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Integrated isolation and quantitative analysis of exosome shuttled proteins and nucleic acids using immunocapture approaches

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ABSTRACT

Clinical implementation of exosome based diagnostic and therapeutic applications is still limited by the lack of standardized technologies that integrate efficient isolation of exosomes with comprehensive detection of relevant biomarkers. Conventional methods for exosome isolation based on their physical properties such as size and density (filtration, ultracentrifugation or density gradient), or relying on their differential solubility (chemical precipitation) are established primarily for processing of cell supernatants and later adjusted to complex biological samples such as plasma. Though still representing gold standard in the field, these methods are clearly suboptimal for processing of routine clinical samples and have intrinsic limits that impair their use in biomarker discovery and development of novel diagnostics. Immunoisolation (IA) offers unique advantages for the recovery of exosomes from complex and viscous fluids, in terms of increased efficiency and specificity of exosome capture, integrity and selective origin of isolated vesicles. We have evaluated several commercially available solutions for immunoplate- and immunobead-based affinity isolation and have further optimized protocols to decrease non-specific binding due to exosomes complexity and matrix contaminants. In order to identify best molecular targets for total exosome capture from diverse biological sources, as well as for selective enrichment in populations of interest (e.g. tumor derived exosomes) several exosome displayed proteins and respective antibodies have been evaluated for plate and bead functionalisation. Moreover, we have optimized and directly implemented downstream steps allowing on-line quantification and characterization of bound exosome markers, namely proteins and RNAs. Thus assembled assays enabled rapid overall quantification and validation of specific exosome associated targets in/on plasma exosomes, with multifold increased yield and enrichment ratio over benchmarking technologies. Assays directly coupling selective immobilization of exosomes to a solid phase and their immune- and or molecular profiling through conventional ELISA and PCR analysis, resulted in easy-to-elaborate, quantitative readouts, with high low-end sensitivity and dynamic range, low costs and hands-on time, minimal sample handling and downscaling of a working plasma volumes to as few as 100 μl.

1. Introduction

Use of exosomes and exosome-displayed molecules as diagnostic sensors for the assessment of a functional state of a parent cell in basic and clinical research is supported by increasing body of evidence suggesting their pivotal role in cell-to-cell communication, as well as their biological significance in health and disease [1–3]. Exosome biomarkers are currently the subject of clinical trials but are not yet in routine clinical use [4,5]. Difficulties in exosome isolation constitute a major obstacle to rapid adoption of exosomal markers from the research laboratories to a diagnostic practice, with particular need to ensure reliable, rapid turnaround and cost-effective combination of novel isolation solutions with state-of art diagnostic assets [4,6,7]. Moreover, the lack of standards and poor consensus on different methodological aspects for characterization of exosomes in biological fluids leads to significant lab-to-lab variations in pursued protocols and impairs the comparison of a large amount of data generated in parallel biomarker discovery efforts across different centers worldwide. This partly explains the inconsistencies concerning the molecular

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composition of both canonical and disease specific exosomes reported in wide range of "omics" studies that employed different isolation approaches [6,8,9].

A variety of methods have been so far reported for isolation of exosomes for the purpose of morphologic and biochemical characterization [10–13]. Most of the currently used approaches are based on exosome differential physical properties such as size, density and solubility. Size and density based methods encompass filtration, differential centrifugation followed by buoyant density or ultra-centrifugation, or combinations thereof. A recent research market analysis reveals that the ultracentrifugation is the absolute gold standard for exosome isolation, accounting for over 56% users in the field (GenReports). Methods based on sedimentation are also well established and acknowledged for obtaining fairly enriched exosome pellets, but are time-consuming and require capital equipment and extensive sample handling, being thus poorly suitable for routine clinical practice. Alternative precipitation methods feature the use of water excluding polymers, such as ExoQuick™ (System BioScience) or Exosome Isolation Reagent (Life Technologies). These approaches are simple and economical, but, similarly to filtration and centrifugation methods, have variable yield, interfere with integrity of exosomes and associated proteins and do not guarantee sufficient purity of a sample, potentially affecting some of the downstream analyses. Co-sedimentation of exosomes with contaminants such as co-precipitating vesicles, protein aggregates and complexes (i.e. immune-complexes or vault ribonucleoprotein particles), or simply large and highly abundant proteins (e.g. complement factors, glycoproteins or immunoglobulines), is evident in complex biological samples such as plasma and serum [6,14,15]. Contamination negatively impacts the identification of *bona fide* exosome-associated proteins in LC/MS/MS analysis, and may exacerbate non-specific reactivity causing false positives or blockade of a specific signal in both conventional immunoassays and emerging platforms with advanced detection technologies. Viscosity and complexity of biological fluids that constitute routine clinical samples for trials and diagnostics impose thus different stringency criteria with respect to culture supernatants used extensively to pin down the exosome composition and roles in fundamental studies. The protocols of choice for exosome isolation and characterization are likely to be slightly different depending on the sample type and the research focus.

In this article we focus on the advantages of an immunoaffinity (IA) capture as an exosome isolation approach. Conversely to precipitation based techniques, this method promises more specific exosome capture, with multifold increased efficiency in recovery of highly pure and intact exosomes from complex biofluids, while reducing capital costs and hands-on time. IA addresses the issue of exosome heterogeneity and leverages the unique feature of these vesicles in co-expressing surface markers that reveal their origin and specialized function, including also disease markers of diagnostic relevance. In addition to enabling a selection of specific exosome subpopulations, IA can be easily coupled with immunoassays and molecular diagnostic assays, for direct quantification and analysis of either vesicle-associated proteins or RNAs. Several recent reports describe the use of the innovative devices such as microfluidic [7,16] nanohole patterned chips [17] or monolithic silica tips [18] that integrate IA with the advanced detection technologies in order to profile exosomes in the blood of cancer patients. Noteworthy, also conventional immunoassays such as ELISA, have been proven to be powerful tool for quantification of disease-related exosomes in patient samples and tumor models [19]. Hereby we illustrate the advantage of easy-to-use immunoplate- or immunobead-based assays, which provide cost-effective and reliable tool for exosome isolation and analysis from both model samples (such as culture supernatants) and "real" plasma samples. We address the yield and selectivity with respect to benchmark protocols such as ultracentrifugation and polymer/peptide based precipitation, and evaluate performance of downstream ELISA and PCR assays for a quantitative analysis of exosome proteins and mRNAa/miRNAs.

2. Materials and methods

2.1. Cell supernatants and human plasma samples

Conditioned media and purified exosomes used in this study derive from human tumor cell line cultures, namely Prostate cancer line, LNCaP (cone FGC, ATCC® CRL-1740™) and Colon cancer line HCT116 (ATCC® CCL-247™). Cells were grown in a complete medium (RPMI-1640, Gibco, supplemented by 1% Pen/Strep and 1% Glutamine, Euroclone, and 10% FCS, Gibco) according to ATCC recommendations, without using exosome depleted FCS as we have demonstrated that anti-human antibodies used in this study do not recognize bovine proteins. Human blood from healthy donors or tumor patients was collected in BD EDTA Vacutainer tubes (367525, Lavender cup). Plasma was separated from blood cells by sedimentation at room temperature. Collected human plasma was stored at −80 °C until use.

2.2. Isolation of exosomes by differential centrifugation and ultracentrifugation (UC)

For isolation of exosomes from both supernatants and plasma samples we have used differential (ultra)centrifugation protocol modified from Thery et al. Conditioned medium (CM) was collected from 80% to 90% confluent cells in sterile conditions, with protease inhibitors added at 1:1.000 concentration (Sigma). CM was filtered immediately using a filter unit (Millipore,) with a 0.22 μm membrane to remove intact cells and cell debris. Ultracentrifugation was performed at 120,000×*g* (Sorvall WX ULTRA SERIES, rotor A-641) for 2 h at 4 °C. Pellet was resuspended and washed in 1 ml of cold PBS and ultracentrifuged again (120,000*g*, 2 h, 4 °C). The pellet was finally resuspended in 100 μl of cold PBS and transferred into low binding protein tube and either used immediately or stored at −80 °C.To measure protein concentration of isolated exosomes, 2 μl sample was loaded on a card and concentration was determined using Direct Detect™ (Millipore).

Before sample processing for exosome extraction, human plasma was supplemented with protease inhibitors at 1:500 concentrations (Sigma). Supernatant was transferred in a new tube and centrifuged at 1200*g* for 20 min at 4 °C. Supernatant was carefully transferred in a new tube without pellet contamination and centrifuged at 10,000*g* for 30 min at 4 °C to remove larger vesicles. Samples could be used at this stage as pre-cleared human plasma (unfractioned – UF) or were further diluted with PBS, filtered on a 0.22 μm syringe filter (Millipore) and ultracentrifuged at 110,000*g* (Sorvall WX ULTRA SERIES, rotor F65L) for 2 h at 4 °C. The pellet was resuspended with cold PBS and ultracentrifuged again (110,000*g*, 1 h, 4 °C). The exosomes pellet was carefully dried and resuspended in cold PBS. Exosomes were used as fresh preparation for immunoisolation or conserved at −80 °C. For long term storage and shipment exosome preparations were lyophilized using optimized internal SOP.

2.3. ExoQuick™ precipitation

ExoQuick™ was used according to manufacturer's instructions (System Biosciences). Briefly, conditioned medium, human plasma or serum was mixed with indicated volume of ExoQuick™ solution by inverting the tube. The sample was incubated overnight at 4 °C

after which it was spin-down twice at 1500 g for 30 and 5 min, respectively. The supernatant was discarded and the pellet was resuspended in 50 μl of PBS and stored at −80 °C.

2.4. ME™ for exosome isolation

Samples (cell culture medium, human plasma or serum) were diluted as indicated and used according to manufacturer's instructions (New England Peptide – NEP). Briefly, samples were pre-cleared by centrifugation at 17,000*g* for 7 min to remove micro-particulate matter following by incubation with ME™ reagent (NEP peptide) and mixed for 15 min at RT with rotation. The incubated samples were centrifuged at 10,000*g* for 7 min at RT using a bench-top microcentrifuge. All samples were washed three times with PBS and final pellet was resuspended in appropriate kit comprised buffer.

2.5. Antibodies

The following primary and secondary antibodies were used for exosomes characterization in Western Blot, ELISA and FACS: mAb aCD9, mAb aCD63, mAb aCD81, mAb aCD63-FITC, aCD81-PE, were from Beckton Dickinson, mAb aCD63-biotin (BioLegend), mAb aAlix (Santa Cruz), mAb aTsg101 (Abcam), pAb aRab5 (Santa Cruz), mrAb aRab5 (Epitomics), mAb aCalnexin (Abcam), secondary antibodies anti-Mouse and anti-Rabbit HRP-conjugated (Dako). Proprietary tumor exosomes (HBM1 and HBM2) binding antibodies were provided by HansaBioMed R&D team.

2.6. Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) was performed using a NanoSight LM10-HS microscope (NanoSight Ltd., Amesbury, UK) equipped with a 405 nm laser. Three 60-s videos were recorded of each sample with camera level set at 16 and detection threshold set at 10. Temperature was monitored throughout the measurements. Videos recorded for each sample were analyzed with NTA software version 2.3 to determine the concentration and size of measured particles with corresponding standard error. For analysis, auto settings were used for blur, minimum track length and minimum expected particle size. The NanoSight system was calibrated with polystyrene latex microbeads of 50, 100, and 200 nm (Thermo Scientific) prior to analysis. PBS was used to dilute the starting material. Plasma samples were used at approximate 10.000-fold starting dilution for healthy donor samples with concentrations adjusted in necessary as to specifically fit the optimal working range (20 particles per frame) of the instrument.

2.7. Western Blot for detection of exosome proteins

Samples were resuspended in an appropriate volume of Protein Loading Buffer (Lonza) at indicated concentration. Exosome proteins were separated by SDS–PAGE on precast gels (Biorad). For tetraspanin analysis not reducing conditions were used. Proteins were transferred onto a nitrocellulose membrane (GE Healthcare). Western Blotting was run with primary antibodies against specific exosome markers and the appropriated secondary antibody HRP conjugated as indicated above. Signal is acquired in the dark room using ECL Plus (Amersham Pharmacia Biotech).

2.8. FACS for detection of exosome proteins

Exosome solutions in PBS or plasma samples were incubated with 4-μm-diameter aldehyde/sulfate latex beads (Invitrogen) coated with aCD9 according to a standard protocol [9,20] or with 0.4 μm-diameter immunobeads (HansaBioMed) for 2 h at 4 °C under gentle agitation. After washing in FACS washing buffer, beads bound exosomes were stained with fluorescent antibodies and analyzed on a FACS Calibur flow cytometer (BD Biosciences) using CellQuest software.

2.9. ELISA for exosomes immunocapture and protein quantification

Screening of exosome binding antibodies for immunocapture from cell supernatants and from human plasma was performed in 96 well-plates (Nunc) coated with different antibodies diluted in carbonate buffer (pH 9.6) and blocked with 0.5% BSA and stabilized with 1% sucrose solution. PBS with 0.05% Tween-20 (PBST) was used as washing buffer, while samples were diluted in PBS if and where indicated. Exosome solutions, conditioned media or plasma samples were added to plate wells (final volume of 100 μl) and incubated either overnight at 4 °C or 2 h at RT. Commercially available pre-coated plates for exosome capture (HansaBioMed) were used for exosome isolation according to manufacturer's instructions as well as for further optimization of ELISA assays. Basic protocol typically comprised incubation with a primary antibody (aCD9 or aCD63) diluted in sample buffer (0.5% BSA PBS) for 2 h at 4 °C. After extensive washing, the plate was incubated with HRP-conjugated Secondary Antibody (BioRad) where necessary, diluted in sample buffer for 1 h at 4 °C. Upon addition of the BM Blue POD Substrate (Roche) optical densities were recorded at 450 nm using a microplate reader Infinite® M1000 (TECAN). QuantaBlu Fluorogenic Peroxidase Substrate (Thermo Scientific) was read in a non-stopped mode using excitation/emission maxima of 325/420. SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific) was incubated for 1 min and read in chemiluminescence mode.

2.10. Bead based exosome immunoaffinity capture

Three types of beads were used for comparative analysis as exosome immobilisation tools. Commercial pre-coated latex beads for total exosome isolation were used according to manufacturer's instructions (HansaBioMed). Briefly, each sample was brought to a volume of 1 ml using PBS and incubated with appropriate beads overnight at 4 °C on a rotator. Upon exosome binding, beads were recovered by top bench centrifugation (5000*g* for 10 min) and washed three times with PBS 0.05% Tween-20 (PBST). Alternatively the use of supermagnetic beads was assessed. We compared 1 μm Streptavidin Magnetic Beads (Thermo Scientific, 88816) to submicron sized supermagnetic beads that were customized in-house. Both types of beads were washed with PBS containing 0.05% Tween-20, and functionalized following one hour incubation with 1.2 μg of antibodies (isotype controls and exosome binding antibody, anti-CD9 from Beckton Dickinson). Biotinylated antibodies were used for streptavidin magnetic beads. The beads were next washed three times, blocked with appropriate blocking reagent (casein, BSA or human serum) for 1 h at RT, resuspended in PBS, and added to the sample (either conditioned media or human plasma), to the final concentration of approximately 10^8/ml, for an incubation overnight at 4 °C with rotation. Beads were recovered by magnetophoresis and washed three times with washing buffer (PBS with 0.1% Tween-20, PBST) prior to quantification of exosomal proteins or RNA extraction. Beads were either lysed in Laemmli buffer and boiled prior to Western Blot analysis, or were used as such in the bead-based ELISA. In the latter case, immunobeads were incubated with the primary antibody for 2 h at RT and washed in ELISA washing buffer (see above). Secondary HRP conjugated antibody was next added for 1 h incubation followed by extensive bead washing. To detect the signal, the particles were incubated with BM Blue POD Substrate (Roche) for 10 min. To stop

the reaction, H_2SO_4 2 N was added and optical density was read at 450 nm.

2.11. RNA analysis

Total RNA was isolated from exosome samples using the Exosome Total RNA Extraction Kit (HansaBioMed), phenol-isopropanol precipitation (Trizol, Invitrogen) or Exosome RNA Isolation Kit (Norgen Biotek) according to the manufacturer's instructions. RNA concentration was measured using a UV–Vis spectrophotometer (Nanodrop Technologies) and RNA integrity was evaluated using the Agilent 2100 Bioanalyzer instrument and Small RNA kit (Agilent Technologies). Expression analysis of beta-actin (β-actin) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) messenger RNAs (mRNA) was performed by reverse transcription polymerase chain reaction (RT-PCR). 200 ng of RNA were retro-transcribed with the QuantiTect Reverse Transcription kit (Qiagen) and 20 ng of cDNA were amplified using the Emerald Mastermix (Takara) and the following primers: β-actin FW: AGAAAATCTGGCACCACACC, RW: GGGGTGTTGAAGGTCTCAAA; GAPDH FW: CAATGACCCCTTCATTGACC, RW: TTGATTTTGGAGGG ATCTCG. PCR cycle conditions were: 98 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s for 35 cycles. PCR products were run on 2% agarose gel and visualized using the UVIdoc instrument (Uvitek Cambridge). Expression analysis of human microRNA-21 (hsa-miR-21) was evaluated via quantitative real-time (RT-qPCR). 50 ng of RNA were retrotranscribed using the miScript II RT kit (Qiagen). The 25 μl PCR reaction mix contained miScript Primer assay (2.5 μl, Hs_miR-21_2; Qiagen), QuantiTect SYBR Green PCR mastermix (12.5 μl), cDNA (2.5 μl corresponding to the cDNA reverse transcribed from approximately 6.25 ng RNA), and nuclease free water (5 μl). The 96-well plate was then run on the CFX 96 Real Time System (Bio-Rad) at 95 °C for 15 min, then 94 °C for 15 s, 55 °C for 30 s and 70 °C for 30 s (for 40 cycles).

3. Results and discussion

3.1. Design and validation of an ELISA immunoassay for exosome capture and quantification from human plasma

Enzyme-linked Immunosorbent Assay (ELISA) with a microtiter plate format, is a common technique for basic research and high throughput screening in laboratory and drug discovery programs. This technology is trusty, cost-effective and compatible with different detection systems, with specificity and sensitivity superior to that of microarrays and other multiplex technologies. Exosomes lend themselves ideally to sandwich immunoassays that make use of the advantage of vesicles over traditional soluble biomarkers, in co-expressing multiple antigens. We have developed a double sandwich ELISA that employs pre-validated exosome-displayed molecules for capturing vesicles from diverse biological samples, including human plasma, serum and urine. Immunocaptured exosomes can be quantified and characterized through immunodetection of specific proteins or downstream extraction and amplification of nucleic acids (Fig. 1A). This assay may be used for a range of applications, from a secondary validation of biomarkers of interest to the development of novel diagnostic tools.

Exosome tags for efficient immunoisolation are ideally selected as membrane bound molecules lacking soluble counterparts, that are exclusively expressed or highly enriched on exosomes from specific biological source, and enable sorting of exosomes from other membranous vesicles or fragments, or among different exosome subpopulations. Currently available commercial tools as well as exosome profiling assays reported in literature [7,16,18] rely on the binding of large-spectrum antibodies (e.g. anti-CD9, -CD63, -CD81) the exosome specificity of which has been questioned [18,21,22]. Indeed, most membrane-bound proteins used to identify exosomes, including CD9 and CD63, flotillin, HSP70 etc, are enriched in vesicles with respect to total cell extracts but are also abundantly present on other non-exosome vesicles (Fig. 1C). Expression of most of these proteins can also vary in a tissue- or condition-dependent manner. On the other side, intrinsic heterogeneity of exosomes prejudice identification of a general exosome hallmarks even when released from a single cellular source [23–25]. For instance, only a portion of total vesicles in cell supernatants contains common exosome markers such as tetraspanins CD63 (47%) and CD81 (21%) [24], and the molecular diversity of circulating exosomes is likely to be further exacerbated by their mixed cellular origin. In this context, effective combinations of antibodies, rather than single-antibody approaches, are likely to improve exosome isolation and analysis.

We have identified several proteins related to exosome biogenesis and function that are exclusively expressed on exosomes and designed microplates functionalized with antibodies selected for exosome binding (Fig. 1B). In particular, Rab5 and other Rab GTPases [13,19] are an example of a class of proteins that have provided a paradigm for exosome identification. In this study we have also used a commercially available exosome-capturing microplate (HBM; HansaBioMed) that employs a combination of antibodies tested for the immobilization of purified exosomes from cell cultures and from human plasma as confirmed by the detection of CD63 or CD9 (Fig. 1B and D). Noteworthy, each immunoassay measured a concentration-dependent signal from exosomal pellets obtained through microfiltration and differential (ultra) centrifugation (Fig. 1B). Conversely, hardly any signal was detected from the two fractions corresponding to microparticles (MP) and cell membrane debris, despite the strong expression of CD63/CD9 in the same MP fraction as measured by Western Blotting (Fig. 1C and D). These data suggest that microplates, if coated with the appropriate combinations of antibodies, can selectively bind exosomes, readily enabling their linear quantification from both cell supernatants and human plasma.

A different trend was observed when comparing the intensity of CD63 and CD9 signal from the immobilized exosomes previously enriched by ultracentrifugation of 1 ml of supernatant or 0.5 ml equivalent of human plasma, and from the same sample directly loaded onto the plate (100 μl of precleared plasma sample/well), (Fig. 2A). The signal obtained from supernatant-derived exosomes was proportional to the starting volume from which exosomes have been precipitated (the signal obtained for exosomes purified from 1 ml of supernatant corresponds to a direct capture from 100 μl of 10× concentrated sample) consistent with reports indicating ultracentrifugation as an efficient method for exosome isolation approach from cell supernatants.

Conversely, direct capturing of exosomes in plasma samples resulted in an overall improved signal-to-noise ratio if compared to capturing after ultracentrifugation of the same samples (Fig. 2B). In fact, the signal obtained directly from 100 μl of plasma was equal or higher than the signal measured from exosomes obtained with ultracentrifugation of 0.5 ml of the same sample, indicating that ultracentrifugation may not be an effective approach for isolating exosomes from viscous and complex samples [26,27].Overall, the plate-based immunocapture provided a time- and effort-saving approach to isolate and quantify exosomes from both sample types, with a clear advantage over ultracentrifugation when plasma samples were used.

Besides the identification of a suitable immunoligand for exosome binding, typically an antibody or mix thereof, the complexity of both plasma as a biological matrix and of exosomes as carriers of multiple target antigens poses technical challenges to an efficient and specific exosome capture and a reliable detection of associated

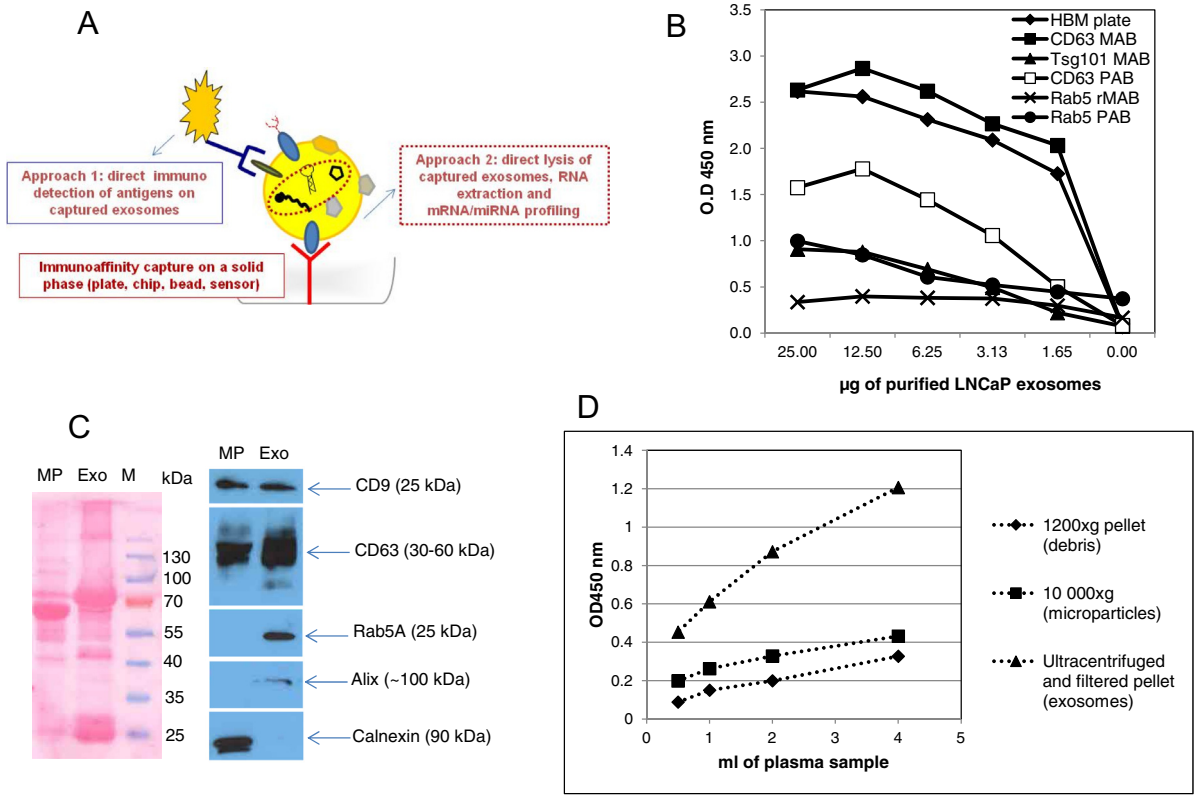

Fig. 1. Optimization of tools for specific exosome immunoisolation. The scheme of a double sandwich assay for targeted capture and identification of exosomal biomolecules. Once exosomes are captured on a solid phase either direct immunodetection of target proteins can be performed, or captured exosomes can be lysed and overall associated RNA extracted for further analysis of mRNAs and miRNAs of interest (A). Comparative screening of exosome tags and relevant antibodies for identification of best capturing agent for recovery of overall exosome population from the sample. Linear dilutions of purified exosomes are loaded on precoated plates (B). Coating antibodies are used at previously determined plate saturation concentrations (up to 1 µg/ml) and CD63 detection used for bound exosome quantification. The effective enrichment of acknowledged exosome proteins – CD9, CD63 and Rab5 in exosomes (exo) and microvesicles (MV) pellets obtained by differential (ultra) centrifugation was analyzed in Western Blot (C) and ELISA (D). Ponceau staining shows total amount of material in different fractions. Exosomes, MV and debris pellets loaded onto the microplate (HBM) were obtained through differential centrifugation from healthy donor plasma, starting from sample volume in a 0.5–4 ml range, resuspended in 100 µl of PBS and loaded onto the plate (D).

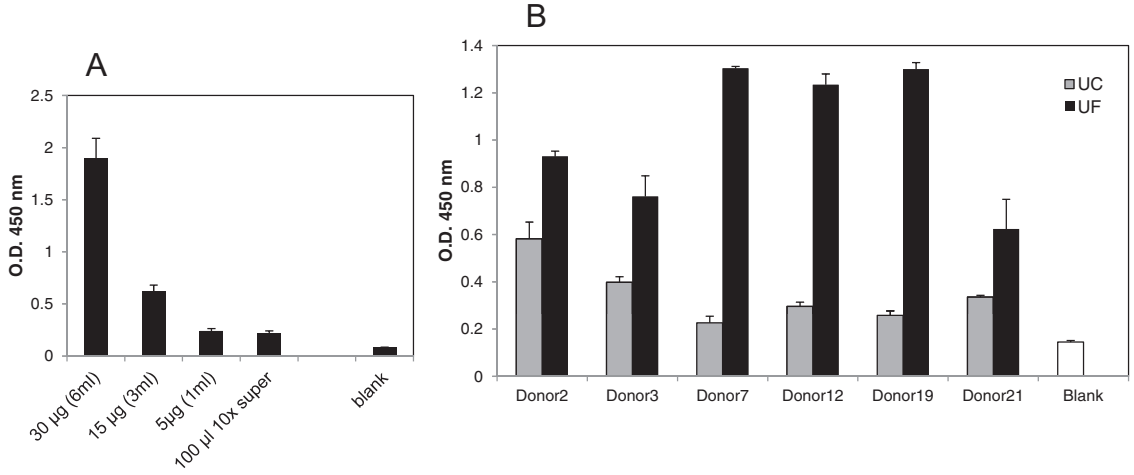

Fig. 2. Comparison of a microplate-based exosome immunocapture efficiency from cell supernatants and human plasma. Exosomes from LNCaP cell supernatants were purified by differential (ultra)centrifugation and loaded onto the exosome-immunocapture plate (A). Amount of loaded exosomes (protein content) is indicated as well as the correspondent volume of supernatant. Alternatively, conditioned supernatant was concentrated 10× and loaded directly onto the plate. The CD63 signal observed in sandwich ELISA was dose-dependent. Direct capture from 100 µl of unfractioned human plasma (UF) appeared more efficient with respect to purified exosomes obtained by ultracentrifugation (UC) from 500 µl of starting plasma volume, as revealed by CD9 measurement. Plasma was pre-cleared prior to loading or ultracentrifugation by two low speed centrifugation steps (1200×*g* and 10,000×*g*). Presented data are representative of at least three independent experiments and are reported as mean ± standard deviation of 3 technical replicates.

proteins. The contamination of vesicle preparations with other plasma components, typically abundant plasma proteins, complement factors and immunoglobulins, has been reported as an artifact of different purification methods, most prominently those based on chemical precipitation and sedimentation [6,14,28]. Moreover, it has been suggested that circulating exosomes might have natural affinity for immunoglobulins [15], promoting their co-precipitation, but also possibly mediating non-specific binding of antibodies in *in vitro* assays. In addition, exosomes have also proven to be "sticky" to most of commercially available plasticware, as well as microplates and beads featuring conventional immunoaffinity supports. Accordingly, we have observed significant binding of purified exosomes to IgGs and IgMs, and to different surfaces functionalized with irrelevant antibodies such as anti-FLAG, or isotype-matched control antibodies (not shown). In this scenario, surface chemistry and fine optimization of blocking and washing steps, are extremely important to ensure both the highest yield and specificity of exosome recovery (Supplementary Fig. S1). For instance, we found that purification of plasma exosomes via ultracentrifugation increases the background signal in downstream immunoassays due to a non-specific binding of secondary antibodies to plasma exosomes (Fig. S1A); however, selection the appropriate blocking reagents (bovine serum albumin; BSA 2% of human serum) abolishes the non-specific portion of the signal. Consistent with our previous data, spurious signals can also be avoided by capturing exosomes directly from plasma (Fig. S1C). This is a second point in favor of immunocapture approach to isolation and characterization of plasma exosomes. Finally, the optimization of a coating chemistry, and customized industrial coating of capture microplates, employing covalent binding of antibodies and suitable inactivation/blocking steps, leads to the increased specificity of antibody-based immobilization, decreased antibody consumption and improved linearity of quantification of exosome displayed proteins (Fig. S1B).

In this article, we have deliberately expressed ELISA results as a function of absorbance (optical density at 450 nm) values as to rapidly compare the expression of known surface markers CD63 and CD9, as an immediate readout of yield and specificity of isolated exosomes. By implementing the assay calibration using standards with known exosome amounts, we can easily extrapolate absorbance values to quantitative units such as overall amount of exosomes, corresponding particle number or an amount of particular protein of interest (Fig. 3A). Noteworthy, this ELISA assay enables a linear titration of exosomes from the sample while NTA (NanoSight) used as independent vesicle counting method, fails to track the sample dilutions in the same linear range (Fig. 3B). In the latter case, samples were prepared and assessed as to fit the working range of LM10 instrument. Noteworthy, clinically relevant alterations of disease biomarkers are often in linear range.

3.2. Exosome immunocapture microplate ensures efficient exosome isolation from small volumes of minimally handled plasma samples with improved yield over precipitation and centrifugation based methods

Next, we have evaluated the specificity and yield of exosome capture by plate-based immunoaffinity approach with respect to ultracentrifugation and a polymer based precipitation (ExoQuick, System Bio). Though we work mainly with plasma, in order to use the conditions recommended for optimal performance of used reagents, for experiments with ExoQuick we have used in parallel serum and plasma samples from the same donor. While ultracentrifugation typically yielded a pellet containing 10–30 μg of total exosome proteins, ExoQuick pellet had a protein concentration of about 10 mg/ml. Considering the overall serum protein concentration (60–80 mg/ml, [29]) this high yield was clearly due to a huge amount of water-insoluble complexes, only partially derived from exosomes. The pellet was rather difficult to solubilize which significantly hampered its analysis in Western Blot (Fig. 4A). We loaded equivalent amounts of ExoQuick- and ultracentrifuge-derived pellets and found little or no expression of the typical exosomal markers in the ExoQuick fraction, indicating that the high yield observed with the polymer-based precipitation was mainly due to spurious protein complexes. Since this method alters the chemical properties of soluble serum complexes, we cannot exclude that some exosomal proteins may also be affected and therefore not recognized by the antibodies of interest.

When the material obtained by precipitation and ultracentrifugation was analyzed on the exosome binding microplate (Fig. 4B), quantification of exosomes in both samples gave similar results despite discrepancies in the overall protein contents as determined by BCA assay. Samples obtained through ExoQuick treatment and ultracentrifugation (UC) from 0.5 ml of serum gave the same CD63 readings, while higher signal was measured for exosomes purified from the same donor's plasma. As expected, the signal for UC plasma exosomes was decreased upon incubation overnight (ON) at +4 °C as to mimic incubation conditions used in ExoQuick precipitation protocols. This is consistent with our prior observation on the impact of exposure to different temperatures on the preserving of exosome proteins. Lyophilization of purified exosomes highly improved the detection of signal in the serum-derived sample, consistent with our observations and SOPs confirming the improved long term stability of exosome proteins in this physical state. These data corroborate the importance of purification and storage conditions for ELISA detection of exosome-derived antigens.

Despite the ExoQuick precipitated sample contained a mix of serum proteins also beyond exosome fraction, our experiments confirm the ability of an immunoaffinity plate to "clean up" the complex sample by selectively binding exosomes. This immunoisolation approach is thus less effected by the presence of other proteins and impurities in the sample. Importantly, when the plasma sample from the same donor was loaded and incubated directly onto the plate, the CD63 signal detected was similar to the signal detected from UC-purified exosomes from 5-fold higher plasma volumes, indicating the superior efficiency of the direct immunocapture over UC (Fig. 4B). It is also important to note that, before any isolation approach, we pre-cleared plasma/serum samples by low speed centrifugation to remove debris and large(r) microparticles and focus on the nanovesicle fraction.

In this study we are reporting a conventional colorimetric ELISA assay with sensitivity for common exosome proteins down to sub-picogram levels (Supplementary Fig. 2). The detection limit assessed by comparison of a detected signal to that of a recombinant CD81 protein immobilized on the plate, was below 1 pg per well (<3.5 pg/ml) that corresponded to 0.1 μg (1 μg/ml) of total exosome proteins, or $2 * 10^8$ of total exosomes ($2 * 10^9$ exosomes/ml). The sensitivity was ultimately dependent on the HRP substrate used for assay development (Supplementary Fig. 2) and the choice was determined by sample type. While potentiated chemiluminescence substrate (Super signal) was indicated for detection of exosomes from cell supernatants, with over 100-fold increased sensitivity in comparison to a colorimetric substrate (TMB), the best signal-to-background ratio in plasma samples was obtained with fluorescent substrate Quanta Blue (5-fold over TMB, and 2.3-fold over ECL; Supplementary Fig. 2).

Low-end sensitivity and dynamic range of a described assay fits well to an estimated concentrations of plasma exosomes. According to our measurements using nanoparticle tracking analysis (NTA) and consistent with the literature reports, the concentration of circulating exosomes in the healthy subject plasma can vary in the 10^8 to 10^{10} particles/ml range, while these values can be

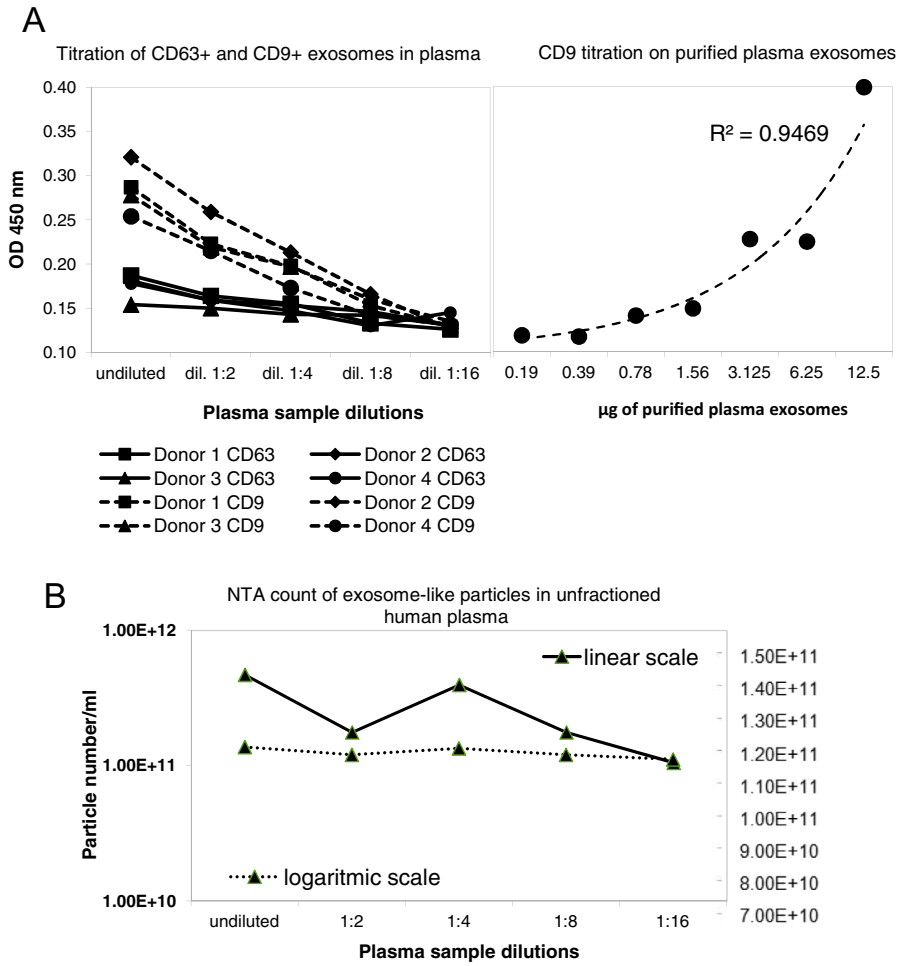

Fig. 3. Exosome titration in human plasma. (A) Precleared human plasma (UF) from four healthy donors was serially diluted and directly loaded on functionalized exosome isolation microplate (HBM), 100 µl per well. Bound exosomes were detected with aCD9 and aCD63 antibodies (A, left). Calibration curve was generated using known protein concentrations of purified exosomes from human plasma pool (from healthy donors), employing the same microplate and detection with anti-CD9 (A, right). (B) In parallel, the same serial dilutions of human plasma were analyzed with NTA (NanoSight) employing the settings that grant the optimal counting of exosome sized particles.

increased 10–1000 times, up to 10^{14} particles/ml in plasma samples from cancer patients. If we consider theoretical mass of a single exosome to be $0.5 * 10^{-6}$ ng, based on its size [25], these values fairly fit the number of exosomes (10^{10}/ml) calculated from an average protein content of exosomes recovered from healthy subjects plasma. Nevertheless, disease specific biomarkers are likely to be associated to only a minor portion of circulating exosomes, that remain extremely diluted in overall blood. Further efforts are needed to increase the selectivity of isolation and the sensitivity of detection methods in order to meet stringent requirements for development of exosome based *in vitro* diagnostics.

3.3. Immunobeads are versatile tools for exosome isolation and downstream analysis

Compared to the plate surface-based capture, the immunoprecipitation using beads as solid phase for affinity binding has higher capture efficiency and increased sensitivity due to the larger surface area. The incubation of functionalized beads with solutions containing exosomes under rotation provides privileged conditions for interaction between vesicles and ligands immobilized on the beads. Moreover, bead-based protocols do not impose the higher limit to the starting sample volume and can be easily scaled-up or down. These advantages prompted us to transfer the sandwich assay principle to bead-based assays for streamlined exosome isolation and rapid quantification of associated molecules. A variety of beads are available on the market, most of which are in micron-size range, representing a popular tool for macromolecules separation from complex matrices as well as for automated *in vitro* diagnostic applications. Some of these have been already adopted for exosome isolation [2,4]. We have tested beads with different properties, including size, material, surface coating and, eventually, magnetic content.

Latex beads are commonly used for passive absorption of purified exosomes for FACS analysis [30]. These beads are cheap, easy to recover from the solution by bench top centrifugation and with well established functionalization protocols. Based on this experience, we have first tested latex beads ranging from 0.4 to 4 µm in size. Similarly to optimization of exosome binding to the plate surface, buffers and in particular surface chemistry were carefully selected in order to minimize bead aggregation, and non-specific binding of contaminants from biofluid matrix. Western Blotting analysis showed almost exclusively antibody-(aCD63 and aCD9 respectively)-mediated capture and antigen detection while no signal was measured from empty beads or beads functionalized with isotype-matched control antibodies in both concentrated supernatants and human plasma (Fig. 5A). The smaller beads size (0.4 µm) improved the specific binding but interfered with efficient bead recovery from the solution (Fig. 5A). The use of magnetic beads facilitated the recovery of bound material and simplified the

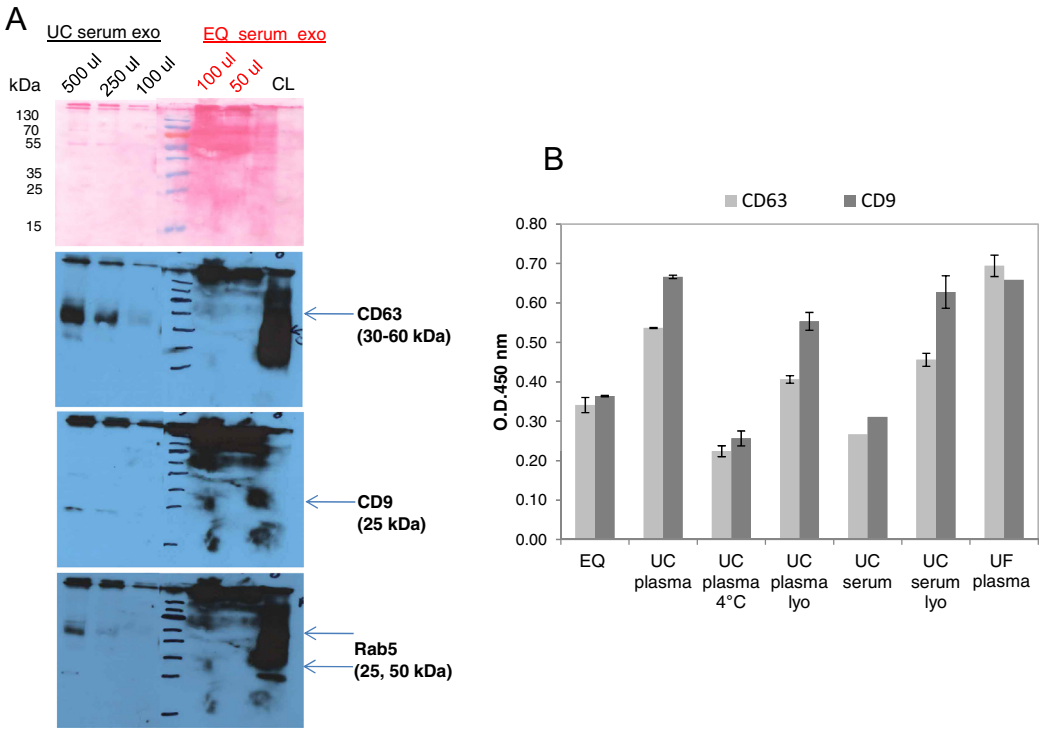

Fig. 4. Detection of exosomal markers on exosomes purified from human plasma/serum by ultracentrifugation, ExoQuick precipitation or direct immunoplate capture. Plasma exosomes were purified from different volumes of human serum by ultracentrifugation (UC) or by ExoQuick (EQ) and analyzed in Western Blot. The nitrocellulose membrane was stained with Ponceau to check the total protein concentration (upper panel) and then blotted with aRab5, aCD63 and aCD9; 30 μg of LNCaP cell lysate (CL) was used as a control (A). Exosomes purified by ultracentrifugation and ExoQuick (UC and EQ) from 500 μl of serum and plasma samples from the same donor were loaded onto the immunoplate (HBM), incubated over-night (ON) and analyzed in a sandwich ELISA for CD63 expression. The signal obtained was compared to that obtained when 100 μl of the same (plasma) sample was precleared (see above) and directly loaded and incubated in the well (UF plasma). UC samples were either lyophilized, or stored at 4 °C ON prior to analysis. Presented data are representative of at least three independent experiments, performed with pooled human plasma samples from healthy subjects and are reported as mean ± standard deviation of 3 technical replicates.

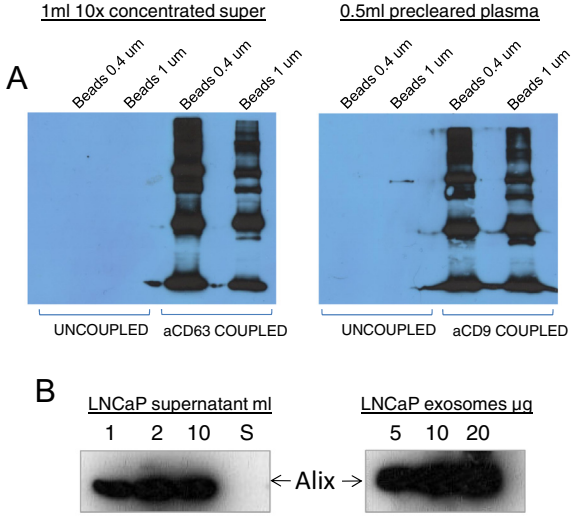

Fig. 5. Use of immunobeads for exosome capture from cell supernatants and human plasma. Two different size of latex immunobeads coupled with anti-CD63 or -CD9 were incubated with 1 ml of 10 times concentrated (spin concentrator, Millipore) LNCaP cell culture supernatant or 500 μl of precleared human plasma respectively (A). Immunoprecipitated material was analyzed by Western Blot (WB) with aAlix to verify the presence of exosomes. Uncoupled beads were used as controls for capture specificity. Submicron size magnetic Beads (SMB) developed in house were incubated with 1, 2 and 10 ml of cell culture supernatant and the Alix signal in immunoprecipitates compared to that in exosomes obtained from the same sample volumes by ultracentrifugation (B). Immunoprecipitation from supernatant after ultracentrifugation (S) failed to reveal any signal.

protocol for both isolation and downstream detection of exosome associated proteins. We have compared commercially available beads such as Dynabeads (Life Technologies) and Streptavidin Magnetic Beads (Pierce) to submicron size supermagnetic beads (SMB) developed in house. SMB could be applied to as few as 1 ml of supernatant sample (Fig. 5B), with capture efficiency comparable to the ultracentrifugation protocol. The fair efficacy of ultracentrifugation for processing of cell media was also confirmed by the lack of any signal for Alix following immuno-pull-down of post-ultracentrifugation supernatant (Fig. 5B). Alix was used throughout this study as an acknowledged exosome specific protein although we acknowledge the presence of exosomal subpopulation that are originated via non ESCRT dependent pathway and thus might lack Alix expression [10]. Despite similar yield, immunoprecipitation still has the advantage of being rapid (over-night incubation can be reduced to few-hours time), easy and cost-effective, and compatible with routine bench equipment. More remarkable advantages are observed when recovering exosomes from plasma where the yield obtained from pulled-down material was 10- to 15-folds higher than ultracentrifuged pellets, as measured by detecting Alix expression in Western Blot, (Fig. 6). This result is consistent with some independent reports describing low recovery rate using ultracentrifugation (5–25%) for processing plasma and serum samples [26,27], while conversely to others [6], we report Alix detection on small volumes of plasma exosomes after either centrifugation or immunoprecipitation. FACS analysis further confirmed multifold increase of exosomes identified as positive for CD63 and CD81 upon direct incubation of plasma samples with functionalized beads, in comparison to exosomes purified via ultracentrifugation from the same

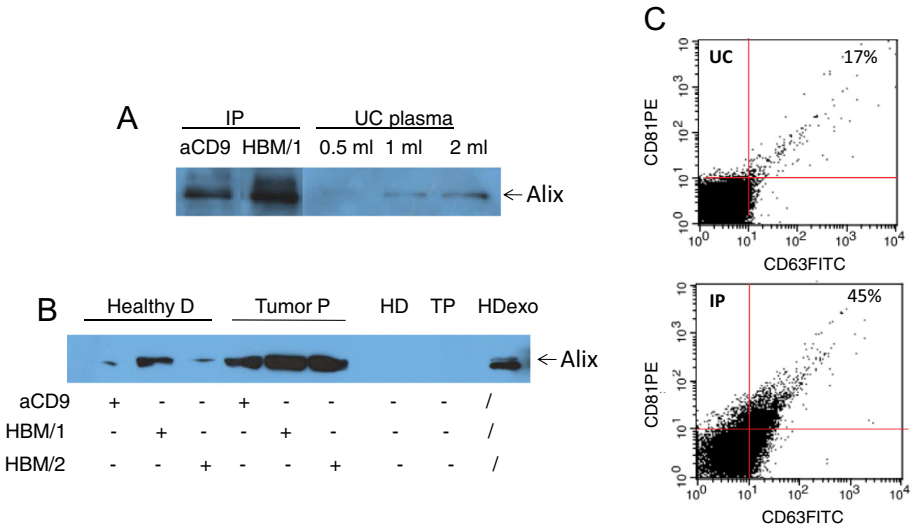

Fig. 6. Immunocapture of exosomes from human plasma shows multifold increase of separation efficiency vs. ultracentrifugation. Exosomes are extracted from pooled sample of human plasma using standard ultracentrifugation protocol (UC) or were immunoprecipitated (IP) using 0.4 μm latex beads coupled with anti-CD9 antibody, or specific exosomes binding – and tumor exosome-binding antibodies (HBM/1 and HBM/2) (A). Pulled down material (IP) from 500 μl of plasma was compared for Alix content to exosomes extracted from different volumes of the same plasma pool by ultracentrifugation (UC). Selective enrichment of exosomes in different samples (healthy donor HD vs tumor patient TP) was dependent on the capture antibody used (B). Plasma exosome isolation efficiency of IP approach with respect to ultracentrifugation was also assessed in FACS. 0.8 μm latex beads coupled with aCD9 antibody were used to pull down CD63+/CD81+ exosomes directly from 500 μl of pre-cleared plasma (C).

sample (Fig. 6C). Another distinctive advantage that immunoaffinity approach has over other purification methods is the possibility to selectively isolate or enrich in exosome subpopulations with specific molecular profile, presumably corresponding to a distinct cellular or subcellular origin. For instance, the use of the beads, functionalized with different antibodies, ensures differential capture of exosomes from healthy and tumor patient's plasma (Fig. 6B). For this purpose we have tested two antibodies from HansaBioMed pipeline that have been generated and tested for binding to specific tumor-over expressed molecules which association to tumor cell derived exosomes has recently been demonstrated (unpublished data).

Overall comparison of ultracentrifugation, peptide based precipitation (ME™ kit, NEP) and aCD9 immunobeads confirms the immunoisolation as a method of choice for plasma exosomes recovery, as shown by Alix content detected in Western Blot (Fig. 7). Pre-treatment of a sample with thrombin prior to incubation with beads significantly improved the yield, likely due to the release of vesicles previously entrapped in fibrin structures. On the other side, it also promoted co-precipitation of large amounts of plasma proteins as evident from gel Ponceau staining. Curiously, we found no correlation between the number of particles/ml measured by NTA before and after exosome precipitation (residual supernatants) by NTA, and the corresponding protein amount in WB. The rate of recovery for ultracentrifugation, ME and aCD9 IA resulted pretty similar (21%, 16.4% and 26%, respectively). Such discrepancy may be explained by intrinsic technical limitations or by the bias introduced when using Alix for exosome identification.

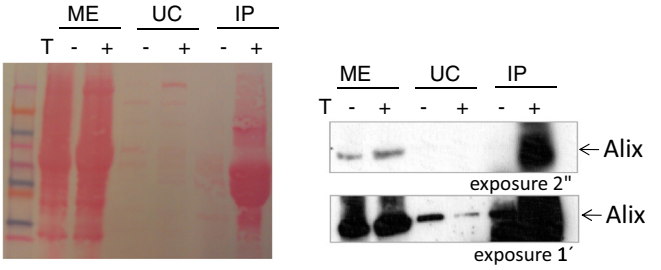

Fig. 7. Comparison of peptide based precipitation (ME), ultracentrifugation (UC), and immunoprecipitation (IP) for exosomes isolation from human plasma. Exosomes were purified from the same volume (1 ml) of human plasma with three technologies: ME™ kit (ME), ultracentrifugation (UC) and immunobeads (IP). Before isolation sample was treated with thrombin (T) where indicated and isolated exosomes were analyzed by Western Blot. The nitrocellulose membrane was stained with Ponceau (left) to check the total protein concentration and then blotted with anti-Alix (right) to detect exosome specific signal. The film was exposed for 2 s or for 1 min as to better evidence the differences in the protein amount in different lanes.

3.4. Immunobead-based ELISA-like assays for quantification of exosome associated proteins

We have further assessed the feasibility and performance of a bead-based ELISA. We used magnetic immunobeads to collect and concentrate exosomes from a 0.5 ml pre-cleared plasma samples prior to quantify the expression of common exosome proteins (e.g. CD9) in a sandwich assay. As expected, increasing amounts of exosome proteins were detected in comparison to plate-based capture from 100 μl of the same plasma sample (Fig. 8A). These beads showed linear titration of exosomes from serially diluted plasma (Fig. 8B). The signal was largely antibody-mediated as demonstrated by the low signal observed from empty (non-coated) beads (Fig. 8B). Comparison between plate- and bead-based ELISA performed on plasma samples spiked with increasing amounts of exosomes purified from LNCaP cultures showed good linearity, signal recovery and sensitivity (Fig. 9A and B). CD63 was chosen as highly expressed marker on LNCaP exosomes but not on plasma exosome (our observation). Western Blot analysis for Alix showed that immunobeads have bound to exosomes, and the signal intensity well correlated to the signal detected by the immunoassay (Fig. 9C).

In summary, bead-based assays allow simple and rapid protocol, diminishing hands-on and turnaround time (<4 h) in comparison to the plate-based ELISA. We are currently working on further improving the bead-based exosome capture and display as a complementary and/or integral step of ELISA-like

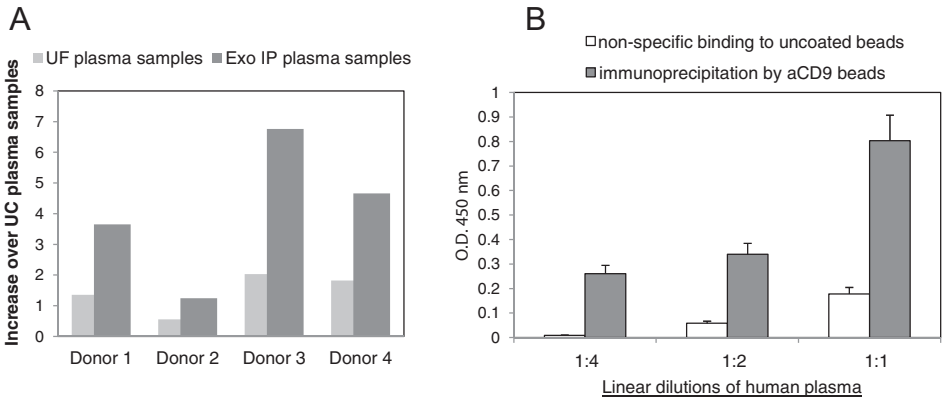

Fig. 8. Immunobead-based ELISA like assays for exosome immunoisolation and quantification from human plasma. We used in-house customized magnetic immunobeads to collect and concentrate exosomes from 500 μl of precleared plasma samples from four healthy donors, following by direct quantification of CD63+ exosomes. An increased amount of exosome proteins were detected with respect to immunoplate based capture from 100 μl of the same plasma sample (A). Bead-based assay enabled linear titration of exosomes from serially diluted plasma with only a small portion of CD9 detected signal due to unspecific exosome binding to empty beads (B). Presented data are representative of at least three independent experiments, performed with human plasma samples from healthy subjects and are reported as mean ± standard deviation of 3 technical replicates.

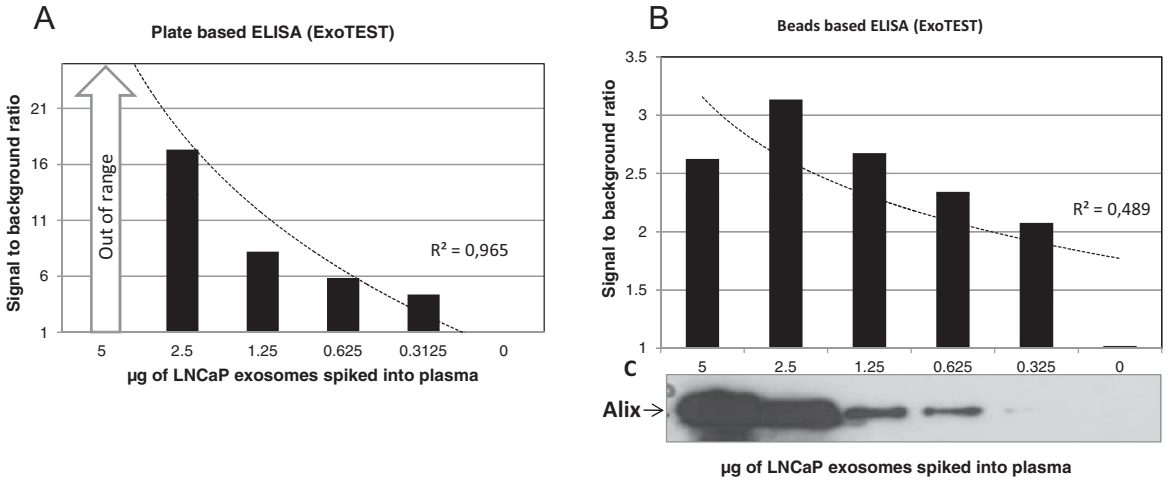

Fig. 9. Comparison of linearity, signal recovery and sensitivity of the plate- and bead-based ELISA assays for exosome quantification in human plasma. Signal recovery experiments are performed on healthy donor plasma samples spiked with increasing amounts of exosomes purified from LNCaP cultures detected with aCD63 antibody (A, B). Precoated immunoplates (A) or immunobeads (B) for overall exosome capture were used in parallel in two independent experiments. Western Blot analysis of bead-bound exosomes (C) correlated to a CD63 signal detected in an immunoassay.

immunoassays in order to increase dynamic range and low-end sensitivity (∼pg/ml of target protein concentration).

3.5. Exosomal RNA extraction and analysis following immunoplate- or bead-based isolation

We have next addressed the advantages and/or disadvantages of using microplates or microbeads for exosome capture and direct extraction and analysis of exosome-derived RNA. We compared the yield, composition and quality of RNA extracted from immunocaptured exosomes vs. exosomes prepared by ultracentrifugation. To determine the best extraction protocol, we have tested different approaches based on phenol-isopropanol precipitation (Trizol, Invitrogen), and column-based technique (HansaBioMed Exosome RNA extraction kit) as well as an alternative exosome isolation followed by RNA extraction solution including RNA binding columns (Norgen Biotek). The yield and purity of RNA extracted from exosomes immobilized on a single microplate strip (thus starting from 800 μl plasma volume) was determined by spectrometric measurement (Nanodrop) revealing the multifold increase of total RNA yield when immunocapture plate was used as integral part of a HBM kit (average for 8 individual plasma samples processed with 2 technical replicates – 920 ng/ml plasma), in comparison to the same immunoplate combined with Trizol (212 ng/ml). Parallel extraction with the Norgen Biotek kit gave 107 ng/ml. These results confirm that although conventional RNA extraction tools can be applied to exosomes independently on the isolation method, specific adjustment of downstream lysis and RNA release/capture steps are important to maximize exosome RNA recovery. In addition, the purity of RNA obtained by integrated immunocapture and RNA extraction solution also appeared high in comparison to the other methods used while avoiding phenol or DNA contamination. These results were consistent with RIN values and RNA size distribution from differently processed samples data obtained using the Agilent Bioanalyzer (Fig. 10A). Total RNA extracted by using different methods was loaded on chips for both total RNA and small RNA Agilent analysis. Consistently with expected composition of exosomal RNA, no contamination with cellular RNAs was observed as no signs of 18S and 28S rRNA peaks were observed. Low mRNA content was revealed (RIIN 2.5), with an enrichment in small RNAs, in particular with HBM kit processed samples (up to 87%). Low RNA yield obtained by Norgen Biotek did not allow reliable profiling. To

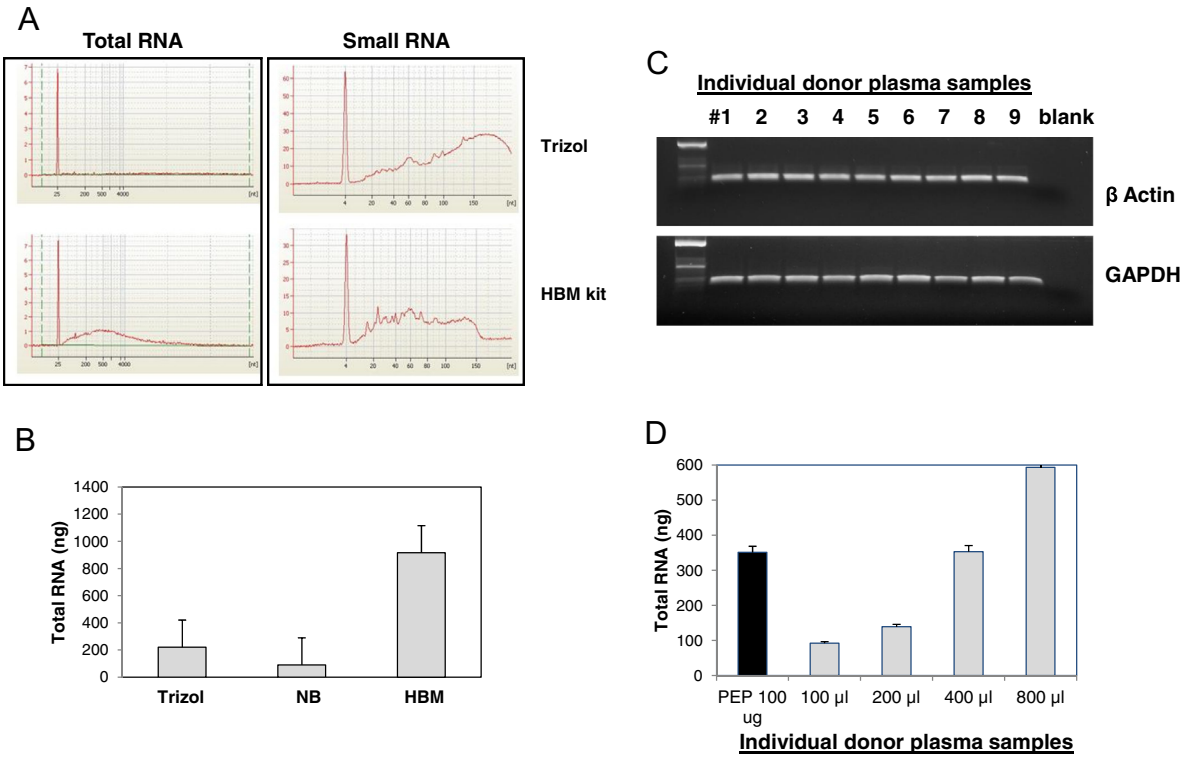

Fig. 10. Analysis of yield and quality of RNA extracted from plasma exosomes immunocaptured on the precoated microplate. Extraction of total RNA from exosomes captured on a single immunoplate strip, from a total volume of 800 µl of healthy donor plasma, was done with Trizol reagent or the Total RNA Extraction Kit (HBM). RNA quality was evaluated by electropherogram with Total RNA and Small RNA microfluidic chips on the 2100 Bioanalyzer instrument (Agilent) (A). The yield of total RNA extracted from immunocaptured plasma exosomes was compared to that obtained using column based Norgen Biotek kit for exosome RNA extraction (NB) (B). RNA extracted from a set of individual donor plasma samples was used in a RT-PCR for amplification of β-actin and GAPDH mRNAs, as confirmed on agarose gel (2%) (C). Recovery of total exosomal RNA from increasing volumes (100–800 µl) of human plasma incubated on the microplate was assessed (D). One hundred micrograms (100 µg) of exosomes purified from plasma by ultracentrifugation protocol (PEP) were used as a control. Presented data are representative of at least three independent experiments. In the panel B data represents a mean ± standard deviation of yields obtained from 4 plasma samples from individual healthy donors while experiment illustrated in the panel D is a representative of 3 independent experiments and reports data as mean ± standard deviation of 3 technical replicates.

demonstrate the suitability of these extracted exosomal RNAs for downstream analyses, conventional RT- PCR was successfully performed for amplification of β-Actin and GAPDH, two mRNAs commonly found in exosomes (Fig. 10C).

The RNA yield obtained from exosomes captured using the microplate was typically higher compared to that obtained from exosomes pulled down from the same sample and volume by ultracentrifugation (Fig. 10D). The independent experiment on 5 randomly chosen healthy donors showed that exosome RNA can be extracted from as few as 100 µl of plasma (corresponds to a single microplate well) and is dependent on the volume of sample. Moreover, the amount of RNA extracted from 100 µg of plasma exosomes (PEP) purified by ultracentrifugation from 2.5 ml of the original sample, was comparable to the RNA extracted from 400 µl of unfractioned plasma directly loaded onto the microplate.

As expected, the use of immunobeads resulted in even more efficient exosome isolation from plasma, which was directly transferred into an improved yield of overall extracted RNA from pulled-down vesicles. We have quantified exosome-derived RNA obtained from plasma in repeated rounds of immunoprecipitation from the same sample, confirming that single incubation step recovers 75–80% of exosomes from precleared plasma. Noteworthy, empty beads showed low non-specific absorption of exosomes and yielded only 15% of RNA with respect to that obtained using aCD9 coated beads.

As shown in Fig. 11, we have first assessed the best protocol for total RNA extraction from bead-immobilized exosomes by using HCT116 cell line (100 µg/sample) as a model, for extraction of exosome-derived RNA. We have applied two gold standard RNA extraction reagents, namely Trizol (Phenol based, Invitrogen) and Qiagen (column based) RNAeasy extraction kit, in comparison to an integrated exosome immunocapture and extraction solution (HBM isolation and extraction RNA kit). The latter one gave multifold increased yields in comparison to a combination of immunobeads with either Trizol or Qiagen Kit. DNAse treatment was included after extraction to avoid genomic DNA carry-over in the sample. Though Qiagen kit gave the best purity, all solutions allowed efficient amplification of β-Actin mRNA starting from 100 ng of total RNA. At the same time, amplification of miR-21 expressed as Ct value per ng of input RNA was similar between RNA samples extracted using HBM or Qiagen extraction kits but the HBM kit was superior in terms of overall RNA yield, (2.5fold increase; Fig. 11B and C). Noteworthy, this was also true for the recovery of HCT116 exosomes spiked into the healthy donor plasma. Moreover, pre-treatment of bead-immobilized material with RNAse (100 µg/ml) prior to RNA extraction did not alter yields of RNA nor the amplification of miR-21 (data not shown), demonstrating that the results likely derive from exosome encapsulated RNA and that immunocaptured exosomes have maintained high integrity. RNAse treatment instead had a moderate effect on ultracentrifuge-derived samples, likely due to a slight damage that can be caused to vesicles by this method.

3.6. Immuno-affinity isolation enables extraction of total RNA and successful amplification of exosomal miRNAs from plasma samples

Finally, we have addressed the challenge of exosomal RNA extraction and analysis from small plasma volumes. Most of

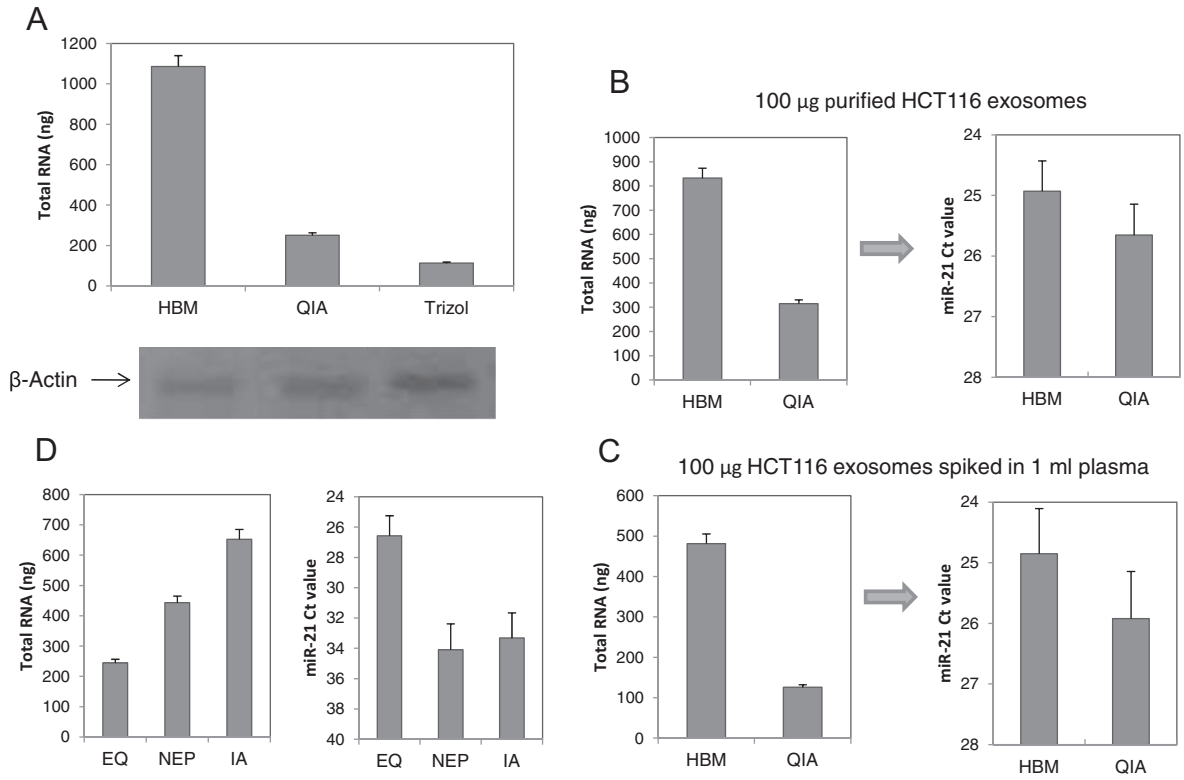

Fig. 11. Extraction of total RNA and amplification of miR-21 from plasma exosomes following beads based immunoisolation. Recovery of total RNA (ng) using Trizol reagent, HansaBioMed (HBM) and Qiagen (QIA) RNA extraction kits from plasma exosomes immunocaptured from 1 ml sample volumes (A). The purity and suitability of thus extracted RNA for β Actin mRNA amplification is shown. Pannel B shows the recovery of total RNA and amplification of miR-21 from 100 µg of ultracentrifugation-purified exosomes from HCT116 colon cancer cell line (B). Recovery of total RNA and amplification of miR-21 from 100ug of immunocaptured HCT116 exosomes spiked into, and recovered from 1 ml of healthy donor plasma using HBM and QIA RNA extraction kits (C). Recovery of total RNA and profiling of miR-21 expression from exosomes captured from 100 µl precleared plasma with different methods: ExoQuick (EQ), ME-kit (NEP), immunoaffinity using exosome capture beads (IA) (D). The levels of miR-21 expression are presented as mean ± standard deviation of 3 runs per sample.

reported omics studies performed on circulating exosomes employed large volumes of plasma, up to hundreds of ml [6,11,30]. Of course, the amount of sample that is normally available from individual donors in both biobanks and in routine clinical laboratories is typically in a several ml range. The possibility to scale-down the volumes for exosome isolation and biomarker validation is likely to have a great impact in accelerating basic and clinical research and the adoption of exosome-derived markers in clinical trials and diagnostics.

When we used immunobeads for exosome capture from 100 µl of plasma samples from healthy donors and patients with Prostate Cancer, we obtained a 5.5-fold increase in RNA yield from diseased subject (from 88 to 480 ng respectively, figure not shown) while no increase was observed when control immunobeads were used (coupled to irrelevant anti-FLAG antibody). Immunobeads used in this experiment were coated with previously described HBM antibodies for specific tumor exosome binding. In addition to increased amount of total RNA obtained from tumor patient samples, we observed an enrichment of miR-21 signal (ΔCt = 2.22). This data strongly support the suitability of the described immunocapture method coupled to optimized extraction protocol for rapid, reliable and specific quantification of mRNA/miRNA of interests in as few as 100 µl of plasma sample.

4. Conclusions

The outburst of interest in physiological and pathological relevance of exosomes and other extracellular vesicles that we have witnessed in the last decade has brought to an elevated number of studies focusing on both fundamental and methodological issues. These reports were mainly focused on *in vitro* samples (cell supernatants), while the advancing concept of the use of exosomes in biomedical applications highlighted the need of identifying appropriate technologies for the isolation of exosomes from body fluids that would take into account efficiency, purity, throughput and reproducibility as crucial factors for the correct implementation of biomarker screening, validation and drug discovery studies [4,18].

We report simple, affordable, yet reliable and efficient methods integrating specific immunoisolation and comprehensive analysis of circulating exosomes. For these purposes, we have compared commercially available and in-house developed immunoaffinity plates and beads that can be used as self-standing tools for highly efficient exosome separation from complex and "crude" samples, as well serve as an upstream step integrated with conventional analytical techniques such as ELISA and PCR. We have performed downscaling of plasma volumes from 1 to 0.1 ml and evaluated yield, purity, and suitability of obtained material for on-line analysis of proteins and mRNAs/miRNAs as most studied exosome-shuttled biomolecules. These assays work with real(istic) samples in terms of volumes and minimal required processing in compliance with the standard procedures of hospital's and diagnostic labs, as well as with the common practice of biorepositories, facilitating thus multicentric trials.

Plasma is notoriously difficult sample for omics studies as its complexity interferes with reliable detection of low abundant molecules. Exosomes are estimated to account for less than 0.1% of plasma proteome [31], while their nanoscale size and intrinsic heterogeneity further impinges efficient recovery. We report

careful optimization of reagents and protocols, including robust antibody selection, test of the solid support, bead size, ligand density, surface chemistry – all properties that ultimately affect overall capture efficiency and specificity, and increase sensitivity of downstream quantitative assays. We have demonstrated that canonical exosome constituents such as tetraspanins in the circulation of healthy subjects reach concentrations in a pg/ml range. Immunoaffinity approach upstream the detection step cleans up the sample and enriches the fraction of interest so that the detection of low density markers can be accomplished using conventional ELISA assays. The integrity of extracted exosomes has been demonstrated in sandwich immunoassays that rely on co-detection of a marker pair on intact vesicles, as well as by the analysis of RNA cargo following the enzymatic treatment of immobilized vesicles. We have also optimized protocols for multifold increase in RNA yield and confirmed the enrichment in small RNAs.

Co-detection of overlapping exosomal protein and RNA markers may realistically allow to accurately distinguish between different sample types and conditions. Both plates and bead-based immunoaffinity methods are versatile and easy to customize for assessing of either total exosome population in the sample or a defined exosome sub-population, likely reducing the background that often masks the quantitative differences in the analysis of both proteins and RNA sequences. This is the unique advantage that immunoaffinity has over other exosome isolation approaches. These methods constantly benefit from innovations in surface modifications and fabrication of novel materials and can be easily coupled with more sophisticated devices (microfluidic chips or specific sensors) [7,16,17]. While these proof-of-concept applications of advanced technologies still are in the form of novel and expensive devices with limited penetration to routine laboratory practice, hereby described assays and tools are cost-effective, feature familiar formats and are compatible with off-the-shelf laboratory equipment such as plate readers or PCR cyclers, as well as with commercial 96-well automatic workstations with high throughput capacity.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ymeth.2015.05.028.

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