SUPPORTING INFORMATION

Tackling CD147 exosome-based cell-cell signaling by electrochemical biosensing for early colorectal cancer detection

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MATERIALS AND METHODS

Apparatus and electrodes

Current-time measurements were performed using a CHI1140A potentiostat controlled by the homonym software (CH Instruments, Inc.). 4-mm ∅ screen-printed carbon electrodes (SPCEs, DRP-110) and the specific cable connector (DRP-CAC 70499) were purchased from Metrohm Hispania, S.L.U. A homemade polymethylmethacrylate (PMMA) casing with embedded neodymium magnet (AIMAN GZ) was employed to capture modified magnetic bioconjugates uniformly and reproducibly onto the surface of the working electrode (WE) from the SPCE. Other instruments used included a vortex (Velp Scientifica), a BioSan TS-100 constant temperature incubation shaker (Thermo), DynaMag®2 magnetic separators (Invitrogen-ThermoFisher Scientific), a magnetic stirrer (Inbea.SL), and a Basic pH meter (Basic 20+, Crison).

Reagents and solutions

KCl, NaCl, $NaH_2PO_4 \times 2H_2O$, Na_2HPO_4 and Tris-HCl were acquired from Scharlab. Carboxylic acid-functionalized microbeads (HO_2C-MBs, Dynabeads M-270) were purchased from Invitrogen-Thermo Fisher. N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC), N-hydroxysulfosuccinimide (NHSS), hydrogen peroxide (H_2O_2) (30 %, w/v), hydroquinone (HQ), ethanolamine, human haemoglobin (Hb), Tween® 20, immunoglobulin G (IgG) from human serum, and human serum albumin (HSA) were from Sigma-Aldrich, while bovine serum albumin (BSA) was acquired from Gerbu Biotechnik. Recombinant human CD147 standard, mouse anti-human CD147 capture antibody (CAb), and biotinylated goat anti-human CD147 detector antibody (bDAb) were purchased as the components from an ELISA Kit (DuoSet®, DY972, R&D Systems Europe). Blocker casein solution (BB solution: phosphate-buffered saline (PBS) containing 1 % w/v casein, pH 7.4), Protein-free (PBS) Blocking Buffer (PFBB) (Catalog Number. 37572), and SuperBlock™ (PBS) Blocking Buffer (SP) (Catalog Number. 37515) were acquired from Thermo Fisher Scientific, and NaOH, from Labkem. Streptavidin peroxidase conjugate (Strep-HRP) was purchased from Roche Diagnostics GmbH. Total E-cadherin (E-Cad), hypoxia-inducible factor 1-α (HIF1α) human standards (DuoSet-IC, Catalog Numbers DYC4225 and DYC1935 respectively, R&D Systems Europe, Ltd.), human tumour necrosis factor alpha (TNF-α) protein (BD Pharmigen, ref. 55,618) and recombinant human cadherin-17 (CDH-17) protein (OriGene Technologies, Inc.) were used as potential interfering compounds. All reagents were of the highest available grade. The following buffer solutions,

prepared with deionized water from a Milli-pore Milli-Q purification system (18.2 MΩ cm) were employed: 0.05 M phosphate buffer (PB), pH 6.0; 0.1 M PB, pH 8.0; phosphate-buffered saline (PBS) containing 137 mM NaCl and 2.7 mM KCl, pH 7.4; and 0.025 M MES buffer, pH 5.0. 0.1 M H_2O_2 and HQ solutions in 0.05 M PB (pH 6.0), respectively, were freshly prepared just before carrying out the amperometric transduction. Wash buffer (0.05 % Tween®20 in PBS), stop solution (2N H_2SO_4), and reagent diluent (RD, 1 % BSA in PBS) were used in ELISA assays.

Colorectal cancer cell lines

Non-metastatic SW480 and their isogenic lymph-node metastatic SW620 CRC cells obtained from the American Type Culture Collection cell repository were used, together with non-metastatic KM12C and their isogenic liver metastatic KM12SM and liver and lung metastatic KM12L4a CRC cells from Fidler's laboratory (MD Anderson Cancer Center). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Lonza) supplemented with 1X L-glutamine (Lonza), 1X penicillin/streptomycin (Lonza), and 10 % fetal bovine serum (FBS, Sigma Aldrich) at 37 °C and 5 % CO_2, according to established protocols.

Exosome isolation and purification

Exosomes secreted by CRC cells were isolated and purified by ultracentrifugation as previously reported (Montero-Calle et al., 2021). Briefly, cells were grown until 90 % confluence, washed with PBS, and incubated in FBS free DMEM for 48 h at 37 °C and 5 % CO_2. Eight 175 cm^2 cell culture flasks (Corning) were seeded per cell line. Supernatants (conditioned mediums) were collected and centrifuged subsequently for 5 min at 500 g and 4 °C to remove the cell debris (pellet) and for 10 min at 2000 g and 4 °C to remove vesicles greater than 1 µm (pellet). Then, conditioned mediums were centrifuged using the Beckman-Coulter ultracentrifuge (Optima AUC) once at 10000 g and 4 °C for 30 min to discard extracellular microvesicles (MVs, pellet), and twice at 100000 g and 4 °C for 60 min to sediment exosomes (pellets). Finally, exosomes were re-suspended in 500 µL of PBS 1X and aliquoted and stored at – 80 °C until use.

Purified exosomes were characterized by transmission electron microscopy (TEM) and with the Nanosight NS300 (Malvern Panalytical), as previously reported (Montero-Calle et al., 2021). The protein concentration of exosome samples was determined using the MicroBCA Protein Assay Kit (Thermo Fisher Scientific).

Protein extraction

Exosomes were lysed with 100 µL of lysis buffer (RIPA, Sigma Aldrich) supplemented with protease and phosphatase inhibitors (MedChemExpress) by mechanical disaggregation using 16-gauge needle syringes for the extraction of exosomal proteins. Protein extracts were centrifuged at 10000 g and 4 ºC for 10 min and supernatants containing exosomal proteins stored at – 80 °C until use.

Western-blot analysis

5 µg of each exosomal lysate were separated under reducing conditions on 10 % SDS-PAGE and transferred to nitrocellulose membranes at 100 V for 90 min. Membranes were incubated with PBS 1X containing 3 % BSA and 0.1 % Tween-20 (blocking buffer) for 1 h at room temperature (RT) in rotation. Next, membranes were incubated overnight with a biotin-functionalized detector anti-CD147 antibody (bDAb, DuoSet®, DY972, R&D Systems Europe) 1:1,000 diluted in blocking buffer at 4 ºC in rotation. Then, membranes were washed three times in PBS 1X containing 0.1 % Tween-20 (wash buffer), incubated with Strep-HRP reagent (EL-HRP, RayBiotech) 1:1,000 diluted in blocking buffer for 30 min at RT in rotation, and washed three times in wash buffer. Signal was developed with the ECL Pico Plus chemiluminescent reagent (Thermo Fisher Scientific) and detected on an Amersham Imager 680 (GE Healthcare).

Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM)

To confirm the correct binding of exosomes to magnetic beads (MBs), the size of particles in suspension before and after incubation of MBs with exosomes was determined by DLS analysis using the Zetasizer Nano ZS (Malvern Panalytical). In brief, MBs, purified exosomes, exosomes incubated with MBs coated with anti-CD147 CAb, and exosomes incubated with MBs without the capture antibody were 1:100 diluted in PBS 1X and measured by triplicate with the Zetasizer to check the size distribution by means of the light scatter intensity of the particles and using the Zetasizer software. The hydrodynamic size and the relatively large polydispersity index (PDI) were obtained (mean ± SD) for each sample.

Purified exosomes and exosomes incubated with MBs were analyzed by electron microscopy with negative staining. Samples were fixed in 4 % paraformaldehyde (1:2 dilution) for 5 min at RT. They were incubated on glow-discharged carbon-coated grids for 5 min and negatively stained with 2 % aqueous uranyl acetate. Then, the samples were analyzed on an FEI Tecnai 12

electron microscope equipped with a LaB6 filament operated at 120 kV, and images were recorded with an FEI Ceta digital camera.

Assembly of sandwich-based immunocomplexes onto magnetic microbeads

Sequential modification of commercial HO_2C-MBs was performed under constant temperature (25 ºC) and shaking (950 rpm). After each modification step, MBs were washed and magnetically decanted for 3 min by using a magnet concentrator.

A 3 µL-aliquot HO_2C-MB suspension was placed in a 1.5 mL eppendorf tube and washed twice with 50 µL of 0.025 M MES buffer, pH 5.0, for 10 min under the conditions stated above. Then, carboxylic groups on the MBs surface were activated for 35 min with 25 µL of a freshly prepared EDC and NHSS solution (50 mg mL^{-1} each, in MES buffer), followed by two washings with 50 µL of MES buffer. Covalent attachment of specific CD147 capture antibodies (CAbs) was performed by re-suspending activated MBs in 25 µL of a 25 µg mL^{-1} CAb solution (in MES buffer) for 15 min, and after two washings with 50 µL of MES buffer, the unreactive activated carboxylic groups were blocked for 60 min in 25 µL of 1 M ethanolamine solution (prepared in 0.1 M phosphate buffer, pH 8.0). Subsequently, one washing with Tris-HCl buffer (0.1 M, pH 7.2), and two more with BB solution or SP were performed before storing CAb-MBs at 4 °C in filtered PBS until they were employed. The determination of the target protein involved the CAb-MBs re-suspension and incubation for 15 min in 25 µL of a solution containing the CD147 standard or the sample to be analysed in the corresponding buffer media (BB was used for standards while SP buffer was employed for the lysed and entire isolated exosomes, respectively) followed by two washings with 50 µL of the corresponding buffer solution. Thereafter, CD147 sandwich immunocomplexes were implemented and subsequently labelled by sequential incubation with 25 µL of 1.0 µg mL^{-1} biotinylated detection antibody (bDAb) and 1/1,000 diluted Strep-HRP solution, both in BB, during 15 min, respectively, and by carrying out two washings with same buffer after each modification step. Just before proceeding with amperometric transduction, MBs bearing the immunocomplexes were re-suspended in 50 µL of 0.05 M phosphate buffer (PB), pH 6.0.

Amperometric readout

A PB-re-suspended MBs aliquot was homogeneously located onto the working electrode (WE) of a SPCE previously fitted in a poly(methyl methacrylate) (PMMA) housing with the embedded Nd magnet to ensure reproducible, stable, and appropriate site capture of the MBs. The ensemble SPCE/magnet holding block was immersed into an electrochemical cell

containing 10 mL of 0.05 M PB (pH 6.0) supplemented with 1 mM HQ and connected to the electrochemical station through a specific cable connector. Amperometric measurements were performed under a constant potential of – 0.2 V applied to the WE *vs.* the Ag pseudo-reference electrode in a continuously stirred solution, when 50 µL of a freshly prepared HRP-substrate (0.1 M H_2O_2) was added to the supporting electrolyte solution. Cathodic current variations were read once steady state was reached. Error bars given along the manuscript were estimated as three times of the standard deviation (SD) of three replicates (n=3).

RESULTS AND DISCUSSION

Optimization of experimental variables

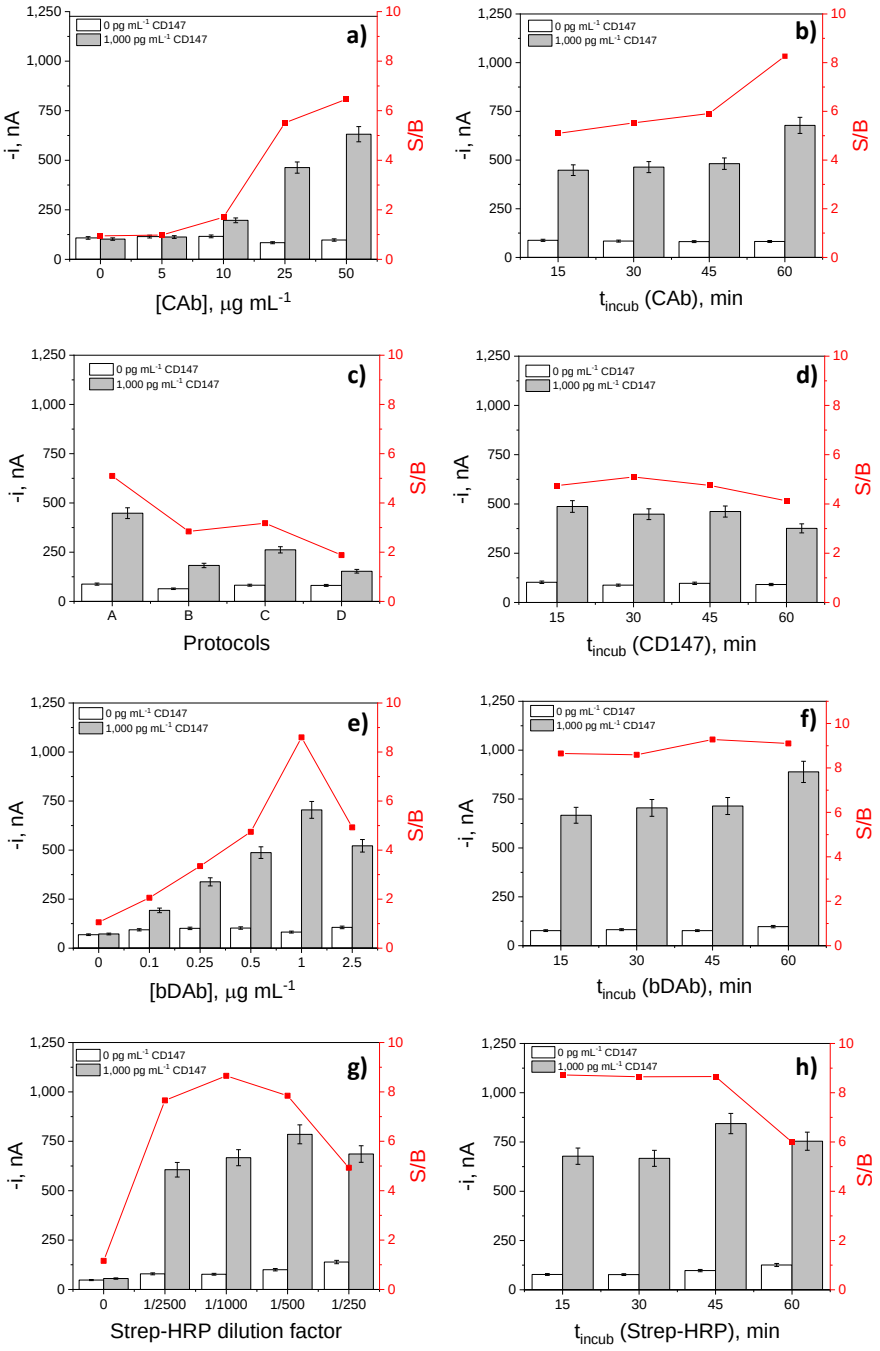

Fig. S1. Dependence of the amperometric responses measured using the sandwich-based immunosensing strategy in the absence (B, white bars) and in the presence (S, grey bars) of 1,000 pg mL^{-1} CD147 standard with: CAb concentration (a) and incubation time (b), number of steps used to perform the assay (c), CD147 incubation time (d), bDAb concentration (e) and incubation time (f), and Strep-HRP conjugate dilution factor (g) and incubation time (h). Resulting S/B ratios are depicted as red squares and lines.

Fig. S1a shows as the S/B ratio increases significantly with the CAb concentration up to 25 µg mL^{-1}, and such increase is slower for larger concentrations. Accordingly, and as a compromise between sensitivity and assay cost, 25 µg mL^{-1} was selected as the CAb concentration for further work. Similarly, although an incubation time of 60 min for the covalent attachment of CAb provided a larger S/B ratio (**Fig. S1b**), 15 min were chosen as a compromise between sensitivity and a shorter test time. Since the immune-sandwich preparation implies several sequential steps, an evaluation of the effect on the response of different working protocols (i.e. the number of steps involved in the formation of sandwich immunocomplexes on the surface of CAb-MBs) was carried out. **Fig. S1c** shows that blanks remained practically constant for all the assayed protocols. However, the specific signal in the presence of CD147 was notably larger when a 3-steps protocol (A), consisting of the sequential incubation of CAb-MBs with the target protein, bDAb, and the enzymatic tracer Strep-HRP solutions, respectively, was employed. The incubation of CAb-MBs immunoconjugates with mixtures of CD147 and bDAb (protocol B), bDAb and Strep-HRP (protocol C), or CD147, bDAb, and Strep-HRP (protocol D), resulted in lower CD147 capture and labelling efficiencies, most likely due to aggregation phenomena or steric hindrance when the corresponding immunoreagents coexist in solution. This was more evident for protocols where the target protein was not previously captured by CAb-MBs. The effect of concentration of bDAb (**Fig. S1e**) and dilution factor of Strep-HRP (**Fig. S1g**) led us to select 1.0 µg mL^{-1} and 1/1,000, respectively, for further work. Interestingly, when larger amounts of bDAb and Strep-HRP were used, a substantial decrease in the specific signal was observed together with a significant increase in the blanks. This behaviour can be explained by the hindrance for an efficient labelling of previously captured CD147 molecules, and the high non-specific adsorption of the enzymatic tracer on CAb-MBs, respectively, resulting in both cases in a remarkable decrease of the S/B ratios. Regarding the incubation times for CD147, bDAb, and Strep-HRP conjugate, **Figs. S1d**, **S1f**, and **S1h**, show as 15 min were sufficient for each incubation step because longer times did not provide a noticeable improvement in sensitivity.

In addition, the variables related to the amperometric transduction such as the applied potential, the concentration level of both the redox mediator and the enzyme substrate, and the concentration and pH of the electrolyte solution were taken from previous works (Eguílaz et al., 2010; Conzuelo et al., 2012; Esteban-Fernández de Ávila et al., 2013).

Table S1

Optimized experimental variables involved in the preparation of the developed amperometric immunosensing strategy for CD147 determination.

Variable	Tested range	Selected value
[CAb], μg mL^{-1}	0 – 50	25
CAb incubation time, min	15 – 60	15
Number of steps	3 – 1	3
CD147 incubation time, min	15 – 60	15
[bDAb], μg mL^{-1}	0 – 2.5	1.0
bDAb incubation time, min	15 – 60	15
Strep-HRP, dilution factor	0 – 1/250	1/1,000
Strep-HRP incubation time, min	15 – 60	15

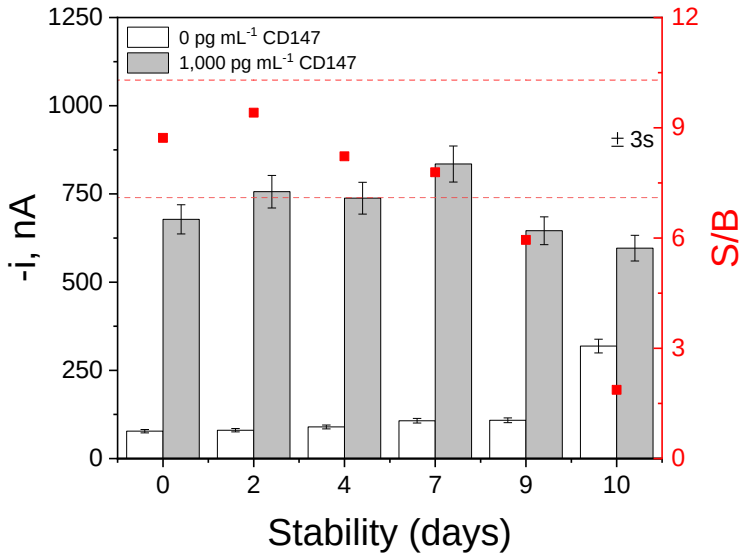

Fig. S2. Storage stability of CAb-MBs immunoconjugates stored in filtered PBS at 4 ºC after their preparation. S/B ratio values obtained for 0 and 1,000 pg mL^{-1} CD147 standards with stored immunoconjugates each control day. Control limits (red dashed lines) were set as ± 3 s of the S/B average value obtained the first day of the study (n = 3).

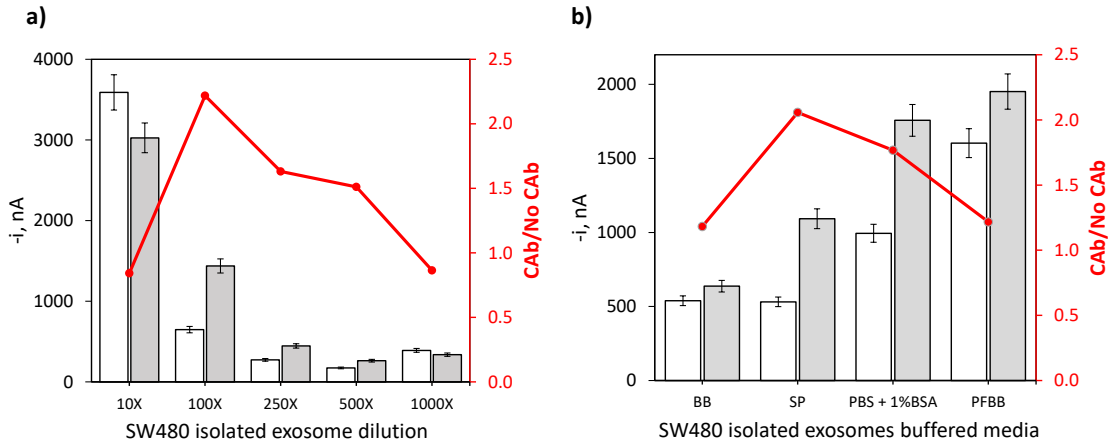

Fig. S3. Direct detection of exoCD147 in isolated SW480 exosomes (as a model EV). Dependence of the amperometric responses obtained with the developed sandwich-based immunoplatform with the exosome dilution factor (a) and the buffered media used (b) after sample incubation with bare MBs (white bars) and anti-CD147-MBs immunoconjugates (grey bars) followed by the enzymatic label with bDAb and Strep-HRP conjugate. Resulting specific/non-specific ratio (CAb/No CAb) is depicted as red circles and lines.

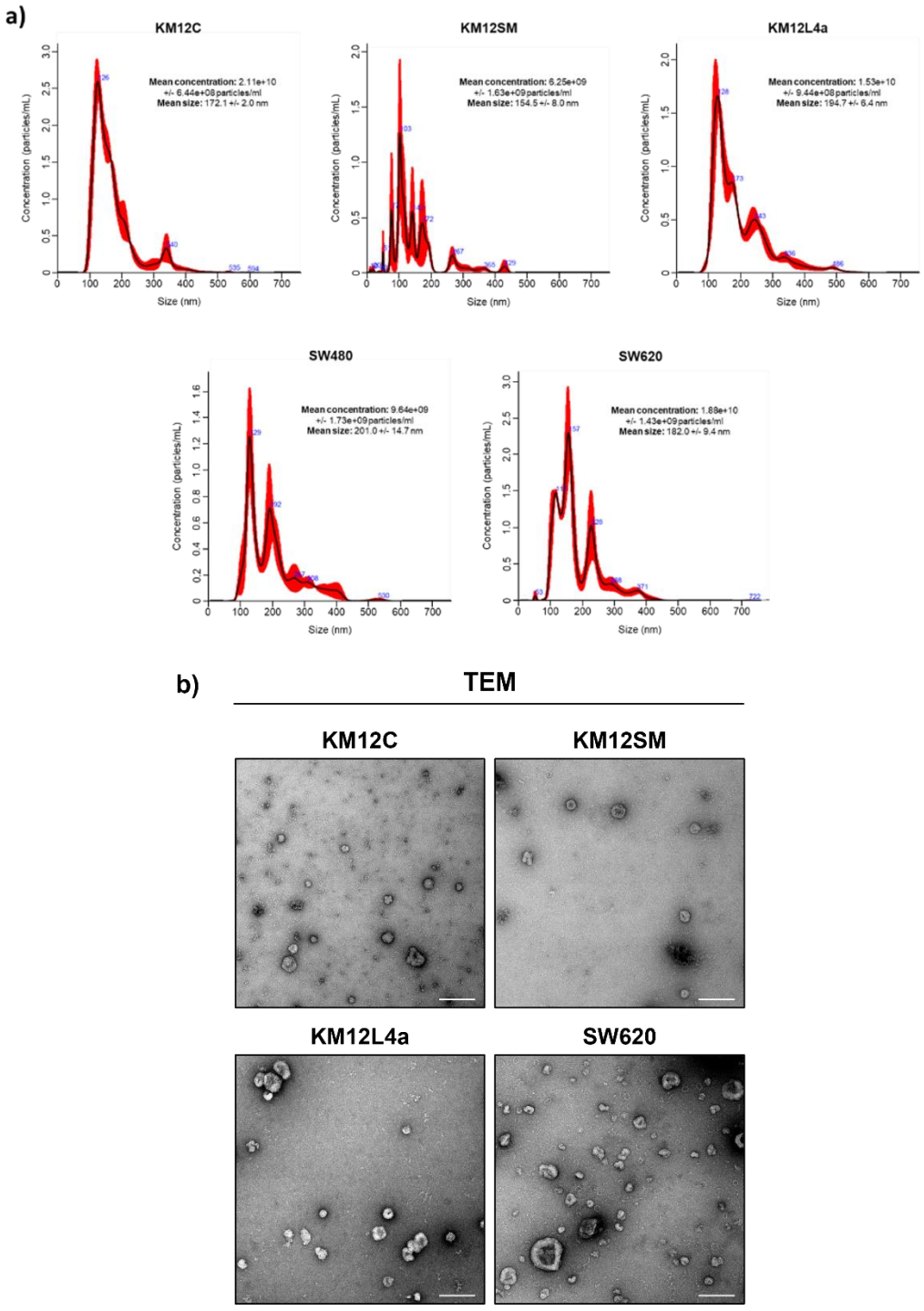

Fig. S4. Characterization of exosomes secreted by CRC cell lines isolated and purified by ultracentrifugation. Size and concentration of EVs secreted by CRC cell lines as obtained with the Nanosight (a). Representative TEM micrographs of exosomes derived from KM12C, KM12SM, KM12L4a and SW620 CRC cell lines isolated by ultracentrifugation (b). Extracellular vesicles purified from CRC cell lines showed a size compatible with exosomes. Exosome samples were 1:100 diluted in PBS 1X or crudely analysed through Nanosight and TEM, respectively. Scale bar: 200 nm.

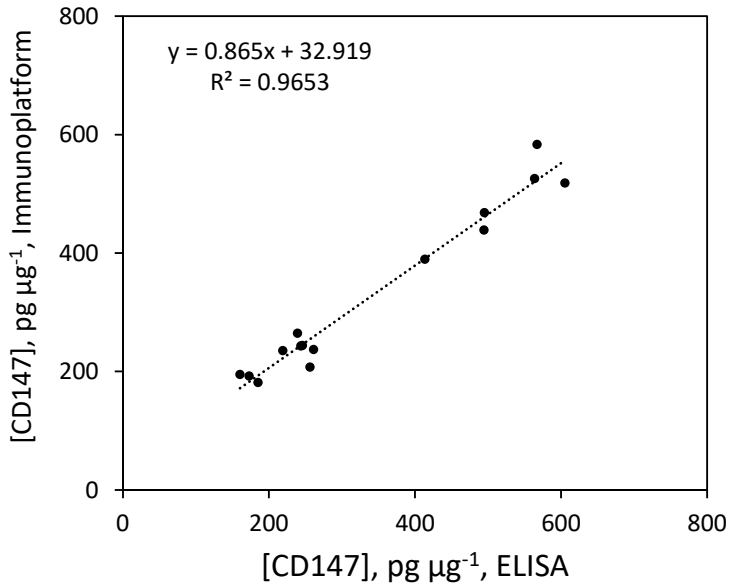

Fig. S5. Correlation between the CD147 concentration obtained with the developed amperometric immunoplatform and the ELISA method in isolated lysed exosomes from different CRC cells (SW480, SW620, KM12C, KM12SM and KM12L4a).

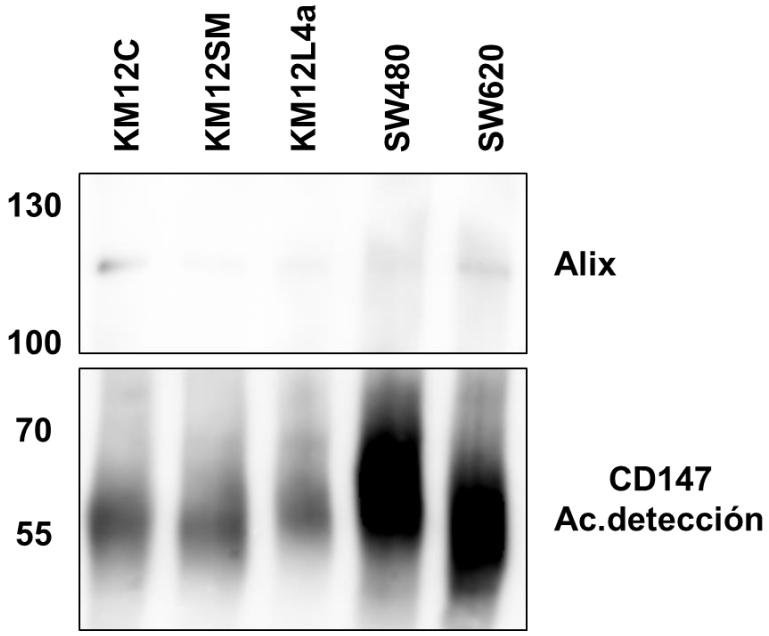

Fig. S6. WB analysis of 1.0 µg of exosomes lysate separated by 10% SDS-PAGE, transferred to nitrocellulose membranes, and proved with the bDAb.

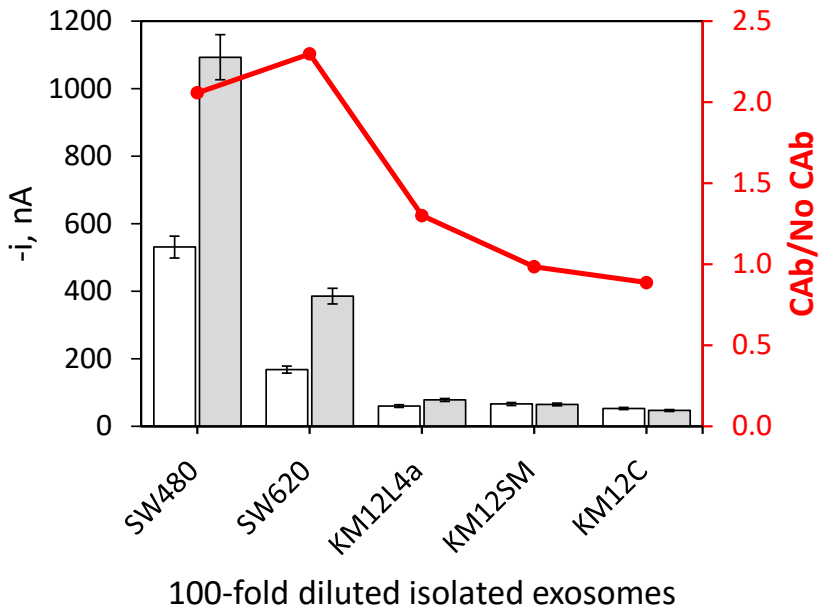

Fig. S7. Amperometric signals obtained for the direct detection of exoCD147 in entire exosomes isolated from the specified CRC cell lines. Suspensions of entire exosomes were incubated with bare MBs (white bars) and anti-CD147-MBs immunoconjugates (grey bars) and subsequently labelled with bDAb and Strep-HRP conjugate. Resulting specific/non-specific ratio (CAb/No CAb) is depicted as red circles and lines.

Table S2

Comparison of the results obtained ([CD147] in pg μg^{-1}) by using the developed amperometric immunoplatform and the ELISA method for the determination of exoCD147 in 0.1 µg of exosome lysates isolated from different CRC cell lines by ultracentrifugation.

CRC isolated exosomes	Immunosensor	RSD $_{(n=3)}$	ELISA	RSD $_{(n=3)}$	|ER*|, %
SW480	(432 ± 99)	9.2	(468 ± 117)	10.0	7.7
SW620	(542 ± 88)	6.6	(579 ± 57)	4.0	6.4
KM12C	(189 ± 18)	3.8	(173 ± 30)	7.1	9.2
KM12SM	(250 ± 30)	4.9	(243 ± 8)	1.4	2.9
KM12L4a	(226 ± 42)	7.4	(245 ± 57)	9.4	7.7

*relative error

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