P-04-Free Flow Electrophoresis (FFE) enables subfractionation of human plasma-derived exosomes (EV) bearing the same antigens.

Simon Staubach[1], Markart Meckel[2], Gerhard Weber[2], Johann Bauer[3], Bernd Giebel[1]
[1]Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany.
[2]FFE Service GmbH, Feldkirchen, Germany; www.ffeservice.com
[3]SiHaTho GmbH, Biedenkopf, Germany.

FFE Service GmbH

Abstract

Exosomes are cell-cell communicators. Purification of the various types of exosomes helps to study this communication. Here we show that FFE is an important tool to support these efforts. FFE was applied to separate macromolecules and extracellular vesicles of the plasma of four human blood donors according to their electric charge densities in a liquid pH gradient into 48 fractions at a preparative scale. Subsequently imaging flow cytometry (IFCM) was performed to analyse the content of the 48 FFE fractions in regard to vesicles bearing CD9 or CD41. The results reproducibly demonstrate that FFE enables subfractionation of EVs bearing the same antigens.

Introduction

FFE is a highly developed technique applicable for preparative separation of different types of molecules and bio-particles with diameters up to 10 µm. (See P01-P03 for basic principles and features.) Proteins and small vesicles are best separated if samples are injected in a chamber fluid consisting of an optimal composition of adjacently flowing media with different pH values.

At the current state of FFE development the electrophoretic migration behavior of exosomes was analysed, in order to see whether all detectable exosomes show equal electrophoretic velocity due to equal negative surface charge density and to learn whether there is any relationship between the expression of the exosome markers CD9 and CD41 and the vesicles' electrophoretic mobility.

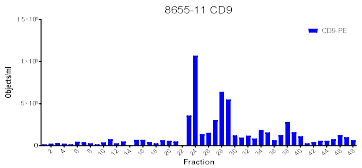
Method

Exosomes were fractionating injecting human plasma into the FFE separation chamber without any pretreatment, which normally causes a tremendous loss of these extracellular vesicles. After separation both, proteins and vesicle, were collected in microtiter plates. There, proteins are quantified measuring light absorption at 280 nm, and autofluorescence at 595 m. In addition, light scattering was determined at 510 nm. Then aliquots are taken from each well for labeling by anti CD9 and anti CD41. Finally, IFCM was performed semiquantitatively at the AMNIS Imaging Stream X II platform.

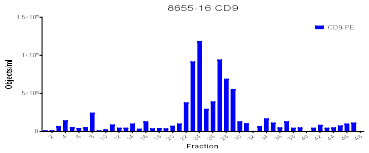
Depending on the purpose of an experiment, samples are injected with a maximal velocity of 1.5 ml/h at one site. Then, 96 different fractions are collected from the chamber edge opposite to the injection edge.

AMNIS-analysis: antibody CD9
48 FFE-fractions

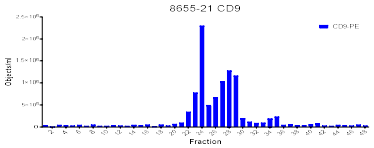
Donor 1



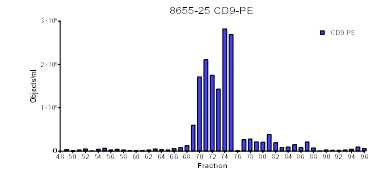
Donor 2



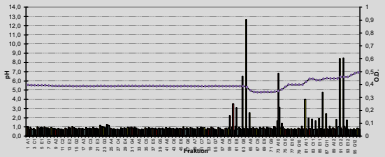
Donor 3



Donor 4

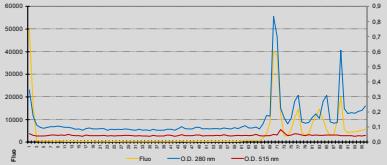


8655p08: FFE-Protocol: 3 pH-steps
pH-profile and Abs at 420, 515 and 595 nm

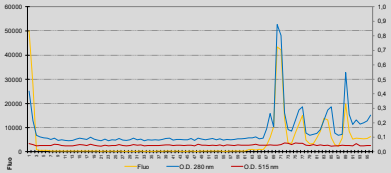


FFE-protocol: 3 pH-steps:
Abs 280 and 595 nm and autofluorescence across all 96 FFE-fractions

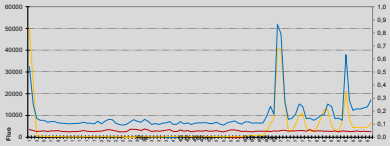
Donor 1: 8655 p09



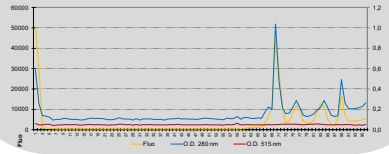
Donor 2: 8655p14



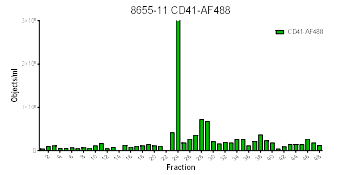
Donor 3: 8655p19

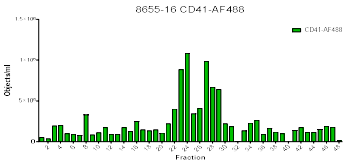


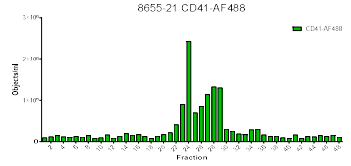
Donor 4: 8655p23

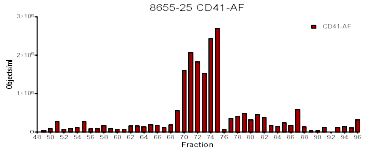


AMNIS-analysis: antobody CD 41
48 FFE-fractons









Method (cont.)

A recent development doubled the throughput, as it is now possible to inject two samples simultaneously at two different sites of one edge and to collect two times 48 fractions at the opposite edge. Here we used the new technology, but injected only once at the right part of the injection edge and collected 48 fractions numbered from 49 to 96. In this way a separation time of 1.5 min is required to obtain enough material for further analysis.

Results

The upper panel of the middle column shows that media with declining pH values are flowing through the right part of the separation chamber. The four panels below indicate that the proteins of the plasmas of four blood donors migrated within the area of pH declination. After separation they were collected in fractions 64 to 96. Spectroscopic analyses of each fraction unveiled the distribution of separated proteins as absorption at 280 nm and autofluorescence at 595 nm of each fraction are measured. The diagrams obtained from the four donors look rather similar representing normal plasma protein distribution and the light scattering shown by the red line is insignificant indicating the lack of larger particles.

The diagrams of the left and the right columns have been obtained by AMNIS measurements of CD9 (left side) and CD41 (right side) antigens found on small particles present in the aliquots taken from fractions 49 to 96. It is evident that exosomes are separated in an FFE. In addition three special features are noteworthy: i) a considerable number of exosomes migrate faster than proteins. Of these exosomes the number of CD41 positive particles is superior. ii) AMNIS analyses of each separation result generated a bimodal distribution curve ranging from fractions 70 to 78. However the relations of the two peaks depend on the blood donor and the antigens determined. iii) in fractions 79 to 96 exosomes with CD9 and CD41 antigens are also found. Their quantities appear to vary depending on blood donor and antigens determined.

Conclusion and outlook

FFE separation of human plasma combined with subsequent AMNIS analysis of each fraction obtained proved that exosomes circulating in the blood have different negative charge densities, which do not only depend on their types of antigens. This makes FFE to an important tool of exosome purification and characterization procedures.

If it is linked to other fractionation techniques, which are applicable to separate exosomes according to their size, density or antigen expression, the FFE's capability to fractionate exosomes according to negative charge density will be a further aid in the search for minorities within the exosomes of various body fluids carrying distinct messages from releasing cells to targets.