Supporting Information

Isolation of Extracellular Vesicles from Small Volumes of Plasma by Microfluidic Aqueous Two-Phase System

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S1. Calculation of optimal length of microfluidic channel

We analyzed the partitioning behavior of proteins from bulk ATPS. As indicated by Kim et al., [1] the recovery efficiency of proteins in DEX phase was measured to be 30%, 9%, and 3% with an increase in the batch number from 1 to 3. The value of 30% was almost similar to the volume ratio of DEX phase as compared to that of PEG phase. Thus, the factors interfacial tension and affinity played no dominant role in the partitioning of proteins. With this hypothesis, we calculated optimal channel length for protein removal in one-phase PEG/PEG/PEG model. Using the Stoke-Einstein equation, the diffusion coefficient of particles was described as

$$D = \frac{k_b T}{6\pi\mu R_h} \quad (1)$$

Where D is diffusion coefficient, k_b is Boltzmann's constant, T is temperature, μ is viscosity of fluid, and R_h is hydrodynamic radius of particle. Under our experimental condition, the viscosity of PEG phase was found to be 4.0×10^{-3} kg·m^{-1}·s−1.[2] We set the hydrodynamic radius of EV and BSA to 50 and 3.5 nm,[3-5] (Fig. S1 and S2) respectively, to calculate the diffusion coefficient of particle at the PEG phase as follows:

$$D_{EV_{PEG}} = 1.09 \times 10^{-12}\, m^2.s^{-1} \quad (2)$$

$$D_{BSA_{PEG}} = 1.56 \times 10^{-11}\, m^2.s^{-1} \quad (3)$$

The maximum diffusion length between side of the channel and the middle phase at a determined flow rate was about 59.0 μm, which is necessary to be an equilibrium state of

diffusion. The diffusion time for the equilibrium state follows the one-dimensional diffusion equation.

$$x_{rms} = \sqrt{2Dt} \quad (4)$$

$$t_{EV} = 306.86\ s \quad (5)$$

$$t_{BSA} = 111.53\ s \quad (6)$$

Finally, the channel length could be calculated from the flow rate using simple calculation.

$$L_{EV} = 91.14\ cm \quad (7)$$

$$L_{BSA} = 33.12\ cm \quad (8)$$

With the simple theoretical calculation, the microfluidic ATPS device was designed with a channel length of 36.7 cm.

S2. Determination of polymer concentration

First, we tried to minimize the amount of polymer because it could interfere the downstream analysis such as mass spectroscopy.[6] In order to form two phases at a DEX concentration less than 1.5 wt %, high concentration of PEG was needed according to binodal curve of PEG (average MW 35 kDa) and DEX (500 Kda)[7] which can further increase the amount of polymer collected in middle outlet. Also, we did not consider the concentration of DEX higher than 1.5 wt %, could directly affect the problem we mentioned. As a result, we determined that 1.5 wt % of DEX was the proper concentration in our system.

Afterwards, we investigated the aggregation behavior of EVs in microfluidic ATPS with three different PEG concentrations. For the prepared EV-protein mixture, we could observe two main peaks at about 10 and 100 nm (Fig. S1-a). After isolation using 1.5 wt% PEG, the aggregated particles were not detected, but several 100 nm particles assumed to be exosomes remained at the middle phase. In fact, the aqueous two-phase system was not maintained from the inlet to

the outlet under this condition, wherein the concentration was below the minimum value required to allow formation of two phases. In the case of 5.5 wt%, the intensity fraction of 10 nm particles significantly increased, and the peak at 100 nm disappeared and was substituted by a larger peak. Thus, the total number of particles significantly decreased with the aggregation of 100 nm particles, but not 10 nm particles. The population of 10 nm particles greatly increased.

The condition with 5.5 wt% PEG was deemed as the best option based on the size distribution graph; however, the microfluidic clogged during the long-term isolation process; hence, the concentration of 3.5 wt% was chosen with our device.

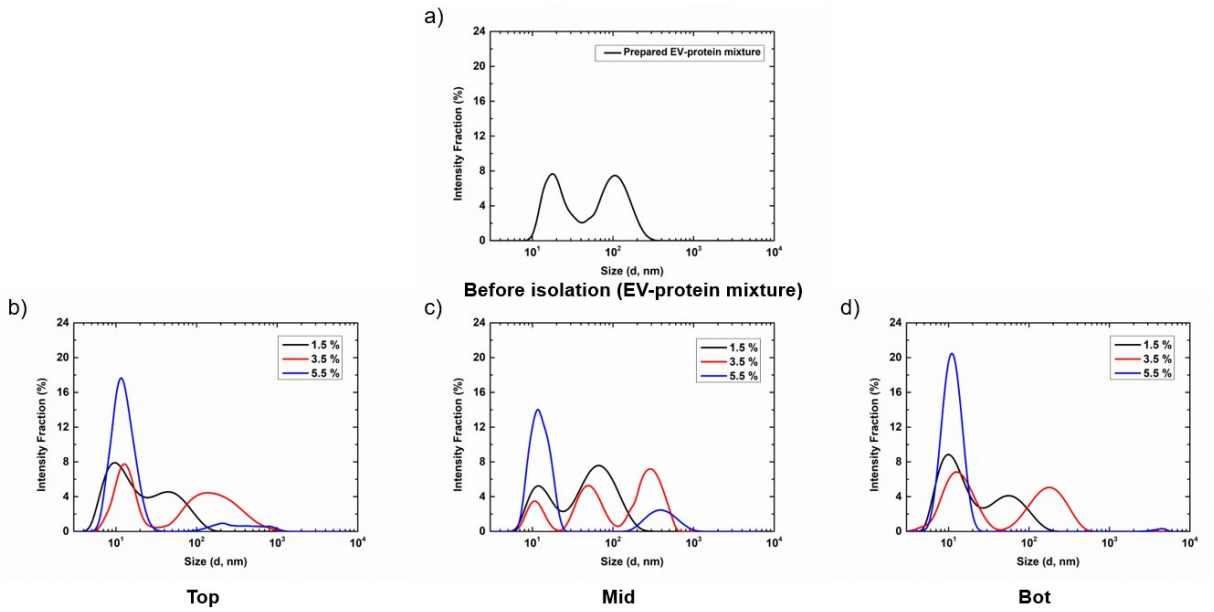

Figure S1. Size distribution of a) EV-protein mixture, collected sample from the b) top, c) middle, d) bottom outlet with three PEG concentrations and 1.5 wt% DEX. All collected samples were analyzed by DLS.

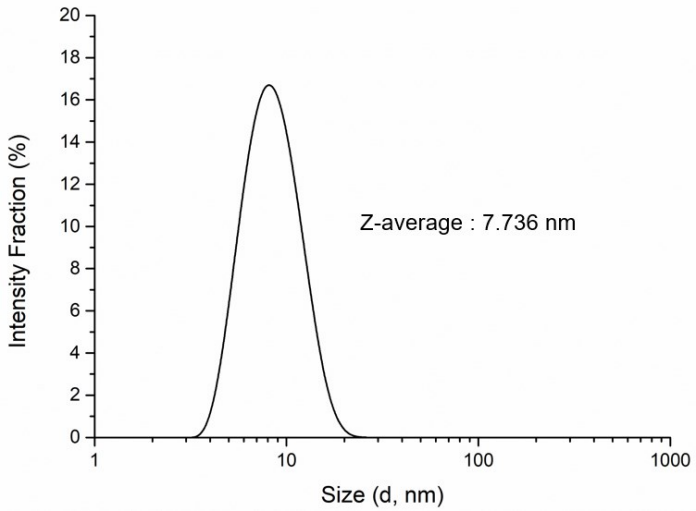

Figure S2. Size distribution of BSA used in our experiment. Hydrodynamic radius was measured about 3.868 nm by DLS which is similar to our assumption 3.5 nm.

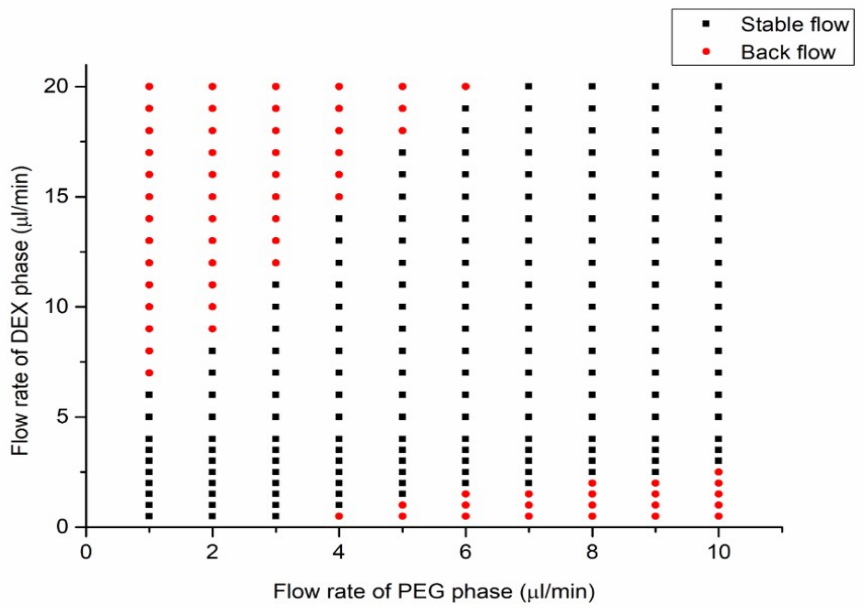

Figure S3. Flow stability plot of the aqueous two-phase system formation in the microfluidic device at different flow rates of PEG and DEX.

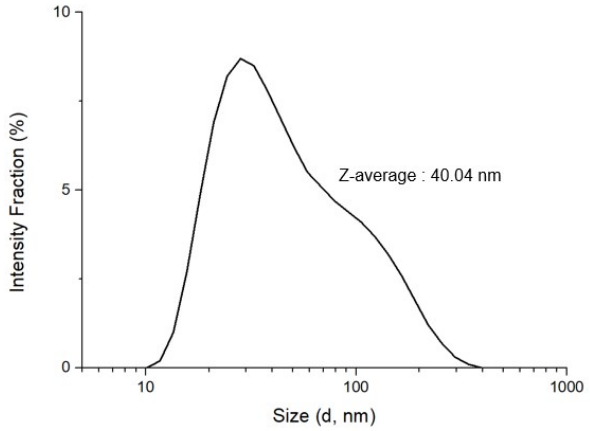

Figure S4. Size distribution of PKH 67-labeled extracellular vesicles measured by DLS. The range of particle size was consistent with the size of exosomes.

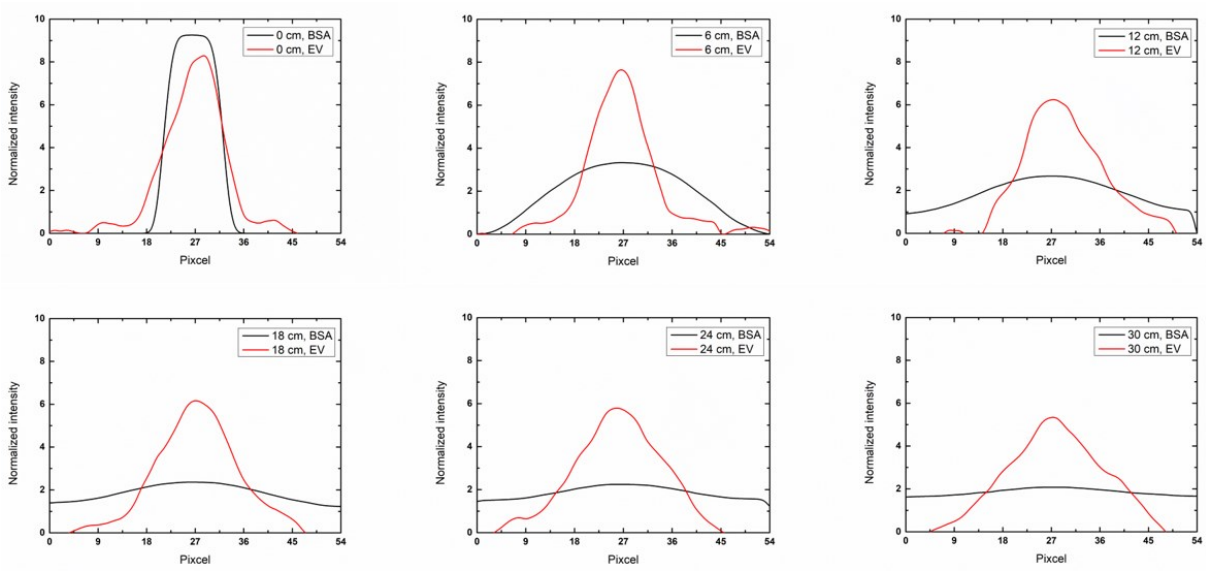

Figure S5. Normalized intensity plot of fluorescently labelled EV and BSA described in Fig. 3. BSA seems almost fully diffused out, while almost all of EVs remained at the middle phase. (Collection outlet, 18-36 pixcel, Area under the curve, ~78.8% at 30 cm).

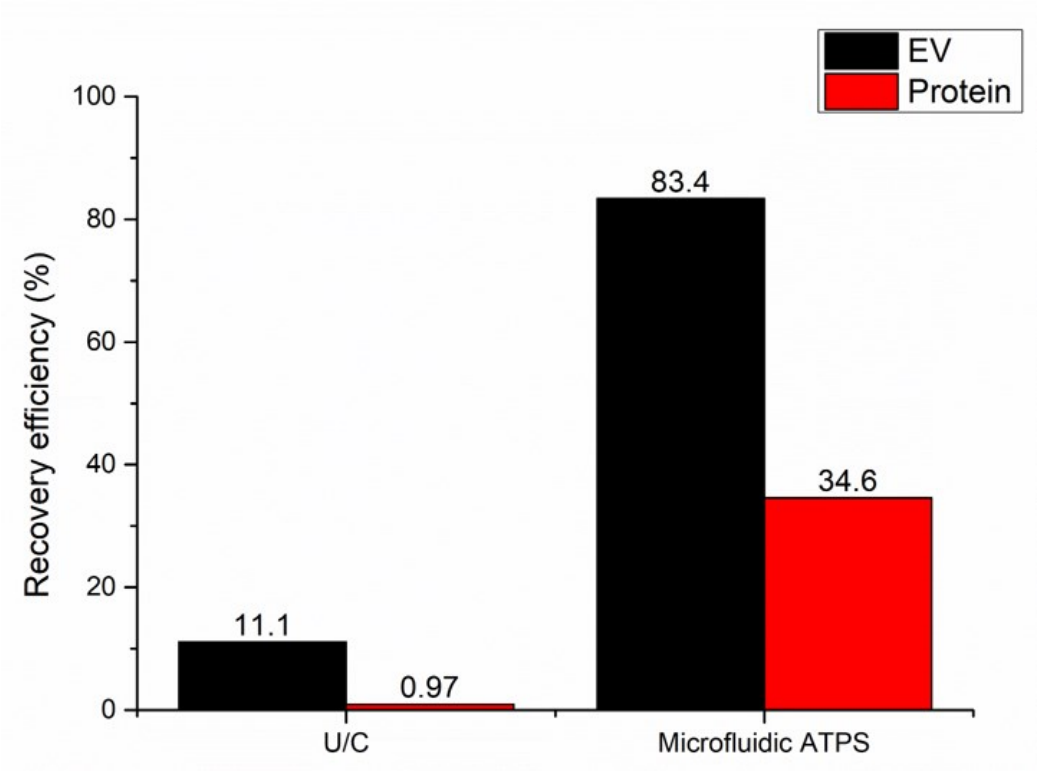

Figure S6. Recovery efficiency of EV and protein from U/C and microfluidic ATPS. Data obtained from EV-protein mixture measured by NTA and Bradford assay.

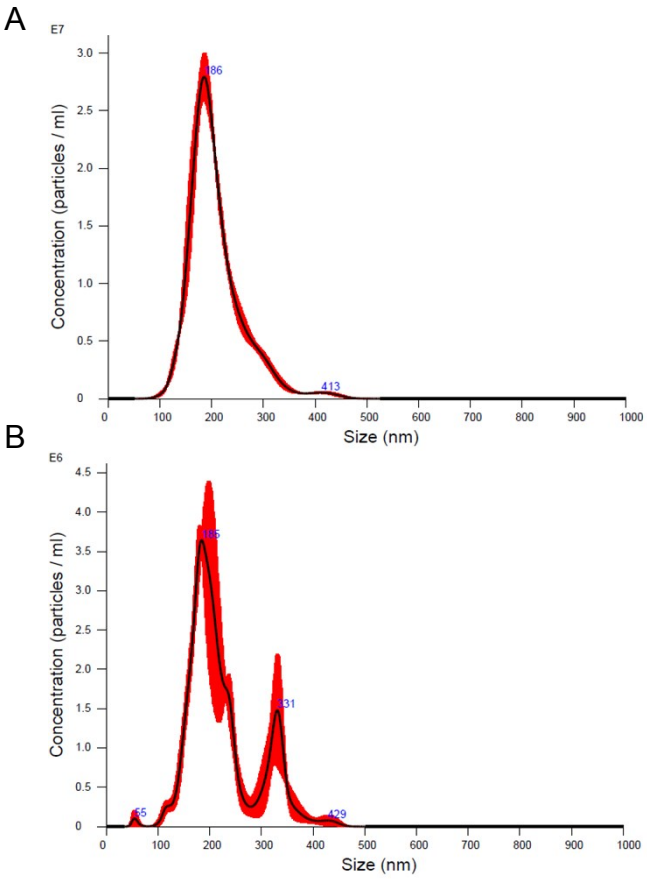

Figure S7. Size distribution of A) EV-protein mixture, EV isolated by B) U/C measured by NTA. Multiplying the measured concentration to dilution factor, calculated concentration of each sample A) $2.47 \times 10^{11} \pm 9.40 \times 10^{9}\ particles/mL$ and B) $1.38 \times 10^{11} \pm 8.07 \times 10^{9}\ particles/mL$. Multiplying the volume of sample, the amount of EV is calculated A) $1.24 \times 10^{11} \pm 4.70 \times 10^{9}\ particles$ and B) $1.38 \times 10^{10} \pm 8.07 \times 10^{8}\ particles$.

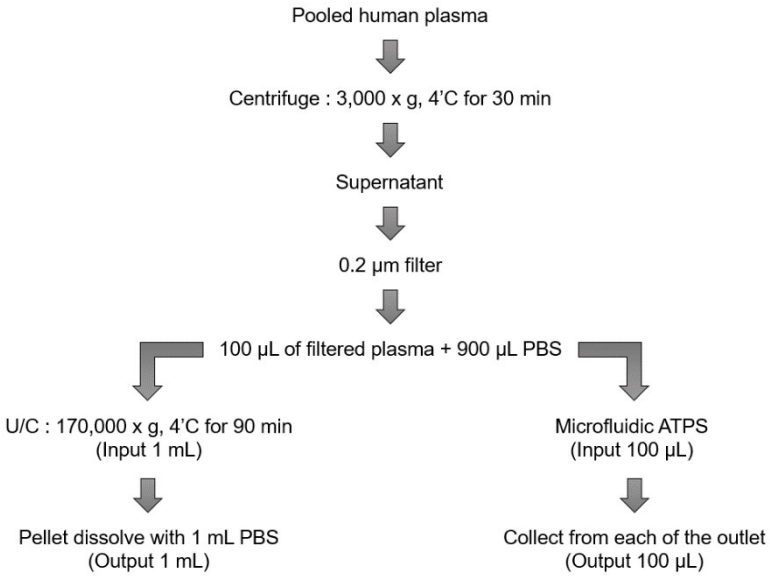

Figure S8. Detailed experimental procedure preparing the sample for western blotting. 10 μL of collected sample was loaded for western blot analysis.

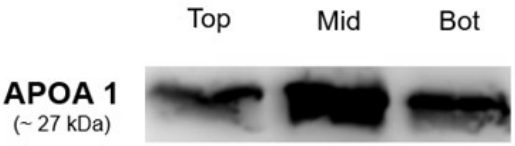

Figure S9. Result of western blot with APOA 1. Large amount of lipoproteins seems remained in the middle outlet.

Batch #	0	1	3	5
Recovery efficiency of EV or protein				
EV	100	77.22	76.71	73.89
Protein	100	29.43	3.57	2.39
Time required for isolation				
Bulk ATPS	0	60	180	300
Microfluidic ATPS	0	30	34	38

Table S1. Expected A) recovery of EV, protein and B) time required for isolation according to repeated separation process with fresh PEG solution. Data from A) : reconstructed from reference[1].

Reference

[1]. J. Kim, H. Shin, J. Kim, J. Kim and J. Park, *PloS one*, 2015, **10**, e0129760.
[2]. S. J. Lee, J. Y. Kang, W. Choi and R. Kwak, *Small*, 2017, **13**, 1601725.
[3]. G. Yohannes, S. K. Wiedmer, M. Elomaa, M. Jussila, V. Aseyev and M.-L. Riekkola, *Analytica chimica acta*, 2010, **675**, 191-198.
[4]. D. Yang, W. Zhang, H. Zhang, F. Zhang, L. Chen, L. Ma, L. M. Larcher, S. Chen, N. Liu and Q. Zhao, *Theranostics*, 2020, **10**, 3684.
[5]. K. O. Jung, H. Jo, J. H. Yu, S. S. Gambhir and G. Pratx, *Biomaterials*, 2018, **177**, 139-148.
[6]. B. O. Keller, J. Sui, A. B. Young and R. M. Whittal, *Analytica chimica acta*, 2008, **627**, 71-81.
[7]. H. Shin, C. Han, J. M. Labuz, J. Kim, J. Kim, S. Cho, Y. S. Gho, S. Takayama and J. Park, *Scientific reports*, 2015, **5**, 13103.