A novel hydrogel-based method for efficient extracellular vesicles isolation from various biological samples

○Ryota Kajihara, Riho Tanigaki , Kenji Yoneoka, Kyohei Tobinaga, Ryosuke Suzuki (Sanyo Chemical Industries, Ltd.)

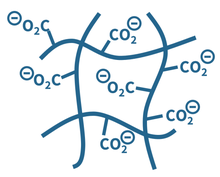
Introduction

Extracellular vesicles (EVs), which are secreted by nearly all living cells and play crucial roles in various physiological processes, have attracted increasing attention from researchers. Numerous protocols for EV isolation such as ultracentrifugation and polymer precipitation have been developed, yet conventional methods each possess distinct strengths and limitations.

Here, we present a novel EV isolation technique, termed **Hydrogel Adsorption Separation (HAS)**. This method selectively isolates EV particles with high purity through adsorption onto a hydrogel surface. EVs obtained from various biofluids using the HAS method have been successfully applied to multiple analytical techniques, including proteomics and flow cytometry, demonstrating its potential for both basic and clinical EV research.

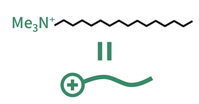
Method Material Design for EV Isolation Using Hydrogel Adsorption Separation (HAS)

Hydrogel: Cross-linked Polyacrylate



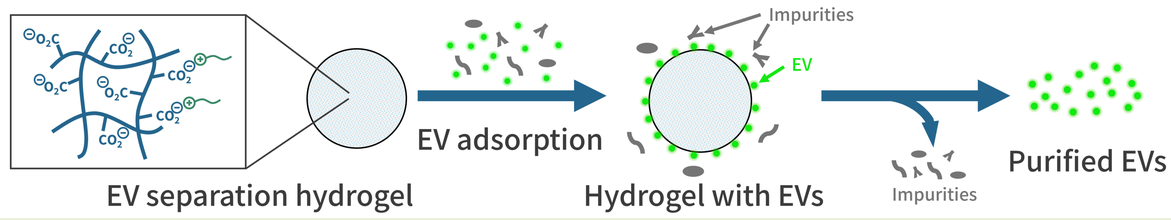
- Negative Surface Potential: Minimizes non-specific binding of impurities (e.g. albumin).
- High and Rapid Water Absorbance: Facilitates rapid sample concentration and separation.
- High Biocompatibility: Minimal interference with biomacromolecules.

EV Adsorption: Lipid bilayer anchoring by palmitoyl group



- EV membrane incorporation via long alkyl chain
- Reversible interactions with the hydrogel

HAS Method Protocol



Results

Adsorption and Elution of EV Model Substrate by HAS

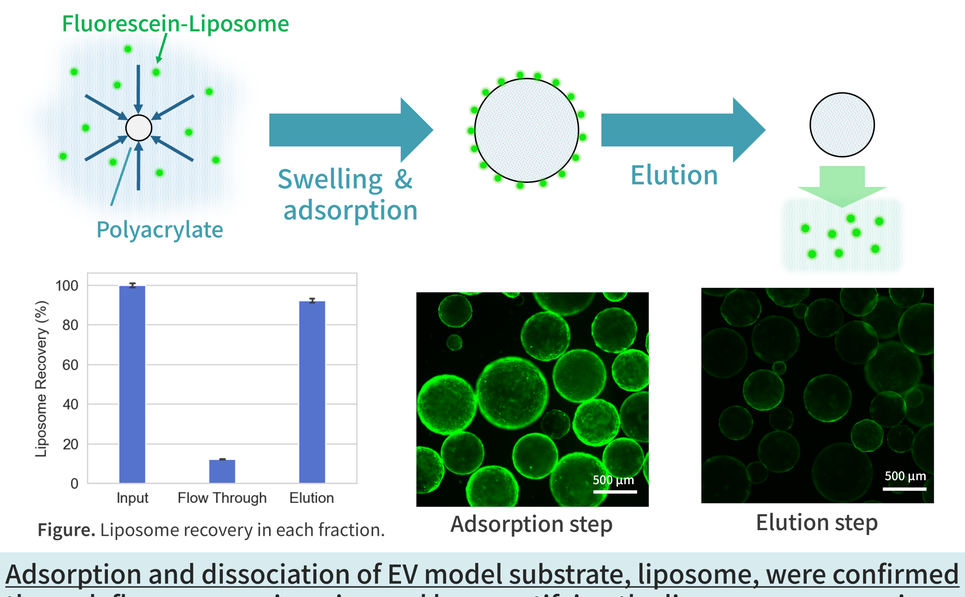


Figure. Liposome recovery in each fraction.

Adsorption and dissociation of EV model substrate, liposome, were confirmed through fluorescence imaging and by quantifying the liposome recovery in each solution during the adsorption and elution processes.

EV isolation by HAS

Conditions
Sample volume: 1mL (urine, conditioned medium)
0.1mL (human serum, plasma)
Isolation Method: EXORPTION™
NTA: NanoSight NS300 (Malvern)

<Nanoparticle tracking analysis (NTA)>

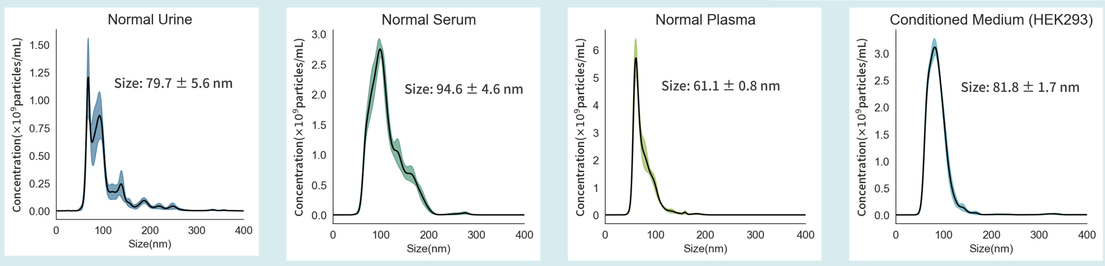


Figure. Size distributions of recovered EV solution from various biofluids.

Small EVs (sEVs, <200 nm) exhibiting sharp size distributions were observed.

<Immunoassay of EV markers>

ELISA

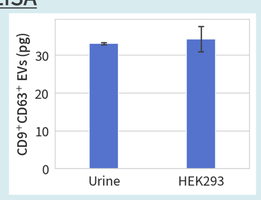


Figure. Quantification of $CD9^+CD63^+$ EVs from EV Solutions.

Western Blot

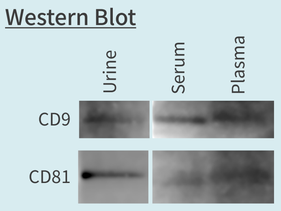


Figure. Western blot analysis of EV solutions.

Conditions
ELISA: CD9/CD63 Exosome ELISA kit (Cosmo Bio)
Western blot: anti-CD9 (clone: 12A12, Cosmo Bio)
anti-CD81 (clone: 12C4, Cosmo Bio)

EV markers (CD9, CD63, CD81) were detected in each EV solution.

<RNA isolation & detection>

Total RNA

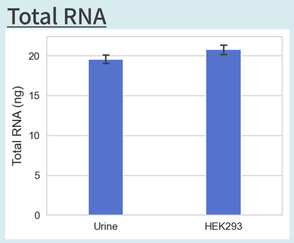


Figure. Total RNA yield from 200 µL of EV solutions (Urine EVs, HEK293 cell EVs).

RT-qPCR

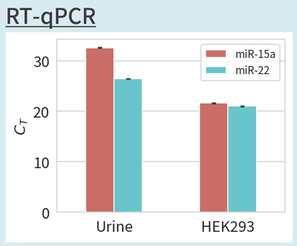


Figure. RT-qPCR of miRNAs isolated from urine EVs and HEK293 cell EVs.

Conditions
RNA Isolation: Exosomal RNA Isolation kit (Norgen)
Quantification: QuantiFluor RNA System (Promega)
RT-PCR: TaqMan assay (Thermofisher Scientific)
Detection: QuantStudio 5 (Thermofisher Scientific)

EV-specific miRNAs were detected in EV solutions using RT-qPCR.

EV Isolation using HAS and other methods

EV isolation was performed using hydrogel adsorption separation (HAS), ultracentrifugation (UC), polymer precipitation (PP), and Tim4-affinity (Tim4) to evaluate their distinct features.

EV yield and purity

EV isolation from urine samples of chronic kidney disease (CKD) patients was conducted to compare recovery yield and purity.

<SDS-PAGE, Western blot>

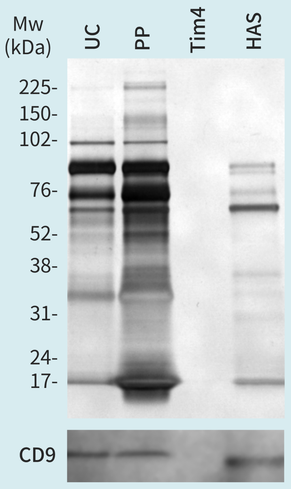


Figure. SDS–PAGE with subsequent silver staining (upper) and Western blot analysis (lower) of EV solutions.
Sample: Urine from a CKD patient (CKD1)

<ELISA>

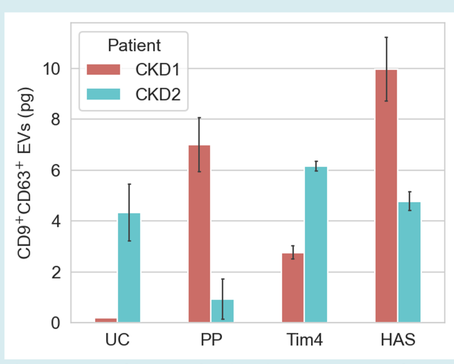


Figure. Quantification of isolated $CD9^+CD63^+$ EVs from EV solutions using four different isolation.
Sample: Urine from CKD patients

<Purity>

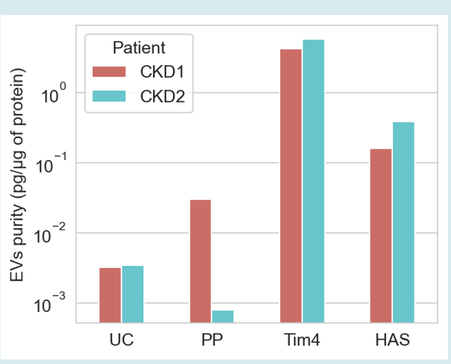


Figure. EV purities of EV solutions isolated using four different methods .Purity was calculated using the equation below.
Purity = ($CD9^+CD63^+$ EV [pg])/(Total Protein [µg])
Sample: Urine from CKD patients

Sample: Provided by Tokushima Univ.

HAS method showed high EV recovery yield and purity.

Proteomics

Shotgun LC-MS/MS-based proteomic analyses were performed on human serum EV solutions isolated by UC, Tim4, and HAS.

Data: Provided by APRO Science Group / Pharma Foods International Co., Ltd.

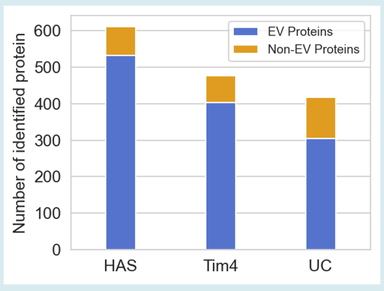


Figure. Numbers of identified proteins in EV solutions with each isolation method (blue: EV proteins, orange: Non-EV proteins)

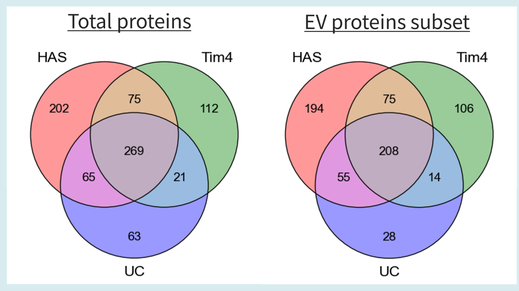


Figure. Venn diagrams showing the complete set of detected proteins (left) and the EV protein subset (right).

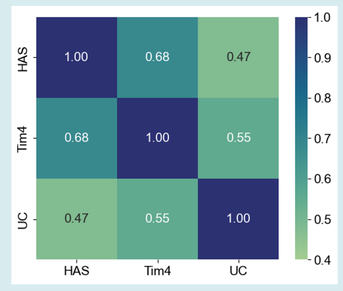


Figure. Correlation heatmap of identified proteins across EV solutions.

Serum EVs isolated using the HAS method yielded the highest number of protein identifications.

Flow Cytometry

EVs from mouse serum, isolated by UC and HAS, were analyzed by flow cytometry using EV-specific markers (CD9, CD81), and nucleic acids in EV (SYBR Gold).

Data: Provided by Nippon Becton Dickinson Company, Ltd.

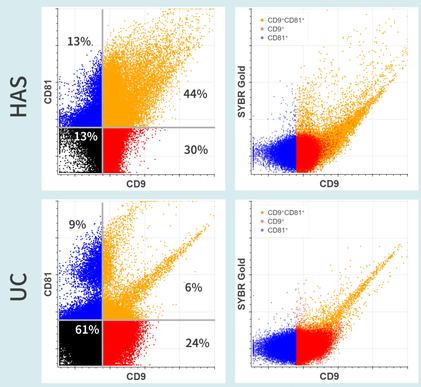


Figure. Flow cytometric analysis of purified mouse serum EVs isolated by HAS (upper panels) and UC (lower panels).

	$CD9^+CD81^+$	$CD9^+CD81^-$	$CD9^-CD81^+$	$CD9^-CD81^-$
HAS	43.6%	30.4%	13.5%	12.5%
UC	5.7%	23.9%	8.8%	61.6%

The $CD9^+CD81^+$ EV subpopulation exhibited the SYBR Gold signal, indicating the presence of nucleic acids within the EVs (HAS, UC).

Serum EVs isolated using the HAS method exhibited higher tetraspanin-positive EVs ratio than those isolated by UC.

Discussion

EV isolation using HAS was compared with UC and other commercially available isolation kits (PP, Tim4).

	UC	PP	Tim4	HAS
Recovery yield	+	++	++	+++
Purity	+	+	+++	++
Total around time	3 h	2.5 h	2 h	1 h
Immunoassay	+	+	+	+
Nucleic acids	NM	NM	NM	+
Proteomics	+	NM	++	++
Flow cytometry	+	NM	NM	+

NM: Not-measured

Other Benefit for HAS method

- Simple protocol for EV isolation
  Purification completes in 3 step procedures.
- Non-specific molecular target for isolation
  Isolation of plant EV and bacterial EV is possible.

Conclusion

- We developed a novel EV isolation system that employs a hydrogel as an EV adsorption material.
- Purified EV solutions obtained via the HAS method exhibit a sharp size distribution ranging from 50 to 200 nm (sEV).
- The HAS method achieves high EV yield and purity, and the resulting EV solutions are suitable for downstream applications such as proteomics, transcriptomics, and single-vesicle analysis by flow cytometry.

EXORPTION™

The EV isolation kit, “EXORPTION™", which utilizes the HAS method, is currently available in Japan and will be launched in the U.S. starting in 2025.