoma [14] and ovarian carcinoma [15]. However, the clinical significance of the LTBP-1 level of peripheral plasma in cancer patients is unknown. In the present study, we measured the plasma levels of LTBP-1 and AFP to investigate whether LTBP-1 could improve the diagnostic performance for HCC, along with AFP.

Materials and methods

Patients and specimens

We recruited peripheral blood plasma samples from 167 HCC patients (33 females and 134 males with the mean age of 54.2 years) before hepatectomy, at the Cancer Hospital, Peking Union Medical College & Chinese Academy of Medical Sciences (PUMC & CAMS). The plasma samples from 50 chronic hepatitis B (CHB) patients (17 females and 33 males with the mean age of 49.0 years) and 50 liver cirrhosis patients (17 females and 33 males with the mean age of 53.8 years, all of these patients were CHB positive) were collected at Beijing You'an Hospital, Capital Medical University. The plasma samples from 104 healthy individuals without viral hepatitis or malignant disease (39 females and 65 males with the mean age of 47.6 years) were taken from a health screening program at the Cancer Hospital, CAMS. Written informed consent was obtained from all patients.

Diagnosis of the HCC cases was based on abdominal ultrasound, computed tomography (CT), or magnetic resonance imaging (MRI), biochemical profiles (AFP serology and liver function enzymes), and was confirmed after surgery by histopathology examination, according to the American Association for the Study of Liver Disease guidelines (AASLD) [16]. The TNM stage of the HCC cases was defined according to the American Joint Committee on Cancer

(AJCC) Cancer Staging Manual (the seventh edition). In this study, stage I (solitary tumor, no vascular invasion) HCC was classified as the early stage [17]. Diagnoses of CHB and liver cirrhosis were according to those previously described [18].

Prior to surgery (for the HCC patients), peripheral blood was collected by venipuncture and kept in an EDTA-coated tube. Blood samples were centrifuged at 4°C for 10 min at 1000 g to separate the plasma and blood cells. The supernatants were collected, divided into aliquots and stored at -80°C until analysis.

Assessment of plasma LTBP-1 and AFP concentrations

The concentrations of LTBP-1 protein in the plasma samples were measured using an ELISA kit (cat.# E01L0245, BlueGene, Shanghai, China) according to the manufacturer's instructions. In brief, 100 μ l of plasma sample (1:1 diluted) was added to each well in the antibody pre-coated microtiter plate, then 50 μ l of conjugate was added to each well and mixed properly. After incubating the plate at 37°C for one hour, excess plasma sample was then washed off, and color development was achieved. The absorbance was measured at wavelengths of 450 nm using a microplate reader (Bio-Rad Laboratory, Hercules, CA, USA) immediately. The coefficient of variation (CV) of intra-assay and inter-assay given by the manufacturer is less than 10%.

AFP levels were tested using a commercial electrochemiluminescent immunoassay kit (Roche Diagnostics, Mannheim, Germany) at the Clinical Laboratory of the Cancer Hospital, CAMS.

Statistical analysis

Differences of LTBP-1 levels in the plasma samples among various groups were tested by Mann-Whitney test (two groups) or Kruskal-Wallis rank sum test (more than two groups) using the R statistic software (<http://www.r-project.org/>). Receiver operating characteristics (ROC) curves, respective areas under the curves (AUCs) with 95% confidence interval (CI), and statistic comparison of two ROC curves were assessed by the R package "pROC". The optimal cut-off value for diagnosis was calcu-

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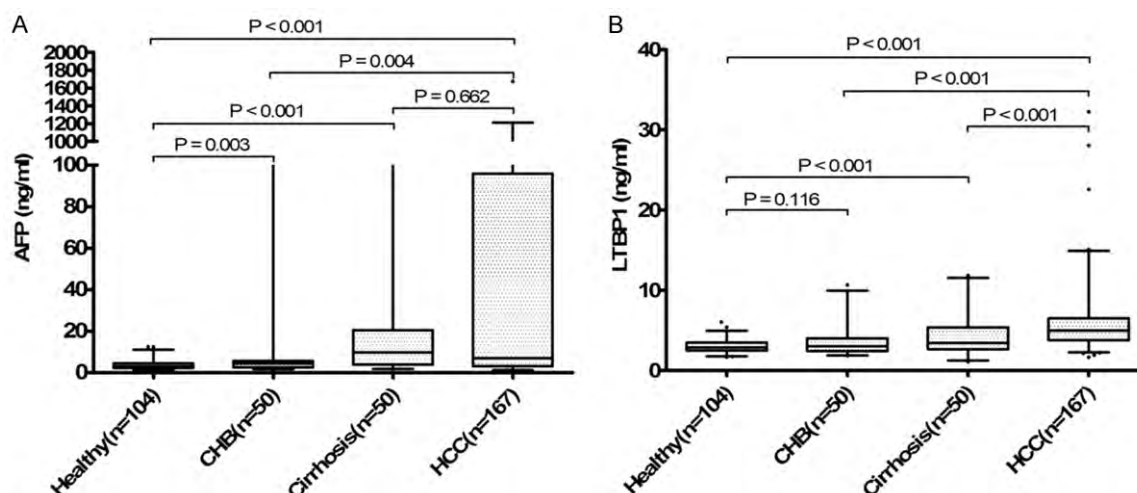


Figure 1. Distribution of plasma levels of AFP and LTBP-1 in HCC and other groups. Box plot shows the distribution of plasma concentrations of AFP (A) and LTBP-1 (B) in each group. Box refers to the 25th and 75th percentile values, with a line indicating median levels, whereas the 95% range is presented by short lines outside the box, points outside the 95% range are outliers. Statistic comparison between HCC and each of other three groups was performed by two-sided Mann-Whitney test, and $P < 0.05$ was considered to be significant.

Table 1. Plasma concentrations of AFP and LTBP-1 in HCC (positive of CHB and cirrhosis) and three other cohorts

†Cohorts	N	AFP (ng/ml)		LTBP-1 (ng/ml)	
		Median (range)	‡P	Median (range)	‡P
Healthy	104	3.0 (1.1-12.5)	7.09E-12	2.9 (1.7-6.0)	2.14E-24
CHB	50	4.5 (1.6-638.0)	4.34E-4	3.0 (1.8-10.7)	2.7E-10
Cirrhosis	50	9.8 (1.8-282.0)	0.199	3.4 (1.1-11.8)	1.74E-4
HCC	120	9.5 (1.2-1674.0)	-	5.0 (1.6-14.3)	-

†CHB, chronic hepatitis B; HCC, hepatocellular carcinoma; ‡P values were assessed by comparison between HCC (positive of CHB and cirrhosis) and each of the other cohorts, two-sided Mann-Whitney test.

lated by minimum distance to the top-left corner of the ROC curve. In combination analysis of AFP and LTBP-1, logistic regression model was employed to estimate the probability of the two combined variables. The sensitivity, specificity and overall accuracy with 95% CI for the optimum cut-off were calculated using the R package "DiagnosisMed". Box plot and scatter plot of AFP and LTBP-1 plasma levels were performed by GraphPad Prism 5. All statistical tests were two-sided, and $P < 0.05$ was considered as statistical significance.

Results

The plasma levels of AFP and LTBP-1 in HCC patients and other groups

As Shown in **Figure 1A**, the protein levels of AFP in HCC patients (median = 7.0 ng/ml, range:

0.9-1674.0 ng/ml) were significantly elevated than that in CHB patients (median = 4.5 ng/ml, range: 1.6-368.0 ng/ml, $P = 0.0037$) and healthy controls (median = 3.0 ng/ml, range: 1.1-12.5 ng/ml, $P < 0.0001$); but had not statistical difference with that of liver cirrhosis patients (median = 9.8 ng/ml, range: 1.8-282 ng/ml, $P = 0.662$); and the AFP levels were higher in both CHB and

cirrhosis patients, when compared with healthy. The LTBP-1 levels were significantly higher in HCC group (median = 5.0 ng/ml, range: 1.6-32.2 ng/ml) than that in the cirrhosis patients (median = 3.4 ng/ml, range: 1.1-11.8 ng/ml, $P = 0.00015$), CHB patients (median = 3.0 ng/ml, range: 1.8-10.7 ng/ml, $P < 0.0001$), and healthy controls (median = 2.9 ng/ml, range: 1.7-6.0 ng/ml, $P < 0.0001$); there was no statistic significance between healthy and CHB patients, and significant difference between healthy and cirrhosis patients (**Figure 1B**).

Furthermore, we also investigated the HCC patients who were CHB positive and suffering cirrhosis ($n = 120$, **Table 1**). Similarly, the protein levels of AFP were significantly elevated in HCC patients, compared with CHB patients ($P = 0.0004$) and healthy controls ($P < 0.0001$); but had not statistical difference with that of liver

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Table 2. Clinical association of the plasma levels of AFP and LTBP-1 in HCC patients

†Variables	‡Phenotypes	N	AFP (ng/ml)		LTBP-1 (ng/ml)	
			Median (range)	§P	Median (range)	§P
Age	< 55 yrs	88	5.8 (1.1-1674.0)	0.523	4.5 (1.6-10.0)	0.003
	≥ 55 yrs	79	7.1 (0.9-1210.0)		5.6 (2.1-32.2)	
Gender	Female	33	33.1 (1.3-1210.0)	0.042	5.6 (2.8-28.1)	0.058
	Male	134	5.7 (0.9-1674.0)		4.8 (1.6-32.2)	
Ves.Inv.	No	106	9.3 (0.9-1210.0)	0.265	5.1 (1.9-32.2)	0.322
	Yes	56	5.7 (1.2-1674.0)		4.7 (1.6-15.1)	
pTNM	I	80	9.3 (0.9-1210.0)	0.737	5.2 (1.9-28.1)	0.207
	II/III/IV	79	6.3 (1.2-1674.0)		4.7 (1.6-32.2)	
Tumor Size	≤ 2 cm	17	4.4 (1.2-359.8)	0.264††	4.8 (1.6-7.5)	0.086††
	2~5 cm	103	9.4 (0.9-1674)		4.8 (2.2-22.6)	
	> 5 cm	45	5.9 (1.3-1210.0)		5.7 (1.9-32.2)	
Tumor Dif.	High	20	4.3 (1.3-1210.0)	0.042††	4.4 (2.4-14.3)	0.853††
	Moderate	109	8.3 (0.9-1674.0)		5.0 (1.6-28.1)	
	Low	32	36.5 (1.3-1210.0)		5.1 (1.9-32.2)	
CHB	No	7	7.1 (0.9-1210.0)	0.326	4.1 (2.4-32.2)	0.647
	Yes	156	6.7 (1.1-1674.0)		5.0 (1.6-28.1)	
Cirrhosis	No	40	4.5 (0.9-1210.0)	0.017	4.9 (1.9-32.2)	0.716
	Yes	121	10.7 (1.2-1674.0)		5.0 (1.6-14.3)	

†CHB, chronic hepatitis B; HCC, hepatocellular carcinoma; Ves.Inv., vessel invasion; Tumor Dif., tumor differentiation. ‡yrs, years. §P values were assessed by two-sided Mann-Whitney test. ††P values were assessed by Kruskal-Wallis rank sum test. The patients with missing value of clinical variables were not listed in this table.

cirrhosis patients ($P = 0.199$). The LTBP-1 levels were higher in HCC patients compared with any of the other groups ($P < 0.01$).

Correlation between the plasma levels of AFP and LTBP-1 and clinical variables in HCC patients

The relationship between clinical characteristics and the plasma protein levels of AFP and LTBP-1 in the HCC were summarized in the **Table 2**. Interestingly, the plasma levels of LTBP-1 were significantly elevated in the older patients (≥ 55 years old, $P = 0.003$), while the AFP levels showed no correlation with age. The two proteins were associated with gender, both raising in female patients (marginally significant for LTBP-1, $P = 0.058$). Plasma levels of

AFP were significantly higher in the HCC patients with low-differentiated tumor ($n = 32$, median = 36.5 ng/ml, range: 1.3-1210 ng/ml, $P = 0.042$), and patients with cirrhosis ($n = 121$, median = 10.7 ng/ml, range: 1.2-1674 ng/ml, $P = 0.017$). In contrast, the LTBP-1 levels showed increased trend along with tumor size (marginal significance, $P = 0.086$), but no significant correlation with the TNM stage, vessel invasion, tumor differentiation, CHB or cirrhosis status.

Performance of LTBP-1 in discriminating HCC from CHB or liver cirrhosis patients

We further evaluated the diagnostic performance of LTBP-1 in distinguishing HCC ($n = 167$) from the patients at high-risk (with CHB or cirrhosis, $n = 100$). ROC analysis revealed that

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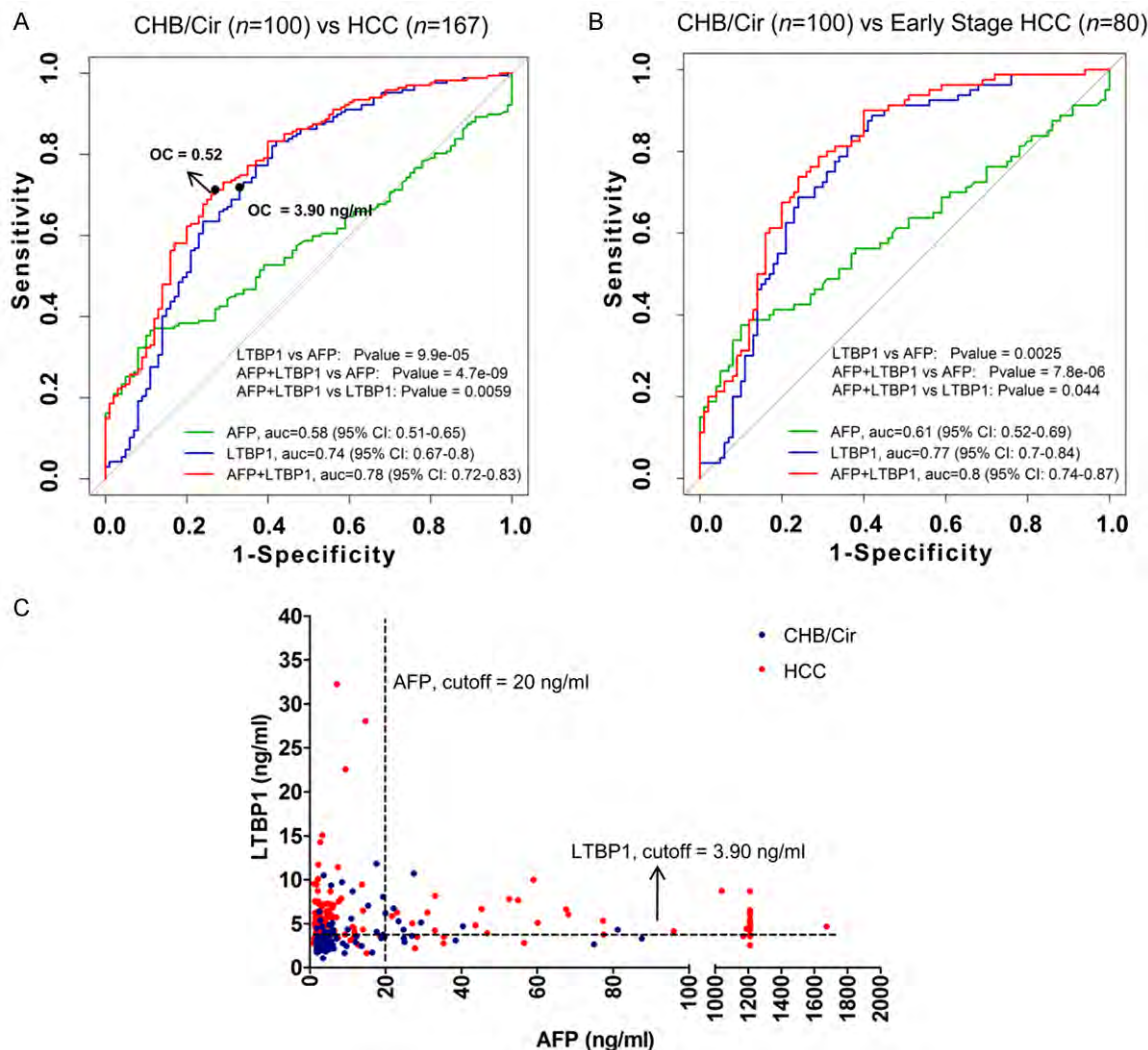


Figure 2. Diagnostic performance of LTBP-1 in discriminating HCC from CHB and liver cirrhosis patients. Receiver operating characteristics (ROC) curve analysis was performed in CHB and liver cirrhosis patients ($n = 100$) versus all HCC patients (A. $n = 167$) and early stage HCC patients (B. $n = 80$). ROC curves and area under the curves (AUC) with 95% confidence interval (CI) are plotted for AFP, LTBP1 and combination of the two proteins (probability of Logistic regression), respectively. Statistic comparison between two ROC curves was performed with “delong” method using the R package “pROC”, $P < 0.05$ was considered to be significant. Optimal cut-offs (OC, the black points) of LTBP-1 and AFP + LTBP-1 were calculated by minimum distance to the top-left corner of the ROC curves in (A). (C) Scatter plot of AFP and LTBP-1 concentrations for each sample point indicates a good diagnostic performance of LTBP-1 in AFP-negative HCC patients, at the optimal cut-off in (A).

the LTBP-1 had a better area under curve (AUC = 0.74, 95% CI = 0.67-0.80) than AFP (AUC = 0.58, 95% CI = 0.51-0.65), with a statistical significance ($P < 0.0001$). Moreover, combination of the two biomarkers showed better diagnostic performance (AUC = 0.78, 95% CI = 0.72-0.83) than LTBP-1 ($P = 0.006$) or AFP ($P < 0.0001$) which were performed alone (**Figure 2A**). The optimal diagnostic cut-offs of LTBP1 (3.9 ng/ml) and LTBP-1 + AFP (probability of Logistic regression, 0.52) were calculated accord-

ing to the ROC curves (**Figure 2A**), and the cut-off of AFP was set as routinely used 20 ng/ml. The diagnostic accuracy of AFP (sensitivity = 38.3%, specificity = 82.0%), LTBP-1 (sensitivity = 71.9%, specificity = 67.0%), and LTBP-1 + AFP (sensitivity = 70.7%, specificity = 73.0%) were 54.7%, 70.0% and 71.5%, respectively (**Table 3**).

In this study, 80 (47.9%) out of the 167 HCC cases were in early-stage. As data shown in

Diagnostic value of LTBP-1 plasma levels in HCC

Table 3. The diagnostic performance of AFP, LTBP-1, and the combination for HCC

†Cohorts	Classifiers	Sensitivity (95% CI)	Specificity (95% CI)	Accuracy (95% CI)
HCC vs. CHB/Cir	AFP	0.383 (0.313-0.459)	0.82 (0.733-0.883)	0.547 (0.487-0.605)
	LTBP-1	0.719 (0.646-0.781)	0.67 (0.573-0.754)	0.700 (0.643-0.752)
	AFP + LTBP-1	0.707 (0.634-0.770)	0.73 (0.636-0.807)	0.715 (0.658-0.766)
Early Stage HCC vs. CHB/Cir	AFP	0.412 (0.311-0.522)	0.82 (0.733-0.883)	0.639 (0.566-0.705)
	LTBP-1	0.775 (0.672-0.853)	0.67 (0.573-0.754)	0.717 (0.647-0.777)
	AFP + LTBP-1	0.750 (0.645-0.832)	0.73 (0.636-0.807)	0.739 (0.670-0.798)
HCC (AFP < 20 ng/ml) vs. CHB/Cir	AFP	-	-	-
	LTBP-1	0.689 (0.595-0.771)	0.67 (0.573-0.754)	0.680 (0.613-0.740)

†CHB, chronic hepatitis B; Cir, cirrhosis; HCC, hepatocellular carcinoma.

Figure 2B and **Table 3**, the concentrations of plasma LTBP-1 improved the diagnostic accuracy on this part of HCC (AUC = 0.77, 95% CI = 0.70-0.84; sensitivity = 77.5%, specificity = 67.0%, accuracy = 70.1%, when cut-off = 3.9 ng/ml) from the cirrhosis or CHB cases, comparing to AFP (AUC = 0.61, 95% CI = 0.52-0.69; sensitivity = 41.2%, specificity = 82.0%, accuracy = 63.9%, when cut-off = 20.0 ng/ml). Combination of the two proteins (AUC = 0.80, 95% CI = 0.74-0.87; sensitivity = 75.0%, specificity = 73.0%, accuracy = 73.9%) also showed better diagnostic result than any protein analyzed alone.

Complementary effect between LTBP-1 and AFP in HCC diagnosis

As what shown in **Figure 2C**, there was no significant correlation (Spearman's correlation rho = 0.029, $P = 0.711$) between the plasma protein levels of LTBP-1 and AFP in HCC, with only 49 out of 167 HCC patients were simultaneously detected by the two proteins at the current cut-offs. Among the 167 HCC cases investigated, 103 (61.7%) were AFP-negative according to the recommended clinical cut-off 20.0 ng/ml, which resulted in a diagnostic sensitivity of 38.3%. Whereas, in this AFP-negative HCC group, LTBP1 (cut-off = 3.9 ng/ml) remained a higher diagnostic sensitivity of 68.9%, specificity of 67.0%, and accuracy of 68.0% (**Table 3**). These results indicated that LTBP-1 was complementary to AFP in diagnosis of HCC.

Discussion

Transforming growth factor- β (TGF- β) is a multifunctional factor that is involved in cell growth

and differentiation [19]. LTBP-1 (latent transforming growth factor- β binding proteins) were first purified by Kanzaki T et al. from human platelets (120-165 kDa) and fibroblast (170-190 kDa), and were found not to bind directly to active TGF- β 1 [20]. The LTBP-1 are structural components of the extracellular matrix (ECM) microfibrils, which associate with elastic fibers. There are four isoforms of LTBP-1 (LTBP-1, -2, -3, -4), which are components of connective tissue microfibrils and regulators of TGF- β tissue deposition and signaling [21]. LTBP-1 is a secreted protein, and may have a structural role in the ECM. A previous study reported that LTBP-1 may be involved in epithelial-mesenchymal cell transformation (EMT) of embryonic heart, suggesting that LTBP-1 likely contributes to malignant transformation of cells [22].

Chronic hepatitis B (CHB) and subsequent liver cirrhosis resulting from Hepatitis B virus (HBV) infection is a major cause of developing hepatocellular carcinoma (HCC) in China [6]. Data obtained in this study show that the concentration of LTBP-1 protein in the circulating plasma is gradually increased from healthy individuals to CHB patients, to cirrhosis patients, and has the highest levels in HCC patients (**Figure 1B**). This indicates that LTBP-1 may play an important role in the process of HBV-induced HCC. Furthermore, LTBP-1 levels in the plasma displayed a good performance in distinguishing HCC from the healthy cohort, and significantly improved the diagnostic efficiency of the routinely used AFP when combining the two biomarkers (data not shown). Thus, LTBP-1 is proposed to be a potential novel biomarker for HCC diagnosis.

To date, a great challenge is to screen HCC in the high-risk populations, such as liver cirrhosis and CHB patients, which is partially due to lack of effective biomarkers. Although raised AFP level in the plasma was a risk factor for HCC development [23, 24], it was unsuitable for HCC screening because of its poor diagnostic sensitivity of 39%-65% [5]. In the present study, the plasma levels of AFP had no statistic difference between the HCC and cirrhosis groups ($P = 0.662$), and thus couldn't distinguish HCC from the cirrhosis patients. In contrast, the plasma levels of LTBP-1 were significantly elevated in the HCC group than that in the cirrhosis group ($P = 0.00015$), and had a rising trend from CHB, cirrhosis to HCC patients (**Figure 1B**). Furthermore, the HCC patients with cirrhosis had higher AFP levels than those without ($P = 0.017$, **Table 2**), while the plasma levels of LTBP-1 in HCC patients were independent of the cirrhosis status. It has been reported that many non-malignant chronic liver disease patients with cirrhosis also have raised AFP concentrations in the serum [18, 25]. In the patients with chronic liver disease background, our data showed that LTBP1 performed well in discriminating HCC from the non-HCC patients (**Supplementary Figure 1A, 1B**).

Early detection was demonstrated to be helpful in improving the prognosis of primary liver cancer including HCC in China during the last decades [26]. Unfortunately, the biomarkers for early detection of HCC are greatly insufficient. Herein we found that the plasma LTBP-1 improved the diagnosis accuracy of AFP for the early-stage HCC, with an increased sensitivity from 41.2% to 75.0%, and overall accuracy from 63.9% to 73.9%. Moreover, among the HCC cases investigated in this study, about 60% were AFP-negative at the cut-off 20 ng/ml, similar with other published data [27]. Our results suggested that measurement of the plasma LTBP-1 could distinguish 68% of AFP-negative HCC cases from the cirrhosis and CHB patients. These data suggested that LTBP-1 could be complementary to AFP in diagnosis of HCC, especially for the cases at early stage.

In the clinical practice, the diagnosis of HCC tumors with small diameters is a prevalent problem currently. Solitary nodules without blood flow in the liver detected by ultrasonogra-

phy (US) screening is usually less than 2 cm [28, 29], making a difficulty in HCC diagnosis. In the present study, despite an increase trend of LTBP-1 levels along with tumor size, there was no statistical difference between tumors less than 2 cm and those with diameters of 2-5 cm (**Supplementary Figure 1C**). Furthermore, the diagnostic performance of LTBP-1 remained better than AFP for HCC less than 2 cm (**Supplementary Figure 1D**). However, these observations need to be validated in large cohort of patients with small HCC.

In the present study, the plasma protein levels of AFP were higher in the female patients with HCC, similar results was also reported in a recently study based on a large cohort [30]. In addition, the LTBP-1 was correlated with age and sex, raising in elderly and female patients too (**Table 2**). However, there were no significant correlation between LTBP-1 and age in the healthy control, CHB nor cirrhosis patients (data not shown). Further, the diagnostic performance of LTBP-1 for HCC was independent of age and gender (**Supplementary Figure 2**). The research groups from Taiwan found that male gender and elder age were independently associated with a higher risk of HCC in a community-based cohort study [31, 32]. In mainland of China, HCC is two to three times more frequent in men than in women [26]. Coincidentally, the expression of LTBP-1 was reported to be hormonally regulated [33], suggesting a potential biological explanation for the differences on HCC incidence between men and women. These observations suggest that the biological function of LTBP-1 in carcinogenesis of HCC needs to be further studied.

In summary, the protein levels of LTBP-1 in the circulating plasma exhibited a gradually increased trend in healthy individuals, CHB, liver cirrhosis and HCC patients. Combination of LTBP-1 and AFP performed better diagnostic ability than AFP in discriminating HCC from CHB or liver cirrhosis patients, especially in the AFP-negative or early-stage HCC cases. The LTBP-1 is proposed to be a promising diagnostic biomarker for HCC, which could complement some of the limitations of the AFP. The diagnostic value of LTBP-1, however, needs to be confirmed by large-scale and multicenter validation in future.

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Disclosure of conflict of interest

None.

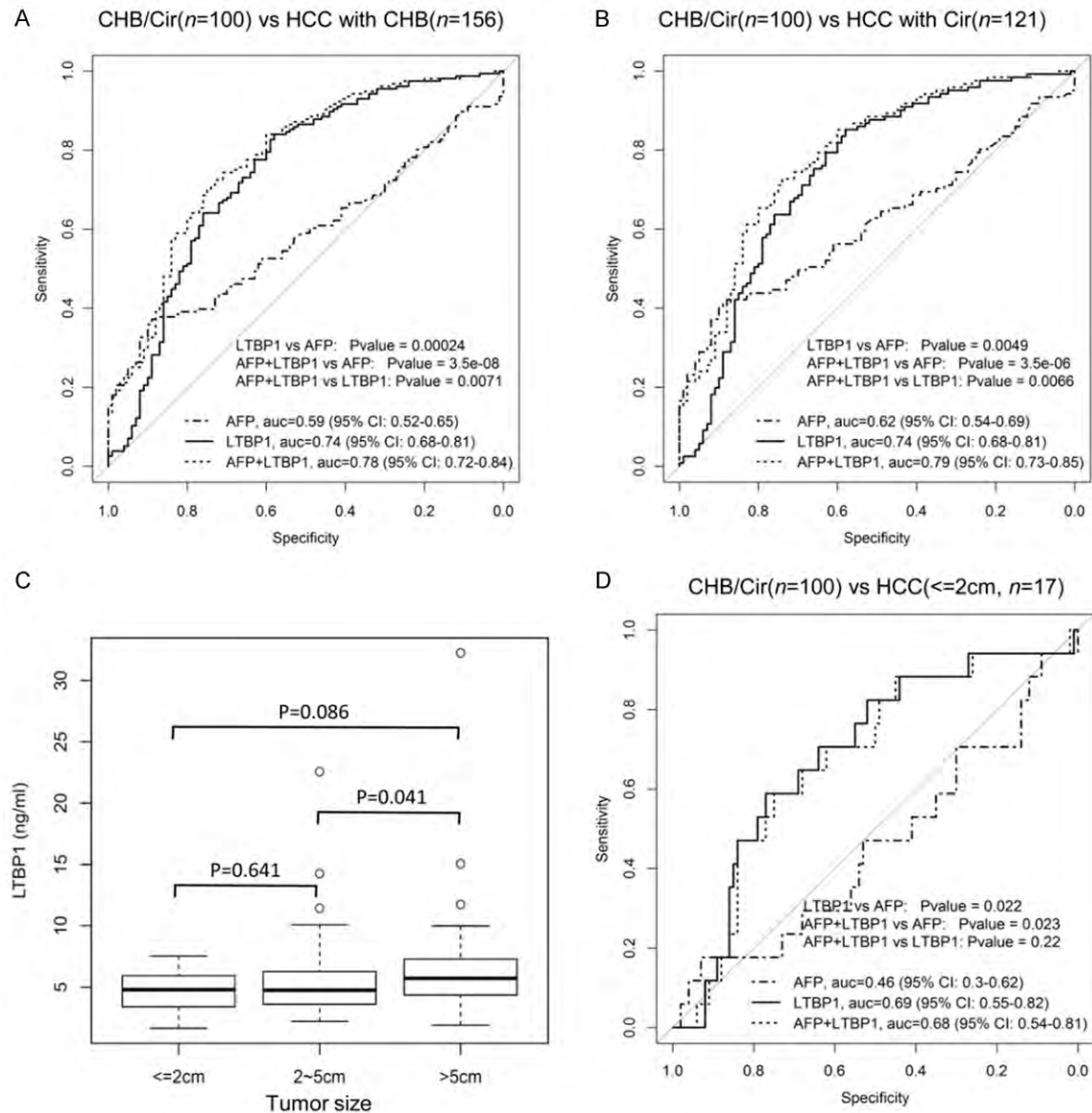
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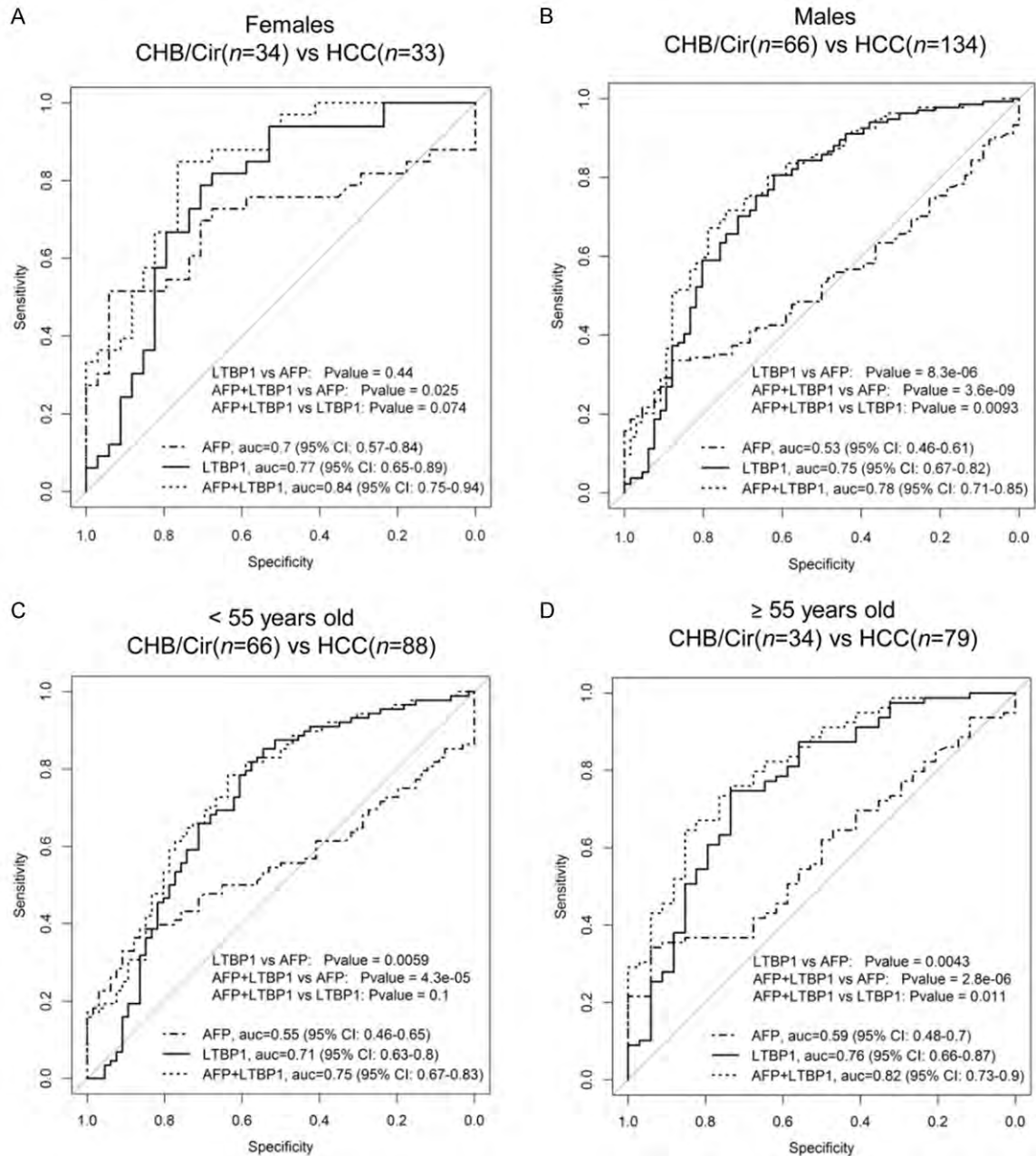
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Diagnostic value of LTBP-1 plasma levels in HCC



Supplementary Figure 1. HCC diagnostic performance of LTBP-1 in different sub-groups. Receiver operating characteristics (ROC) curve analysis was performed in CHB and liver cirrhosis patients versus HCC patients in different groups: A. HCC patients with CHB ($n = 100$ vs. $n = 156$); B. HCC patients with liver cirrhosis ($n = 100$ vs. $n = 121$); D. HCC patients with tumors ≤ 2 cm ($n = 100$ vs. $n = 17$). ROC curves and area under the curves (auc) with 95% confidence interval (CI) are plotted for AFP, LTBP-1 and combination of the two proteins (probability of Logistic regression), respectively. Statistic comparison between two ROC curves was performed with “delong” method using R package “pROC”, $P < 0.05$ was considered to be significant. C. Box plot showing LTBP-1 levels in different HCC groups with diverse tumor size. Box refers to the 25th and 75th percentile values, with a line indicating median levels, whereas the 95% range is presented by short lines outside the box, points outside the 95% range are outliers. Statistic comparisons between each of two groups and all three groups were performed by two-sided Mann-Whitney and Kruskal-Wallis rank sum test, respectively.

Diagnostic value of LTBP-1 plasma levels in HCC



Supplementary Figure 2. HCC diagnostic performance of LTBP-1 with stratification on gender and age. Receiver operating characteristics (ROC) curve analysis was performed in CHB and liver cirrhosis patients versus HCC patients in different groups: A. Females ($n = 34$ vs. $n = 33$); B. Males ($n = 66$ vs. $n = 134$); C. Individuals who < 55 years old ($n = 66$ vs. $n = 88$); D. Individuals who ≥ 55 years old ($n = 34$ vs. $n = 79$). ROC curves and area under the curves (AUC) with 95% confidence interval (CI) are plotted for AFP, LTBP1 and combination of the two proteins (probability of Logistic regression), respectively. Statistic comparison between two ROC curves was performed with “delong” method using R package “pROC”, $P < 0.05$ was considered to be significant.

Exhibit H

Screenshot of Taylor and Francis database record for Zaccaria

The screenshot displays the Taylor & Francis Online interface for a specific article. The browser's address bar shows the URL: <https://www.tandfonline.com/doi/full/10.1080/10964654.2015.1021470>. The page header includes the Taylor & Francis logo, navigation links (Browse, Search, Publish), and user options (Login, Register). The breadcrumb trail indicates the article's location: Home > All Journals > International Journal of Nanomedicine > List of Issues > Volume 10, Issue > Accessing to the minor proteome of red b....

The article details are as follows:

- Journal:** International Journal of Nanomedicine > Volume 10, 2015 - Issue
- Article Title:** Accessing to the minor proteome of red blood cells through the influence of the nanoparticle surface properties on the corona composition
- Author:** Affif Zaccaria (with an email icon)
- Co-authors:** Florence Roux-Dalval, Ali Bouamrani, Adrien Mombrun, Pascal Mossuz, Bernard Monsarrat & ...show all
- Publication Info:** Pages 1869-1883 | Published online: 09 Mar 2015
- Metrics:** 58 Views, 6 CrossRef citations to date, 12 Altmetric
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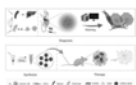
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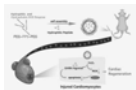
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Exhibit J

Screenshot of ScienceDirect database record for Hu

The screenshot shows a web browser window with multiple tabs open. The active tab is displaying a ScienceDirect article. The browser's address bar shows the URL: <https://www.sciencedirect.com/elsevier/libraries/pennstate/article/pii/S0927776514001014>. The ScienceDirect logo is in the top left, and the PennState University Libraries logo is in the top right. The article title is "Nanoparticle size matters in the formation of plasma protein coronas on Fe₃O₄ nanoparticles". The journal is "Colloids and Surfaces B: Biointerfaces", Volume 121, 1 September 2014, Pages 354-361. The authors listed are Zhengyan Hu, Hongyan Zhang, Yi Zhang, Ren'an Wu, and Hanfa Zou. The article has a DOI of 10.1016/j.colsurfb.2014.06.016. The left sidebar contains a table of contents with links to Outline, Highlights, Abstract, Graphical abstract, Keywords, Introduction, Materials and methods, Results and discussion, Conclusions, Acknowledgements, Appendix A. Supplementary data, and References. The right sidebar shows recommended articles and article metrics. The bottom of the page features a Windows taskbar with various application icons and a system clock showing 1:20 PM on 9/5/2024.

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Nanoparticle size matters in the formation of plasma protein coronas on Fe₃O₄ nanoparticles

Zhengyan Hu ^{a, b}, Hongyan Zhang ^{a, b}, Yi Zhang ^{a, b}, Ren'an Wu ^{a, b}, Hanfa Zou ^{a, b}

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Supporting Information

Nanoparticle Size Matters **in** the Formation of Plasma Protein Coronas on Fe₃O₄ nanoparticles

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Table S1 Total identified plasma proteins in the protein coronas of the Fe₃O₄ NPs of different sizes and their isoelectric points (pI), molecular weights (MW) and relative protein abundance (blank spaces indicate that the corresponding proteins were not identified in the analysis). **Because** the proteins were detected in more than 2 replicates, the standard deviation was from the mean (n=2 or n = 3).

IP1	Names	pI	MW (kD)	FeO _x 30					FeO _x 400					FeO _x 400				
				MWSpC ₋₁	MWSpC ₋₂	MWSpC ₋₃	Average	SD	MWSpC ₋₁	MWSpC ₋₂	MWSpC ₋₃	Average	SD	MWSpC ₋₁	MWSpC ₋₂	MWSpC ₋₃	Average	SD
IP100021842.1	Apolipoprotein E	5.65	36.13	5.52	5.38	5.52	5.47	0.08	4.27	4.26	4.27	4.27	0.01	8.55	7.66	8.43	8.21	0.48
IP100298971.1	Vitronectin	5.55	54.27	4.09	4.07	3.94	4.03	0.08	3.75	3.73	3.66	3.71	0.05	6.37	6.64	6.79	6.60	0.21
IP100022371.1	Histidine-rich glycoprotein	7.09	59.54	0.26	0.26	0.28	0.27	0.01	3.14	3.17	3.18	3.16	0.02	4.42	5.16	4.59	4.72	0.39
IP100022445.1	Platelet basic protein	9.04	13.89	3.71	3.75	4.18	3.88	0.26	3.00	3.33	3.07	3.14	0.17	4.32	4.55	4.51	4.46	0.12
IP100022446.1	Platelet factor 4	8.93	10.84	8.95	8.36	8.39	8.57	0.33	3.55	3.32	3.64	3.51	0.16	4.09	4.08	3.87	4.01	0.12
IP100022394.2	Complement C1q subcomponent subunit C	8.61	25.76	3.47	2.81	3.28	3.19	0.34	0.82	0.86	0.72	0.80	0.07	4.08	3.96	3.76	3.93	0.16
IP100022392.1	Complement C1q subcomponent subunit A	9.26	26.00	2.30	2.39	2.56	2.42	0.13	0.62	0.76	0.80	0.73	0.09	3.44	3.82	3.58	3.61	0.19
IP100026314.1	Isoform 1 of Gelsofin	5.90	85.64	0.61	0.57	0.74	0.64	0.09	2.01	2.19	2.18	2.13	0.10	3.40	3.49	3.41	3.44	0.05
IP100021855.1	Apolipoprotein C-I	8.01	9.33	0.95	0.51	0.69	0.72	0.22	5.83	6.81	6.19	6.28	0.50	3.07	3.50	3.13	3.23	0.23
IP100783987.2	Complement C3 (Fragment)	6.02	187.03	1.99	1.99	2.01	2.00	0.01	1.53	1.59	1.59	1.57	0.04	2.65	2.60	2.70	2.65	0.05
IP100019568.1	Prothrombin (Fragment)	5.64	69.99	2.76	2.56	2.78	2.70	0.13	1.41	1.51	1.47	1.46	0.05	2.47	2.34	2.70	2.51	0.18
IP100745872.2	Serum albumin	5.92	69.32	1.33	1.24	1.25	1.27	0.05	8.83	9.32	8.89	9.01	0.27	2.53	2.52	2.45	2.50	0.04
IP100967849.1	C1QB Uncharacterized protein	8.83	26.44	1.48	1.52	1.71	1.57	0.12		0.23	0.38	0.30	0.11	2.24	2.11	2.31	2.22	0.10
IP100785094.2	IGHG1	8.91	51.05													2.10	2.10	0.01
IP100876888.1	IGHG1	8.44	51.56	3.21		3.07	3.14	0.10	0.67	0.73		0.72	0.04	2.00		2.07	2.03	0.05
IP100784817.1	IGHG1	8.64	52.33		2.83	2.92	2.88	0.07						1.89	2.04		1.96	0.11
IP100298828.3	APOH Beta-2-glycoprotein 1	8.34	38.27	1.92	1.79	2.06	1.92	0.13	0.96	0.94	0.93	0.94	0.02	2.07	1.83	1.84	1.91	0.13

IP100969456.1	IGKC Putative uncharacterized protein	8.17	25.79							0.93	0.93	0.81	0.89	0.07	1.85	1.95	1.88	1.89	0.05
IP100887154.2	Complement component 4B	6.89	192.63	0.64	0.63	0.73	0.67	0.05	0.46			0.42	0.44	0.03	1.80	1.81		1.81	0.01
IP100653045.1	IGKC Anti-RND monoclonal T125 kappa light chain	8.69	25.68	3.62	3.55		3.58	0.03							1.75	1.88	1.77	1.80	0.07
IP100021841.1	Apolipoprotein A-I	5.56	30.76	2.21	2.21	2.41	2.28	0.11	9.44	10.11	9.63	9.73	0.35	0.35	1.77	2.03	1.50	1.77	0.27
IP100657670.1	Apolipoprotein C-III variant 1	7.90	12.81	3.37	2.91	2.82	3.04	0.30	6.67	6.15	6.05	6.29	0.34	1.22	1.43	1.78	1.48		0.28
IP100032328.2	Isoform HMW of Kininogen-1	6.33	71.91	1.46	1.35	1.51	1.44	0.08	2.96	2.93	2.84	2.91	0.06	1.41	1.37	1.33	1.37		0.04
IP100032179.3	SERPINC1	6.32	52.57	1.01	1.16	1.18	1.12	0.09	0.69	0.56	0.64	0.63	0.06	1.45	1.32	1.27	1.35		0.09
IP100017696.1	Antithrombin-III Complement C1s subcomponent	4.86	76.63	0.55	0.63	0.66	0.61	0.06	0.16	0.17	0.18	0.17	0.01	1.21	1.30	1.19	1.23		0.06
IP100021856.3	Apolipoprotein C-II	4.72	11.28	1.05	1.31	1.26	1.21	0.14	1.16	1.10	1.08	1.11	0.04	1.15	1.09	1.20	1.14		0.06
IP100418153.1	IGHM Putative uncharacterized protein	8.39	56.98	1.85	1.59	1.86	1.77	0.15	0.46	0.50	0.54	0.50	0.04	1.02	1.23	1.12	1.12		0.11
IP100021727.1	DKFZp686I15212 CAb-binding protein alpha chain	7.15	66.99	0.28	0.34	0.37	0.33	0.04	0.88	0.92	0.88	0.89	0.02	1.01	1.17	1.14	1.10		0.08
IP100296165.6	C1R cDNA FLJ54471, highly similar to Complement C1r subcomponent	5.96	81.84	0.52	0.53	0.54	0.53	0.01	0.14	0.13	0.16	0.14	0.02	1.04	1.13	1.11	1.09		0.05
IP100399007.7	IGHG2 Putative uncharacterized protein DKFZp686I04196 (Fragment)	7.63	46.03											1.04		1.13	1.08		0.06
IP100654888.4	K1KB1 Plasma kallikrein	8.60	71.32	1.10	0.97	1.19	1.09	0.11	0.82	0.88	0.84	0.85	0.03	1.02	1.09	1.12	1.08		0.05
IP100792115.1	C1EC3B Putative uncharacterized protein DKFZp686H17246	4.96	17.78	2.36	2.26	2.21	2.28	0.08	0.95	1.03	0.79	0.92	0.13	1.00	1.15	1.00	1.05		0.09
IP100827875.1	Lambda-chain	7.54	24.64	1.27	1.56	1.73	1.52	0.23	0.67	0.80	0.60	0.69	0.10	1.05	0.97		1.01		0.06
IP100021854.1	Apolipoprotein A-II	6.27	11.17	1.32	1.43	1.39	1.38	0.05	1.87	2.15	1.93	1.98	0.15	0.79	1.04	1.15	0.99		0.18
IP100011264.2	Complement factor	7.39	37.63	0.52	0.50	0.48	0.50	0.02	0.85	0.76	0.87	0.83	0.06	0.83	0.87	0.83	0.84		0.02

IP100478809.4	F5 Coagulation factor V	5.68	251.55	0.12	0.13	0.12	0.12	0.01	0.08	0.08	0.08	0.08	0.00	0.16	0.15	0.13	0.15	0.01
IP10087931.1	SERPINC1 cDNA FLJ58826, highly similar to Plasma protease C1 inhibitor	6.28	59.45							0.00	0.00	0.00	0.00	0.10	0.18	0.14	0.05	
IP100656092.1	Isoform F of Proteoglycan 4	9.50	146.38	0.08	0.09	0.13	0.10	0.03	0.11	0.11		0.11	0.00	0.15	0.12	0.14	0.02	
IP100964840.1	IGJ Uncharacterized protein	5.92	17.67							0.00	0.00	0.00	0.00	0.15	0.12	0.13	0.02	
IP100010295.1	Carboxypeptidase N catalytic chain	6.86	52.25						0.03	0.05	0.07	0.05	0.02	0.09	0.12	0.14	0.02	
IP100847179.1	apolipoprotein A-IV precursor	5.28	45.34						0.87	0.89	0.86	0.87	0.01	0.12	0.09		0.02	
IP100553177.1	SERPINA1 Isoform 1 of Alpha-1-antitrypsin	5.37	46.71	0.09	0.10	0.07	0.09	0.02	0.29	0.32	0.32	0.31	0.01	0.10	0.13	0.08	0.03	
IP100027462.1	Protein S100-A9	5.71	13.23		0.18	0.20	0.19	0.01		0.00	0.00	0.00	0.00	0.10	0.10	0.10	0.00	
IP100019359.4	Keratin, type I cytoskeletal 9	5.14	62.03						0.19	0.19	0.19	0.19	0.00	0.10	0.10	0.10	0.00	
IP100843942.1	Complement factor H-related protein 5	6.81	64.38	0.17		0.15	0.16	0.02	0.16	0.16	0.13	0.15	0.02	0.07	0.08	0.13	0.10	0.03
IP100292532.6	Cathelicidin antimicrobial peptide precursor	9.48	19.58	0.06		0.07	0.06	0.00	0.05	0.07	0.04	0.05	0.02	0.07	0.10	0.11	0.09	0.02
IP100896419.3	Isoform 1 of Inter-alpha-trypsin inhibitor heavy chain H4	6.51	103.29						1.54	1.59	1.53	1.56	0.03	0.11	0.07	0.09	0.09	0.02
IP100879573.1	SERPIND1 Heparin cofactor 2	6.41	57.03		0.08	0.08	0.08	0.00	0.09	0.12	0.11	0.11	0.01	0.06		0.07	0.07	0.01
IP100641737.1	Hapoglobin	6.28	46.69						0.18	0.17	0.25	0.20	0.04	0.10	0.04	0.05	0.06	0.03
IP100019576.1	FT10 Coagulation factor X	5.68	54.70	0.45	0.52	0.51	0.49	0.03	0.05	0.03	0.03	0.03	0.01	0.06	0.06	0.07	0.06	0.00
IP100925635.1	IGFALS insulin-like growth factor-binding protein complex acid labile subunit isoform 1 precursor	6.48	70.19						0.04	0.05	0.05	0.05	0.01	0.05	0.09	0.05	0.06	0.02
IP100554504.1	KRIT1 krev interaction trapped protein 1 isoform 2	8.97	78.60								0.00	0.00	0.00	0.06		0.06	0.06	0.00

IP100021364.1	CFP Propertidin	8.32	51.24	0.09	0.08	0.11	0.10	0.02	0.02	0.01	0.01	0.04	0.04	0.07	0.06	0.05	0.01
IP100025204.1	CD5 antigen-like	5.28	38.06	0.03		0.03	0.03	0.00	0.06	0.07	0.08	0.08	0.07	0.01	0.04	0.05	0.03
IP100914948.1	apolipoprotein L1 isoform c precursor	5.58	42.13	0.07	0.07	0.06	0.07	0.01	0.08	0.10		0.09	0.01	0.06	0.03	0.05	0.02
IP100032311.4	Lipopolysaccharide-binding protein	6.23	53.35	0.02	0.08	0.07	0.06	0.03	0.08	0.08	0.08	0.08	0.08	0.00	0.05	0.05	0.02
IP100032291.2	Complement C5	6.11	188.19	0.06	0.08	0.05	0.06	0.01	0.04	0.03	0.03	0.03	0.03	0.00	0.04	0.04	0.00
IP100947307.1	CP gDNA FLJ58075, highly similar to Ceruloplasmin	5.49	108.75							0.00	0.00	0.00	0.00	0.04	0.03	0.04	0.01
IP100967086.1	HCFAC Uncharacterized protein	6.69	71.44						0.03	0.03	0.04	0.03	0.00	0.05	0.02	0.03	0.02
IP100645849.1	ECM1 Isoform 4 of Extracellular matrix protein 1	6.43	63.52						0.01	0.01	0.02	0.01	0.00	0.04	0.02	0.03	0.01
IP100029168.1	Apolipoprotein(a)	5.58	501.00	0.03	0.03	0.03	0.03	0.00		0.00	0.00	0.00	0.00	0.03	0.03	0.03	0.00
IP100478003.3	Alpha-2-macroglobulin	6.03	163.19						0.04	0.05	0.05	0.05	0.00	0.03	0.03	0.03	0.01
IP100298497.3	Fibrinogen beta chain	8.54	55.89	0.06	0.10	0.10	0.09	0.02	0.08	0.04	0.07	0.06	0.02	0.02	0.02	0.02	0.00
IP100297550.8	F13A1 Coagulation factor XIII A chain	5.75	83.21							0.00	0.00	0.00	0.00	0.02	0.02	0.02	0.00
IP100679709.3	Complement component 6 precursor	6.41	105.68	0.03	0.02	0.05	0.04	0.02	0.07	0.09	0.09	0.08	0.01	0.03	0.02	0.02	0.01
IP100215894.1	Isoform LMW of Kininogen-1	6.29	47.85	2.00		2.02	2.01	0.01	4.30	4.26	4.15	4.24	0.07				
IP100944960.1	ITIH4 protein	6.43	103.82	0.04		0.06	0.05	0.02	1.48	1.47	1.47	1.48	0.01				
IP100896413.1	inter-alpha (globulin) inhibitor H4 isoform 2 precursor	6.06	99.80						1.48	1.47	1.47	1.47	0.00				
IP100784985.1	IGK@ IGK@ protein	6.14	25.50	3.73		3.69	3.71	0.03	0.97	0.86	0.91	0.91	0.08				
IP100550731.2	Putative uncharacterized protein	8.24	26.22	3.57	3.50		3.53	0.03	0.90	0.92		0.91	0.01				
IP100304273.2	Apolipoprotein A-IV	5.28	45.37	0.22		0.24	0.23	0.01	0.87	0.92	0.89	0.89	0.02				
IP100552578.2	SAAI	6.28	13.52						0.57	0.55	0.61	0.58	0.03				
IP100953689.1	Alpha-2-HS-glycoprotein	5.43	39.30	0.48	0.44	0.46	0.46	0.02	0.58	0.49	0.55	0.54	0.04				

IP100847635.1	SERPINA3 Isoform 1 of Alpha-1-antitrypsin	5.33	47.62			0.02	0.04	0.03	0.01	0.01	0.03	0.02	0.02	0.01						
IP100908365.1	AGT cDNA FLJ52255, highly similar to Angiotensinogen	5.64	44.38							0.02	0.02		0.02	0.00						
IP100973177.1	L1TBPI Isoform 4 of Latent-transforming growth factor beta-binding protein 1	5.63	186.74								0.01	0.01	0.01	0.00						
IP100783931.3	COL18A1 Isoform 1 of Collagen alpha-1(XVII) chain	5.67	178.08							0.00		0.01	0.01	0.00						
IP100384938.1	IGHG1	8.75	52.82	3.05			2.88	2.96	0.12											
IP100022391.1	APCS Serum amyloid P-component	6.10	25.37	1.45	1.26		1.37	1.36	0.09											
IP100887169.2	ICL V1-44 Putative uncharacterized protein	7.59	25.01	1.09			1.37	1.23	0.20											
IP100930072.1	IGHG2 Putative uncharacterized protein DKFZp686E23209	7.51	51.50	1.24	1.13		1.25	1.21	0.07											
IP100829640.1	ICL V3-19 ICL@ protein	6.29	24.84	0.71			0.88	0.80	0.12											
IP100017601.1	CP Ceruloplasmin	5.44	122.13	0.72	0.70		0.70	0.71	0.01											
IP100827906.1	Anti-mucin1 light chain variable region (Fragment)	8.64	11.61	0.41			0.50	0.45	0.07											
IP100743194.2	Kappa light chain variable region (Fragment)	6.70	13.17	0.36	0.45			0.40	0.05											
IP100291866.5	SERPINC1 Plasma protease C1 inhibitor	6.09	55.12	0.40	0.38		0.42	0.40	0.02											
IP100940493.1	cDNA FLJ51179, highly similar to Vitamin K-dependent protein C	8.24	46.03	0.41	0.35		0.42	0.39	0.04											
IP100827829.1	HRV Fab NB-VL (Fragment)	6.54	12.80		0.46		0.20	0.33	0.18											
IP100627891.2	Immunoglobulin V-set domain containing protein	7.74	13.03	0.14			0.25	0.19	0.08											

IP100007047.1	S100A8 Protein S100-A8	6.51	10.83	0.16	0.22		0.19	0.04											
IP100453473.6	HIST1HAD	11.36	11.36		0.10	0.23	0.17	0.09											
IP100926941.2	KRIT1 Uncharacterized protein	7.23	64.77	0.18	0.15		0.16	0.03											
IP100973032.1	V1-17 protein	5.55	12.28	0.14		0.16	0.15	0.01											
IP100873210.1	FN1 263 kDa protein	5.38	262.39	0.05	0.05	0.05	0.05	0.00											
IP100643034.3	PLTP cDNA FLJ39690 fls, clone SMINT2010639, highly similar to PHOSPHOLIPID TRANSFER PROTEIN	6.19	56.60		0.02	0.06	0.04	0.03											
IP100328609.3	SERPINA4 Kallistatin	7.34	48.51		0.02	0.03	0.03	0.00											
IP100236556.1	Isoform H7 of Myeloperoxidase	9.29	87.19		0.03	0.01	0.02	0.01											
IP100925547.1	Lactoferrin	8.51	77.93		0.02	0.02	0.02	0.00											
IP100607777.2	KALRN kalirin isoform 1	5.72	340.05	0.01	0.01		0.01	0.00											

Exhibit M

Screenshot of database record for Cai, Ramalingam in ScienceDirect database

The screenshot displays a web browser window with multiple tabs open, including 'Home | Micros...', 'Databases A-Z', 'EJP Search Res...', 'Characterizatio...', 'Bruker IPR - Re...', 'Project Diary...', and 'bitable hours'. The address bar shows the URL: <https://www.sciencedirect.com/elsevier/libraries/psu.edu/science/article/pii/S1549963412005977>. The ScienceDirect logo is visible in the top left, and 'PennState University Libraries' is in the top right. Below the navigation bar, there are buttons for 'View PDF' and 'Download full issue'. The main content area features a sidebar on the left with a table of contents: Outline, Abstract, Graphical Abstract, Abbreviations, Key words, Methods, Results, Discussion, Acknowledgment, Appendix A. Supplementary data, References, Show full outline, Cited by (88), and Figures (9). The central article section is titled 'Nanomedicine: Nanotechnology, Biology and Medicine' and includes the article title 'Characterization of carbon nanotube protein corona by using quantitative proteomics'. The authors listed are Xiaoning Cai PhD ^{a,1}, Rajkumar Ramalingam PhD ^{a,1}, Hau San Wong PhD ^b, Jinping Cheng PhD ^a, Paul Ajuh PhD ^c, Shuk Han Cheng PhD ^{a,2}, and Yun Wah Lam PhD ^{a,2}. Below the authors, there are links for 'Add to Mendeley', 'Share', and 'Cite', along with the DOI: <https://doi.org/10.1016/j.nano.2012.08.004>. The abstract text reads: 'The protein corona of a nanomaterial is a complex layer of proteins spontaneously and stably formed when the material is exposed to body fluids or intracellular environments. In this'. To the right of the article, there is a 'Recommended articles' section with three entries: 'Physiological and behavioural impacts of Pacific ciguatera toxin-1 (P-CTX-1) on marine...', 'Acute toxicity of functionalized single wall carbon nanotubes: A biochemical...', and 'Characterization of the interaction between carbon black and three important antioxidants...'. Each recommended article has a 'View PDF' link. At the bottom right, there is an 'Article Metrics' section and a 'FEEDBACK' button. The Windows taskbar at the bottom shows the search bar, taskbar icons, and system tray with weather (77°F, Mostly cloudy) and date/time (3:46 PM, 8/31/2024).

Exhibit N



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Exhibit O

Supplementary Table 2: Data reported in Figure 3

Column explanation:

Accession number (1)	Accession number (2)	Gene symbol	Gene name	No of detected peptide	Total Mascot score	smPAI	Medium:Light ratio	StDev	Heavy:Light ratio	StDev	log2(M/L)	log2(H/L)
Accession numbers of identified proteins in IPi (International Protein Index)				Accession numbers of identified proteins in other commonly used formats (as recommended by the Proteincenter database)								
Gene symbols of the identified proteins (pls note some identified proteins have no official gene symbols)				Commonly used gene names of the identified proteins								
No of detected peptides				Number of quantified peptides								
Total Mascot score				Combined Mascot scores of the identified proteins								
smPAI				Empirical Protein Abundance Indices of the identified proteins								
Medium:Light ratio				SILAC ratio of the identified proteins (medium isotope: light isotope). Average of all the ratios of the detected peptides for the proteins. StDev are the standard deviations for the averaged ratios.								
log2(M/L)				Log2 of the above ratio								
Heavy:Light ratio				SILAC ratio of the identified proteins heavy isotope: light isotope). Average of all the ratios of the detected peptides for the proteins. StDev are the standard deviations for the averaged ratios.								
log2(H/L)				Log2 of the above ratio								

Accession number (1)	Accession number (2)	Gene symbol	Gene name	No of detected peptide	Total Mascot score	empAI	Medium:Light ratio	StDev	Heavy:Light ratio	StDev	log2(M/L)	log2(H/L)
IP00167259	Q8N7Q0	FLJ40504	cDNA FLJ40504 fs, clone TEST02045509, highly	3	103	0.25	19.278	3.56	0.795	0.23	4.268883482	-0.33097323
IP00874197	ABMY52		Uncharacterized protein ENSP00000380179	5	215	1.11	10.017	5.46	0.86	0.48	4.11226904	-0.2159144
IP00873425	ABMZU		Uncharacterized protein ENSP00000380005	7	334	8.98	16.84	7.64	0.727	0.25	4.07382023	-0.45097273
IP00869238.1	IP00869238.1	PTPN14	Similar to Keratin 18	6	291	3.14	15.272	7.1	0.706	0.51	3.932817103	-0.50252991
IP00217437	Q8MWY7	TTBK2	Tau-tubulin kinase	5	192	0.13	14.673	6.65	0.729	0.35	3.875091965	-0.45600928
IP00871933	ABMWZ1		Uncharacterized protein ENSP00000381433	6	329	3.96	14.665	7.52	0.75	0.51	3.874305166	-0.4150375
IP00554788	P05783	KRT18	Keratin, type I cytoskeletal 18	51	3175	16164.62	14.504	17.46	1.147	1.5	3.8583718925	-1.97865391
IP00871144	ABMVV2		Uncharacterized protein ENSP00000380940	8	380	2.54	14.471	5.94	0.529	0.2	3.855092716	-0.91866037
IP00170800	Q7RTW8	OTOA	Otoacortin	2	69	0.05	14.437	0.28	0.014	0.05	3.851699077	-1.27229733
IP00879559	IP00879559.1		17 kDa protein	3	101	0.2	14.419	5.63	0.546	0.21	3.849899208	-0.87302714
IP00795725	IP00795725.1	KRT8	17 kDa protein	19	1184	186987.9	14.046	8.91	0.638	0.3	3.812087435	-0.64837167
IP00871514	ABMWY		Uncharacterized protein ENSP00000382824	13	624	13.03	14.023	5.21	6.385	20.12	3.809723119	2.87486862
IP00005859	B4DQJ4	KRT75	cDNA FLJ36809, highly similar to Homo sapiens c	9	372	1.12	13.736	6.62	0.742	0.51	3.779890039	-0.43050891
IP00872547	ABMYN2		Uncharacterized protein ENSP00000381396	5	250	1.41	13.732	8.11	0.716	0.24	3.779496857	-0.48196851
IP00737822	B9047319	KRT18P8	PREDICTED: similar to Keratin, type I cytoskeletal 3	3	128	5.72	13.613	1.44	0.494	0.02	3.766913134	-1.01741705
IP00554648	P05787	KRT18	Keratin, type II cytoskeletal 8	59	3472	130673	13.492	7.64	1.094	2.26	3.754032318	0.12961278
IP00871770	Q9H552	KRT18P11	Keratin-8-like protein 1.	14	756	20.59	13.482	4.3	0.568	0.2	3.752962625	-0.81603717
IP00794807	IP00794807.1	KRT18	15 kDa protein	15	1012	6322.95	13.144	7.6	1.571	2.26	3.71633248	0.651683181
IP00306959	P08729	KRT7	Keratin, type II cytoskeletal 7	23	1323	6.77	13.083	7.34	0.945	1.12	3.709621491	-0.08161377
IP00787323	IP00787323.2	HCCR1	Similar to Keratin, type I cytoskeletal 8	26	1506	273.13	13.077	8.11	0.563	0.25	3.707611554	-0.62878317
IP00873523	ABMW44		Uncharacterized protein ENSP00000380980	8	406	11.23	13.018	4.94	0.543	0.2	3.702435914	-0.8809759
IP00873732	113420397	HCG_1988300	PREDICTED: similar to Keratin, type II cytoskeletal 16	16	111.51	12.724	8.89	0.533	0.28	3.669480372	-0.90779256	
IP00872735	ABMY66		Uncharacterized protein ENSP00000381884	8	409	5.03	12.618	3.23	0.498	0.12	3.657411351	-1.00578235
IP00793572	IP00793572.1	KRT7	8 kDa protein	2	169	1.45	12.558	3.44	2.733	3.01	3.650534812	1.45048546
IP00847342	IP00847342.1	KRT7	keratin 7	23	1320	5.87	12.492	6.52	0.985	1.14	3.642932569	-0.02180437
IP00792642	IP00792642.1	KRT8	25 kDa protein	28	1780	2254683	12.418	7.02	0.646	0.31	3.634360932	-0.60339393
IP00796330	IP00796330.1	KRT6A	18 kDa protein	6	283	4.53	12.099	4.06	0.59	0.21	3.596815906	-0.76121314
IP00872585	ABMW47		Uncharacterized protein ENSP00000381179	12	555	6.69	11.794	4.72	0.58	0.22	3.5599861194	-0.78587519
IP00794122	IP00794122.1	KRT7	13 kDa protein	5	189	0.58	11.594	4.79	0.737	0.24	3.558388486	-0.44026348
IP00009867	P13641	KRT5	Keratin, type II cytoskeletal 5	15	465	1.52	11.565	0.779	0.534	0.28	3.531393831	-0.90518835
IP00294649	94538345	KRT35	keratin 35	3	117	0.56	11.424	5.52	0.315	0.21	3.513995979	-1.66657627
IP00871464	ABMU77		Uncharacterized protein ENSP00000383774	17	985	221.39	11.401	6.04	0.54	0.2	3.511088466	-0.88896869
IP00299145	P48668	KRT6C	Keratin, type II cytoskeletal 6C	10	436	1.76	10.752	3.03	0.511	0.16	3.426533138	-0.9686048
IP00300725	P02039	KRT6A	Keratin, type II cytoskeletal 6A	10	436	1.75	10.752	3.03	0.511	0.16	3.426533138	-0.9686048
IP00297452	Q8TDJ5	TFG	Tyrosine-protein kinase receptor	11	561	0.6	10.65	5.44	1.307	1.57	3.412781525	-0.38259141
IP00796921	Q05BK6	TFG	TFG protein	11	561	1.77	10.65	5.44	1.307	1.57	3.412781525	-0.38259141
IP00013164	P41219	PRPH	Peripherin	4	261	1.17	10.591	5.18	0.4	0.25	3.40476691	-1.32192809
IP00294619	Q9ET34	TFG	TFG protein	12	615	2.01	10.4	5.23	1.194	1.53	3.378511623	0.255802837
IP00220007	IP00220007.6	APOL2	cDNA FLJ55764, highly similar to Apolipoprotein-I	9	401	0.57	10.383	4.93	0.66	0.65	3.376151442	-0.59946207
IP00106913	32483352	DHRS4	peroxisomal short-chain alcohol dehydrogenase	49	338	0.58	10.359	7.44	0.447	0.44	3.372812835	-1.16165326
IP00793646	IP00793646.1		cDNA FLJ61294, highly similar to Keratin, type I c	5	198	0.93	10.352	3.41	0.782	0.31	3.371837617	-0.35475949
IP00168554	Q98YH0	SRXN1	Sulfiredoxin-1	4	214	1.93	10.258	5.45	0.267	0.31	3.358677571	-1.90580835
IP00241841	Q04K45	KRT79	Keratin, type II cytoskeletal 79	9	401	0.57	10.211	3.94	0.541	0.17	3.352052259	-0.78810595
IP00873742	ABMWV3		Uncharacterized protein ENSP00000382295	12	688	18.63	10.2	5.5	0.507	0.21	3.350497247	-0.97994235
IP00290078	109255249	KRT4	keratin 4	4	219	0.49	9.919	1.15	0.58	0.22	3.31019468	-0.78587519
IP00376379	194018511	KRT77	keratin 77	3	159	0.37	9.893	1.12	0.451	0.04	3.304086077	-1.14880066
IP00300552	Q9NSB2	KRT84	Keratin, type II cuticular Hb4	3	142	0.56	9.951	1.79	0.413	0.01	3.300270184	-0.27853161
IP00642542	IP00642542.1	CNN3	DJ539P13.2.2	3	180	0.85	9.806	2.18	0.357	0.13	3.2393664762	-1.48500402
IP00642078	Q5TDQ0	TPD52L1	Tumor protein D52-like 1	3	193	1.16	9.806	6.3	0.953	0.42	3.239664762	-0.06945188
IP00032831	095721	SNAP29	Synaptosomal-associated protein 29	5	300	0.55	9.667	5.66	0.54	0.51	3.273068242	-0.88896869
IP00166205	25140706	KRT78	TPA: TPA_exp: keratin 5b	2	84	0.18	9.547	1.4	0.482	0.11	3.255047459	-1.05284994
IP00308359	Q001546	TFG	Keratin, type II cytoskeletal 2 oral	4	247	0.46	9.448	1.1	0.435	0.04	3.240008965	-1.20091269
IP00290857	P12035	KRT3	Keratin, type II cytoskeletal 3	5	166	0.35	9.448	1.1	0.435	0.04	3.240008965	-1.20091269
IP00787216	113414151	LOC650864	PREDICTED: similar to Keratin, type II cytoskeletal 21 kDa protein	4	238	7.25	9.212	2.57	0.443	0.1	3.203514411	-1.1746214
IP00794644	IP00794644.1	KRT19	21 kDa protein	5	264	4.16	8.725	5.79	0.428	0.39	3.125155131	-1.2243173
IP00791852	IP00791852.1	KRT17	42 kDa protein	19	1013	4.83	8.723	3.28	0.722	0.34	3.142844938	-0.46949262
IP00472076	ABMU17	TPD52L1	Putative uncharacterized protein TPD52L1	4	274	0.57	8.57	0.9	0.35	0.27	3.082928202	-0.10783529
IP00009866	P13646	KRT13	Keratin, type I cytoskeletal 13	5	229	0.38	8.568	5.4	0.385	0.08	3.09895848	-1.37706965
IP00060181	Q96C19	EFHD2	EF-hand domain-containing protein D2	9	483	3.12	8.41	4.39	0.51	0.09	3.0721058	-2.73695955
IP00225363	P41436	GFAP	Glia fibrillary acidic protein	5	267	0.67	8.378	1.72	0.378	0.26	3.06665884	-1.40354186
IP00303815	P14136-2	GFAP	Isform 2 of Glial fibrillary acidic protein	5	267	0.67	8.378	1.72	0.378	0.26	3.06665884	-1.40354186
IP00443478	P14136-3	GFAP	Isform 3 of Glial fibrillary acidic protein	5	267	0.67	8.378	1.72	0.378	0.26	3.06665884	-1.40354186
IP00180956	ABMW45	LOC650611	Uncharacterized protein ENSP00000382160	10	495	0.93	8.337	4.11	0.628	0.31	3.059528335	-0.67116354
IP00171196	IP00171196.2	KRT13	keratin 13 isoform b	6	273	0.63	8.306	4.44	0.331	0.13	3.054153872	-1.59509688
IP00015262	Q98439	CNN2	Calponin-2	6	158	0.66	8.257	3.16	0.724	0.56	3.045641705	-0.58271044
IP00065671	Q9CZK2	UCK2	Ubrin-cyclidine kinase 2	4	252	1.93	8.251	4.29	0.351	0.27	3.044586891	-1.5104706
IP00465084	P17661	DES	Desmin	7	359	1.45	8.218	5.23	0.355	0.23	3.038787331	-1.49410907
IP00450768	Q04695	KRT17	Keratin, type I cytoskeletal 17	29	1510	7.36	8.175	3.88	0.618	0.33	3.033334895	-0.64611216
IP00240503	IP00240503.7		48 kDa protein	11	577	1.21	8.175	3.88	0.618	0.3	3.032128731	-0.69432126
IP00877836	IP00877836.1	SNAP29	31 kDa protein	3	216	0.68	8.054	5.1	0.29	0.12	3.007010542	-0.73216461
IP00873410	IP00873410.1	CNN3	46 kDa protein	3	503	0.88	7.935	3.1	0.432	0.32	2.982230223	-1.21089678
IP00745035	IP00745035.2		15 kDa protein	2	73	0.51	7.889	0.52	0.237	0.1	2.979842438	-0.20770414
IP00747707	47939651	KRT17	KRT17 protein	22	1213	6.78	7.888	3.03	0.623	0.32	2.979695952	-0.68269503
IP00879145	IP00879145.1	KRT18	Keratin, type I cytoskeletal 18	7	338	1.38	7.681	3.53	0.401	0.35	2.94129415	-1.31835886
IP00025277	Q7S340	PCDC6	Programmed cell death protein 6	7	342	7.5	7.349	3.47	0.476	0.62	2.877547952	-1.07096652
IP00009862	ABMU2	CNN2	Putative uncharacterized protein CNN2 (Calponin	4	251	1.11	7.318	1.11	0.317	0.05	2.871448416	-0.93702737
IP00644674	Q9Y5V2	NUPB2	Nucleotide-binding protein 2	2	121	0.24	7.314	1.68	2.817	3	2.870650627	1.494159564
IP0018471	P08670	VM	Vimentin	48	2842	1007.01	7.203	2.45	0.283	0.16	2.848597904	-1.22126604
IP00029640	O14737	PCDC5	Programmed cell death protein 5	6	350	4.51	7.124	3.28	0.098	0.1		

IP00399053	Q9BU61	C3orf50	Uncharacterized protein C3orf50	4	229	0.85	4.097	1.61	0.433	0.29	2.034567893	-1.20756107
IP00420084	P59597-2	BID	Isoform 2 of BH3-interacting domain death agonist	3	222	0.6	4.078	1.09	0.332	0.13	2.027861775	-1.59074485
IP00784154	P10089	HSPD1	60 kDa heat shock protein, mitochondrial	45	343	337.11	4.067	1.43	0.328	0.46	2.023964909	-1.60823228
IP00880053	IP00880053.1		Mitochondrial heat shock 60kD protein 1 variant 1	18	1064	6.61	4.057	1.48	0.226	0.27	2.020413303	-2.14566532
IP00861639	P51148	RAB5C	Ras-related protein Rab-5C	10	604	6.42	4.028	0.73	0.266	0.24	2.010063683	-1.91050185
IP00854538	AY543	TPD52	CLMPRPS28 fusion protein	6	444	1.49	3.908	0.62	0.331	0.22	1.966430467	-1.59509688
IP00815398	P17980	PSMC3	26S protease regulatory subunit 6A	14	795	3.17	3.902	1.49	0.255	0.18	1.964213778	-1.97143085
IP00787692	169211725	PREDICTED: similar to 40S ribosomal protein S2i	2	130	8.45	3.852	4.074	0.02	0.12	0.02	1.945607103	-3.75623092
IP00023510	P20339	RAB5A	Ras-related protein Rab-5A	7	371	3.29	3.805	1.7	0.284	0.27	1.927896454	-1.81603717
IP00794769	IP00794769.1	HSPD1	18 kDa protein	14	882	103.06	3.674	1.08	0.198	0.28	1.977351626	-2.33642766
IP00794046	IP00794046.1	CC14	20 kDa protein	5	262	2.57	3.664	0.59	0.566	0.88	1.873419503	-0.82112604
IP00003419	C11orf58		Small acidic protein	2	111	0.17	3.522	0.02	1.308	0.34	1.815334909	-1.69899774
IP00619951	7060814	P5287	tumor protein D52 isoform 2	9	369	3.63	3.519	1.09	0.48	0.31	1.815165514	-1.05889369
IP00873344	Q9UCX8	TPD52	N8 protein long isoform	9	580	2.7	3.519	1.09	0.48	0.31	1.815165514	-1.05889369
IP00844412	A4D286		Similar to Nuclear protein Hcc-1 (HSPC316) (Proti	3	194	0.57	3.512	2.58	0.719	1.1	1.812292485	-0.47593632
IP00017344	P61020	RAB5B	Ras-related protein Rab-5B	6	315	1.88	3.451	1.03	0.174	0.11	1.771084474	-2.52224079
IP00301354	P63208	SKP1	S-phase kinase-associated protein 1	4	191	1.3	3.291	1.09	0.58	0.61	1.718526026	-0.78587519
IP00030243	P61289	PSME3	Proteasome activator complex subunit 3	10	572	2.6	3.291	0.49	0.253	0.25	1.718526026	-1.98279071
IP00219445	P61289-2	PSME3	Isoform 2 of Proteasome activator complex subunit	10	572	2.41	3.291	0.49	0.253	0.25	1.718526026	-1.98279071
IP00076042	Q96R13	LOC642237	Short heat shock protein 60 Hsp60s2	8	491	6.2	3.276	1.13	0.294	0.32	1.711935357	-1.76611194
IP00554681	Q15718	NUDUFAS	NADH dehydrogenase lubeinone1 alpha subcc	2	106	0.57	3.263	0.43	0.319	0.02	1.706189887	-1.64837167
IP00795769	119617241		NC02016179, isoform CRA_c	6	305	0.36	3.227	1.93	0.517	0.65	1.687084688	-0.95176381
IP00060650	Q8WV12-2	PCPN	Isoform 2 of PEST proteolytic signal-containing nu	2	85	0.57	3.211	0.86	0.148	0.01	1.683022665	-2.75633092
IP00796456	IP00796456.1	PCPN	Isoform 2 of PEST proteolytic signal-containing nu	2	85	0.57	3.211	0.86	0.148	0.01	1.683022665	-2.75633092
IP00505746	Q9Y266	NUDC	Nuclear migration protein nudC	8	457	1.11	3.2	0.81	0.143	0.09	1.678071905	-2.80591295
IP00298559	Q9BU61.8	POD10	Programmed cell death protein 10	4	249	0.68	3.191	0.24	0.219	0.05	1.674006619	-1.78519719
IP00022462	P02786	STRC	Transferin receptor protein 1	3	193	0.12	3.173	1.84	0.142	0.03	1.665847521	-2.81603717
IP00334190	Q9JUZ1	STOML2	Stomatin-like protein 2	3	199	0.39	3.168	0.51	0.868	1.16	1.663572335	-0.20423305
IP00738655	113413200	LOC653781	PREDICTED: similar to protein expressed in prost	7	327	0.64	3.155	0.98	0.286	0.05	1.667400005	-1.80591295
IP00786945	IP00786945.1	PREDICTED: similar to actin-like protein	7	247	0.55	3.155	0.98	0.286	0.05	1.667400005	-1.80591295	
IP00790339	IP00790339.1	ACTG1	22 kDa protein	9	549	173.56	3.118	0.91	0.297	0.07	1.640620928	-1.75146516
IP00796881	IP00796881.1	ACTG1	16 kDa protein	9	549	889.49	3.118	0.91	0.297	0.07	1.640620928	-1.75146516
IP00555809	IP00555809.3	TBC1D22A	Pseudogene candidate	2	67	0.13	3.109	0.23	2.763	3.46	1.638450617	-1.466235562
IP00740545	88953571	LOC653269	PREDICTED: similar to protein expressed in prost	8	397	0.75	3.103	0.92	0.286	0.05	1.633663697	-1.79589528
IP00784029	AB757	HTATP2	cDNA FLJ75232, highly similar to Homo sapiens I	4	295	0.79	3.086	0.28	0.283	0.07	1.625713802	-1.82112604
IP00739539	AS4362	POTEF	ANKRD26-like family C member 1B	8	458	0.94	3.084	0.93	0.276	0.05	1.624802765	-1.85725983
IP00016610	Q15365	PCBP1	Pol(y)C-binding protein 1	14	834	4.43	3.082	1.45	0.235	0.22	1.623866862	-0.8926734
IP00021370	P16086	UBE2K	Ubiquitin-conjugating enzyme E2 K	4	230	0.52	3.072	0.81	0.376	0.13	1.619178216	-1.41119543
IP00478743	Q63214	POTEE	ANKRD26-like family C member 1A	10	521	1.67	3.069	0.29	0.281	0.05	1.617288483	-1.8312604
IP00030297	P50991	CC14	T-complex protein 1 subunit delta	24	1300	6.72	2.978	1	0.24	0.37	1.574343754	-2.05889369
IP00027681	P40261	NNMT	Nicotinamide N-methyltransferase	4	217	0.38	2.94	0.41	0.151	0.12	1.555816155	-2.72737955
IP00383665	Q9BUP3-2	HTATP2	Isoform 2 of Oxidoreductase HTATP2	4	183	0.85	2.903	0.45	0.31	0.06	1.537545472	-1.68965988
IP00008603	P62736	ACTG2	Actin, aortic smooth muscle	16	837	80.28	2.88	0.78	0.585	1.23	1.526068812	-0.77349147
IP00025416	P62736	ACTG2	Actin, gamma-enteric smooth muscle	16	837	80.28	2.88	0.78	0.585	1.23	1.526068812	-0.77349147
IP00021428	P68133	ACTA1	Actin, alpha skeletal muscle	17	885	107.75	2.876	0.75	0.57	1.2	1.524063676	-0.81096618
IP00023006	P68032	ACT1	Actin, alpha cardiac muscle 1	17	885	109.08	2.876	0.75	0.57	1.2	1.524063676	-0.81096618
IP00848058	IP00848058.1	ACTB	Actin, cytoplasmic 2	22	1454	716.06	2.858	0.64	0.411	0.38	1.510505916	-1.2827897
IP00878919	Q56275	LOC389036	Actin-like protein	2	88	3.85	2.785	0.03	0.322	0.03	1.482848283	-1.63486741
IP00871744	Q56274	P704P	Actin-like protein	2	88	3.79	2.795	0.03	0.322	0.03	1.482848283	-1.63486741
IP00640817	Q57987	AK1	Adenylyl kinase 1	5	319	2.8	2.786	0.67	0.182	0.15	1.478195258	-1.54798964
IP00010158	Q9NRG0	CHRA21	Chromatin accessibility complex protein 1	2	88	0.52	2.777	0.29	0.211	0.22	1.473527177	-2.2446851
IP00555900	Q9Y8Y7	ACTBL3	Kappa-actin	5	297	2.92	2.766	0.56	0.289	0.05	1.467801156	-1.7308586
IP00024095	P12129	ANKA3	Annexin A3	4	249	0.89	2.762	0.29	0.06	0.34	1.467801156	-1.7308586
IP00006643	Q68616	BAG2	BAG family molecular chaperone regulator 2	8	317	1.52	2.755	1.33	0.331	0.29	1.462052319	-1.59509688
IP00030143	A7YDY3	BOLA2B	BoA homolog 2 (E. coli)	4	174	1.49	2.726	0.05	0.466	0.39	1.446785562	-1.10159814
IP00791573	Q9Y220-2	SUGT1	Isoform 2 of Suppressor of G2 allele of SKP1 hom	2	76	0.18	2.628	0.48	0.259	0.26	1.391217808	-1.948976
IP00412525	ABWZ1	LOC13757335	Isoform 2 of Suppressor of G2 allele of SKP1 hom	2	76	0.18	2.628	0.48	0.259	0.26	1.391217808	-1.948976
IP00016179	Q96868	S100A13	Protein S100-A13	4	165	1.87	2.524	0.3	0.115	0.02	1.33571191	-3.12029428
IP00167750	IP00167750.5	PCBP3	Pol(y)C-binding protein 3 (Alpha-CP3), isoform 5	5	218	0.53	2.483	0.84	0.155	0.01	1.312084262	-2.68956988
IP00505365	IP00505365.2	PCBP3	Putative uncharacterized protein PCBP3	5	218	0.52	2.483	0.84	0.155	0.01	1.312084262	-2.68956988
IP00003269	Q562R1	ACTBL2	Beta-actin-like protein 2	5	357	12.17	2.482	0.35	0.282	0.03	1.311503115	-1.82623293
IP00015148	RAPIB		Ras-related protein Rap-1b	4	294	3.48	2.467	0.38	0.414	0.05	1.302757716	-1.27229733
IP00019345	P62834	RAPIA	Ras-related protein Rap-1A	4	294	3.41	2.467	0.38	0.414	0.05	1.302757716	-1.27229733
IP00877120	Q5U0C3	RAPIA	RAPIA, member of RAS oncogene family	4	294	3.41	2.467	0.38	0.414	0.05	1.302757716	-1.27229733
IP00874082	ABMY02		Uncharacterized protein ENSP00000382574	2	77	0.59	2.438	0.53	0.33	0.02	1.296898126	-1.59946027
IP00018671	Q9Y266	DUSP3	Dual specificity protein phosphatase 3	3	184	0.58	2.401	0.19	0.121	0.05	1.286365404	-3.04692105
IP00021836	196533965	PSMC6	proteasome 26S, AT1Pase subunit 6	4	277	0.82	2.375	0.585	0.85	1.23	1.247827513	-1.73219447
IP00303882	Q60664	MPRBP1	Mannose-6-phosphate receptor-binding protein 1	7	521	0.84	2.312	0.56	0.315	0.16	1.209141398	-1.66657627
IP00797742	IP00797742.1	CIFP29	5 kDa protein	2	82	2.29	2.288	0.08	0.201	0.18	1.194087052	-2.31473259
IP00789551	ABMXP9	MATR3	Putative uncharacterized protein MATR3	2	161	0.17	2.255	0.71	0.161	0.01	1.173127433	-2.63486741
IP00303319	FAM149B	FAM149B	Protein FAM149B	6	224	0.22	2.227	0.22	0.121	0.09	1.151858891	-3.04692105
IP00021590	P63173	RPL38	60S ribosomal protein L38	7	421	551.556	2.195	0.57	0.391	0.41	1.13422094	-1.84348563
IP00100796	Q9NZ23	CHMP5	Charged multivesicular body protein 5	3	123	0.29	2.168	1.96	0.047	0.34	1.11364757	-1.0862014
IP00004902	P38117	ETFB	Electron transfer flavoprotein subunit beta	3	107	0.57	2.04	0.71	0.101	0.01	1.028699152	-3.7075728
IP00554541	P38117	ETFB	Isoform 2 of Electron transfer flavoprotein subunit i	3	107	0.4	2.04	0.71	0.101	0.01	1.028699152	-3.7075728
IP00796442	IP00796442.1	PCBP2	18 kDa protein	3	107	0.4	1.995	0.78	0.076	0.04	0.986389746	-3.72519477
IP00798360	IP00798360.1	CIFP29	18 kDa protein	4	187	1.33	1.978	1.41	0.128	0.14	0.984042426	-2.96578478
IP00016372	P51151	RAB9A	Ras-related protein Rab-9A	3	88	0.31	1.835	1.7	0.245	0.24	0.975780063	-2.02914635
IP000218918	P40483	ANXA1	Annexin A1	11	786	1.59	1.826	0.47	0.117	0.19	0.888868765	-0.95041957
IP00012066	ABQ174	ANXA1	cDNA FLJ177432, highly similar to Homo sapiens i	11	786	1.5	1.826	0.47	0.117	0.19	0.888868765	-0.95041957
IP000184525	Q9BV20-2	MC3207	Isoform 2 of Translation initiation factor eIF-2B sub	2	115	0.32	1.802	0.19	0.126	0.0	0.849599011	-2.98850436
IP00006052	Q9UHV9	PFDC2	Prefoldin subunit 2	2	151	1.1	1.794	0.29	0.082	0.04	0.84317989	-3.60823228
IP00329801	P08758	ANXA5	Annexin A5	20	1170	39.54	1.793	0.49	0.151	0.22	0.842375488	-2.72737955
IP00295098	Q9YF98	ANXA5	Signal recognition particle receptor subunit beta	3	177	0.34	1.787	0.49	0.151	0.02	0.8375339634	-1.0086822
IP00022597	P16081	UBE2M	NEDD8-conjugating enzyme UbcL2	4	177	0.82	1.778	0.47	0.13	0.07	0.830255324	-2.94341647
IP00033143	Q9UBQ5	EIF3K	Eukaryotic translation initiation factor 3 subunit K	3	223	0.87						

IP000301277	P34931	HSPA1L	Heat shock 70 kDa protein 1L	6	453	0.58	1.102	0.29	0.095	0.07	0.140124224	-3.9592868
IP000300341	Q15369	TCEB1	Transcription elongation factor B polypeptide 1	3	101	0.63	1.098	0.19	0.064	0.04	0.134878054	-3.96578428
IP000109551	P07455/2735	EPK1	epikotin 1	4	222	0.93	1.021	0.21	0.164	0.12	0.125651132	-4.00823228
IP000085529	P05387	RPLP2	60S acidic ribosomal protein P2	7	432	0.25	1.093	0.33	0.041	0.09	0.125651102	-2.82623335
IP000295386	P16152	SETD4	Carbonyl reductase [NADPH] 1	7	500	2.14	1.081	0.29	0.68	1.33	0.112366523	-0.55639335
IP000169383	P00558	PGK1	Phosphoglycerate kinase 1	24	1290	46.23	1.067	0.26	0.104	0.26	0.093560176	-2.26534477
IP000021347	P68036	UBE2L3	Ubiquitin-conjugating enzyme E2 L3	2	123	1	1.047	0.31	0.105	0.09	0.066261442	-3.25153877
IP000174448	P65240	RPS21	60S ribosomal protein S21	4	192	1.65	1.041	0.17	0.121	0.15	0.057070059	-3.04651005
IP00643115	A2A202	STMN1	Stathmin	2	85	0.97	0.981	0.21	0.09	0.11	-0.02767496	-3.47393119
IP00744618	IP00744618.2	STMN1	Stathmin	2	85	0.84	0.981	0.21	0.09	0.11	-0.02767496	-3.47393119
IP00017454	Q9H853	TUBA4B	Putative tubulin-like protein alpha-4B	3	193	2.52	0.977	0.51	0.047	0.04	-0.03366953	-4.41119543
IP00792673.1	ETFA	24 kDa protein	24 kDa protein	7	450	0.68	0.974	0.58	0.031	0.01	-0.03806132	-5.01158797
IP000216691	P07737	PFN1	Profilin-1	3	509	37.66	0.968	0.14	0.059	0.07	-0.04692105	-4.08314124
IP000024282	Q92930	RAB8B	Ras-related protein Rab-8B	2	118	0.7	0.966	0.09	0.098	0.02	-0.04904941	-3.35107444
IP000028481	P61006	RAB8A	Ras-related protein Rab-8A	2	118	0.7	0.966	0.09	0.098	0.02	-0.04904941	-3.35107444
IP000300096	Q15286	RAB35	Ras-related protein Rab-35	2	118	0.72	0.966	0.09	0.098	0.02	-0.04904941	-3.35107444
IP00336449	P59190-2	RAB15	Ras-related protein Rab-15	2	118	0.67	0.966	0.09	0.098	0.02	-0.04904941	-3.35107444
IP00394882	P59190	RAB15	Ras-related protein Rab-15	2	118	0.67	0.966	0.09	0.098	0.02	-0.04904941	-3.35107444
IP00556414	Q59EP4	RAB8A	Mel transforming oncogene variant	2	118		0.966	0.09	0.098	0.02	-0.04904941	-3.35107444
IP00790414.1	RAB8B	10 kDa protein	10 kDa protein	2	118		0.966	0.09	0.098	0.02	-0.04904941	-3.35107444
IP00797126.1	NACA	nascent polypeptide-associated complex alpha sub	7	570	0.78		0.963	0.4	0.36	0.43	-0.0542923	-1.47393119
IP00017726	Q98744	HS017B10	3-hydroxyacyl-CoA dehydrogenase type-2	11	884	17.65	0.953	0.2	0.1	0.08	-0.06945188	-3.32192809
IP00872214.1	IP00872214.1	TBCA	16 kDa protein	2	114	0.81	0.945	0.1	0.117	0.09	-0.08161377	-3.09541957
IP000018146	P27348	YWHAQ	14-3-3 protein theta	13	722	12.58	0.933	0.25	0.057	0.06	-0.10005107	-1.43289427
IP00740010	113415150	LOC643412	PREDICTED: similar to basic transcription factor 3	2	136	0.11	0.922	0.27	0.345	0.09	-0.11716134	-1.53533173
IP00221005	P20290	BTFC3	Transcription factor BTFC3	3	127	0.76	0.922	0.17	0.345	0.09	-0.11716134	-1.53533173
IP00795676	IP00795676.1	RAB11A	22 kDa protein	6	286	2.05	0.921	0.15	0.07	0.06	-0.11872694	-3.83650127
IP00798247	IP00798247.1	HSPA8	20 kDa protein	4	296	1.97	0.918	0.41	0.111	0.05	-0.12343394	-3.17136842
IP00291922	P28066	PSMA5	Proteasome subunit alpha-type-5	7	317	1.3	0.914	0.36	0.079	0.06	-0.12973393	-3.66200354
IP00549248	NP1M1	NP1M1	Nucleophosmin	12	667	10.37	0.922	0.22	0.667	0.06	-0.13131323	-3.14560532
IP000297579	Q13185	LOC653972	Chromobox protein homolog 3	3	199	0.82	0.911	0.11	0.087	0.05	-0.13447704	-3.52284079
IP00658013	Q9BYG9	NP1M1	Nucleophosmin (Nucleolar phosphoprotein B23, n	11	638	14.97	0.908	0.24	0.12	0.12	-0.1392358	-3.05889369
IP00016572	P62308	SNRPG	Small nuclear ribonucleoprotein G	2	99	1	0.899	0.06	0.247	0.03	-0.15360698	-2.01741705
IP00291928	P61106	RAB1A	Ras-related protein Rab-1A	3	180	0.69	0.896	0.19	0.17	0.01	-0.15842936	-3.06889369
IP00005969	P52907	CAPZL1	F-actin-capping protein subunit alpha-1	2	112	0.82	0.884	0.25	0.057	0.02	-0.18114944	-2.11587977
IP000218667	Q93045	STMN2	Stathmin-2	2	86	0.82	0.881	0.36	0.118	0.07	-0.18278608	-3.08314124
IP00216049	P61788	HNRNPDK	Heterogeneous nuclear ribonucleoprotein K	17	1066	5.98	0.876	0.47	0.074	0.06	-0.19099723	-3.75633092
IP00013679	P33316	DUT	Deoxyuridine 5-triphosphate nucleotidohydrolase, a	4	264	1.57	0.874	0.08	0.05	0.06	-0.19429482	-3.32192809
IP00749113	P33316.1	DUT	Deoxyuridine 5-triphosphate nucleotidohydrolase, a	4	264	1.57	0.874	0.08	0.05	0.06	-0.19429482	-3.32192809
IP00793322	IP00793322.1	DUT	Deoxyuridine 5-triphosphate nucleotidohydrolase, a	4	264	1.79	0.874	0.08	0.05	0.06	-0.19429482	-3.32192809
IP00514561	Q5T6W5	HNRNPDK	Heterogeneous nuclear ribonucleoprotein K	15	997	6.01	0.871	0.49	0.079	0.09	-0.19925538	-3.66200354
IP00001639	Q14974	PKNB1	Importin subunit beta-1	4	223	0.22	0.868	0.34	0.166	0.12	-0.20423305	-2.59074485
IP00298547	Q99487	PKP1	Protein D3-1	6	295	6.64	0.865	0.15	0.11	0.08	-0.20922796	-3.18442457
IP00477997	P16649	STMN1	Stathmin	2	86	0.84	0.884	0.25	0.057	0.02	-0.21069678	-3.07546866
IP00029631	P84090	ERH	Enhancer of rudimentary homolog	2	75	0.28	0.859	0.18	0.084	0	-0.2126996	-3.57346866
IP00021700	P12004	PCNA	Proliferating cell nuclear antigen	10	544	8.99	0.855	0.19	0.136	0.08	-0.22600367	-2.87832144
IP00010810	P13804	ETFA	Electron transfer flavoprotein subunit alpha, mitocho	4	203	0.88	0.835	0.48	0.041	0.02	-0.2601519	-4.60823228
IP00418235.4	IP00418235.4	GSTM5	Glutathione S-transferase U2 subunit	5	144	0.92	0.82	0.04	0.82	0.04	-0.28630474	-3.64585619
IP00396468	Q10396468	PRPF19	Pre-mRNA-processing factor 19	3	105	0.19	0.809	0.13	0.222	0.24	-0.30578839	-3.17136842
IP00004968	Q9UM54	EEF1D	eukaryotic translation elongation factor 1 delta isofo	9	596	0.96	0.809	0.23	0.121	0.03	-0.30578839	-3.04692105
IP00642971	194239723	PRPF19	Pre-mRNA-processing factor 19	3	105	0.19	0.809	0.13	0.222	0.24	-0.30578839	-3.17136842
IP00216319	Q04917	YWHAH	14-3-3 protein eta	6	311	1.73	0.805	0.14	0.025	0.04	-0.31293931	-5.32192809
IP00604504	Q5C263	CLEC2D	Putative uncharacterized protein	5	145	0.8	0.797	0.14	0.057	0.02	-0.32192809	-5.26534477
IP00246975	P21266	GSTM3	Glutathione S-transferase Mu 3	8	418	4.91	0.797	0.23	0.08	0.09	-0.32734837	-3.64385619
IP00868801	8903164	LOC647504	PREDICTED: similar to Acidic leucine-rich nucleo	6	275	4.19	0.795	0.27	0.126	0.18	-0.33097323	-2.98850436
IP00179964	P26599	PTBP1	Poly(pyrimidine tract-binding protein 1	7	462	1.19	0.792	0.17	0.156	0.1	-0.33642766	-3.68038207
IP00872407	YWHAB	YWHAB	Yeast protein phosphatase 2B	5	263	1.68	0.786	0.09	0.263	0.03	-0.34739878	-3.92139017
IP00007423	Q92682	ANP2B	Acidic leucine-rich nuclear phosphoprotein 32 fam	7	343	0.38	0.781	0.24	0.058	0.04	-0.35646505	-2.85725683
IP000010796	P07327	P4HB	Protein disulfide-isomerase	10	513	0.96	0.78	0.23	0.141	0.16	-0.35845397	-2.82623293
IP00017334	P35232	PHB	Prohibitin	8	449	2.57	0.776	0.19	0.189	0.03	-0.36587144	-2.40351474
IP00291005	P40925	MDH1	Malate dehydrogenase, cytoplasmic	9	504	2.99	0.775	0.19	0.107	0.05	-0.36773178	-2.40351474
IP00216319	YWHAB	YWHAB	Yeast protein phosphatase 2B	5	263	1.68	0.786	0.09	0.263	0.03	-0.34739878	-3.92139017
IP00759832	P31946-2	YWHAB	Isoform Short of 14-3-3 protein beta/alpha	15	891	20.12	0.775	0.11	0.079	0.14	-0.36731738	-3.66200354
IP00797206	IP00797206.1	CCT8	Protein	6	416	1.45	0.756	0.23	0.038	0.18	-0.40354186	-2.45008445
IP00027350	P32119	PRDX2	Peroxiredoxin-2	9	515	25.63	0.756	0.14	0.038	0.02	-0.40354186	-2.47178567
IP00878440	IP00878440.1	RANBP1	19 kDa protein	2	107	0.93	0.749	0.02	0.038	0.03	-0.41686238	-2.71785677
IP00796294	EEF1D	EEF1D	cDNA FLJ57945, highly similar to Elongation fact	6	274	1.24	0.749	0.12	0.241	0.04	-0.42692547	-2.04025104
IP00220642	P61981	YWHAG	14-3-3 protein gamma	9	527	4.93	0.742	0.12	0.071	0.12	-0.43050891	-3.81603717
IP00793442	ABMUL1	PHB	Putative uncharacterized protein PHB	6	307	2	0.725	0.15	0.183	0.15	-0.4639471	-2.50080445
IP00791634	Q53FV0	PHB	Prohibitin variant	7	359	1.6	0.725	0.15	0.183	0.15	-0.4639471	-2.50080445
IP00302925	LOC620925	CCT19	Chaperonin containing TCP1, subunit 8 (theta), iso	5	245	2.46	0.725	0.15	0.183	0.15	-0.4639471	-2.50080445
IP00795516	IP00795516.1	YWHAE	cDNA FLJ51975, moderately similar to 14-3-3 pro	5	255	4.35	0.713	0.12	0.043	0.04	-0.48802602	-4.53951953
IP00869193	88952419	LOC644131	PREDICTED: similar to chaperonin containing TC	2	134	0.43	0.711	0.61	0.026	0.06	-0.49027854	-3.92139017
IP00013890	P31947	SFN	14-3-3 protein sigma	9	331	1.78	0.709	0.15	0.052	0.07	-0.49614247	-2.26534477
IP00395627	Q9HB71	CACYPB	Calcycin-binding protein	2	127	0.82	0.705	0.12	0.157	0.02	-0.50430484	-2.67116354
IP00794673.1	IP00794673.1	CCT19	Chaperonin containing TCP1, subunit 8 (theta), iso	5	245	2.46	0.725	0.15	0.183	0.15	-0.4639471	-2.50080445
IP00024175	Q14818	PSMA7	Proteasome subunit alpha-type-7	5	393	1.47	0.705	0.24	0.072	0.05	-0.50430484	-3.95858928
IP00010137	119604798	EEF1D1P1	hCG2000329	3	201	0.84	0.698	0.37	0.12	0.02	-0.51870106	-3.05889369
IP00015954	SAR1A	SAR1A	GTP-binding protein SAR1A	3	132	0.75	0.694	0.53	0.229	0.23	-0.52699243	-2.12568059
IP00032826	ST13	ST13	HSP70-interacting protein	2	102	0.36	0.692	0.07	0.067	0.02	-0.53115606	-2.13298427
IP00218038	Q8Q22	FAM104A	Protein FAM104A	2	133	0.58	0.692	0.04	0.057	0.02	-0.53115606	-2.13298427
IP00216298	P10599	TXN	Thioredoxin	5	301	6.78	0.692	0.09	0.077	0.12	-0.53115606	-3.68899774
IP00448725	Q6PWK3	RAB4B	RAB4B protein	2	114	0.96	0.691	0.48	0.339	0.32	-0.53324238	-1.56064282
IP00399212	116921776	LOC389842	PREDICTED: similar to RanBP1	2	95	0.99	0.691	0.1	0.034	0.02	-0.53324238	-1.87832144
IP00414127	P43487	RANBP1	Ran-specific GTPase-activating protein	3	145	1.24	0.691	0.1	0.034	0.02	-0.53324238	-1.87832144
IP00878075	119623394	RANBP1	RAN binding protein 1, isoform CRA_d	3	145	1.25	0.691	0.1	0.034	0.02	-0.53324238	-1.87832144
IP00879359	IP00879359.1	AK2	28 kDa protein	13	764							

IP00418813	IP00794205.1		Similar to Ribosomal protein S27a	4	188	0.87	0.472	0.09	0.036	0.04	-1.08314124	-4.79585928
IP00794205			26 kDa protein	4	188	0.84	0.472	0.09	0.036	0.04	-1.08314124	-4.79585928
IP00641244	IP00793584.1	PRDX1	peroxiredoxin 1	7	329	2.38	0.455	0.04	0.011	0.05	-1.08314124	-4.79585928
IP00871646	Q8NHV5	NAPF1-like	60S acidic ribosomal protein P0-like	6	338	1.3	0.466	0.05	0.008	0.02	-1.10559814	-3.64855619
IP00023860	P55209	RAPL11	Nucleosome assembly protein 1-like 1	4	211	0.32	0.465	0.01	0.063	0.02	-1.10469738	-3.98585046
IP000220014	B4E155	ID1	cDNA FLJ56330, highly similar to Homo sapiens t	3	154	0.22	0.463	0.24	0.063	0.06	-1.1109159	-3.98850436
IP00010896	O00299	CLC1	Chloride intracellular channel protein 1	5	326	1.02	0.463	0.13	0.063	0.07	-1.1109159	-3.98850436
IP00795465.1	IP00795465.1	CLCBP	Protein	8	141	0.85	0.46	0.02	0.078	0.01	-1.12052943	-3.68027077
IP000218820	P07951-3	TPM2	isoform 3 of Tropomyosin beta chain	6	229	1.16	0.459	0.39	0.283	0.08	-1.12343394	-1.82112604
IP000220709	P07951-2	TPM2	isoform 2 of Tropomyosin beta chain	6	229	0.96	0.459	0.39	0.283	0.08	-1.12343394	-1.82112604
IP00793002	IP00793002.1	RPLP0	18 kDa protein	5	293	3.39	0.455	0.04	0.101	0.05	-1.13661655	-3.3075728
IP00793584		RPLP0	23 kDa protein	5	293	2.38	0.455	0.04	0.101	0.05	-1.13661655	-3.3075728
IP00686999	IP00686999.1	RPLP0-like	60S acidic ribosomal protein P0	4	244	1.68	0.453	0.05	0.081	0.03	-1.12421704	-3.62534328
IP00000874	Q06830	PRDX1	Peroxiredoxin-1	14	664	91.04	0.447	0.24	0.069	0.04	-1.16165326	-3.85725983
IP000218474	P13929	ENO3	Beta-enolase	2	173	0.31	0.443	0.09	0.016	0.01	-1.1746214	-5.96578428
IP000216171	P09104	ENO2	Gamma-enolase	3	201	0.5	0.443	0.09	0.016	0.01	-1.1746214	-5.96578428
IP00011229	P07339	C10P8	Cathepsin D	5	240	0.65	0.437	0.01	0.101	0.01	-1.19429462	-3.3075728
IP000224933	P30050	RPL12	60S ribosomal protein L12	6	257	4.76	0.421	0.24	0.065	0.01	-1.20756107	-3.94341647
IP000215919	P84085	ARF5	ADP-ribosylation factor 5	7	356	4.3	0.427	0.16	0.08	0.02	-1.22769203	-3.64385619
IP000224911	P30040	ERP29	Endoplasmic reticulum protein ERp29	5	252	0.72	0.426	0.27	0.05	0.03	-1.23107466	-4.32192909
IP00555614	Q589F7	HSP90AB3P	Putative heat shock protein HSP 90-beta-3	7	275	0.33	0.424	0.18	0.08	0.01	-1.27229733	-4.47393119
IP00397609	1669211240	LOC388339	PREDICTED: similar to ribosomal protein	6	257	4.76	0.421	0.24	0.065	0.22	-1.24810786	-1.30400519
IP00414676	P08238	HSP90AB1	Heat shock protein HSP 90-beta	21	1112	1.53	0.42	0.23	0.148	0.19	-1.25153877	-2.75633092
IP000216975	P67936-2	TPM4	isoform 2 of Tropomyosin alpha-4 chain	7	254	0.97	0.417	0.38	0.02	0.08	-1.26188071	-1.88363524
IP00792139	IP00792139.1	RP527A	Ubiquitin C	4	174	2.66	0.414	0.17	0.036	0.04	-1.27229733	-4.79585928
IP00010262	Q42423	ANP32C	Acidic leucine-rich nuclear phosphoprotein 32 fam	2	86	0.12	0.413	0.08	0.219	0.23	-1.17517861	-2.19097217
IP00456429	Q3MHM3	UBA52	Ubiquitin A-52 residue ribosomal protein fusion pr	5	232	3.27	0.412	0.12	0.026	0.04	-1.27928376	-5.26534457
IP00798923	IP00798923.1	RPS27A	16 kDa protein	5	232	2.81	0.412	0.12	0.026	0.04	-1.27928376	-5.26534457
IP00794211	IP00794211.1	RPS27A	18 kDa protein	5	232	2.43	0.412	0.12	0.026	0.04	-1.27928376	-5.26534457
IP00796600	IP00796600.1	RPS27A	18 kDa protein	5	232	2.43	0.412	0.12	0.026	0.04	-1.27928376	-5.26534457
IP00075248	P62156	Calmodulin	Calmodulin	3	131	0.73	0.401	0.07	0.912	0.12	-1.51825986	-0.13298427
IP00015018	Q15181	PPA1	Inorganic pyrophosphatase	5	193	0.62	0.393	0.17	0.062	0.02	-1.34739878	-0.1158797
IP00646864	IP00646864.1	SSR4	Protein	3	146	1.01	0.385	0.19	0.154	0.05	-1.37706965	-2.69899774
IP00647421	AGNL M8	SSR4	Putative uncharacterized protein SSR4	3	146	0.77	0.385	0.19	0.154	0.05	-1.37706965	-2.69899774
IP00013719	Q20422	ENO1B	Alpha-enolase, lung specific (2-phospho-D-glyceri	4	255	0.91	0.382	0.06	0.029	0.01	-1.38855546	-5.10780329
IP00793498	IP00793498.1	PA2GAP4	Pseudogene candidate	3	204	0.55	0.381	0.05	0.071	0.03	-1.3921371	-3.81603717
IP00794875	IP00794875.1	PA2G4	41 kDa protein	10	581	1.33	0.379	0.1	0.126	0.15	-1.39973025	-2.98850436
IP00382470	153792590	HSP90AA1	heat shock protein 90kDa alpha (cytosolic), class I	16	769	0.75	0.377	0.17	0.131	0.17	-1.40736357	-2.93236128
IP00799847	IP00799847.1	Protein	Protein	16	769	0.75	0.377	0.17	0.131	0.17	-1.40736357	-2.93236128
IP00010779	P67936	TPM4	Tropomyosin alpha-4 chain	9	373	1.7	0.373	0.34	0.248	0.09	-1.42275246	-0.10158797
IP00477279	1131418061	LOC464675	PREDICTED: similar to ribosomal protein L12	3	132	0.55	0.37	0.02	0.076	0.01	-1.43440282	-3.71785677
IP00738605	89036532	LOC463550	PREDICTED: similar to 60S ribosomal protein L12	4	154	2.5	0.37	0.02	0.076	0.01	-1.43440282	-3.71785677
IP003101021	P43307	SSR1	Transferrin-associated protein subunit alpha	2	134	0.34	0.366	0.05	0.09	0.01	-1.45008445	-3.47393119
IP00793851	IP00793851.1	SSR1	17 kDa protein	2	134	0.32	0.366	0.05	0.09	0.01	-1.45008445	-3.47393119
IP00688616	IP00688616.1	LOC45684	Pseudogene candidate	3	138	4.35	0.365	0.03	0.06	0.01	-1.45403163	-0.05898369
IP00788737	IP00788737.1	GAPDH	39 kDa protein	17	862	11.7	0.364	0.25	0.017	0.07	-1.45798964	-3.2243173
IP00790307	IP00790307.1	PA2G4	20 kDa protein	3	111	0.59	0.362	0.14	0.215	0.26	-1.45693984	-2.21759144
IP00019370	Q5R0WV1	RPS27A	cDNA FLJ39793, Homo sapiens ribosomal protein	3	117	2.53	0.361	7	0.14	0.023	-1.46992826	-4.42222233
IP00333476	P06571	ATP5B	ATP synthase subunit beta, mitochondrial	18	111	0.38	0.359	0.09	0.142	0.33	-1.47393119	-5.6346741
IP00018853	Q15657	TPM1	Tropomyosin isoform	5	211	0.74	0.353	0.1	0.472	0.32	-1.50225991	-1.08314124
IP000216134	119598039	TPM1	tropomyosin 1 (alpha), isoform CRA_m	5	211	0.73	0.353	0.1	0.472	0.32	-1.50225991	-1.08314124
IP000216135	P08493-3	TPM1	isoform 3 of Tropomyosin alpha-1 chain	5	211	0.62	0.353	0.1	0.472	0.32	-1.50225991	-1.08314124
IP00745267	IP00745267.1	TPM1	isoform 2 of Tropomyosin alpha-1 chain	5	211	0.57	0.353	0.1	0.472	0.32	-1.50225991	-1.08314124
IP00873694	ABMTF8	Uncharacterized protein ENSP00000383857	2	78	0.78	0.352	0.01	0.066	0.02	-1.50635267	-3.92139017	
IP00022645	ABNKJ6	Uncharacterized protein ENSP00000375058	4	120	0.68	0.352	0.01	0.066	0.02	-1.50635267	-3.92139017	
IP00478310	113422026	RPL12P8	PREDICTED: similar to 60S ribosomal protein L12	5	142	0.36	0.352	0.01	0.066	0.02	-1.50635267	-3.92139017
IP00791052	IP00791052.1	LOC388720	31 kDa protein	12	734	1.74	0.351	0.18	0.155	0.31	-1.50635267	-2.69899774
IP00791968	169162368	LOC388720	PREDICTED: similar to ubiquitin	9	333	2.36	0.349	0.08	0.005	0.01	-1.51870106	-3.64385619
IP00013296	P62269	LOC100130553	40S ribosomal protein S18	11	549	45.05	0.348	0.26	0.328	0.22	-1.52284079	-1.60823228
IP000219018	P04406	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	20	1082	29.8	0.348	0.23	0.099	0.07	-1.52284079	-1.60823228
IP00790847	IP00790847.1	ATP5B	15 kDa protein	5	303	2.47	0.347	0.1	0.334	0.41	-1.52699243	-5.8027999
IP00211917	P07195	LDHB	L-lactate dehydrogenase B chain	10	656	11.07	0.345	0.06	0.058	0.01	-1.53535317	-1.10729299
IP00479722	Q06323	PSME1	Proteasome activator complex subunit 1	5	262	0.93	0.342	0.05	0.095	0.07	-1.54793177	-3.95929868
IP000218319	P06753-2	TPM3	isoform 2 of Tropomyosin alpha-3 chain	11	573	2.31	0.342	0.08	0.305	0.06	-1.54793177	-1.71311885
IP00011916	Q13155	JTV1	Multisynthetase complex auxiliary component p38	2	82	0.2	0.339	0.1	0.116	0.03	-1.56064282	-3.90289495
IP00019329	P63136	ATP5B	Dynamin light chain 1, cytoplasmic	3	99	1.38	0.337	0.05	0.211	0.03	-1.5681795	-2.05299429
IP00798157	IP00798157.1	ATP5B	ATP synthase subunit beta, mitochondrial	10	656	2.32	0.337	0.05	0.211	0.03	-1.5681795	-2.05299429
IP000220766	Q04760	GL01	Lactylglutathione lyase	3	128	0.35	0.331	0.05	0.087	0.03	-1.59509688	-3.52284079
IP00796844	Q86U12	HSP90AA1	Full-length cDNA clone SC0CAP007YF18 of Thyr	10	478	0.91	0.331	0.15	0.068	0.06	-1.59509688	-3.52284079
IP000183968	IP000183968.1	TPM3	Tropomyosin 3 isoform 1	6	268	0.96	0.329	0.07	0.03	0.02	-1.60384051	-1.64385619
IP00317005	Q5R0WV1	Uncharacterized protein ENSP00000340627	6	142	0.34	0.329	0.04	0.249	0.05	-1.62594246	-2.0053025	
IP00062037	Q96F32	DYNNL2	Dynamin light chain 2, cytoplasmic	2	70	0.34	0.323	0.06	0.225	0.03	-1.63039393	-2.15200309
IP00031523	Q14568	HSP90AA2	Putative heat shock protein HSP 90-alpha-2	5	212	0.56	0.323	0.07	0.224	0.29	-1.63039393	-2.15200309
IP00064537	63252896	TPM1	tropomyosin 1 alpha chain isoform 3	4	181	0.62	0.321	0.08	0.332	0.06	-1.6393548	-1.59074495
IP00794332	IP00794332.2	TUBA3D	Tubulin alpha-2 chain	4	288	2.45	0.321	0.08	0.332	0.06	-1.6393548	-1.59074495
IP00844078	Q0844078	TPM3	Putative tropomyosin alpha-3 chain-like protein	4	209	0.27	0.318	0.12	0.287	0.05	-1.65290133	-1.64585619
IP00450555	Q8NAH0	TPM3	cDNA FLJ35373 fs. clone SKMUS2001740, highl	5	205	0.4	0.318	0.07	0.334	0.07	-1.65290133	-1.64585619
IP000383751	Q9UDG2	CALRETICULIN-CALCIUM binding protein	4	225	1.17	0.317	0.317	0.11	0.049	0.03	-1.65744525	-3.5107444
IP007919171	IP00791917.1	NME1	9 kDa protein	2	179	11.34	0.316	0.05	0.039	0.01	-1.66200354	-0.88038207
IP00005199	Q102905	IF2	Interleukin enhancer-binding factor 2	4	372	0.15	0.315	0.21	0.113	0.23	-1.66200354	-0.88038207
IP000641665	55962124	LOC463751	Interleukin enhancer binding factor 2, 45kDa	4	234	1.09	0.315	0.14	0.318	0.23	-1.66657627	-1.65290133
IP000616786	P09953-2	CD2	isoform 2 of Cell division control protein 42 homol	2	112	0.55	0.314	0.03	0.121	0.09	-1.67116354	-3.04692105
IP00070778	119613212	LOC463751	hCG39634, isoform CRA_a	2	112	0.55	0.314	0.03	0.121	0.09	-1.67116354	-3.04692105
IP00017672	Q8NHV5	NP	cDNA FLJ25678 fs. clone TST04067, highly simil	11	627	14.09	0.313	0.19	0.069	0.02	-1.67116354	-3.04692105
IP000215914	P84077	ARF1	ADP-ribosylation factor 1	11	627	14.09	0.313	0.19	0.069	0.02	-1.67116354	-3.04692105

IP00212093	P08708	TPS17	40S ribosomal protein S17	5	276	2.99	0.186	0.05	0.109	0.08	-2.4662547	-3.19759996
IP000783641	IP000783641.4	TXNRD1	Isomform 3 of Thoredoxin reductase 1, cytoplasmic	5	274	0.2	0.184	0.14	0.105	0.1	-2.4422233	-3.25153877
IP00552072	ABMW48	CTT7	Putative uncharacterized protein CTT7	5	203	0.29	0.183	0.04	0.132	0.05	-2.4508485	-3.21139017
IP00003881	P52597	HNRNPF	Heterogeneous nuclear ribonucleoprotein F	4	248	0.52	0.177	0.11	0.036	0.02	-2.49817873	-4.79588026
IP000030296	Q9NZ66	E1FAA2	BM-010	7	145	0.177	0.05	0.026	0.026	0.02	-2.49817873	-5.26534457
IP000000811	P28072	PSMB6	Proteasome subunit beta type-6	3	145	0.28	0.176	0.01	0.056	0.01	-2.50635267	-4.15842936
IP00513829	Q5SX90	GDJ2	GDP dissociation inhibitor 2	2	116	0.74	0.171	0.05	0.154	0.03	-2.54793177	-2.69899774
IP00203867	P30046	DOT	D-6-dephospho decarboxylase	3	147	0.171	0.05	0.171	0.234	0.29	-2.54793177	-2.06535267
IP00395865	Q16576	RBBP7	Histone-binding protein RBBP7	2	173	0.14	0.171	0.14	0.077	0.01	-2.54793177	-3.69899774
IP00552530.2	IP00552530.2	RBBP7	Retinoblastoma binding protein 7	2	173	0.15	0.171	0.14	0.077	0.01	-2.54793177	-3.69899774
IP00646512	Q5J3P0	RBBP7	cDNA FLJ3592.5s, clone KIDNE200887, highly	2	173	0.13	0.171	0.14	0.077	0.01	-2.54793177	-3.69899774
IP00025491	E1FAA1	E1FAA1	Eukaryotic initiation factor 4A-II	17	309	0.69	0.141	0.13	0.048	0.05	-2.55480485	-4.38082178
IP00328328	Q14240	E1FAA2	Eukaryotic initiation factor 4A-II	10	148	1.62	0.168	0.05	0.024	0.01	-2.57346686	-5.38082178
IP00791157	IP00791157.1		16 kDa protein	3	174	1.63	0.162	0.05	0.148	0.05	-2.62593428	-2.75633092
IP00218493	P00492	HPRT1	Hypoxanthine-guanine phosphoribosyltransferase	5	286	0.67	0.162	0.07	0.062	0.04	-2.62593428	-4.01158797
IP00555602	B4DKP9	E1FAA1	cDNA FLJ58012, moderately similar to Eukaryotic	13	798	2.86	0.159	0.03	0.047	0.05	-2.65290133	-4.41119543
IP00186290	P12093	EEF2	Elongation factor 2	12	625	0.45	0.159	0.14	0.07	0.05	-2.66200354	-3.83650127
IP00382885	Q8WTY3	RPL27	60S ribosomal protein L27	4	174	2.08	0.155	0.15	0.046	0	-2.68965988	-4.4222233
IP00219155	P61353	RPL27	60S ribosomal protein L27	6	242	2.91	0.155	0.15	0.046	0	-2.68965988	-4.4222233
IP00788730	IP00788730.1	E1FAA2	25 kDa protein	5	269	1.1	0.155	0.03	0.021	0.02	-2.68965988	-5.57346686
IP00029733	Q04828	PKR1C1	Ado-ko reductase family 1 member C1	2	133	1.52	0.155	0.13	0.033	0.06	-2.68965988	-4.01780329
IP00607708	IP00607708	LDHA	Isomform 2 of L-lactate dehydrogenase A chain	21	1028	28.87	0.147	0.07	0.026	0.03	-2.76611194	-3.26534457
IP00375704	31874085	ASMB1	hypothetical protein	5	343	0.73	0.142	0.12	0.075	0.02	-2.81603717	-3.73696559
IP00444262	Q6ZS99	NCL	cDNA FLJ45706 f1s, clone FEBRA2028457, highl	11	616	0.79	0.141	0.13	0.088	0.09	-2.82623293	-3.50635267
IP00604620	P13338	NCL	Nucleolin	12	638	0.65	0.141	0.13	0.088	0.09	-2.82623293	-3.50635267
IP00627674.1	IP00627674.1	NCL	Nucleolin, isomform CRA_c	12	638	0.68	0.141	0.13	0.088	0.09	-2.82623293	-3.50635267
IP00217966	P00338	LDHA	L-lactate dehydrogenase A chain	24	1190	57.53	0.141	0.06	0.024	0.03	-2.82623293	-5.38082178
IP00873466	ABMSU4	HPRT1	Putative uncharacterized protein HPRT1	4	194	0.47	0.14	0.07	0.049	0.04	-2.83650127	-4.35107444
IP00789078	ABMS173	SDO1	Superoxide dismutase [Cu-Zn]	2	90	0.55	0.137	0.01	0.224	0.2	-2.8677522	-2.15842936
IP00218733	SDO1	SDO1	Superoxide dismutase [Cu-Zn]	3	159	0.79	0.137	0.224	0.07	0.02	-2.8677522	-2.15842936
IP00872233	ABMW59		Uncharacterized protein ENSP00000375452	3	146	2.85	0.135	0.13	0.279	0.26	-2.88989689	-1.39973020
IP00031461	B4DLV7	GDJ2	cDNA FLJ60299, highly similar to Rab GDP disso	5	285	0.46	0.134	0.07	0.113	0.07	-2.89969509	-3.14560532
IP00646762	ABNFX8	NUDT5	Putative uncharacterized protein NUDT5	4	205	0.83	0.129	0.07	0.108	0.07	-2.95455703	-3.21089678
IP00419373	P51991	HNRNPA3	Heterogeneous nuclear ribonucleoprotein A3	5	294	0.49	0.121	0.1	0.1	0.06	-3.04682105	-3.32192809
IP00872250	IP00872250.1	HNRNPA3	7 kDa protein	2	191	0.12	0.121	0.11	0.1	0.06	-3.04682105	-3.32192809
IP00002520	P34897	SHMT2	Serine hydroxymethyltransferase, mitochondrial	2	168	0.12	0.119	0.02	0.15	0.03	-3.07096652	-2.73696559
IP00012074	Q43390	HNRNPR	Heterogeneous nuclear ribonucleoprotein R	3	139	0.15	0.117	0.04	0.048	0.04	-3.09541957	-4.38082178
IP00000494	P48777	RPL5	60S ribosomal protein L5	4	182	0.32	0.114	0.1	0.062	0.05	-3.13298427	-4.01158797
IP00259571	IP00259571.1	RPL5	Isomform 2 of Protein disulfide-isomerase A6	6	142	0.14	0.114	0.06	0.04	0.02	-3.13298427	-3.87828144
IP00021840	P62753	RP56	Isomform 1 of Protein disulfide-isomerase A6	7	315	0.55	0.112	0.09	0.131	0.06	-3.15842936	-2.9326128
IP00879622	IP00879622.1		18 kDa protein	2	94	2.96	0.111	0.01	0.215	0.07	-3.17136842	-2.21759144
IP00478277	169165866	LOC100128936	PREDICTED: similar to ribonucleoprotein L10a	4	217	1.78	0.108	0.05	0.04	0.03	-3.21089678	-4.05889369
IP00008433	P45872	RP56	40S ribosomal protein S6	2	92	0.31	0.104	0.05	0.113	0.07	-3.26534457	-3.14560532
IP00101405	P14421	FDPS	Farnesyl pyrophosphate synthetase	3	114	0.43	0.104	0.04	0.032	0.02	-3.26534457	-4.865725983
IP00025019	P20618	PSMB1	Proteasome subunit beta type-1	3	135	0.43	0.104	0.03	0.18	0.2	-3.26534457	-2.47933119
IP00011913	Q13151	HNRNPA0	Heterogeneous nuclear ribonucleoprotein A0	3	207	0.36	0.103	0.04	0.054	0.03	-3.27928376	-4.21089678
IP00689052.1	IP00689052.1	TMSL3	Similar to Elongation factor 1-alpha 1	6	261	5.19	0.102	0.02	0.22	0.05	-3.29358894	-2.3075728
IP00026271	P62263	GDJ2	Ado-ko reductase family 1 member C1	2	113	1.13	0.101	0.4	0.01	0.02	-3.3075728	-3.86725983
IP00027569	Q06812	HNRNPLC1	Heterogeneous nuclear ribonucleoprotein C-like 1	2	291	0.81	0.101	0.04	0.048	0.01	-3.3075728	-4.38082178
IP00735540	B2RXH8	LOC440563	LOC440563 protein	5	291	0.81	0.101	0.02	0.048	0.01	-3.3075728	-4.38082178
IP00871838	ABMW44		Uncharacterized protein ENSP00000383818	5	291	0.74	0.101	0.02	0.048	0.01	-3.3075728	-4.38082178
IP00003918	P39578	RPL4	60S ribosomal protein L4	14	571	1.09	0.1	0.06	0.113	0.07	-3.32192809	-3.314560532
IP00117964	P002319	SNRPD3	Small nuclear ribonucleoprotein Sm D3	2	193	0.93	0.099	0.102	0.127	0	-3.33642766	-2.9770996
IP00879750	IP00879750.1	SNRPD3	19 kDa protein	2	89	0.64	0.099	0.02	0.127	0	-3.33642766	-2.9770996
IP00003217	Q99436	PSMB7	Proteasome subunit beta type-7	3	150	0.52	0.099	0.01	0.052	0.01	-3.33642766	-2.65534457
IP00640006	Q5SX88	GDJ2	GDP dissociation inhibitor 2 (cDNA FLJ60305, hig	4	232	0.32	0.099	0.05	0.105	0.1	-3.33642766	-3.25153877
IP00440662	Q649M8		Antigen ML4A-42	3	142	0.73	0.093	0.03	0.087	0.07	-3.35107444	-3.60823228
IP00029623	P62909	PSMA6	Proteasome subunit alpha type-6	4	242	0.58	0.098	0.06	0.303	0.4	-3.35107444	-1.7226103
IP00755956	P07910-4	HNRNRC	Isomform 4 of Heterogeneous nuclear ribonucleopro	9	573	2.48	0.098	0.03	0.041	0.01	-3.38082178	-4.60823228
IP00873616	ABMW42		Uncharacterized protein ENSP00000381868	2	84	0.53	0.093	0.02	0.147	0.03	-3.42662547	-2.76611194
IP00216592	P07910-2	HNRNPC	Isomform C1 of Heterogeneous nuclear ribonucleopro	10	612	2.24	0.092	0.03	0.041	0.01	-3.4422233	-4.60823228
IP00350706	Q93541	NONO	Activator of 90 kDa heat shock protein ATPase ho	2	104	0.18	0.091	0.02	0.137	0	-3.45798964	-3.60823228
IP00304596	Q15233	NONO	Non-POU domain-containing octamer-binding pro	5	200	0.27	0.091	0.04	0.086	0.01	-3.45798964	-3.53951953
IP00479191	IP00479191.2	HNRNPH1	51 kDa protein	3	210	0.37	0.09	0.04	0.032	0.01	-3.47393119	-4.96578428
IP00216730	Q8UJ66	HIST2H2AB	Histone H2A type 2-B	6	346	10.0	0.09	0.09	0.087	0.07	-3.47393119	-3.52284079
IP00419585	P62937	PP1A	Peptidyl-prolyl cis-trans isomerase A	11	520	12.2	0.089	0.02	0.049	0.06	-3.49005085	-4.35107444
IP00016862	P01380	CSF1	Guanidine nucleotides, mitochondrial	2	115	0.82	0.084	0.05	0.087	0.01	-3.50635267	-2.86725983
IP00219156	P62888	RPL30	60S ribosomal protein L30	5	218	3.16	0.088	0.07	0.138	0.01	-3.50635267	-2.86725983
IP00872940	ABMYX5		Putative uncharacterized protein RPL30	5	218	3.11	0.088	0.07	0.138	0.01	-3.50635267	-2.86725983
IP00180730	CD1380		Similar to Elongation factor 1-alpha 1	6	266	1.95	0.088	0.03	0.187	0.04	-3.50635267	-2.47889892
IP00010157	MA1724		3'-adenosylmethionine synthetase isomform type-2	8	393	1.36	0.085	0.06	0.134	0.01	-3.55639335	-4.21089678
IP00514814.2	IP00514814.2	AKR1C1	Ado-ko reductase family 1, member C1 (Dihydr	8	396	0.95	0.085	0.06	0.101	0.08	-3.55639335	-3.3075728
IP00642560	ABNHU4	AKR1C1	Putative uncharacterized protein AKR1C1	5	295	1.57	0.085	0.06	0.101	0.08	-3.55639335	-3.3075728
IP00291483	P42330	AKR1C3	Ado-ko reductase family 1 member C3	8	426	1.36	0.085	0.06	0.101	0.08	-3.55639335	-3.3075728
IP00736657	113420853	LOC648517	PREDICTED: similar to Ado-ko reductase family	8	466	1.72	0.085	0.06	0.101	0.07	-3.55639335	-3.3075728
IP00219525	P62909	PCD	6-phosphogluconate dehydrogenase, decarboxyla	5	306	0.62	0.083	0.07	0.096	0.02	-3.59017444	-2.68989689
IP00688835.2	IP00688835.2		Similar to Heterogeneous nuclear ribonucleopro	5	303	0.9	0.082	0.03	0.045	0.01	-3.60823228	-4.7933119
IP00453476	ABNJ80	hCG_2015269	Uncharacterized protein ENSP00000348237	9	426	1.67	0.082	0.05	0.04	0.04	-3.60823228	-4.64385619
IP00872802	ABMU41		Uncharacterized protein ENSP00000375462	3	146	0.78	0.081	0.06	0.049	0.01	-3.62593428	-4.35107444
IP00433834	ABN290	LOC730343	Putative uncharacterized protein RPL26P6	6	263	2.29	0.081	0.06	0.249	0.01	-3.62593428	-4.35107444
IP00412579	P62906	RPL10A	60S ribosomal protein L10a	8	343	1.13	0.08	0.06	0.053	0.03	-3.64385619	-4.23786383
IP00827508	IP00827508.2	RPL10A	25 kDa protein	8	343	1.12	0.08	0.06	0.053	0.03	-3.64385619	-4.23786383
IP00215780	P39019	RP519	40S ribosomal protein S19	8	402	3.61	0.08	0.03	0.204	0.12	-3.64385619	-2.29358894
IP00236378	HNRNPA2B1		Heterogeneous nuclear ribonucleoproteins A2B1&	13	681	2.04	0.08	0.03	0.03	0.02	-3.66200354	-4.4422233
IP00023503	119609761	CDK5	Cyclin-dependent kinase 5, isomform CRA_a	2	144	0.18	0.078	0.02	0.157	0.02	-3.68038207	-3.25153877
IP00031681	P24941	CDK2	Cell division protein kinase 2	3	188	0.3						

IP00411329	A6NCA2	Uncharacterized protein ENSP0000022956	8	433	1.38	0.041	0.02	0.051	0.02	-4.60823228	-4.29335894
IP00513971	P42677	40S ribosomal protein S27	2	117	0.87	0.04	0.01	0.104	0.04	-4.64385619	-3.26534457
IP00738640	169184563	PREDICTED: similar to v-fos transformation effect	5	250	0.7	0.04	0.04	0.074	0.09	-4.64385619	-3.75633092
IP00647546	IP00647546.1	16 kDa protein	5	208	1.15	0.039	0.01	0.061	0.05	-4.68038207	-4.03504695
IP00478127	119580810	SLC5A1	6	237	0.74	0.039	0.01	0.061	0.05	-4.68038207	-4.03504695
IP00789127	IP00789127.1	LOC645001	5	295	0.52	0.038	0.02	0.053	0.02	-4.71785677	-4.23786383
IP00874005	ABMW77	Uncharacterized protein ENSP00000381249	4	165	0.32	0.037	0.02	0.046	0.02	-4.75633092	-4.44222233
IP00031804	Q9Y277	VDAC3	5	233	0.67	0.037	0.02	0.065	0.02	-4.75633092	-3.94341647
IP00294779	Q9Y277-2	VDAC3	5	233	0.67	0.037	0.02	0.065	0.02	-4.75633092	-3.94341647
IP00871813	IP00871813.1	VDAC3	5	233	0.67	0.037	0.02	0.065	0.02	-4.75633092	-3.94341647
IP00871473	ABMW77	Uncharacterized protein ENSP00000381626	3	162	0.39	0.036	0.02	0.049	0.02	-4.79585928	-4.35107444
IP00397611	169212066	LOC388344	4	153	0.08	0.03	0.02	0.019	0.01	-4.79585928	-5.71785677
IP00465361	P26373	RPL13	8	357	1.82	0.036	0.02	0.019	0.01	-4.79585928	-5.71785677
IP00144171	119616214	HCG_2015956	10	427	0.86	0.036	0.02	0.02	0.01	-4.79585928	-5.64385619
IP00472171	119607406	ribosomal protein L7, isoform CRA_a	12	527	0.99	0.036	0.02	0.02	0.01	-4.79585928	-5.64385619
IP00871827	ABMVV7	Putative uncharacterized protein RPL7	12	527	1.19	0.036	0.02	0.02	0.01	-4.79585928	-5.64385619
IP00872387	ABMY04	Uncharacterized protein ENSP00000381447	12	527	1.19	0.036	0.02	0.02	0.01	-4.79585928	-5.64385619
IP00061178	Q96E39	CCBL2	2	160	0.16	0.035	0.02	0.049	0	-4.83650127	-4.35107444
IP00643486	Q5T9T9	CCBL2	2	160	0.44	0.035	0.02	0.049	0	-4.83650127	-4.35107444
IP00304692	P38159	RBMX	3	212	0.25	0.035	0.01	0.046	0.01	-4.83650127	-4.44222233
IP00879848	119598703	HCG2022018	4	153	1.08	0.034	0.02	0.067	0.02	-4.87832144	-3.89969509
IP00847163	IP00847163.1	THOC3	3	189	1.63	0.034	0.02	0.03	0.03	-4.87832144	-5.05889369
IP00299573	P62424	RPL7A	11	521	1.58	0.033	0.02	0.278	0.39	-4.92139017	-1.84684321
IP00397676	169213130	LOC388474	7	396	1.09	0.032	0.03	0.056	0.07	-4.96578428	-4.15842936
IP00736803	119570872	HCG1988775	3	155	0.47	0.031	0.03	0.043	0.03	-5.01158797	-4.53951953
IP00645201	Q5JUR5	RPS58	7	403	2.61	0.031	0.02	1.477	3.77	-5.01158797	0.56269826
IP00790291	IP00790291.1	RPL7	5	215	1.76	0.03	0.02	0.017	0.01	-5.05889369	-5.87832144
IP00221091	P62244	RPS15A	5	216	0.85	0.03	0.02	0.037	0.01	-5.05889369	-4.75633092
IP00738970	A6NIE4	Uncharacterized protein ENSP00000304033	6	258	0.39	0.03	0.02	0.017	0.01	-5.05889369	-5.87832144
IP00879238	B5MCT8	Putative uncharacterized protein RPS9 (Ribosome)	7	317	2.04	0.03	0.02	0.047	0.04	-5.05889369	-4.41119543
IP00816796	Q8N8Y7	cDNA FLJ136696 fs, clone KIDNE2001931, highly	2	128	0.17	0.029	0.01	0.045	0.01	-5.10780329	-4.47393119
IP00556601	Q59EP7	Solute carrier family 25 member 4 variant	4	232	0.54	0.027	0.02	0.058	0.02	-5.21089678	-4.10780329
IP00398673	113429532	LOC388885	4	253	0.87	0.026	0.02	0.117	0.05	-5.26534457	-3.09541957
IP00221088	P46781	RPS9	10	409	1.64	0.026	0.02	0.041	0.04	-5.26534457	-4.60823228
IP00008293	169161603	LOC149224	3	98	0.54	0.025	0.02	0.051	0	-5.32192809	-4.29335894
IP00008438	P46783	RPS10	6	323	1.27	0.025	0.01	0.109	0.05	-5.32192809	-3.19759996
IP00011253	P23396	RPS3	16	768	4.87	0.025	0.01	0.04	0.02	-5.32192809	-4.64385619
IP00450975	Q6IPX4	RPS16	3	159	0.72	0.024	0.01	0.045	0.01	-5.38082178	-4.47393119
IP00413641	P15121	AKR1B1	6	262	0.7	0.024	0.01	0.146	0.18	-5.38082178	-2.77595973
IP00478986	IP00478986.2	CGB8	3	105	0.23	0.023	0.01	0.054	0.05	-5.44222233	-4.21089678
IP00075558	169166935	LOC152663	3	124	0.31	0.023	0.02	0.364	0.51	-5.44222233	-1.45798964
IP00012772	P62917	RPL8	8	359	0.96	0.021	0.01	0.016	0.01	-5.57346686	-5.96578428
IP00641950	37724561	GNB2L1	7	379	0.8	0.02	0.01	0.02	0.01	-5.64385619	-5.64385619
IP00847774	B40M74	RPL18A	3	125	0.79	0.018	0.01	0.022	0.02	-5.79585928	-5.50635267
IP00792352	119618921	RAN	8	454	3.69	0.018	0.01	0.121	0.02	-5.79585928	-3.04692105
IP00796462	IP00796462.1	RAN	8	454	3.61	0.018	0.01	0.121	0.02	-5.79585928	-3.04692105
IP00397017	88948852	LOC644790	3	115	0.23	0.017	0.01	0.031	0.01	-5.87832144	-5.01158797
IP00784408	38570357	GNB2L1	5	278	0.7	0.017	0.01	0.025	0	-5.87832144	-5.32192809
IP00217465	P18403	HIST1H1C	11	553	4	0.015	0.01	0.063	0.11	-6.05889369	-3.98850436
IP00010420	Q9H0C2	SLC25A31	3	180	0.31	0.014	0.01	0.043	0	-6.15842936	-4.53951953
IP00217466	P16402	HIST1H1D	7	422	2.52	0.013	0.01	0.075	0.13	-6.26534457	-3.73696559
IP00217467	P10412	HIST1H1E	10	523	3.81	0.013	0.01	0.075	0.13	-6.26534457	-3.73696559
IP00453473	P62805	HIST2H4B	10	585	765.54	0.013	0.01	0.033	0.01	-6.26534457	-4.92139017
IP00249267	IP00249267.2	13 kDa protein	3	197	2.9	0.01	0.01	0.076	0	-6.64385619	-3.71785677

Exhibit P

Screenshot of ACS Publications record for Bryk

The screenshot displays the ACS Publications record for the article "Quantitative Analysis of Human Red Blood Cell Proteome" by Agata H. Bryk and Jacek R. Wiśniewski. The article is published in the Journal of Proteome Research, Volume 16, Issue 7, July 2017, pages 2752-2761. The DOI is 10.1021/acs.jproteome.7b00025. The article is an open access article published under an ACS AuthorChoice License.

Journal of proteome research

Quantitative Analysis of Human Red Blood Cell Proteome

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[Supporting Information](#)

ABSTRACT: Red blood cells (RBCs) are the most abundant cell type in the human body. RBCs and, in particular, their plasma membrane composition have been extensively studied for many years. During the past decade proteomics studies have extended our knowledge on RBC composition; however, these studies did not provide quantitative insights. Here we report a large-scale proteomics investigation of RBCs and their "white ghost" membrane fraction. Samples were processed using the multicatalytic digestion filter-aided sample preparation (MED-FASP) and analyzed using Q-Exactive mass spectrometer. Protein abundances were computed using the total protein approach (TPA). The validation of the data with stable isotope-labeled peptide-based protein quantification followed. Our in-depth analysis resulted in the identification of 2650 proteins, of which 1890 occurred at more than 100 copies per cell. We quantified 41 membrane transporter proteins spanning an abundance range of five orders of magnitude. Some of these, including the drug transporter ABCA7 and choline transporters SLC44A1 and SLC44A2, have not previously been

Figure 1: A bar chart showing the Log Copy Number for various proteins in Erythrocytes and White ghosts. The chart compares the Log Copy Number for Erythrocytes (red bars) and White ghosts (blue bars) for several proteins: ABCA7, SLC44A1, SLC44A2, and SLC44A3. The Y-axis represents the Log Copy Number, ranging from 0 to 4. The X-axis lists the proteins. The chart shows that ABCA7, SLC44A1, and SLC44A2 have higher Log Copy Numbers in Erythrocytes compared to White ghosts, while SLC44A3 has a higher Log Copy Number in White ghosts.

Figure 2: A bar chart showing the Log Copy Number for various proteins in Erythrocytes and White ghosts. The chart compares the Log Copy Number for Erythrocytes (red bars) and White ghosts (blue bars) for several proteins: ABCA7, SLC44A1, SLC44A2, and SLC44A3. The Y-axis represents the Log Copy Number, ranging from 0 to 4. The X-axis lists the proteins. The chart shows that ABCA7, SLC44A1, and SLC44A2 have higher Log Copy Numbers in Erythrocytes compared to White ghosts, while SLC44A3 has a higher Log Copy Number in White ghosts.

Figure 3: A bar chart showing the Log Copy Number for various proteins in Erythrocytes and White ghosts. The chart compares the Log Copy Number for Erythrocytes (red bars) and White ghosts (blue bars) for several proteins: ABCA7, SLC44A1, SLC44A2, and SLC44A3. The Y-axis represents the Log Copy Number, ranging from 0 to 4. The X-axis lists the proteins. The chart shows that ABCA7, SLC44A1, and SLC44A2 have higher Log Copy Numbers in Erythrocytes compared to White ghosts, while SLC44A3 has a higher Log Copy Number in White ghosts.

Figure 4: A bar chart showing the Log Copy Number for various proteins in Erythrocytes and White ghosts. The chart compares the Log Copy Number for Erythrocytes (red bars) and White ghosts (blue bars) for several proteins: ABCA7, SLC44A1, SLC44A2, and SLC44A3. The Y-axis represents the Log Copy Number, ranging from 0 to 4. The X-axis lists the proteins. The chart shows that ABCA7, SLC44A1, and SLC44A2 have higher Log Copy Numbers in Erythrocytes compared to White ghosts, while SLC44A3 has a higher Log Copy Number in White ghosts.

Exhibit Q



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Exhibit R

Table S1

WG	Sample	Targeted sequence	Amount of heavy peptide spiked-in (pmol)	Uniprot entry #	Gene name	Protein name	Raw file	MS/MS m/z [m]	Charge	m/z [L]	Mass	Mass Error [ppm]	Mass Error [Da]	Retention time	Intensity ratio L/H	PEP	Content of targeted protein (pmol/mg)	Copy number	Average copy number	Standard deviation copy number
Sample 2	TYVPEETGER	0.5	P02724	GYPB		Glycophorin-A	20160812_Oep7_JRW_SA_T_T61	709.33	2	703.8253	1405.636	-0.52845	-0.000372	28.27	0.383	0.000614	38	4.15E+04		
Sample 2	TYVPEETGER	0.5	P02724	GYPB		Glycophorin-A	20160812_Oep7_JRW_SA_T_T62	708.83	2	703.8253	1405.636	-1.327	-0.000934	28.46	0.385	6.63E-05	39	4.18E+04		
Sample 2	TYVPEETGER	1	P02724	GYPB		Glycophorin-A	20160812_Oep7_JRW_SA_T_T63	709.33	2	703.8253	1405.636	-1.0417	-0.000733	28.57	0.287	2.81E-107	57	6.38E+04		
Sample 2	TYVPEETGER	1	P02724	GYPB		Glycophorin-A	20160812_Oep7_JRW_SA_T_T64	708.83	2	703.8253	1405.636	-1.246	-0.000877	28.32	0.196	5.74E-07	39	4.28E+04		
Sample 2	TYVPEETGER	2	P02724	GYPB		Glycophorin-A	20160812_Oep7_JRW_SA_T_T65	708.83	2	703.8253	1405.636	-0.17412	-0.000123	28.15	0.102	5.74E-07	41	4.64E+04		
Sample 2	TYVPEETGER	5	P02724	GYPB		Glycophorin-A	20160812_Oep7_JRW_SA_T_T67	708.83	2	703.8253	1405.636	-0.53514	-0.000377	27.64	0.039	3.51E-35	39	4.22E+04		
Sample 2	TYVPEETGER	5	P02724	GYPB		Glycophorin-A	20160812_Oep7_JRW_SA_T_T68	708.83	2	703.8253	1405.636	0.036156	2.54E-05	27.81	0.039	5.74E-07	39	4.18E+04	4.52E+04	7.60E+03
Sample 3	TYVPEETGER	0.5	P02724	GYPB		Glycophorin-A	20160812_Oep7_JRW_SA_T_T69	708.83	2	703.8253	1405.636	0.12475	0.000279	28.13	0.520	5.74E-07	52	5.68E+04		
Sample 3	TYVPEETGER	0.5	P02724	GYPB		Glycophorin-A	20160812_Oep7_JRW_SA_T_T70	708.83	2	703.8253	1405.636	0.66959	0.000472	28.38	0.476	2.30E-25	48	5.16E+04		
Sample 3	TYVPEETGER	1	P02724	GYPB		Glycophorin-A	20160812_Oep7_JRW_SA_T_T71	708.83	2	703.8253	1405.636	0.68881	0.000485	28.55	0.263	3.51E-35	53	5.70E+04		
Sample 3	TYVPEETGER	1	P02724	GYPB		Glycophorin-A	20160812_Oep7_JRW_SA_T_T72	708.83	2	703.8253	1405.636	-0.13622	-5.95E-05	28.88	0.274	3.51E-35	55	5.93E+04		
Sample 3	TYVPEETGER	2	P02724	GYPB		Glycophorin-A	20160812_Oep7_JRW_SA_T_T73	708.83	2	703.8253	1405.636	-0.24964	-0.000176	28.84	0.131	3.51E-35	53	5.69E+04		
Sample 3	TYVPEETGER	2	P02724	GYPB		Glycophorin-A	20160812_Oep7_JRW_SA_T_T74	472.89	3	469.5377	1405.636	3.8705	0.001817	29.48	0.113	4.69E-60	45	4.90E+04		
Sample 3	TYVPEETGER	2	P02724	GYPB		Glycophorin-A	20160812_Oep7_JRW_SA_T_T74	708.83	2	703.8253	1405.636	0.60367	0.000425	29.44	0.152	0.000614	61	6.58E+04		
Sample 3	TYVPEETGER	5	P02724	GYPB		Glycophorin-A	20160812_Oep7_JRW_SA_T_T75	708.83	2	703.8253	1405.636	-1.3194	-0.000929	28.84	0.051	5.74E-07	51	5.57E+04		
Sample 3	TYVPEETGER	5	P02724	GYPB		Glycophorin-A	20160812_Oep7_JRW_SA_T_T76	708.83	2	703.8253	1405.636	-0.45306	-0.000319	28.50	0.052	1.33E-05	52	5.68E+04	5.72E+04	4.98E+03
Sample 2	TFDEIASGFR	0.5	P11166	SLC2A1		Solute carrier family 2, facilitated glucose transporter member 1	20160812_Oep7_JRW_SA_T_T62	576.78	2	571.7775	1141.54	2.0187	0.001154	51.35	1.599	0.004054	160	1.79E+05		
Sample 2	TFDEIASGFR	1	P11166	SLC2A1		Solute carrier family 2, facilitated glucose transporter member 1	20160812_Oep7_JRW_SA_T_T63	576.79	2	571.7775	1141.54	0.91585	0.000524	51.42	0.872	0.004515	174	1.89E+05		
Sample 2	TFDEIASGFR	1	P11166	SLC2A1		Solute carrier family 2, facilitated glucose transporter member 1	20160812_Oep7_JRW_SA_T_T64	576.78	2	571.7775	1141.54	0.36531	0.000209	51.12	0.882	0.003162	166	1.80E+05		
Sample 2	TFDEIASGFR	2	P11166	SLC2A1		Solute carrier family 2, facilitated glucose transporter member 1	20160812_Oep7_JRW_SA_T_T65	576.78	2	571.7775	1141.54	0.67765	0.000387	51.00	0.413	0.000905	165	1.79E+05		
Sample 2	TFDEIASGFR	2	P11166	SLC2A1		Solute carrier family 2, facilitated glucose transporter member 1	20160812_Oep7_JRW_SA_T_T66	576.78	2	571.7775	1141.54	0.17487	1.0E-04	50.93	0.414	8.73E-10	166	1.79E+05		
Sample 2	TFDEIASGFR	5	P11166	SLC2A1		Solute carrier family 2, facilitated glucose transporter member 1	20160812_Oep7_JRW_SA_T_T67	576.78	2	571.7775	1141.54	1.6261	0.00093	50.21	0.166	3.30E-09	166	1.80E+05		
Sample 2	TFDEIASGFR	5	P11166	SLC2A1		Solute carrier family 2, facilitated glucose transporter member 1	20160812_Oep7_JRW_SA_T_T68	576.78	2	571.7775	1141.54	1.3824	0.00079	50.55	0.167	7.25E-12	167	1.81E+05	1.80E+05	4.62E+03
Sample 3	TFDEIASGFR	0.5	P11166	SLC2A1		Solute carrier family 2, facilitated glucose transporter member 1	20160812_Oep7_JRW_SA_T_T69	576.78	2	571.7775	1141.54	1.1225	0.000642	51.79	1.834	0.002395	183	1.99E+05		
Sample 3	TFDEIASGFR	0.5	P11166	SLC2A1		Solute carrier family 2, facilitated glucose transporter member 1	20160812_Oep7_JRW_SA_T_T70	576.79	2	571.7775	1141.54	-0.16764	-9.59E-05	52.32	1.696	0.002395	170	1.84E+05		
Sample 3	TFDEIASGFR	1	P11166	SLC2A1		Solute carrier family 2, facilitated glucose transporter member 1	20160812_Oep7_JRW_SA_T_T71	576.79	2	571.7775	1141.54	0.041457	2.37E-05	52.57	0.927	3.30E-09	185	2.01E+05		
Sample 3	TFDEIASGFR	1	P11166	SLC2A1		Solute carrier family 2, facilitated glucose transporter member 1	20160812_Oep7_JRW_SA_T_T72	576.79	2	571.7775	1141.54	1.9883	0.001137	52.80	0.983	0.002728	197	2.13E+05		
Sample 3	TFDEIASGFR	2	P11166	SLC2A1		Solute carrier family 2, facilitated glucose transporter member 1	20160812_Oep7_JRW_SA_T_T73	576.79	2	571.7775	1141.54	0.31396	0.00018	52.66	0.445	3.30E-09	178	1.93E+05		
Sample 3	TFDEIASGFR	2	P11166	SLC2A1		Solute carrier family 2, facilitated glucose transporter member 1	20160812_Oep7_JRW_SA_T_T74	576.79	2	571.7775	1141.54	1.9818	0.001133	53.10	0.506	0.002395	202	2.19E+05		
Sample 3	TFDEIASGFR	5	P11166	SLC2A1		Solute carrier family 2, facilitated glucose transporter member 1	20160812_Oep7_JRW_SA_T_T75	576.78	2	571.7775	1141.54	-0.1026	-5.87E-05	52.63	0.184	1.81E-42	184	2.00E+05		
Sample 3	TFDEIASGFR	5	P11166	SLC2A1		Solute carrier family 2, facilitated glucose transporter member 1	20160812_Oep7_JRW_SA_T_T76	576.78	2	571.7775	1141.54	1.0717	0.000613	52.38	0.184	1.81E-42	184	2.00E+05	2.01E+05	1.19E+04
Sample 2	TISFDIPPEILTK	0.5	P27105	STOM		Erythrocyte band 7 integral membrane protein	20160812_Oep7_JRW_SA_T_T61	805.45	2	801.4429	1600.871	0.39283	0.000315	70.87	12.788	0.000859	1279	1.39E+06		
Sample 2	TISFDIPPEILTK	0.5	P27105	STOM		Erythrocyte band 7 integral membrane protein	20160812_Oep7_JRW_SA_T_T62	805.96	2	801.4429	1600.871	0.88902	0.000713	70.67	11.641	0.014229	1164	1.26E+06		
Sample 2	TISFDIPPEILTK	1	P27105	STOM		Erythrocyte band 7 integral membrane protein	20160812_Oep7_JRW_SA_T_T63	805.45	2	801.4429	1600.871	0.58402	0.000468	70.72	6.680	0.007718	1336	1.45E+06		
Sample 2	TISFDIPPEILTK	2	P27105	STOM		Erythrocyte band 7 integral membrane protein	20160812_Oep7_JRW_SA_T_T65	805.45	2	801.4429	1600.871	0.69006	4.01E-05	70.56	3.146	2.18E-07	1258	1.36E+06		
Sample 2	TISFDIPPEILTK	2	P27105	STOM		Erythrocyte band 7 integral membrane protein	20160812_Oep7_JRW_SA_T_T66	805.45	2	801.4429	1600.871	0.31055	0.000249	70.55	3.226	4.58E-05	1290	1.40E+06		
Sample 2	TISFDIPPEILTK	5	P27105	STOM		Erythrocyte band 7 integral membrane protein	20160812_Oep7_JRW_SA_T_T67	805.45	2	801.4429	1600.871	0.46998	0.000377	70.28	1.304	4.78E-05	1304	1.41E+06		
Sample 2	TISFDIPPEILTK	5	P27105	STOM		Erythrocyte band 7 integral membrane protein	20160812_Oep7_JRW_SA_T_T68	805.45	2	801.4429	1600.871	0.19708	0.000158	70.44	1.289	0.001227	1289	1.40E+06	1.38E+06	5.86E+04
Sample 3	TISFDIPPEILTK	0.5	P27105	STOM		Erythrocyte band 7 integral membrane protein	20160812_Oep7_JRW_SA_T_T69	805.45	2	801.4429	1600.871	0.12516	0.0001	71.10	11.112	0.017212	1131	1.23E+06		
Sample 3	TISFDIPPEILTK	0.5	P27105	STOM		Erythrocyte band 7 integral membrane protein	20160812_Oep7_JRW_SA_T_T70	805.45	2	801.4429	1600.871	0.19565	0.000157	71.44	10.549	0.009098	1055	1.14E+06		
Sample 3	TISFDIPPEILTK	1	P27105	STOM		Erythrocyte band 7 integral membrane protein	20160812_Oep7_JRW_SA_T_T71	805.45	2	801.4429	1600.871	-0.689	-0.000552	71.49	5.605	0.013964	1121	1.21E+06		
Sample 3	TISFDIPPEILTK	1	P27105	STOM		Erythrocyte band 7 integral membrane protein	20160812_Oep7_JRW_SA_T_T72	805.45	2	801.4429	1600.871	0.24905	0.0002	71.53	6.165	0.004752	1233	1.34E+06		
Sample 3	TISFDIPPEILTK	2	P27105	STOM		Erythrocyte band 7 integral membrane protein	20160812_Oep7_JRW_SA_T_T73	805.45	2	801.4429	1600.871	-0.36857	-0.000215	71.46	2.789	0.003253	1115	1.21E+06		
Sample 3	TISFDIPPEILTK	2	P27105	STOM		Erythrocyte band 7 integral membrane protein	20160812_Oep7_JRW_SA_T_T74	805.45	2	801.4429	1600.871	-0.3689	-0.000296	71.56	3.147	0.013964	1259	1.36E+06		
Sample 3	TISFDIPPEILTK	5	P27105	STOM		Erythrocyte band 7 integral membrane protein	20160812_Oep7_JRW_SA_T_T75	805.45	2	801.4429	1600.871	-0.14555	-0.000117	71.48	1.175	8.72E-11	1175	1.27E+06		
Sample 3	TISFDIPPEILTK	5	P27105	STOM		Erythrocyte band 7 integral membrane protein	20160812_Oep7_JRW_SA_T_T76	805.45	2	801.4429	1600.871	0.29559	0.000237	71.48	1.218	3.85E-07	1218	1.32E+06	1.27E+06	8.01E+04
Sample 2	YLQITTTIAAEK	0.5	P27105	STOM		Erythrocyte band 7 integral membrane protein	20160812_Oep7_JRW_SA_T_T61	680.39	2	676.377	1350.739	1.6295	0.001102	52.07	9.091	6.33E-74	909	9.85E+05		
Sample 2	YLQITTTIAAEK	0.5	P27105	STOM		Erythrocyte band 7 integral membrane protein	20160812_Oep7_JRW_SA_T_T62	680.39	2	676.377	1350.739	0.74983	0.000507	51.79	10.204	3.47E-47	1010	1.11E+06		
Sample 2	YLQITTTIAAEK	1	P27105	STOM		Erythrocyte band 7 integral membrane protein	20160812_Oep7_JRW_SA_T_T63	680.39	2	676.377	1350.739	1.4985	0.001014	51.90	5.672	3.64E-60	1134	1.23E+06		
Sample 2	YLQITTTIAAEK	1	P27105	STOM		Erythrocyte band 7 integral membrane protein	20160812_Oep7_JRW_SA_T_T64	680.39	2	676.377	1350.739	0.43761	0.000296	51.56	4.257	3.05E-25	851	9.23E+05		
Sample 2	YLQITTTIAAEK	2	P27105	STOM		Erythrocyte band 7 integral membrane protein	20160812_Oep7_JRW_SA_T_T65	680.39	2	676.377	1350.739	-0.6267	-0.000424	51.42	2.152	3.64E-60	861	9.33E+05		
Sample 2	YLQITTTIAAEK	2	P27105	STOM		Erythrocyte band 7 integral membrane protein	20160812_Oep7_JRW_SA_T_T66	680.39	2	676.377	1350.739	-0.61232	-0.000414	51.40	2.104	5.53E-173	922	9.90E+05		
Sample 2	YLQITTTIAAEK	5	P27105	STOM		Erythrocyte band 7 integral membrane protein	20160812_Oep7_JRW_SA_T_T67	680.39	2	676.377	1350.739	0.47678	0.000322	50.59	1.024	3.47E-47	1024	1.11E+06		
Sample 2	YLQITTTIAAEK	5	P27105	STOM		Erythrocyte band 7 integral membrane protein	20160812_Oep7_JRW_SA_T_T68	680.39	2	676.377	1350.739	0.6995	0.000473	51.03	0.969	7.21E-76	969	1.05E+06	1.05E+06	1.09E+05
Sample 3	YLQITTTIAAEK	0.5	P27105	STOM		Erythrocyte band 7 integral membrane protein	20160812													

Table S2

Supplemental data 2: Title: Quantitative analysis of human red blood cell proteome; Authors: Agata Bryk and Jacek R. Wisniewski (Biochemical Proteomics Group, Department of Proteomics and Signal Transduction, Max-Planck-Institute of Biochemistry, Am Klopferspitz 18, 82152 Martinsried, Germany)

Protein IDs	Protein names	Gene name:	Peptides	Enrichment 'White Ghost'/whole erythrocyte			
				Donor 1	Donor 2	Donor 3	Donor 4
P02549;A0A087WZE4;P02549-2	Spectrin alpha chain, erythrocytic 1	SPTA1	924	6.2	6.6	6.7	6.9
P11277-2;H0YJE6	Spectrin beta chain, erythrocytic	SPTB	828	5.9	6.4	6.6	6.5
P02730;A0A0A0MS98	Band 3 anion transport protein	SLC4A1	177	2.7	3.6	3.4	3.1
P16157-12;P16157-14;P16157-16	Ankyrin-1	ANK1	485	6.4	6.7	7.2	7.3
P11171-2	Protein 4.1	EPB41	207	12.0	9.5	10.2	9.8
P16452-2;P16452;P16452-3;F5H563;H3BTE6;H3BUR	Erythrocyte membrane protein band 4.2	EPB42	171	4.7	7.6	10.1	10.0
P23634;P23634-8;P23634-6;P23634-7	Plasma membrane calcium-transporting ATPase 4	ATP2B4	135	13.4	12.7	18.5	26.4
P11166;A0A0D9SFK9;A0A0D9SG74;A6NL68	Solute carrier family 2, facilitated glucose transporter member 1	SLC2A1	55	6.3	15.3	13.3	14.7
Q00013;Q00013-2;Q00013-3;C9J9J4;A8MTH1;C9JB3	55 kDa erythrocyte membrane protein	MPP1	115	16.5	19.5	17.4	18.9
A0A0A0MQS8;Q02094;A0A087WZZ4;Q02094-2	Ammonium transporter Rh type A	RHAG	13	4.5	8.3	10.9	8.2
			Average	7.8	9.6	10.4	11.2
			Deviation	4.5	4.8	4.8	7.0

Table S3

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Volume 42, Issue D1
1 January 2014

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Journal of Separation Science / Volume 32, Issue 5-6 / p. 771-798

Sample Preparation |  Full Access

Sample preparation and fractionation for proteome analysis and cancer biomarker discovery by mass spectrometry

Farid E. Ahmed 

First published: 10 March 2009

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Abstract

Sample preparation and fractionation technologies are one of the most crucial processes in proteomic analysis and biomarker discovery in solubilized samples. Chromatographic or electrophoretic proteomic technologies are also available for separation of cellular protein components. There are, however, considerable limitations in currently available proteomic technologies as none of them allows for the analysis of the entire proteome in a simple step because of the large number of peptides, and because of the wide concentration dynamic range of the proteome in clinical blood samples. The results of any undertaken experiment depend on the condition of the starting material. Therefore, proper experimental design and pertinent sample preparation is essential to obtain meaningful results, particularly in comparative clinical proteomics in which one is looking for minor differences between experimental (diseased) and control (nondiseased) samples. This review discusses problems associated with general and specialized strategies of sample preparation and fractionation, dealing with samples that are solution or suspension, in a frozen tissue state, or formalin-preserved tissue archival samples, and illustrates how sample processing might influence detection with mass spectrometric techniques. Strategies that dramatically improve the

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Latent transforming growth factor-beta binding protein-1 in circulating plasma as a novel biomarker for early detection of hepatocellular carcinoma

[Bangrong Cao](#),¹ [Lei Yang](#),¹ [Weiqi Rong](#),² [Lin Feng](#),¹ [Naijun Han](#),¹ [Kaitai Zhang](#),¹ [Shujun Cheng](#),¹ [Jianxiong Wu](#),² [Ting Xiao](#),¹ [Yanning Gao](#)¹

Abstract

This study aimed to assess the diagnostic value of the latent transforming growth factor-beta binding protein-1 (LTBP-1) in distinguishing hepatocellular carcinoma (HCC) from patients with hepatitis or liver cirrhosis. The protein levels of LTBP-1 or AFP in circulating plasma were measured by enzyme-linked immunosorbent assay (ELISA) or chemiluminescence in four cohorts: HCC ($n = 167$), liver cirrhosis ($n = 50$), chronic hepatitis B (CHB, $n = 50$), and healthy individuals ($n = 104$). Receiver operating characteristics (ROC) curves and area under the curves (AUC) of the proteins were calculated. Results showed that plasma levels of LTBP-1 were significantly higher in HCC patients than those in other three groups. LTBP-1 showed a better diagnostic performance (AUC = 0.74, 95% CI: 0.67-0.80) in distinguishing HCC from the CHB or cirrhosis patients, compared to AFP (AUC = 0.59, 95% CI: 0.52-0.65). In the early-stage HCCs investigated, diagnostic performance of LTBP-1 (AUC = 0.77, 95% CI: 0.70-0.84) remained better than that of AFP (AUC = 0.61, 95% CI: 0.52-0.69). Combination of LTBP-1 and AFP showed increased diagnostic efficiency than any of the two proteins performed alone, for both all HCC (AUC = 0.78, 95% CI: 0.72-0.83) and early-stage HCC (AUC = 0.80, 95% CI: 0.74-0.87). These findings proposed that LTBP-1 may be a promising biomarker for distinguishing HCC from the CHB or liver cirrhosis patients, especially for the early-stage HCC.

Keywords: LTBP-1, biomarker, hepatocellular carcinoma, early detection, AFP

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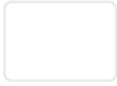
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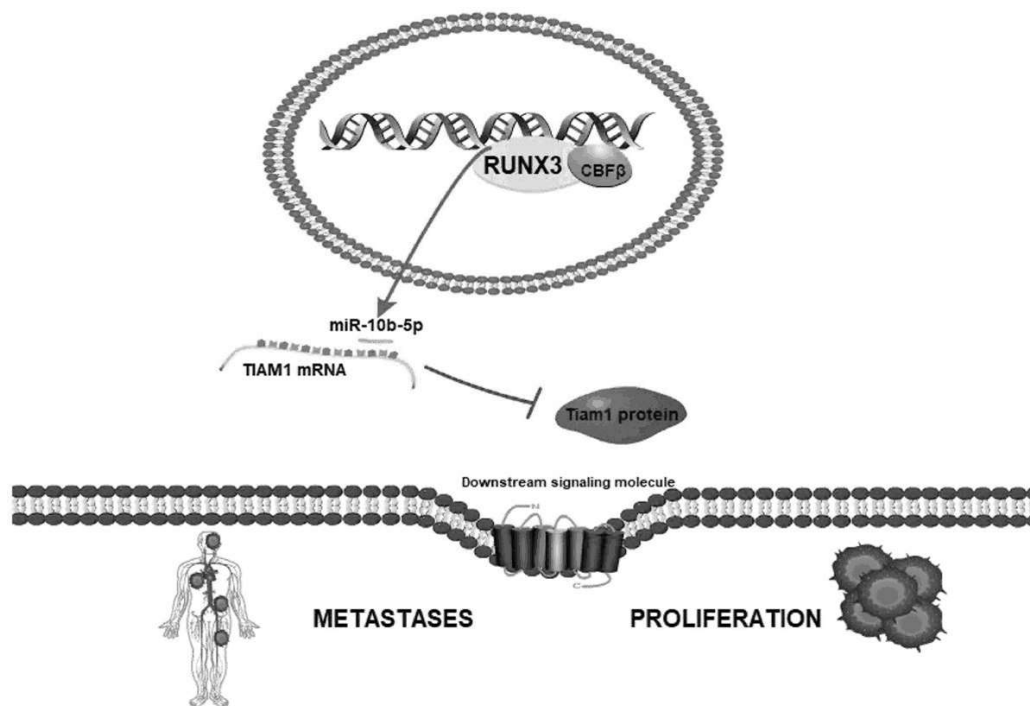
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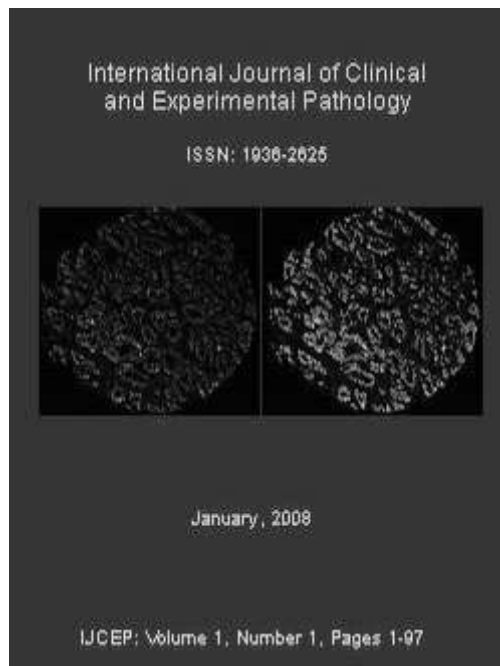
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Exhibit Z

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Quantitative analysis of human red blood cell proteome

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²Present affiliation: Institute of Cardiology, Jagiellonian University Medical College; John Paul II Hospital, 80 Pradnicka Str., 31-202, Cracow, Poland

Content:

Figure S1. Western blot analysis of selected proteins

Table S1. Stable isotope labeled peptide based protein quantification

Table S2. Enrichment of membrane proteins in the *white ghost* fraction

Table S3. List of identified proteins and their concentrations in the whole red cells and *white ghosts*

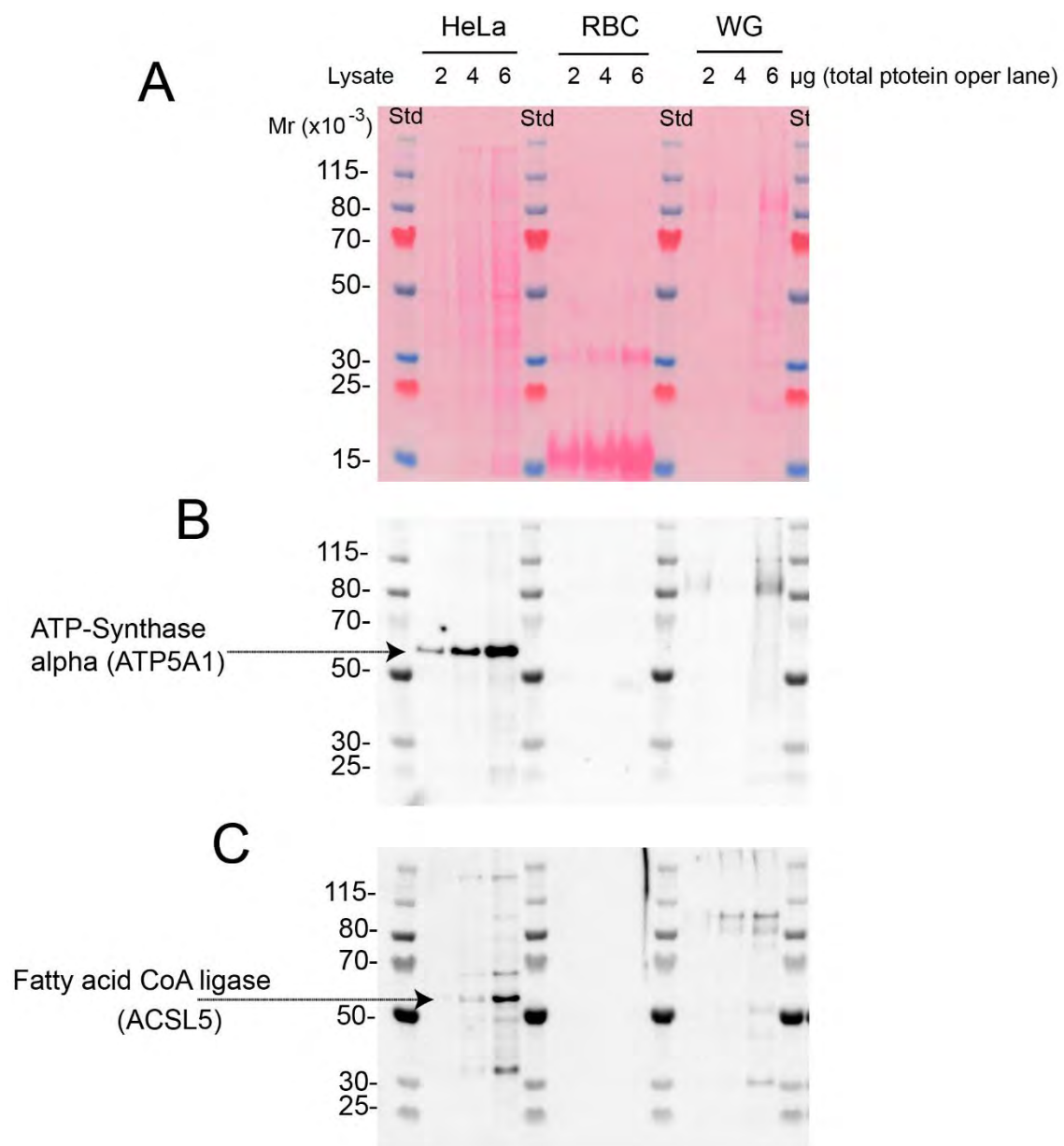


Figure S1. Western blot analysis. (A) Electrophoretically separated proteins were transferred onto nitrocellulose membrane and were stained with Ponceau S. After destaining the blots were probed with antibodies against ATP synthase (B) and Fatty acid CoA ligase (C). HeLa lysate was used as positive control.