Free flow electrophoresis allows quick preparation of extracellular vesicles from cell culture supernatants and human plasma

Simon Staubach[1], Tobias Tertel[1], Verena Börger[1], Christian Grätz[3], Michael Pfaffl[3], Oliver Drews[4], Gerhard Weber[2], Bernd Giebel[1]

[1] Institute for Transfusion Medicine, University Hospital Essen, Germany [2]FFE Service GmbH, Feldkirchen, Germany (www.ffeservice.com)

[3]Institute of Animal Physiology and Immunology, Technical University of Munich (TUM), Germany [4]Institute for Chemistry, University Hospital Mannheim, Mannheim, Germany

Contact: simon.staubach@uk-essen.de; gerhard.weber@ffeservice.com; bernd.giebel@uk-essen.de

Abstract

Despite increasing interests in extracellular vesicles (EVs), it remains a long procedure to prepare EVs to high purity. Neither fractionation by density nor by size alone is sufficient to separate EVs from most contaminants including lipoproteins. For now, a time-consuming combination of two methods (density and size separation) is required to enrich EVs to high purity at the expense of time. During the recent years, we qualified Free Flow Electrophoresis (FFE) as an efficient method for EV separation. FFE is a well-established (semi-) preparative method to separate analytes with inherent difference in charge density and/or their pI-values into up to 96 different fractions. Upon applying imaging flow cytometry analyses to identify EV containing fractions, we have optimized FFE protocols for the preparation of bona fide EVs from conditioned cell culture media and demonstrated the reproducibility of the method.

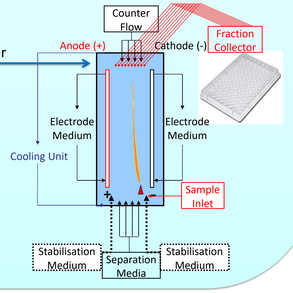
Applying a comparable strategy, we now have improved FFE protocols for the preparation of EVs from human plasma samples. Notably, EVs from plasma show a much higher EV complexity than of cell culture supernatants. Specifically, plasma EVs are recovered in more than three FFE fractions. Several of these fractions also contain relatively high protein contents which could effectively be reduced by subsequent ultra-filtration (UF). The whole procedure takes approximately 40 min per plasma sample and compared to other technologies can be considered as relative quick. Currently, we are characterizing the EVs obtained from different pooled FFE and ultra-filtrated fractions by RNA and proteome analyses.

Principal of FFE Separation

The central unit of the FFE is a separation chamber, which basically consists of a separation plate and a 0.2 mm spaced front plate with a longitudinally arranged anode and cathode. After assembly, buffers with defined pH values are loaded into the vertically arranged media inlets at the bottom edge of the separation chamber. The buffers are continuously transported by a laminar flow along the longitudinal axis of the separation chamber, forming concrete longitudinal buffer lanes. Likewise, the sample to be separated is applied at a specific vertical position at the bottom of the horizontal lane and transported by the laminar flow together with the respective buffer. Typically, buffer application schemes are designed so that the buffer with the lowest pH is closest to the anode and that pH values of the buffers gradually increase towards the cathode. Driven by a vertical electric field and depending on their electric charge, sample components migrate vertically through different buffer zones. The migration speed and distance of each sample component depends on its charge density and/or its isoelectric point (pI). Components, with higher negative charge densities or lower pIs migrate quicker to the anode than those with lower charge densities and higher pIs. Still transported by the horizontal laminar flow, the vertically separated sample components continuously approach the top of the separation chamber, where they are collected into 96 vertically arranged fractions.

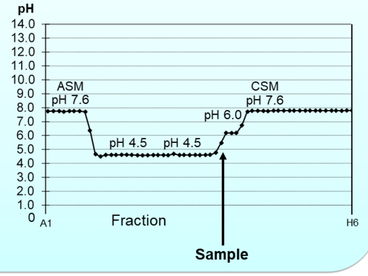
Free Flow Electrophoresis Instrument

Fluids and Media Paths in Free Flow Electrophoresis



Development of a MSC EV separation protocol

Beside an applied current, Free-flow electrophoresis separates analytes, by their isoelectric points at different pH. Here, we developed and optimized the buffer recipes according to the presented pH's. The buffers were applied side by side to the FFE separation chamber. The entire Free Flow Zone Electrophoresis (FF-ZE) protocol was developed for the purification and isolation of MSC-EVs from conditioned media that contained human platelet lysate as a supplement of 10%.



Experimental procedure

Part I

Conditioned MSC media were harvested of six independent cell cultures to obtain samples for six biological replicates. The samples of the conditioned media were separated in three independent runs. Three technical replicates were performed of the whole set of six.

Samples
→ Conditioned MSC medium produced independently on different days
→ 6 biological replicates

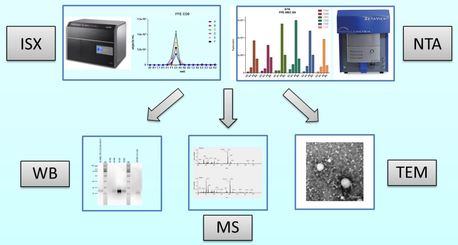
Sample Separations
→ Whole Set, 3 times separated by FFE on 3 subsequent days
→ 3 technical replicates

Part II

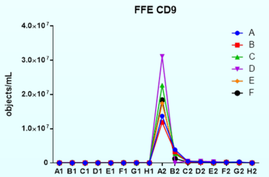
Protocols evaluated by FFE separation of conditioned media were partially transferred and improved to separate EVs derived from human plasma samples. First attempts indicate enrichment of CD9⁺ EVs, but distributed to some fractions. To unravel the heterogeneity of plasma EVs, certain fractions were pooled, washed, and volume reduced by 100 kD Ultrafiltration for further proteomic analysis.

EV analysis according to the MISEV criteria

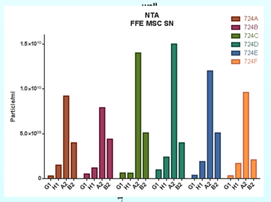
As a proof of principle we aim to get evidence that we indeed purified EVs that fulfill the requirements for the MISEV criteria. Therefor we further analyzed the EV enriched fractions. We chose the four fraction containing most of our vesicles for further analysis by image stream flow cytometry (ISX) Nanoparticle Tracking Analysis (NTA), for Western Blot (WB) and the most vesicle enriched fraction for proteomics (MS) and Transmission Electron Microscopy (TEM).



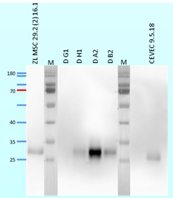
MISEV: Analysis by ISX, NTA, WB



According to the FCM Amnis image stream (ISX) analysis, CD9 positive EVs are reproducibly enriched in fraction A2

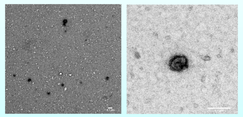


According to NTA most recordable particles are reproducibly enriched in fraction A2



WB analysis recovered the EV specific antigen CD9 abundantly in fraction A2

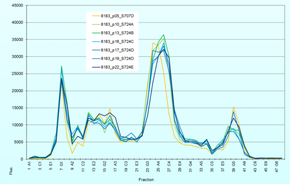
MISEV : Analysis by TEM



Transmission Electron microscopy (TEM) reveals typical double layer membrane structures of vesicles (uranylacetate staining)with characteristic size of EVs that exhibit CD9 positive marker represented by immunogold staining

Pherograms prove FFE high reproducibility

Pherograms reflect the protein content of the separated fractions, measured at 280 nm by an Elisa Plate reader. Every sample has its own characteristic protein fingerprint represented by the corresponding pherogram



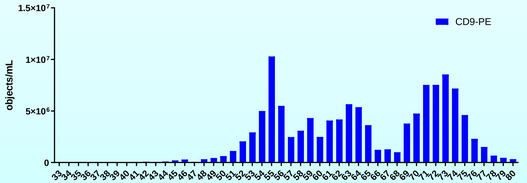
Seven different supernatants were separated by FFE and correspondent pherograms presented in an overlay. The similarity demonstrates the reproducibility of the method.

Proteomic Analysis

The one most vesicle enriched fraction of a selected FFE separation was analyzed by Liquid chromatography–mass spectrometry (LC-MS-MS) to identify common EV protein marker:
523 proteins were identified by more than one peptide.
Amongst others we selected 5 CD marker proteins identified, as representatives for genuine sEV marker: CD166; CD226, CD44; CD5 and CD9.

Plasma EVs

Plasma is high enriched in vesicles. Amongst other one most abundant EV marker is CD9. Our aim is to investigate whether different maxima belong to different entities of EVs. Three to four fractions adjacent to the maxima will be pooled and analyzed by proteomics in a future goal.



Conclusion

- FFE is a reproducible technique to isolate EVs from protein rich sources, the purity of EVs reaches a high grade
- The process is quick in comparison to other methods
- Contaminants like serum albumin are still recovered in reduced quantities by proteomic profiling
- The FFE separation depends on the quality of the sample to be processed: serum free samples result in higher EV purities than serum or hPL containing samples (data not shown)
- Evaluated protocols can be transferred by minor modifications from supernatant to other samples like plasma

Outlook

- FFE is a method that can be adapted to given requirements; conditions and buffers for example can be optimized to increase yields and/or purities
- Our next goals is to use FFE to separate different EV subtypes from plasma derived samples
- Proteomic analysis each other finally becoming able to comprehensively study the heterogeneity of EVs in given preparations.

Conflict of interest:
G. Weber, is CEO of FFE Service GmbH, the manufacturer of the FFE instrument