Exosomes are endocytic lipid-membrane bound bodies with potential to be used as biomarkers in cancer and neurodegenerative disease. The limitations and scarcity of current exosome characterisation approaches has led to a growing demand for translational techniques, capable of determining their molecular composition and physical properties in physiological fluids. Here, we investigate label-free immunosensing, using a quartz crystal microbalance with dissipation (QCM-D), to detect exosomes by exploiting their surface protein profile. Exosomes expressing the transmembrane protein CD63 were isolated by size-exclusion chromatography from cell culture media. QCM-D sensors functionalised with anti-CD63 antibodies formed a direct immunoassay towards CD63-positive exosomes, exhibiting a limit-of-detection of $1.7 \times 10^8$ and $1.1 \times 10^8$ exosome sized particles (ESPs) ml$^{-1}$ for frequency and dissipation response respectively, i.e., clinically relevant concentrations. Our proof-of-concept findings support the adoption of dual-mode acoustic analysis of exosomes, leveraging both frequency and dissipation monitoring for use in diagnostic assays.
Acoustic immunosensing of exosomes using a quartz crystal microbalance with dissipation monitoring.

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Abstract

Exosomes are endocytic lipid-membrane bound bodies with potential to be used as biomarkers in cancer and neurodegenerative disease. The limitations and scarcity of current exosome characterisation approaches has led to a growing demand for translational techniques, capable of determining their molecular composition and physical properties in physiological fluids. Here, we investigate label-free immunosensing, using a quartz crystal microbalance with dissipation (QCM-D), to detect exosomes by exploiting their surface protein profile. Exosomes expressing the transmembrane protein

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CD63 were isolated by size-exclusion chromatography from cell culture media. QCM-D sensors functionalised with anti-CD63 antibodies formed a direct immunoassay towards CD63-positive exosomes, exhibiting a limit-of-detection of $1.7 \times 10^8$ and $1.1 \times 10^8$ exosome sized particles (ESPs)/ml for frequency and dissipation response respectively, i.e., clinically relevant concentrations. Our proof-of-concept findings support the adoption of dual-mode acoustic analysis of exosomes, leveraging both frequency and dissipation monitoring for use in diagnostic assays.

**Introduction**

Extracellular vesicles (EVs) are heterogeneous, biomolecular structures enclosed by a lipid bilayer. They are secreted by nearly all eukaryotic cells into the extracellular space and most bodily fluids.\(^1\) Of particular interest are exosomes, a subset of EVs with a nanoscale size range (30-150 nm), originating from invaginations of early endosomes and are released upon the fusion of multi-vesicular bodies with the cell membrane.\(^2\) They are enriched in nucleic acids, surface proteins such as tetraspannins (CD63, CD81 and CD9) and cytosolic proteins including, heat shock proteins (HSP90 and HSP70) and TSG101.\(^3,4\)

Traditionally thought to function as cellular waste-bins, the roles of exosomes in intercellular communication,\(^5\) disease propagation\(^6\) and regenerative processes\(^7\) are now well established. Crucially, exosome concentrations and phenotype have shown to vary between healthy and diseased states, reflecting their parental cell of origin.\(^8,9\) Thus, exosomes have attracted widespread interest as a concentrated source of biomarkers for minimally invasive, point-of-care (POC) liquid biopsies.\(^10,11\)

Typically, exosomes are characterised via nanoparticle tracking analysis (NTA). Here, the imaging of light scattered from particles moving under Brownian diffusion is used to determine the hydrodynamic size and concentration.\(^12\) Alternatively, tunable elastomeric pore sensing analyses individual particles via the electrical impedance they impart at an aperture.\(^13\) These methods are often coupled with total protein quantification via colorimet-
ric assays such as microBCA and Bradford.\textsuperscript{14} One limitation of the above techniques is that they do not selectively distinguish between exosomes and other EVs, protein aggregates and lipoproteins. This lack of discrimination is compounded by the choice of exosome isolation technique, where commonly adopted centrifugation and polymer precipitation methods co-isolate non-exosomal artefacts from complex media.\textsuperscript{15} Thus, there is a difficulty in defining subsets within a heterogenous exosome population, which hinders these techniques in sensing specific markers in complex biological matrices.\textsuperscript{16} By contrast, flow cytometry\textsuperscript{17,18} and fluorescence-based NTA\textsuperscript{19} has been successfully employed to quantify exosomes and determine their phenotypes via selective tagging of their surface epitopes. Nonetheless, labelling approaches are restricted by the strength of interaction between label and exosome. Furthermore, these techniques are largely destructive, limiting downstream applications. Enzyme-linked immunosorbent assay (ELISA) is the current gold standard for exosomal protein quantification, with sensitivity in the picomolar range.\textsuperscript{20} However, ELISAs can suffer from a lack of multiplexing, cross-contamination and limited potential for POC application.

There is an increasing interest in automation and miniaturisation of exosome screening through microfluidics and lab-on-a-chip approaches to match a clinical demand of minimally invasive patient stratification.\textsuperscript{21,22} Examples of advanced exosomal analytical approaches include, interferometry,\textsuperscript{23} electrochemistry\textsuperscript{24,25} and, in particular, optical sensors utilising nanoplasmonics.\textsuperscript{26,27} Recently, Rupert et al. successfully demonstrated surface plasmon resonance (SPR) based sensing of CD63-positive exosomes through surface based immunocapture.\textsuperscript{28} Collectively, these techniques provide a sensitive, label-free and real-time assessment of exosomes. Despite these attributes, electrochemical approaches require electrolytes, which may affect the structural integrity of some bio-molecules. Interferometry can be hindered by the turbidity of samples, whilst plasmonic approaches have yet to address cost and portability issues. Crucially, a potential drawback of these methods is the ability to distinguish between exosome and artefactual binding phenomena. This is essential, as not all isolated particles are exosomal in composition, potentially leading to false positive results.
To overcome the issue of selectivity, this study employs quartz crystal microbalance with dissipation (QCM-D) monitoring, to leverage differences in mechanical properties between exosomes and associated contaminants in colloidal suspensions. QCM-D is capable of characterising interfacial structure, binding kinetics, molecular affinity and mechanical properties of the adsorbent. Advantages of the acoustic technique include label-free, real-time measurements and ease of miniaturisation. Nonetheless, QCM-D has yet to be fully exploited for exosome biosensing. With the acoustic wave typically possessing a penetration depth of 250 nm from the oscillator surface, which matches the size of exosomes. QCM-D transduced immunosensing has been explored to build assays where an antibody serves as a bioreceptor towards a target analyte. For instance, Uludag et al. demonstrated its applicability for the detection of a prostate specific antigen. Pirincci et al. evaluated a competitive immunoassay for the detection of mycotoxin Ochratoxin A. The addition of gold nanoparticles has also been used as a signal amplifier as part of QCM-D sandwich immunoassays.

In this work, we establish a direct immunoassay of CD63-positive exosomes using QCM-D. After successfully isolating and characterising exosomes, we first identify an optimal antibody immobilisation approach. The sensor performance is then validated by assessing its sensitivity towards spiked samples of CD63 proteins. Increasing concentrations of exosome sized particles (ESPs) serve to determine limit-of-detection (LOD) and limit-of-quantification (LOQ). We further compare the platform performance to a control surface to determine whether the process successfully discriminates between exosomes and contaminating particles and validate our findings by complementary in-liquid atomic force microscopy (AFM).
Experimental

Exosome isolation and characterisation

Size-exclusion chromatography. Size-exclusion chromatography (SEC) was chosen as the isolation technique for exosomes from human umbilical cord mesenchymal stem cell culture media (HUMSCCM), based on previous work in the field. HUMSSCM was first filtered with a 0.45 \( \mu \)m filter (Merck Millipore, USA). 30 ml of clarified media was subsequently concentrated using Amicon Ultra-15 centrifugal filters with a 10 kDa pore size cut-off (Merck Millipore, USA). The filters were spun at 4000 x G for 30 minutes at 4 °C. Post-spin, 0.5 ml of concentrated filtrate was loaded onto a qEV SEC column (Izon Science, UK). 0.2 \( \mu \)m filtered HEPES buffered saline (HBS) was used as the eluting buffer at a flow rate of 1 ml/min. 20 x 1 ml fractions were collected and stored at -80 °C.

NTA analysis of SEC fractions. The concentration and hydrodynamic size of particulates for each fraction was assessed using nanoparticle tracking analysis (NTA) with the Nanosight LM10 instrument (Malvern Instruments, UK). The machine was calibrated with 100 nm polystyrene beads (Thermofisher Scientific, UK) prior to fraction assessment. Measurement specifications were as follows: 532 nm green laser, 5 videos per fraction, 60 second video length, shutter speed of 25-32 ms, camera gain of 400, camera level 15, lower threshold of 910 and higher threshold of 11180. Captured videos were processed using the NTA software version 3.2, a detection threshold of 5, auto settings for blur, minimum track length and minimum particle size. Measurements were carried out in static mode at room temperature.

Protein content analysis. Total protein concentration of SEC fractions were determined using the Pierce micro bicinchoninic acid (microBCA) protein assay kit (ThermoFisher, UK) as per the manufacturer’s instruction (see Supporting Information for details).

Western blot analysis of final isolate. To validate the SEC isolation, exosome
presence was verified through Western blot analysis via capillary gel electrophoresis format, using a WES® instrument from Protein Simple (Biotechnne Ltd, USA). SEC fraction 4 was selected for analysis as it possessed the highest ESP purity of $1.57 \times 10^{10} \text{ESPs/µg of protein}$ (Figure S1). Exosomal proteins Alix (97 kDa) and tetraspannin CD63 (57 kDa) were probed by chemiluminescent immunoassay, using mouse monoclonal anti-Alix (634502, Biolegend UK) and mouse monoclonal anti-CD63 (353013, Biolegend UK) as primary antibodies. The WES run was conducted as per the manufacturer’s instruction (see Supporting Information for details).

**Gold immuno-electron microscopy.** Microscopy images were captured on a Jeol 2100 TEM instrument (Japan). SEC fraction 4 was diluted 100-fold and 20 µl was spotted onto parafilm. A formvar/carbon coated copper grid (200 mesh) was incubated on top of the sample for 7 minutes. The grid was washed 3x with filtered PBS buffer. The grid was then incubated with 20 µl of normal serum block (927501, Biolegend UK), matching the species in which the secondary antibody was generated, for 30 minutes. Grids were washed 3x with PBS. 20 µl of a 1:20 mouse monoclonal anti-CD63 solution (353013, Biolegend UK) in PBS/5% normal serum block was incubated with the grids for 1 hour. The grid was washed 6x with PBS. Thereafter, the grids were incubated with goat anti-mouse IgG conjugated gold nanoparticles (Insight Biotechnology, UK), at a 1:20 dilution in PBS/5% normal serum block, for 1 hr in the dark. The grids were washed 6x with PBS and incubated with 20 µl of 1% uranyl acetate negative stain, mixed with 2% phosphotungstic acid in a 1:10 ratio in DI water, for 5 minutes. Excess dye was removed using filter paper at 45° and grids were then left to dry before analysis.

**QCM-D measurements**

All QCM-D measurements were carried out using a Q-Sense E4 instrument (Bolin Scientific, Sweden). Analysis of frequency and dissipation response was conducted using the QTools software, version 3.0.17.560 (Bolin Scientific, Sweden). Changes in resonance frequency
were recorded from the third, fifth, seventh, ninth and eleventh overtones. Data are presented from the 5th overtone, with variation of \((\Delta f)\) between overtones being 10% or less. In all instances, samples were degassed prior to exchange in the QCM flow module and AT-cut gold coated quartz crystal sensors (Biolin, Sweden) were used.

An optimal antibody immobilisation procedure was first investigated, comparing a covalent and affinity based approach. The covalent approach involved the formation of a self-assembled monolayer (SAM). A 1 mM ethanolic solution of SH-PEG(2 kDa)-COOH and SH-PEG(800 Da)-CH\(_3\) (Laysan Bio, USA) in a 1:3 mixture was flowed across the sensor surface at 7.5 µl/min overnight. Carboxylic acid end groups were activated with an equal parts solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) crosslinker (0.4 M) and N-hydroxysuccinimide (NHS) (0.1 M) in pH 5.5 MES coupling buffer (50 mM) at 7.5 µl per minute. 20 µg/ml of mouse monoclonal antiCD63 (353013, Biolegend UK) was immobilised on the surface at 10 µl/min, in pH 7.4 HBS buffer. Unbound active groups were de-activated with 1 M ethanolamine for 20 minutes at 40 µl/min. This preceded a rinse step and response stabilisation for 30 minutes prior to sample addition.

The affinity based approach utilised an alternative SAM. Here, a 1 mM ethanolic solution of SH-PEG(2 kDa)-Biotin (Laysan Bio, USA) and SH-OEG(800 Da)-COOH (Polypure, Norway) at a 1:9 ratio was flowed across the sensor surface at 7.5 µl/min overnight. A 100 µg/ml solution of streptavidin (SAv) (Sigma Aldrich, USA) was flowed across the sensor surface at 10 µl/min, followed by a rinse step of HBS at 80 µl/min. 20 µg/ml of mouse monoclonal biotinylated-antiCD63 (353017, Biolegend UK) was immobilised on the surface at 10 µl/min, followed by another rinse step and response stabilisation for 30 minutes prior to sample addition.

The affinity based approach was chosen as the method of choice for the following investigations. Immuno-sensor functionality towards spiked CD63 and exosomal CD63 was assessed. Spiked samples of CD63 (Sinobiological, China) of concentrations: 1.25, 2.5, 5, 10, 20, 40, 80, 160 and 320 nM determined the platform’s sensitivity towards the target antigen.
Performance towards exosomal CD63 was initially assessed by measuring responses to SEC fractions 3, 4, 5, 6 and 7, following a 100-factor dilution. Sensitivity towards CD63-positive exosomes was tested using dilutions of fraction 4 from SEC isolations with concentrations being verified by NTA. The following concentrations were assessed: 1x10^8, 2.5x10^8, 5x10^8, 7.5x10^8, 1x10^9, 5x10^9, 1x10^{10} and 5x10^{10} ESPs/ml. Determination of signal-to-noise ratio (SNR) required response comparison with a control sensor. Here, biotin-IgG isotype control antibody (400103, Biolegend UK) was used instead of biotin-antiCD63. Binding response was compared to a target sensor against a 5x10^9 ESPs/ml sample. For the purpose of this work, limit-of-detection (LOD) and limit-of-quantification (LOQ) were defined as the concentration eliciting a SNR of 3 and 10 respectively, as governed by best practice. SNR was calculated by a ratio of the response seen on the target sensor and on a control sensor.

All analytes were prepared using the same HBS stock solution to minimise impact of buffer properties during sample exchange in observed responses. In all cases, the analyte was flowed at 10µl/min and a sensor was reserved for baseline measurement, to account for drift and background changes induced by buffer exchange. Frequency and dissipation responses are reported post-HBS rinse, to account for the removal of weakly bound analytes.

**AFM measurements**

AFM was carried out on ultra-flat gold substrates (Platypus Tech, USA), using a Multimode 8 instrument (Bruker, Santa Barbara, USA), using imaging based on fast force spectroscopy (PeakForce Nanomechanical mapping), with 1 kHz oscillation frequency and 5 nm amplitude. Imaging was conducted with a PF-HR-B probe (Spring constant = 0.12 N/m, Resonance frequency = 100 kHz in air). All imaging was performed in solution. Filtered HBS was used as an imaging buffer. Substrates were incubated overnight in the SAM solution at room temperature. Surfaces were functionalised with identical streptavidin and antibody solutions as used in the affinity approach, by introducing and washing directly within the liquid-cell. 100 µl of 5x10^9 ESPs/ml was introduced into the chamber and incubated for
30 minutes, prior to washing with 1 ml of HBS buffer and image capture.

Images were processed using Gwyddion software (Version 2.5) by first aligning rows using a median of differences correction. Images were then flattened using mean plane subtraction. A 3-pixel (≈6 nm) Gaussian filter was applied to reduce image noise. Height and diameter profiles of particles were taken as an average from 15 detected particles across 3 micrographs.

**Results and discussion**

**SEC isolation of CD63-positive exosomes**

The need for advanced analytical techniques is predicated on reproducible and efficient isolation from complex biological matrices. Moreover, the resulting exosomal samples must possess a high degree of structural and biological integrity. Thus, SEC was chosen as the isolation technique for its low impact on the nanosized vesicles.\(^{38}\)

NTA analysis identified the 4\(^{th}\) out of 20 collected fractions to have the highest yield of ESPs, with \(4.4 \times 10^{11}\) ESPs/ml. The ESP number reduced sequentially in the following fractions, which coincided with an increase in total protein (Figure 1A). Taken together, these results suggest that SEC successfully isolated the large majority of ESPs from contaminating protein in the HUMSCCM source material. Size distribution analysis of fraction 4 confirmed that over 90% of the particles were within the ESP size range (Figure 1B). The isolation protocol was validated by western blot, which identified exosome-enriched proteins, Alix and CD63, consistent with the presence of exosomes in the final sample. As evidenced by immuno-EM analysis (Figure 1C,D), the CD63 protein was shown to be accessible and present at the membrane surface. These results underpin the validity of surface-based immuno-capture of CD63-positive exosomes, as exploited herein.
Immuno-sensor performance towards spiked-CD63

Prior to sensing exosomes, an appropriate antibody immobilisation procedure was investigated. A covalent approach utilising amine-coupling to directly bind anti-CD63 antibody was compared with an affinity driven approach, exploiting the association between SAv and biotinylated-antiCD63. The frequency response during the layer formation of the respective approaches is shown in Figures 2A,B. Figure S2 demonstrates the in-situ overnight formation of the SAM at the sensor surface.
A significant reduction in frequency of the sensor oscillation (∼-11 Hz) was seen for the affinity approach in response to the CD63 sample (Figure 3A). This frequency response is a result of the protein binding to the sensor surface, effectively increasing the crystal thickness and modifying the crystal resonance during thickness-shear oscillation. This was contrasted by a negligible response for the covalently functionalised sensor (∼-0.8 Hz), which represents only a marginally larger frequency shift than found for the control surface functionalised with non-specific antibodies (∼-0.5 Hz). Both of these responses are not significantly different from frequency drift witnessed with the running buffer alone, suggesting almost no CD63 adsorption to the sensor surface.

The poor performance of the covalent approach may be caused by sub-optimal antibody orientation. As discussed by Tan et al., the amine coupling approach has the risk of random antibody orientation via abundantly present lysine residues, often preventing antigen access to the binding site. Conversely, the binding observed with the affinity approach
Figure 3: Immuno-sensing of spiked CD63 protein. (A) Comparative frequency responses to 2 µg/ml of CD63 across antibody immobilisation approaches. Affinity immobilisation appears optimal. (B) Response curve for spiked-CD63 using optimised affinity functionalisation method.

was likely due to superior orientation of antibody via Fc-bound biotin. This is in line with previous studies, which reported benefits of improved antibody orientation, higher binding site availability and improved antigen sensitivity by utilising SAv-biotin interaction for antibody immobilisation as compared to covalent alternatives.\textsuperscript{40,41} Having identified the affinity immobilisation approach as offering optimal performance, this method was employed for the rest of the study.

The performance of the immuno-sensor was subsequently assessed towards exosome-free, spiked CD63 samples (Figure 3B). Our platform displayed a LOD of 3.0 nM and LOQ of 7.9 nM, with a linear dynamic range of 7.9 nM - 160 nM. This demonstrated the high sensitivity of the immuno-sensor towards the target antigen, aligning well with sensitivities reported by other practitioners of acoustic bio-sensing.\textsuperscript{42,43}
Sensitivity and selectivity assessment towards CD63-positive exosomes

For initial screening of CD63-positive exosomes, the various SEC fractions were compared for their acoustic response (Figure 4). QCM-D responses align well with the relative ESP/ml concentrations as identified by NTA (Figure 1A), as frequency reductions were higher for those fractions possessing higher ESP concentrations. ESP-rich fractions 4 and 5 exhibited significant responses (∼-18 and ∼-14 Hz respectively) compared to fractions 3, 6 and 7. These responses are larger than found for fractions 6 and 7, in spite of these latter fractions containing higher total amounts of protein (Figure 1A). These larger responses can be due to the larger size of the adsorbed material causing more liquid at the surface to be moved, a high degree of hydrodynamic co-solvation and/or the greater mass of the fluid-filled vesicles.

Figure 4: Example QCM-D profile responses to eluted SEC fractions. (A) Frequency response to SEC fractions 3-7, and (D) corresponding dissipation profiles.

An important component of acoustic exosome analysis is dissipation. As exosomes are not inherently rigid and prone to deformation, one expects significant friction in the newly formed adlayer during oscillation of the sensor, leading to regions of energy loss. In direct comparison, fractions 4 and 5 exhibited most pronounced dissipative losses of on average 5.2 and 3.9, respectively (Figure 4B). This phenomenon, with similar response magnitude, has been previously reported by groups sensing synthetic vesicles, where the viscoelastic
structures resulted in energy storage (elastic) and loss (viscous) during oscillation. This is of interest, as it provides another discriminating factor to determine whether bound adsorbates are exosomal (vesicular and dissipative) or artefacts (non-vesicular and rigid). Hence, minimal responses seen for fractions 6 and 7 are likely a combination of smaller-sized vesicles expressing CD63, lower ESP concentrations and the relative rigidity of the contaminating protein.

Figure 5: QCM-D immuno-sensor performance against varying concentrations of ESPs. (A) Frequency response curves and example QCM-D profiles. Standard deviation determined from 3 independent experiments. (B) Corresponding dissipation response curves and QCM-D profiles.

Having demonstrated successful immuno-sensing of spiked-CD63 and CD63-positive exosomes, response curves for both frequency and dissipation towards varying ESP concentrations were obtained (Figures 5A,B). LOD and LOQ for the frequency mode of measurement
were found to be $1.7 \times 10^8$ and $8.2 \times 10^8$ ESPs/ml, respectively. The dissipation mode of measurement showed a marginally higher sensitivity, $1.1 \times 10^8$ and $3.3 \times 10^8$ ESPs/ml for LOD and LOQ respectively.

In order to determine the selective nature of our sensing platform, QCM-D responses to ESP samples were compared between a target and a control surface, where the latter was functionalised with non-specific control antibodies (Figure 6A,B). An average SNR of 34.2 and 44.8 was demonstrated by the frequency and dissipation modes of measurement, respectively (with exosomes in the picomolar concentration range). Interestingly, our findings highlight dissipation as a more sensitive and selective measurement over frequency. We propose that sensing CD63 protein as part of a vesicular structure, provides an amplified and sensitive dissipation response, without the need for a secondary probe. The control measurements did reveal some signatures of binding, but this response was nearly overcome upon rinsing with buffer. Therefore, it is likely this binding was weak in nature. Conversely, little change was seen in the response curves of the target sensor during the buffer rinse, suggesting a more robust binding between exosome and anti-CD63.

These QCM-D responses were supported by in-liquid AFM measurements, as particles within the exosome size range were detected (Figure 6D) on the targeted surface, whilst almost no vesicular material was seen to be bound on the substrate functionalised with control-IgG. Detected particles on the target surface had an average height of $60 \pm 17$ nm (mean ± standard deviation, n = 15 particles), however, one must not discount the surface-induced deformation that exosomes undergo upon adsorption, which likely reduces the observed height from the nominal value. Average particle diameter was determined to be $79 \pm 13$ nm (n = 15 particles, thus suggesting that the majority of exosomes expressing CD63 are smaller than the mean (93.7 nm) and modal (86.5 nm) particle size of the ESP sample (Figure 1B), supporting previous findings by Rupert et al. To verify that the responses are not limited by the binding reaction, the flow rate of sample introduction was increased from 10 µl/min to 80 µl/min and 120 µl/min (Figure S3A,B). The increase in adsorption rate
Figure 6: Selectivity of the QCM-D immuno-sensing approach. (A) Example frequency profiles comparing target (anti-CD63) vs control surfaces (IgG control) against 5x10⁹ ESPs/ml. (B) Corresponding dissipation response. (C) Average QCM-D data taken from 3 independent experiments. Student t test (two-tailed) compared responses from the control and target sensor (***p < 0.001). (D) AFM micrographs comparing control and target surfaces post-exosome incubation. Scale bar: 250 nm. Colour scale (height): 100 nm).
was linearly proportional to the cube root of the flow rate, indicating mass-transport limited
binding (Figure S3C).\textsuperscript{47} Thus, the rate limiting step during adsorption was the diffusion of
exosomes to the detection antibody and not the binding kinetics.

The QCM-D results are superior in sensitivity to some commercially available immunoases-
says towards exosomal CD63 by an order of magnitude.\textsuperscript{48} The detection limits demonstrate
similar sensitivity with other recently reported approaches, including interferometric plas-
monic imaging of exosomes.\textsuperscript{49} Only a few detection platforms have shown superior sensitivity
in terms of particle concentration. Ko et. al devised an optofluidic device with sensitivity of
up to \(1.1 \times 10^7\) total particles/ml.\textsuperscript{50} Zhou et al. combined electrochemistry with an aptamer
probe to sense exosomes down to \(1 \times 10^6\) total particles/ml.\textsuperscript{24} Nevertheless, with exosome
counts in native biological samples in the range of \(1 \times 10^8\) to \(1 \times 10^{12}\) particles per ml,\textsuperscript{51} the
acoustic immunosensor developed in this work could reliably detect exosomes from biologi-
cal samples, hence underlining its clinical suitability. Additionally, the process consumes a
mere 100\(\mu\)l for analysis, similar to commercially available ELISAs. Improvements such as
reducing the volume of the sample chamber, tubing dead volume and blocking non-sensing
surfaces by pre-incubation with bovine serum albumin (BSA), can reduce the sample require-
ment down to 0.1-5\(\mu\)l as seen with some SPR and electrochemical set-ups.\textsuperscript{25} Moreover, the
platform offers a dual mode assessment of exosomes, with dissipation describing the stiffness
of the adsorbate relative to the frequency change, lending a superior degree of selectivity to
the procedure.

The SNRs obtained in this work are on par with some SPR based approaches; Liu et. al
reported an SNR of 27 using a higher concentration of exosomes (\(2 \times 10^{10}\) exosomes/ml).\textsuperscript{52}
This is analytically significant as it ensures the platform will not deliver false positive results
as it sufficiently discriminates between CD63-positive vesicles and other colloidal contami-
nants such as albumin and lipoproteins. This can largely be attributed to the PEG based
SAM functionalisation, which decreases the propensity for non-specific protein binding, as
demonstrated in Figure S4.
Beyond serving as a useful quality control tool, the clinical utility of sensing exosomal CD63 has been highlighted by earlier work from Logozzi et al., which demonstrated how CD63-positive exosomes can serve as biomarkers due to their increased abundance in melanoma patients. More recently, Miki et al. identified exosomal CD63 as a potential prognostic marker in gastric cancer. Thus, QCM-D serves as a robust platform which can analytically supplement these research areas, whilst even being applied to other exosomal biomarkers. Additional efforts within this field should focus on two key areas: i) advancing the current sensitivity to match plasmonic approaches, and ii) converting QCM-D responses into quantitative information about exosome concentrations. The former can be achieved through devising novel surface functionalisation with improved binding capacity and exosome entrapment. The latter is met with challenges of current models such as the Sauerbrey or Voigt methods which both assume homogeneous layer formation. The introduction of a formalism which accounts for the discrete and dissipative nature of exosome binding, along with the coupled solvated mass, would only further enhance the analytical insights offered by QCM-D. However, this is a non-trivial task, due to the difficulty in distinguishing between coupled water from the bulk solvent. A possible approach is to replace water with D$_2$O. This would increase the shear viscosity of the bulk liquid whilst not affecting the kinetic or equilibrium state. Hence, discerned differences in frequency between water and D$_2$O allows one to determine mass contribution by the particle and coupled water fractions respectively. Alternatively, combining the QCM-D measurement with optical techniques such as SPR have shown advantages in not being influenced by layer hydration, thus determining the proportion of the QCM-D response which is attributed to the species of interest.

**Conclusion**

This study establishes a QCM-D transduced immuno-sensing approach as a complementary technique for exosome characterisation. Unlike other methods, we exploit a combination of
mass, viscoelasticity and surface antigens of exosomes. This allows sensitive and selective
detection of CD63-positive exosomes without the need of a secondary label. At concentra-
tions of 5x10^8 ESPs/ml we achieved signal-to-noise ratios of 34.2 and 44.8 compared to a
control surface for frequency and dissipation measurements, respectively. With a detection
limit as low as 1.1x10^8 ESPs/ml, acoustic biosensing offers a direct route to phenotypically
subtype exosomes at native concentrations, thus potential to integrate with real-time liquid
biopsies for clinical diagnostics.

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Supporting Information Available

Cell-culture; microBCA protein assay; Western blot; SEC fraction particle purity; Overnight
in-situ formation of SAM; Demonstration of diffusion limited conditions for exosome sensing;
Anti-fouling properties of sensor monolayers.

References

(1) Théry, C.; Boussac, M.; Véron, P.; Ricciardi-Castagnoli, P.; Raposo, G.; Garin, J.;
Amigorena, S. Proteomic Analysis of Dendritic Cell-Derived Exosomes: A Secreted
Subcellular Compartment Distinct from Apoptotic Vesicles. *The Journal of Immunology* 2001, *166*, 7309–7318.

(2) Théry, C.; Zitvogel, L.; Amigorena, S. Exosomes: Composition, biogenesis and function. *Nature Reviews Immunology* 2002, *2*, 569–579.

(3) Van Niel, G.; Mallegol, J.; Bevilacqua, C.; Candalh, C.; Brugière, S.; Tomaskovic-Crook, E.; Heath, J. K.; Cerf-Bensussan, N.; Heyman, M. Intestinal epithelial exosomes carry MHC class II/peptides able to inform the immune system in mice. *Gut* 2003, *52*, 1690–1697.

(4) Kowal, J.; Arras, G.; Colombo, M.; Jouve, M.; Morath, J. P.; Primdal-Bengtson, B.; Dingli, F.; Loew, D.; Tkach, M.; Théry, C. Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. *Proceedings of the National Academy of Sciences* 2016, *113*, E968–E977.

(5) Colombo, M.; Raposo, G.; Théry, C. Biogenesis, Secretion, and Intercellular Interactions of Exosomes and Other Extracellular Vesicles. *Annual Review of Cell and Developmental Biology* 2014, *30*, 255–289.

(6) Coleman, B. M.; Hill, A. F. Extracellular vesicles - Their role in the packaging and spread of misfolded proteins associated with neurodegenerative diseases. *Seminars in Cell and Developmental Biology* 2015, *40*, 89–96.

(7) Ibrahim, A. G. E.; Cheng, K.; Marbán, E. Exosomes as critical agents of cardiac regeneration triggered by cell therapy. *Stem Cell Reports* 2014, *2*, 606–619.

(8) Saman, S.; Kim, W. H.; Raya, M.; Visnick, Y.; Miro, S.; Saman, S.; Jackson, B.; McKee, A. C.; Alvarez, V. E.; Lee, N. C.; Hall, G. F. Exosome-associated tau is secreted in tauopathy models and is selectively phosphorylated in cerebrospinal fluid in early Alzheimer disease. *Journal of Biological Chemistry* 2012, *287*, 3842–3849.
(9) Gauthier, S. A.; Pérez-González, R.; Sharma, A.; Huang, F. K.; Alldred, M. J.; Pawlik, M.; Kaur, G.; Ginsberg, S. D.; Neubert, T. A.; Levy, E. Enhanced exosome secretion in Down syndrome brain - a protective mechanism to alleviate neuronal endosomal abnormalities. *Acta neuropathologica communications* 2017, *5*, 65.

(10) Taylor, D. D.; Gercel-Taylor, C. Exosome platform for diagnosis and monitoring of traumatic brain injury. *Philosophical Transactions of the Royal Society B: Biological Sciences* 2014, *369*, 165–171.

(11) Lin, S. Y.; Chang, C. H.; Wu, H. C.; Lin, C. C.; Chang, K. P.; Yang, C. R.; Huang, C. P.; Hsu, W. H.; Chang, C. T.; Chen, C. J. Proteome profiling of urinary exosomes identifies alpha 1-antitrypsin and H2B1K as diagnostic and prognostic biomarkers for urothelial carcinoma. *Scientific Reports* 2016, *6*.

(12) Gardiner, C.; Ferreira, Y. J.; Dragovic, R. A.; Redman, C. W.; Sargent, I. L. Extracellular vesicle sizing and enumeration by nanoparticle tracking analysis. *Journal of Extracellular Vesicles* 2013, *2*, 1–11.

(13) Vogel, R. et al. A standardized method to determine the concentration of extracellular vesicles using tunable resistive pulse sensing. *Journal of Extracellular Vesicles* 2016, *5*, 31242.

(14) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 1976, *72*, 248–254.

(15) Lobb, R. J.; Becker, M.; Wen, S. W.; Wong, C. S.; Wiegmans, A. P.; Leimgruber, A.; Möller, A. Optimized exosome isolation protocol for cell culture supernatant and human plasma. *Journal of Extracellular Vesicles* 2015, *4*.

(16) Abramowicz, A.; Widlak, P.; Pietrowska, M. Proteomic analysis of exosomal cargo: The challenge of high purity vesicle isolation. *Molecular BioSystems* 2016, *12*, 1407–1419.
(17) Erdbrügger, U.; Lannigan, J. Analytical challenges of extracellular vesicle detection: A comparison of different techniques. *Cytometry Part A* 2016, 89, 123–134.

(18) Lannigan, J.; Erdbruegger, U. Imaging flow cytometry for the characterization of extracellular vesicles. *Methods* 2017, 112, 55–67.

(19) Lee, S.; Zhu, X.; Sha, Q. Recent studies of Kell and XK: Expression profiles of mouse Kell and XK mRNA. *Neuroacanthocytosis Syndromes II* 2008, 7, 107–114.

(20) Ferguson, S. W.; Nguyen, J. Exosomes as therapeutics: The implications of molecular composition and exosomal heterogeneity. *Journal of Controlled Release* 2016, 228, 179–190.

(21) He, M.; Crow, J.; Roth, M.; Zeng, Y.; Godwin, A. K. Integrated immunoisolation and protein analysis of circulating exosomes using microfluidic technology. *Lab on a Chip* 2014, 14, 3773–3780.

(22) Wang, Y.; Yuan, W.; Kimber, M.; Lu, M.; Dong, L. Rapid Differentiation of Host and Parasitic Exosome Vesicles Using Microfluidic Photonic Crystal Biosensor. *ACS Sensors* 2018, 3, 1616–1621.

(23) Daaboul, G. G.; Gagni, P.; Benussi, L.; Bettotti, P.; Ciani, M.; Cretich, M.; Freedman, D. S.; Ghidoni, R.; Ozkumur, A. Y.; Piotto, C.; Prosperi, D.; Santini, B.; Ünlü, M. S.; Chiari, M. Digital Detection of Exosomes by Interferometric Imaging. *Scientific Reports* 2016, 6.

(24) Zhou, Q.; Rahimian, A.; Son, K.; Shin, D. S.; Patel, T.; Revzin, A. Development of an aptasensor for electrochemical detection of exosomes. *Methods* 2016, 97, 88–93.

(25) Doldán, X.; Fagúndez, P.; Cayota, A.; Laíz, J.; Tosar, J. P. Electrochemical Sandwich Immunosensor for Determination of Exosomes Based on Surface Marker-Mediated Signal Amplification. *Analytical Chemistry* 2016, 88, 10466–10473.
(26) Im, H.; Shao, H.; Park, Y. I.; Peterson, V. M.; Castro, C. M.; Weissleder, R.; Lee, H. Label-free detection and molecular profiling of exosomes with a nano-plasmonic sensor. *Nature Biotechnology* 2014, 32, 490–495.

(27) Raghu, D.; Christodoulides, J. A.; Christophersen, M.; Liu, J. L.; Anderson, G. P.; Robitaille, M.; Byers, J. M.; Raphael, M. P. Nanoplasmonic pillars engineered for single exosome detection. *PLoS ONE* 2018, 13.

(28) Rupert, D. L.; Lässer, C.; Eldh, M.; Block, S.; Zhdanov, V. P.; Lotvall, J. O.; Bally, M.; Höök, F. Determination of exosome concentration in solution using surface plasmon resonance spectroscopy. *Analytical Chemistry* 2014, 86, 5929–5936.

(29) Hook, F.; Rodahl, M.; Keller, C.; Glasmastar, K.; Fredriksson, C.; Dahlqvist, P.; Kasemo, B. Dissipative QCM-D technique: Interfacial phenomena and sensor applications for proteins, biomembranes, living cells and polymers. *Proceedings of the Annual IEEE International Frequency Control Symposium* 1999, 2, 966–972.

(30) Li, X.; Song, S.; Shuai, Q.; Pei, Y.; Aastrup, T.; Pei, Y.; Pei, Z. Real-time and label-free analysis of binding thermodynamics of carbohydrate-protein interactions on unfixed cancer cell surfaces using a QCM biosensor. *Scientific Reports* 2015, 5.

(31) Tao, W.; Xie, Q.; Wang, H.; Ke, S.; Lin, P.; Zeng, X. Integration of a miniature quartz crystal microbalance with a microfluidic chip for amyloid beta-Aβ42 quantitation. *Sensors (Switzerland)* 2015, 15, 25746–25760.

(32) Johannsmann, D.; Reviakine, I.; Richter, R. P. Dissipation in films of adsorbed nanospheres studied by quartz crystal microbalance (QCM). *Analytical Chemistry* 2009, 81, 8167–8176.

(33) Uluda, Y.; Tothill, I. E. Development of a sensitive detection method of cancer biomarkers in human serum (75%) using a quartz crystal microbalance sensor and nanoparticles amplification system. *Talanta* 2010, 82, 277–282.
(34) Pirinçci, e. e.; Ertekin, Ö.; Laguna, D. E.; Özen, F. e.; Öztürk, Z. Z.; Öztürk, S. Label-free QCM immunosensor for the detection of ochratoxin A. *Sensors (Switzerland)* 2018, 18, 1161.

(35) Ventura, B. D.; Iannaccone, M.; Funari, R.; Ciamarra, M. P.; Altucci, C.; Capparelli, R.; Roperto, S.; Velotta, R. Effective antibodies immobilization and functionalized nanoparticles in a quartzcrystal microbalance-based immunosensor for the detection of parathion. *PLoS ONE* 2017, 12.

(36) Böing, A. N.; van der Pol, E.; Grootemaat, A. E.; Coumans, F. A.; Sturk, A.; Nieuwland, R. Single-step isolation of extracellular vesicles by size-exclusion chromatography. *Journal of Extracellular Vesicles* 2014, 3.

(37) Shrivastava, A.; Gupta, V. Methods for the determination of limit of detection and limit of quantitation of the analytical methods. *Chronicles of Young Scientists* 2011, 2, 21.

(38) Gámez-Valero, A.; Monguió-Tortajada, M.; Carreras-Planella, L.; Franquesa, M.; Beyer, K.; Borràs, F. E. Size-Exclusion Chromatography-based isolation minimally alters Extracellular Vesicles’ characteristics compared to precipitating agents. *Scientific Reports* 2016, 6, 33641.

(39) Tan, Y. H.; Liu, M.; Nolting, B.; Go, J. G.; Gervay-Hague, J.; Liu, G. Y. A nanoengineering approach for investigation and regulation of protein immobilization. *ACS Nano* 2008, 2, 2374–2384.

(40) Trilling, A. K.; Harmsen, M. M.; Ruigrok, V. J.; Zuilhof, H.; Beekwilder, J. The effect of uniform capture molecule orientation on biosensor sensitivity: Dependence on analyte properties. *Biosensors and Bioelectronics* 2013, 40, 219–226.

(41) Balevicius, Z.; Ramanaviciene, A.; Baleviciute, I.; Makaraviciute, A.; Mikoliumaite, L.; Ramanavicius, A. Evaluation of intact- and fragmented-antibody based immunosensors
by total internal reflection ellipsometry. *Sensors and Actuators, B: Chemical* **2011**, *160*, 555–562.

(42) Hwang, S. S.; Chan, H.; Sorci, M.; Van Deventer, J.; Wittrup, D.; Belfort, G.; Walt, D. Detection of amyloid $\beta$ oligomers toward early diagnosis of Alzheimer’s disease. *Analytical Biochemistry* **2019**, *566*, 40–45.

(43) Chen, Q.; Tang, W.; Wang, D.; Wu, X.; Li, N.; Liu, F. Amplified QCM-D biosensor for protein based on aptamer-functionalized gold nanoparticles. *Biosensors and Bioelectronics* **2010**, *26*, 575–579.

(44) Grieshaber, D.; De Lange, V.; Hirt, T.; Lu, Z.; Vörös, J. Vesicles for signal amplification in a biosensor for the detection of low antigen concentrations. *Sensors* **2008**, *8*, 7894–7903.

(45) Jackman, J. A.; Avsar, S. Y.; Ferhan, A. R.; Li, D.; Park, J. H.; Zhdanov, V. P.; Cho, N. J. Quantitative profiling of nanoscale liposome deformation by a localized surface plasmon resonance sensor. *Analytical Chemistry* **2017**, *89*, 1102–1109.

(46) Rupert, D. L.; Shelke, G. V.; Emilsson, G.; Claudio, V.; Block, S.; Lässer, C.; Dahlin, A.; Lötvall, J. O.; Bally, M.; Zhdanov, V. P.; Höök, F. Dual-Wavelength Surface Plasmon Resonance for Determining the Size and Concentration of Sub-Populations of Extracellular Vesicles. *Analytical Chemistry* **2016**, *88*, 9980–9988.

(47) Van Der Meulen, S. A.; Dubacheva, G. V.; Dogterom, M.; Richter, R. P.; Leunissen, M. E. Quartz crystal microbalance with dissipation monitoring and spectroscopic ellipsometry measurements of the phospholipid bilayer anchoring stability and kinetics of hydrophobically modified DNA oligonucleotides. *Langmuir* **2014**, *30*, 6525–6533.

(48) Logozzi, M. et al. High levels of exosomes expressing CD63 and caveolin-1 in plasma of melanoma patients. *PLoS ONE* **2009**, *4*. 
(49) Zeng, X.; Yang, Y.; Zhang, N.; Ji, D.; Gu, X.; Jornet, J. M.; Wu, Y.; Gan, Q. Plasmonic interferometer array biochip as a new mobile medical device for cancer detection. *IEEE Journal of Selected Topics in Quantum Electronics* **2018**, *25*.

(50) Ko, J.; Hemphill, M. A.; Gabrieli, D.; Wu, L.; Yelleswarapu, V.; Lawrence, G.; Pennycooke, W.; Singh, A.; Meaney, D. F.; Issadore, D. Smartphone-enabled optofluidic exosome diagnostic for concussion recovery. *Scientific Reports* **2016**, *6*.

(51) De Vrij, J.; Maas, S. L.; Van Nispen, M.; Sena-Esteves, M.; Limpens, R. W.; Koster, A. J.; Leenstra, S.; Lamfers, M. L.; Broekman, M. L. Quantification of nanosized extracellular membrane vesicles with scanning ion occlusion sensing. *Nanomedicine* **2013**, *8*, 1443–1458.

(52) Liu, C.; Zeng, X.; An, Z.; Yang, Y.; Eisenbaum, M.; Gu, X.; Jornet, J. M.; Dy, G. K.; Reid, M. E.; Gan, Q.; Wu, Y. Sensitive Detection of Exosomal Proteins via a Compact Surface Plasmon Resonance Biosensor for Cancer Diagnosis. *ACS Sensors* **2018**, *3*, 1471–1479.

(53) Miki, Y.; Yashiro, M.; Okuno, T.; Kuroda, K.; Togano, S.; Hirakawa, K.; Ohira, M. Clinico-pathological significance of exosome marker CD63 expression on cancer cells and stromal cells in gastric cancer. *PLoS ONE* **2018**, *13*.

(54) Reviakine, I.; Johannsmann, D.; Richter, R. P. Hearing what you cannot see and visualizing what you hear: Interpreting quartz crystal microbalance data from solvated interfaces. *Analytical Chemistry* **2011**, *83*, 8838–8848.

(55) Craig, V. S.; Plunkett, M. Determination of coupled solvent mass in quartz crystal microbalance measurements using deuterated solvents. *Journal of Colloid and Interface Science* **2003**, *262*, 126–129.

(56) Höök, F.; Kasemo, B.; Nylander, T.; Fant, C.; Sott, K.; Elwing, H. Variations in coupled water, viscoelastic properties, and film thickness of a Mefp-1 protein film during
adsorption and cross-linking: A quartz crystal microbalance with dissipation monitoring, ellipsometry, and surface plasmon resonance study. *Analytical Chemistry* 2001, 73, 5796–5804.
MSC isolation and cell culture

Umbilical cord tissue was first manually dissected and then enzymatically and mechanically digested to isolate MSCs via plastic adherence to cell culture-treated flasks. Passage-0 cultures were cultured in serum-containing alphaMEM (Gibco) with antibiotic/antimycotic, then expanded subsequently in serum-free, xeno-free medium (Biological Industries) supplemented with 2.5% human pooled platelet lysate (Stemulate, Cook Regentec). Cell culture
supernatant was aspirated, aliquoted and dump frozen at -80°C.

**Total protein assay**

Total protein concentration of SEC isolated EVs was determined using the micro-BCA protein assay (Thermo-Fisher Scientific, UK). Briefly, 5 µl of EV sample was incubated for 15 minutes on ice with 15 µl of RIPA buffer. The sample was then sonicated for 5 minutes, before 10 µl of the lysed solution was pipetted into a 96-well plate. The micro-BCA working reagent was created and incubated with the sample as per manufacturer’s instructions. The final absorbance readings were measured at 562nm.

**Western Blot methodology**

40 µl of deionized water was added to dithiothreitol (DTT) to make a 400mM solution. 10 µl of SEC fraction 4 was lysed in 20 µl RIPA buffer as described above. The lysed solution was then diluted to a working concentration between 0.2-1.0 µg per µl with 0.1X sample buffer, before 4 µl was mixed with 1 µl of loading buffer (fluorescent 5x master mix). The loading buffer was prepared by adding 20 µl of 10x sample buffer, and 20 µl of 400mM DTT solution. Since the detection of tetra-spannin proteins requires non-reducing/native conditions, an additional 20 µl of 0.1X sample buffer was added in place of the DTT solution for these samples. Thus, Alix and CD63 were detected in separate capillaries. The samples and biotinylated ladder were then denatured on a 95°C heat block for 5 minutes, before being briefly centrifuged and loaded onto the detection module assay plate. Anti-alix and anti-CD63 primary antibodies were used at 1:20 and 1:10 dilutions respectively and loaded onto the primary antibody. Corresponding mouse-reactive secondary antibodies were then added, followed by the chemi-luminescent substrate, comprising 200 µl luminol-s and 200 µl peroxide. The fully loaded plate was then centrifuged at 2000RPM for 5 minutes before being inserted into the WES system, in conjunction with a 13-capillary cartridge. Detection and quantification was conducted via a CCD camera and the Compass software, version
3.1.7 (Protein Simple, USA). Anti-Calnexin (W17077C, Biolegend) was used as a negative control to ensure that no cellular protein was present in the chosen SEC fraction.

Figure S1: SEC fraction particle purity, as calculated by a ESP:total protein concentration ratio. Fraction 4 possessed the highest purity of $1.57 \times 10^{10}$ ESPs/μg, hence was chosen as the exosome source in this study unless otherwise stated.
Figure S2: Frequency and dissipation profiles for overnight formation of a SAM using a 1 mM ethanolic solution of SH-PEG(2kDa)-Biotin and SH-OEG(800 Da)-COOH in a 1:9 ratio, at 7.5 µl/min. The decrease in frequency and increase in dissipation upon addition of ethanolic SAM solution from a plain ethanol baseline is indicative of monolayer adsorption and the dissipative nature of the polymer chain now present at the surface.
Figure S3: Verification of diffusion limited conditions through increasing sample flow rate. (A) Frequency and (B) corresponding dissipation response. (C) Computed derivatives plotted as a function of $Q^{1/3}$. Derivatives were fitted with a linear model, with quality of fit determined by $R^2$ as 0.96 and 0.98 for dissipation and frequency respectively.
Figure S4: QCM-D frequency analysis of non-fouling properties for various gold sensor monolayers after testing with 0.1% BSA solution. The SAM used as part of this study (PEG:Biotin / OEG-COOH) was found to have significant protein repellent properties compared to MUA SAM or with SH-PEG-CH₃ as a spacer molecule.
