Microfluidic-based capture and release of cancer-derived exosomes via peptide-nanowire hybrid interface

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EXPERIMENTAL

Preparation of peptide array

A library of eight-mer peptides was derived from the amino acid sequence of human EWI-2 (accession number: Q8R366) by two residue-frame shifted epitope mapping (Fig. S1). By using peptide auto-spotter (MultiPep RSi; Intavis, Cologne, Germany), spot peptide array was synthesized on a cellulose membrane (Grade 542; Whatman, Maidstone, UK), activated with Fmoc-β-alanine (Watanabe Chemical, Hiroshima, Japan) as the N-terminus. Each amino acid elongation was started by deprotecting Fmoc group at the N-terminus with 20% piperidine in N,N-dimethylformamide (DMF) before washing the membrane with DMF and ethanol, respectively. The carboxyl group of the Fmoc-amino acid (0.5 M) was activated by a mixture of 1.1 M hydroxybenzotriazole and 1.1 M diisopropylcarbodiimide before coupling to the N-terminus. Unreacted amino groups were later capped by 4% acetic anhydride in DMF before washing the membrane with DMF and ethanol, respectively. After the final elongation, the synthesized peptide array was stored in a dry place at 4°C. Synthesis was conducted according to the manufacturer’s instructions with some modifications. The scale was 100 nmol/cm² and the peptide spot was 4 mm in diameter. Prior to use, Fmoc and side chain-protecting groups were removed with 20% piperidine in DMF and a mixture of ultrapure water, triisopropylsilane (TIPS), and trifluoroacetic acid (TFA) (2:3:95, v/v/v), respectively. The membrane was finally washed with dichloromethane (DCM), DMF, ethanol, and PBS (pH 7.4), respectively.
**Preparation of peptide powder**

Peptide was synthesized in solid or powder form on copolymer-grafted resin (TentaGel resin; Intavis) with peptide multicolumn synthesizer (ResPep SLi; Intavis) using 0.5 M O-(1H-benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate as an activator and 0.5 M N,N-diisopropylethylamine (DIEA) as a base (Watanabe Chemical). In each amino acid elongation, Fmoc group at the N-terminus was deprotected by 20% piperidine in DMF before washing the resin with DMF. Then, the carboxyl group of Fmoc-amino acid (0.5 M) was activated and coupled to the N-terminus. Acetic anhydride (5%) in DMF was applied to cap the unreacted amino groups, and the resin was washed with DMF. Synthesis was conducted at 5 μmol/column according to the manufacturer’s instructions with some modifications. After the final elongation, the Fmoc group was deprotected by 20% piperidine in DMF before washing the resin with DMF and DCM, respectively. Side chain-protecting groups were deprotected and the peptide was cleaved from the resin using a mixture of ultrapure water, TIPS, and TFA (2.5:5:92.5, v/v/v). Next, the peptide was precipitated and washed by chilled diethyl ether. After centrifugation at 0°C and 5,000 rpm for 10 min, the peptide pellet was dissolved in 30% acetonitrile and freeze-dried. The peptide powder was purified and confirmed by high-performance liquid chromatography (HPLC) and mass spectrometry (Shimadzu) to ensure >90% purity (Fig. S7 and S8). The peptide powder was stored in a dry place at 4°C until use. To obtain the FITC-conjugated peptide, FITC 'Isomer I' (Thermo Fisher Scientific) was incubated with peptide powder in sodium bicarbonate buffer (pH 8.7) (2:1 mole ratio, FITC:peptide) for overnight at room temperature before purifying with HPLC.
Preparation of cancer-derived exosomes

Cancer-derived exosomes were prepared from MDA-MB-231 human breast cancer cell line (HTB-26TM; American Type Culture Collection, Manassas, VA, USA). MDA-MB-231 cells (2×10^6 cells) were cultured in a T-75 flask using 20 mL Dulbecco's modified Eagle medium supplemented with 10% exo-depleted fetal bovine serum, 1 mM sodium pyruvate, and 100 units/mL of penicillin-streptomycin (Thermo Fisher Scientific) under 37°C, 5% CO₂, and a humidified atmosphere. After 48 h, the cultured medium was collected and filtered through a 0.2-μm syringe filter (Merck Millipore, Billerica, MA, USA). Exosomes were isolated by ultracentrifugation at 4°C and 110,000 × g for 80 min (Optima XE-90 ultracentrifuge, Type 70 Ti rotor; Beckman Coulter, Indianapolis, IN, USA). The exosome pellet was washed with 0.2-μm-filtered PBS before ultracentrifugation and redispersion with 0.2-μm-filtered PBS. The suspension of purified exosomes was characterized by NTA system (Malvern) to determine average exosome size and particle number. The exosome suspension was stored in a low-protein binding tube at -80°C until use.
Evaluation of peptide bifunctionality

ZnO microparticles (1–2 µm in diameter; Kasei Optonix, Ltd., Kanagawa, Japan) and FITC-conjugated bifunctional peptide were mixed to obtain 400 µg/mL of particles and 100 µM of peptide in PBS, and incubated with strong agitation for 1 h. The mixture was centrifuged at $18,000 \times g$ for 10 min to remove excess peptide in the supernatant. The pellet was rinsed with PBS three times and redispersed in PBS. The particles modified with FITC-conjugated peptide were mixed with MDA-MB-231-derived exosomes to obtain 400 µg/mL of modified particles and $1 \times 10^9$ particles/mL of exosomes before incubation with strong agitation for 1 h. The mixture was centrifuged at $18,000 \times g$ for 10 min to remove excess exosomes in the supernatant. The pellet was rinsed with PBS three times and redispersed in PBS. The exosomes captured on the particles were stained by CellMask Orange Plasma Membrane Stain (Thermo Fisher Scientific) at 1× concentration with strong agitation for 10 min. Excess CellMask Orange dye in the supernatant was removed after centrifugation at $18,000 \times g$ for 10 min. The pellet was rinsed with PBS three times and redispersed in PBS. The particles were observed under fluorescence microscope (Leica Camera AG). All steps were performed at room temperature or 25°C.
**FIGURES AND TABLE**

![Image of peptide array design from the amino acid sequence of EWI-2 protein](image)

**Fig. S1** Schematic illustration of the peptide array design from the amino acid sequence of EWI-2 protein.
Fig. S2 Fluorescence intensity profile of each peptide spot after the binding assay with fluorescently labeled MDA-MB-231-derived exosomes, determined by image analysis software (ImageQuant).
**Table S1** A list of the candidate peptides.

<table>
<thead>
<tr>
<th>Peptide&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sequence</th>
<th>pI&lt;sup&gt;b&lt;/sup&gt;</th>
<th>GRAVY&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Charge&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>P238</td>
<td>RSHRLRLH</td>
<td>12.4</td>
<td>-1.6</td>
<td>+3.2</td>
</tr>
<tr>
<td>P169</td>
<td>RTYRLRLE</td>
<td>10.9</td>
<td>-1.4</td>
<td>+2.0</td>
</tr>
<tr>
<td>P178</td>
<td>RCLAKAYV</td>
<td>9.5</td>
<td>0.6</td>
<td>+1.9</td>
</tr>
<tr>
<td>P237</td>
<td>GPRSHRLR</td>
<td>12.4</td>
<td>-2.0</td>
<td>+3.1</td>
</tr>
<tr>
<td>P177</td>
<td>TYRCLAKA</td>
<td>9.5</td>
<td>-0.1</td>
<td>+1.9</td>
</tr>
<tr>
<td>P239</td>
<td>HRLRLHSL</td>
<td>12.1</td>
<td>-0.6</td>
<td>+2.2</td>
</tr>
<tr>
<td>P182</td>
<td>RGSGLTRLR</td>
<td>12.4</td>
<td>-1.5</td>
<td>+3.0</td>
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<tr>
<td>P168</td>
<td>ASRTYRLR</td>
<td>11.8</td>
<td>-1.3</td>
<td>+3.0</td>
</tr>
<tr>
<td>P170</td>
<td>YRLRLLEAA</td>
<td>9.6</td>
<td>-0.3</td>
<td>+1</td>
</tr>
</tbody>
</table>

<sup>a</sup>Peptides with spot fluorescence intensity above two standard deviations from the overall mean.

<sup>b</sup>Isoelectric point (pI), <sup>c</sup>Grand average of hydropathy (GRAVY), and <sup>d</sup>Charge were obtained from ProtParam tool in ExPASy (http://web.expasy.org/protparam/).
Fig. S3 Display of the first rank binding site of P238 on CD9 protein surface ($p=0.011$), predicted by PepSite software (http://pepsite2.russelllab.org/).
**Fig. S4** Evaluation of the peptide bifunctionality on ZnO microparticles using (a) the bifunctional peptide (HCVAHRGGGRSHRLRLH) and (b) the control peptide (HCVAHRGGGAAAA). Left top is bright-field image. Left bottom is fluorescence image for FITC-conjugated peptide (green). Right top is fluorescence image for CellMask-stained exosomes (red). Right bottom is overlay image. Scale bar, 20 μm.
Fig. S5 Fabrication of a microfluidic platform containing ZnO nanowires. (a) Photoresist coating. (b) Patterning (c) ZnO layer sputtering. (d) Hydrothermal growth of nanowires. (e) Photoresist removal. (f) PDMS covering.
Fig. S6 One-pot bifunctional peptide modification and elution of ZnO nanowires. (a) FESEM image of the grown ZnO nanowires. (b) FESEM image of the peptide-modified nanowires. (c) Fluorescence intensity of FITC-conjugated bifunctional peptide on nanowire substrate against NaCl concentration during the elution. The fluorescence intensity was quantified from the insert images by ImageJ software. (d) FESEM image of the nanowires after the elution. Scale bar, 500 nm. Error bars denote the standard deviation (N=3).
Fig. S7 HPLC chromatograms of (a) RSHRLRLH (P238), (b) HCVAHRGGGRSHRLRLH (bifunctional peptide) and (c) HCVAHRGGGAAAA (control peptide).
Fig. S8 Mass spectrums of (a) RSHRLRLH (P238), (b) HCVAHRGGGRSHRLRLH (bifunctional peptide) and (c) HCVAHRGGGAAAA (control peptide).