Electronic Supplementary Material (ESI) for Sensors & Diagnostics.

Supporting Information

Extracellular vesicles analysis in supramolecular 3D hydrogels: a proof-of-concept

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Supplementary experimental protocols

1. Peptide synthesis and characterization

Materials

HMPB resin, *N*-α-Fmoc-L-amino acids and building blocks used during chain assembly were purchased from Iris Biotech GmbH (Marktredwitz, Germany). Ethyl cyanoglyoxylate-2-oxime (Oxyma) was purchased from Novabiochem (Darmstadt, Germany), *N,N'*-dimethylformamide (DMF) and trifluoroacetic acid (TFA) were from Carlo Erba (Rodano, Italy). N,N'-diisopropylcarbodiimide (DIC), dichloromethane (DCM) and all other organic reagents and solvents, unless stated otherwise, were purchased in high purity from Sigma-Aldrich (Steinheim, Germany). All solvents for solid-phase peptide synthesis (SPPS) were used without further purification. HPLC grade acetonitrile (ACN) and ultrapure 18.2 Ω water (Millipore-MilliQ) were used for the preparation of all buffers for liquid chromatography. The chromatographic columns were from Phenomenex (Torrance CA, USA). HPLC eluent A: 97.5% H_2O, 2.5% ACN, 0.7%TFA; HPLC eluent B: 30% H_2O, 70% ACN, 0.7%TFA

Peptide Synthesis: General Procedures

Resin loading

Resin (0.5 mmol/g loading) was swollen in CH_2Cl_2 for 30 min then washed with DMF (3 × 3 mL). A solution of entering Fmoc- amino acid, DIC and Oxyme (5:5, 5 eq over resin loading) and 5% of DMAP in DMF (3 mL) was added and the resin shaken at rt for 4 h. The resin was washed with DMF (2 × 3 mL) and capping was performed by treatment with acetic anhydride/ DIEA in DCM (1 x 30 min). The resin was then washed with DMF (2 × 3 mL), CH_2Cl_2 (2 × 3 mL), and DMF (2 × 3 mL). The resin was subsequently submitted to fully automated iterative peptide assembly (Fmoc-SPPS).

Peptide Assembly via Iterative Fully Automated Microwave Assisted SPPS

Peptides were assembled by stepwise microwave-assisted Fmoc-SPPS on a Biotage ALSTRA Initiator+ peptide synthesizer, operating in a 0.1 mmol scale. Activation of entering Fmoc-protected amino acids (0.3M solution in DMF) was performed using 0.5M Oxyma in DMF / 0.5M DIC in DMF (1:1:1 molar ratio), with a 5 equivalent excess over the initial resin loading. Coupling steps were performed for 7 minutes at 75°C. Fmoc- deprotection steps were performed by treatment with a 20% piperidine solution in DMF at room temperature (1 x 10 min). Following each coupling or deprotection step, peptidyl-resin was washed with DMF (4 x 3.5 mL). Upon complete chain assembly, resin was washed with DCM (5 x 3.5 mL) and gently dried under a nitrogen flow.

Cleavage from the Resin

Resin-bound peptide was treated with an ice-cold TFA, TIS, water, thioanisole mixture (90:5:2.5:2.5 v/v/v/v, 4mL). After gently shaking the resin for 2 hours at room temperature, the resin was filtered and washed with neat TFA (2 x 4 mL). The combined cleavage solutions were worked-up as indicated below.

Work-up and Purification

Cleavage mixture was concentrated under nitrogen stream and then added dropwise to ice-cold diethyl ether (40 mL) to precipitate the crude peptide. The crude peptide was collected by centrifugation and washed with further cold diethyl ether to remove scavengers. Residual diethyl ether was removed by a gentle nitrogen stream and the crude peptide was purified by RP-HPLC and pure fractions combined and analysed by ESI-MS. Collected peptide was quantified by UV spectroscopy and aliquoted. Peptide aliquots were stored at -80°C, then lyophilized before use.

Synthesis of Cyanine-labelled peptides

Cysteine-bearing peptides were conjugated to bifunctional MAL-Cy3 or MAL-Cy5 (Lumiprobe GmbH, Germany) as follows: peptide (1 eq.) was dissolved in phosphate buffer (Na_2HPO_4 0.4M, pH 7.8). The resulting solution was ice-cooled and mixed with MAL-FAM solution (1.2 eq., 50:50 acetonitrile/water mixture). The reaction mixture was left to react for under gentle shaking until full reagents conversion (RP-HPLC monitoring). Upon reaction completion, conjugation products were isolated by preparative RP-HPLC and lyophilized.

RP-HPLC analysis and purification

Analytical RP-HPLC was performed on a Shimadzu Prominence HPLC (Shimadzu) using a Shimadzu Shimpack GWS C18 column (5 micron, 4.6 mm i.d. x 150 mm). Analytes were eluted using a binary gradient of mobile phase A (100% water, 0.1% trifluoroacetic acid) and mobile phase B (30% water, 70% acetonitrile, 0.1% trifluoroacetic) using the following chromatographic method: 10% B to 100% B in 14 min; flow rate, 1 ml/min. Preparative RP-HPLC was performed on a Shimadzu HPLC Prominence system using a Gemini Shimadzu C18 column (10 micron, 21.2 mm i.d. x 250 mm) using the following chromatographic method: 0% B to 90% B in 45 min; flow rate, 14 ml/min. Pure RP-HPLC fractions (>95%) were combined and lyophilized.

Electro-spray ionization mass spectrometry (ESI-MS)

Electro-spray ionization mass spectrometry (ESI-MS) was performed using a Bruker Esquire 3000+ instrument equipped with an electro-spray ionization source and a quadrupole ion trap detector (QITD).

Table S1. Peptide list

Code	Sequence
CD63_Cy3	RHSQMTVTSRL-$(O2Oc)_2$-C(Cy3)
CD81_Cy3	SPQYWTGPA-$(O2Oc)_2$-C(Cy3)
uPAR _Cy5	KGSGSGGD-Cha-FsrYLWS-$(O2Oc)_2$-C(Cy5)

Standard amino acids are represented by conventional one letter code. (O2Oc): 8-amino-3,6-dioxaoctanoic acid; Cha: β.cyclohexyl-L-alanine; s: D-serine; r:D-arginine; C(Cy3): Cyanine3-conjugated cysteine; C(Cy5): Cyanine5-conjugated cysteine

Table S2: Peptide characterization

Code	Rt(min)	MS calc.	MS found
CD63_Cy3	12.40	$[M+1]^+$=2288.2 $[M+2]^{2+}$=1144.6 $[M+3]^{3+}$=763.4	$[M+2]^{2+}$=763.2
CD81_Cy3	13.61	$[M+1]^+$=1978.9 $[M+2]^{2+}$=989.9 $[M+3]^{3+}$=660.3	$[M+2]^{2+}$=990.0
uPAR_Cy5	13.99	$[M+1]^+$=2755.4 $[M+2]^{2+}$=1378.2 $[M+3]^{3+}$=919.1	$[M+2]^{2+}$=1378.4 $[M+3]^{3+}$=919.2

2. Hydrogel and EVs characterization

EVs-Hydrogel preparation

Ag-Q3 hydrogel were prepared as already reported in our previous paper [1]. Freshly lyophilized Q3 aliquots were dissolved in PBS buffer (pH=7.4) to 1mM concentration, sonicated for 5 min and eventually diluted in PBS or agarose solution to final desired concentration and ratios. The obtained samples were then incubated at 25°C overnight and the resulting hydrogels directly used. Samples containing EVs were freshly prepared dissolving 6 uL of EVs stock solution (10^{11} part/mL) in 54uL of Ag-Q3 to achieve a final concentration of 10^{10} part/mL.

Hydrogel rheology

All rheology tests were performed using a KINEXUS Pro+ rheometer (MalvernPanalytical, UK). Samples were pre-formed and directly transferred on the bottom rheometer plate. The upper geometry Cone 60mm, was lowered until it was in conformal contact with the top surface of the hydrogel, corresponding to gap distances of 1.0-1.5 mm. Temperature was controlled with a Peltier device and maintained at 25°C. All the oscillatory

measurements were performed within the linear viscoelastic range. Each analysis was repeated at least 3 times, and representative measures are reported.

Droplets volume analysis

A hydrogel droplet of Aga-Q3 0.05% w/v -25μM containing EVs at 10^{10} EVs/mL was deposited on SiO_2 slide, let spontaneously dehydrate, briefly rinsed in water to remove any unbound material and let dehydrate again. The gel droplet was then rehydrated by a brief immersion in PBS buffer and then monitored by a CAM 200 instrument (KSV Ltd), which utilizes video capture and subsequent image analysis to calculate the droplet volume for approximately 80 seconds. Drying and rehydration cycles were repeated for three times.

Cells culture

HEK293 cells were purchased from the American Collection of Cell Cultures (ATCC, Manassas, VA, USA). Cell line was cultured in DMEM supplemented with 10% FCS, 2 mM L-Glutamine and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin-sulfate).

EVs isolation

EVs isolation was performed as previously described [2]. Briefly, 5 x 10^6 HEK cells were seeded on 150 mm dishes in DMEM supplemented with 10% EV-depleted FCS, 2 mM L-Glutamine and antibiotics. After 72 hours incubation, cell culture media were collected and centrifuged (1500 rpm) for 25 min to remove cell debris. Supernatants were filtered through 0.22 μm filter and ultracentrifugation at 150.000 g for 2 hours at 4° C (Beckman Coulter). The EV-containing pellet was resuspended in PBS.

Transmission Electron Microscopy.

Isolated EV were absorbed on glow discharged carbon coated formvar copper grids, washed with water, contrasted with 2% uranyl acetate and air-dried. Grids were observed with a Zeiss LEO 512 transmission electron microscope. Images were acquired by a 2k x 2k bottom-mounted slow-scan Proscan camera controlled by EsivisionPro 3.2 software.

Nanoparticle Tracking Analysis

Nanoparticle tracking analysis (NTA) was performed according to the manufacturer's instructions using a NanoSight NS300 system (Malvern Technologies, Malvern, UK) configured with a 532 nm laser. EVs samples were diluted in filtered PBS to a final volume of 1 mL. Ideal measurement concentrations were found by pre-testing the ideal particle per frame value (20–100 particles/frame). The following settings were adjusted according to the manufacturer's software manual. A syringe pump with constant flow injection was used and three videos of 60 s were captured and analyzed with NTA software version 3.2. From each video, the mean, mode, and median EVs size was used to calculate samples concentration, expressed in nanoparticles/mL.

BCA assay

EV samples and BSA standards for calibration curve were diluted in BCA solution (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, Rockford, USA) in 1:9 ratio and incubated for 30 min at 37◦C. Samples were analysed by a spectrophotometer (HiPo MPP-96 Microplate Photometer, Biosan, Riga, LV) at 562 nm wavelength.

SDS-PAGE and Western Blot Analysis

Laemmli buffer was added to EVs and sample boiled for 5min at 95 ℃. Specifically, 10 μg of EVs' proteins (determined by BCA assay) were prepared in non-reducing conditions for tetraspanins detection, while 10 μg were used for ALIX and TSG101 detection. Proteins were resolved by SDS-PAGE (4–20%, Mini-Protean TGX Precast protein gel, Bio-Rad) and transferred onto a nitrocellulose membrane (BioRad, Trans-Blot Turbo). Nonspecific sites were saturated with a blocking TBS-T solution (tris-buffered saline: 150 mM NaCl, 20 mM TrisHCl, pH 7.4, and 0.5% Tween 20) with 5% w/v BSA for 1 h. Membranes were incubated overnight at 4 °C with the following antibodies: mouse anti-CD9 (1:1000, BD Pharmingen), mouse anti-CD63 (1:1000; BD Pharmingen, San Jose, CA, USA), mouse anti-Alix (1:1000, Santa Cruz, CA, USA), mouse anti-TSG101 (1:1000, Novus Bio, Centennial, CO, USA). After washing with a solution of TBS-T with 0.05% Tween20, membranes were incubated with the horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, Tucker, GA, USA) diluted 1:3000 for 1 h. After washing, the signal was detected by the enhanced chemiluminescence method using Bio-Rad Clarity Western ECL Substrate (Bio-Rad) and imaged using a Chemidoc XRS+ (BioRad).

3. 3D and 2D Microarrays: general procedure

3D Microarrays

Silicon slides (SVM, Sunnyvail, CA) were coated with MCP2 (Lucidant Polymers) according to previous protocols [3] and hydrogels spotted using a noncontact microarray spotter (Scienion sciFLEXARRAYER S12) using an 80μM noozle, 20 droplets are deposited for each spot (approximately volume 8 nL). Spotted slides are incubated in a humid chamber for two hours before blocking with 50 mM ethanolamine in Tris/HCl 1M pH 9 for 1 hours, washed with water and dried under a stream of nitrogen. To prepare the gel-matrix loaded with EVs, 54uL of hydrogel was mixed with 6 uL of EVs preparations (10^{11} part/mL) to achieve a final concentration of 10^{10} part/mL; the empty matrix was spotted without any addition.

Model Immunoassay

3D microarrays produce as descried above, were incubated with a solution of antibody anti-CD63 (Ancell, Bayport, MN, USA), anti-CD81 (Ancell, Bayport, MN, USA) or peptides probes against tetraspanin CD63 and CD81, at different concentration. Incubation with labelling solution was performed for 1 hour and then washed three times (15 minutes) with washing buffer (Tris/HCl 0.05 M pH 9, NaCl 0.25 M, Tween 20 0.05%) in order to remove the non-specific sigla provide by interaction between labeled probes and the hydrogel matrix. Antibodies and peptides probes were diluted in filtered incubation Buffer (Tris/HCl 0.05 M pH 7.6, NaCl 0.15 M, Tween 20 0.02%, 1%BSA). Antibodies were in house labelled by Cyanine3 NHS ester from Biotium. Fluorescent probes were tested at 500nM, 100nM, 20nM, 10nM and 2nM on 3D gel microarray; after immuno-staining incubation slides were washed, dried and analyzed by TECAN power scanner.

uPAR Immunoassay

3D microarrays produce as descried above, were incubated with peptide probe against uPAR; incubation with labelling solution was performed for 1 hour and then washed three times (15 minutes) with washing buffer (Tris/HCl 0.05 M pH 9, NaCl 0.25 M, Tween 20 0.05%) in order to remove the non-specific sigla provide by interaction between labeled probes and the hydrogel matrix. uPAR peptides probes were diluted in filtered incubation Buffer (Tris/HCl 0.05 M pH 7.6, NaCl 0.15 M, Tween 20 0.02%, 1%BSA) and tested, at 20nM, on 3D gel microarray; after immuno-staining incubation slides were washed, dried and analyzed by TECAN power scanner.

2D microarrays and model immunoassay

Silicon slides (SVM, Sunnyvail, CA) were coated with MCP-2 polymer (Lucidant Polymers). The microarrays were produced using a non-contact S12 Spotter (Scienion Co., Berlin, Germany), depositing a drop for each spot (400 pL). Final concentration of antibodies spot solution (a mixture of mouse monoclonal anti-CD9, ant-CD63, anti-CD81 from Ancell) is 1 mg/ml in PBS and 50 mM Trehalose. Printed slides were placed in humid chamber overnight at room temperature and subsequently were washed in a blocking solution (ethanolamine 50mM, Tris HCl 0.1 M, pH 9). EV samples were diluted (10^{10} part/mL) in filtered PBS and incubated on slides for 2 h at room temperature in static conditions, washed three times and dried. Slides were then incubated with peptides probes against tetraspanin CD63 and CD81, at different concentration as previously described. After peptide-staining, chips were washed, dried, and analyzed by TECAN power scanner.

4. Supplementary Figures

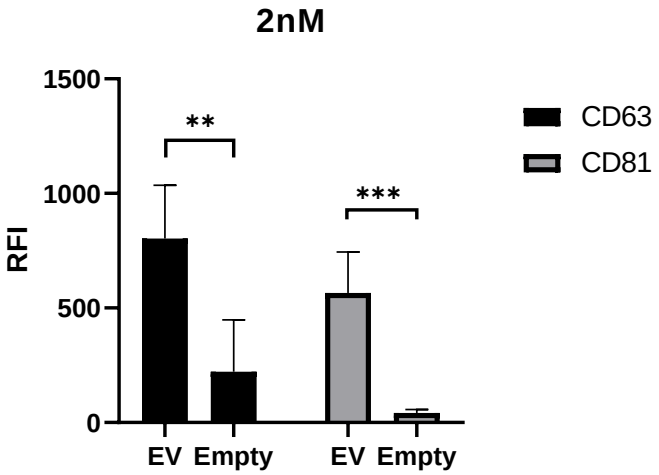

Figure S1. At 2nM of labelled peptide, EV-gel was compared to an empty matrix; both targets CD63 and CD81 demonstrated a statistically significant difference. Data were obtained from three replicates.

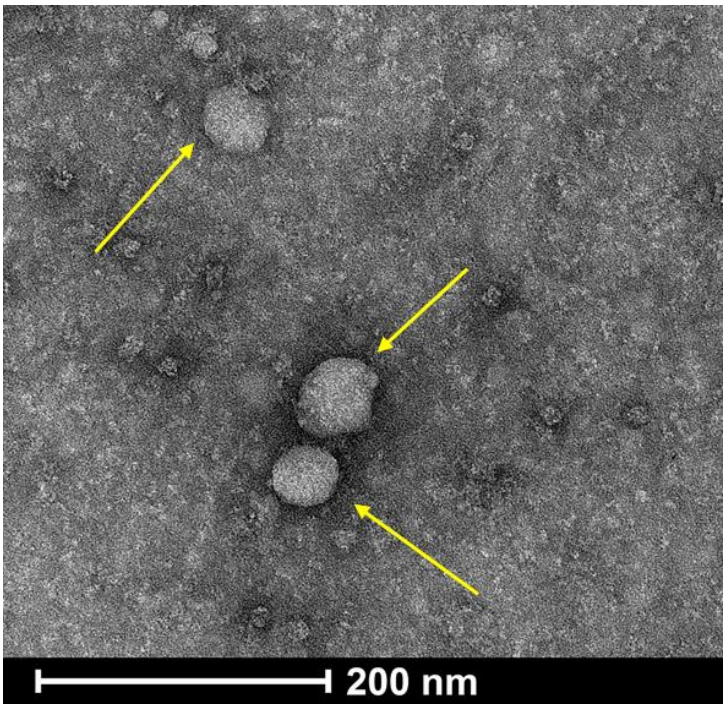

Figure S2. TEM images of 10^{10} EVs/mL.

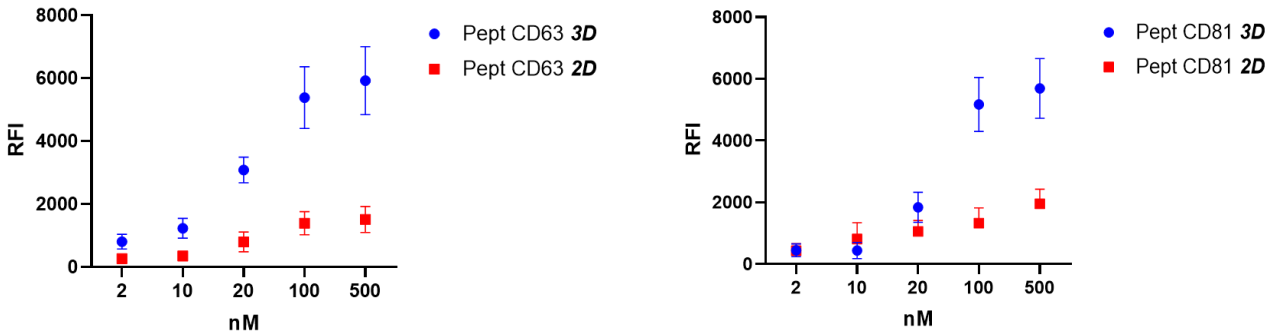

Figure S3. Comparison of detection of surface markers (CD63 and CD81) of EVs (10^{10} EVs/mL) entrapped in the hydrogel (3D) versus capture by pan-tetraspanin CD9/CD63/CD81 antibody spots in a 2D microarray. The fluorescent signals for the 2D and 3D assays are compared, showing an increased signal-to-noise ratio for the 3D system.

SI References

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