P-03-Free-flow electrophoresis provides a new and effective way of isolating extracellular vesicles from plasma and ascites.

Christian Preußer[1], Tobias Tertel[2], Markart Meckel[3], Gerhard Weber[3], Bernd Giebel[2], Elke Pogge von Strandmann[1]

[1] Center for Tumor and Immunbiology (ZTI), Institute for Tumor Immunology, EV Core Facility, Philipps University Marburg, Marburg, Germany

[2] Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany

[3] FFE Service GmbH, Feldkirchen, Germany, www.ffeservice.com

FFE Service GmbH

Abstract

The combination of Free Flow Electrophoresis (FFE) as a separation technique and AMNIS as an analytical technique can display differences in charge density of extracellular vesicles (subfractions), as we have already shown previously.
Here in addition we use Nano Flow Cytometry (NanoFCM) to further characterize EV derived from various bio fluids (plasma, ascites) isolated using FFE.
FFE can offer various separation protocols, differing in the distance of electrophoresis or time of electrophoresis.
The separation protocol with a prolonged distance of electromigration was elected for these experiments.

The actual separation protocol of FFE proves:

- Perfect discrimination of analytes with different values of charge density
- Excellent reproducibility of the pherograms of the extracellular vesicles, separated by FFE
- Concentration of EVs into distinct fractions derived from highly complex bio fluids.
- Convincing separation of particles and most free circulating proteins
- Possibility of differentiation of several EV subpopulation

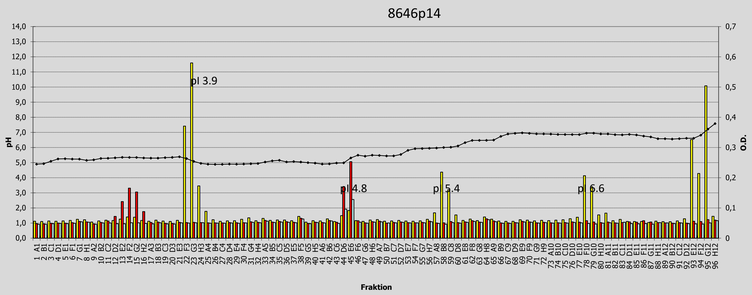
Introduction

The basic features of FFE were described in an earlier presentation (please see here in P01) and visit our homepage: https://www.ffeservice.com/
The FFE-protocol of the continuous process of FF-ZE-pH with elongated distance is using a set of buffer media of different pH-values ranging from pH 4.8 to pH 7. The actual values of sample capacity of the protocol is 1.5 ml/h.
The 96 fractions from the FFE-instrument were collected and the 96 cavities of MTP were analyzed. First a performance test was done with the FFE-instrument (please see the pherogram "pH-profile + pI Marker") before the final experiments with the samples of human plasma and ascites fluid. The quality of the FFE-separation was analyzed by measurement of fluorescence (280/350 nm), UV-280 nm and absorbance at 510 nm. The volumes of FFE-fractions, needed for the final analysis with AMNIS, were collected inside MTPs within 4.5 min.

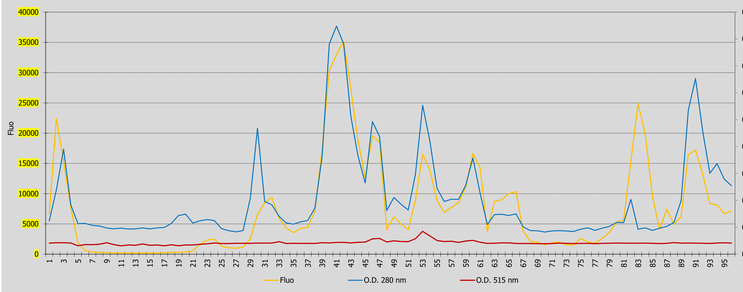
According imaging flow cytometry analyses with EV-specific antibodies on an AMNIS ISXII platform, EVs subtypes were detected with different charge densities across the area of electrophoretic migration. Only some subtypes of EVs were free of proteins, others were still associated with proteins. Upon combining FFE with subsequent ultrafiltration (300 KD-UF-membranes) more than 99 % of the protein can be removed (shown by the upcoming next presentation).

Separation of EVs from plasma and ascites using a 6-step pH FFE protocol

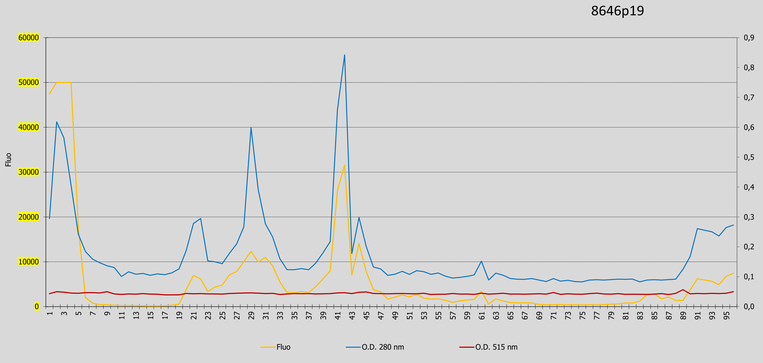
pH-Profile + pI Marker



Human Plasma – FFE Pherogram (Autofluorescence, absorbance at 280 nm and 510 nm

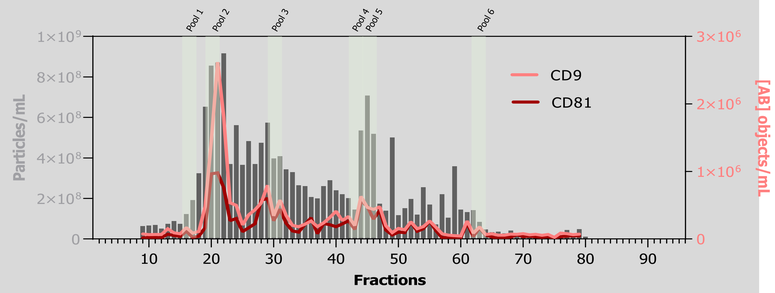


Ascites– FFE Protein pherogram (Autofluorescence, absorbance at 280 nm and 510 nm

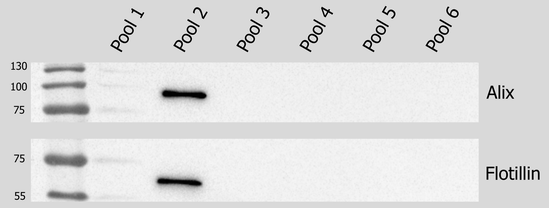


Analysis of separated fractions

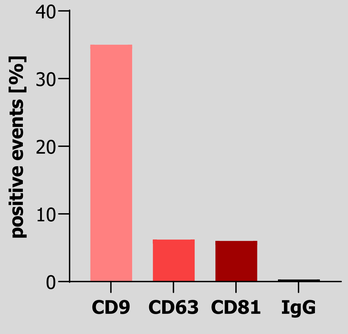
Particle concentrations (NanoFCM) and phenotypic characterization by Amnis ImageStream



Western blot analyses for intravesicular markers



Single-vesicle phenotyping of EVs from Pool 2 by nano-flow cytometry (NanoFCM)



Results

The analysis of the individual fractions isolated by FFE clearly shows that a larger number of extracellular vesicles and their subpopulations with high charge density can be detected when the FFE protocol with extended electrophoretic migration distance and extended electrophoresis time was used.

In addition, FFE has been found to be helpful in separating heterogeneous subpopulations of EVs, based on more detailed analytics using Amnis Image Stream as well as Nano Flow Cytometry (NanoFCM) when the EVs were fluorescently labeled with FITC-conjugated antibodies specific to CD9 and CD81 or PE-conjugated CD63

Also the detection of well-known intravesicular markers as Alix (98 kDa), FLOT-1 (47 kDa) and TSG101 (44 kDa) by western blot analysis further confirmed the specific isolation of extracellular vesicles.

Conclusion/ Outlook

The optimization of the FFE separation protocol, which can lead to a larger number of representable subfractions, was successful.
A new separation protocol was evaluated as well for an improved discrimination of exosomes with high negative charge density in ascites fluid.

Conflict of interest:

G. Weber, is CEO, M. Meckel is employee of FFE Service GmbH, the manufacturer of the FFE instrument