Characterization and specific isolation strategies of prostate cancer exosomes

Preliminary work for the establishment of an integrated microfluidic platform for exosome isolation and analysis

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I. Abstract

Exosomes are nanosized cell-secreted lipid vesicles that represent their tissue of origin by displaying surface markers and by carrying protein and nucleic acid cargo similar to their origin cell. Due to this correlation, high availability and accessibility in bodily fluids, exosomes show great promise in Diagnostics. Exosome analysis is however complicated due to size and limited resolution of the commonly used methods, which are also highly manual and time consuming. For future development of a microfluidic device for prostate cancer (PCa) diagnosis, exosomes from PCa cell lines, LNCaP and PC-3, must be characterized. Protein expression was assessed by fluorescently labeled secondary antibodies anti-PSMA (Prostate Specific Membrane Antigen) and self-fluorescent exosomes for specific capture experiments. These were carried out by measuring bead-bound exosomes, on a FACS-like device, the iQUE screener, and free exosomes on carboxyl-activated glass slides and lateral flow strips. Morphology of vesicles was confirmed by SEM imaging. The iQUE method was optimized in terms of centrifugation duration, tubes, bead concentration and blocking solution for antibodies. It was possible to confirm the expression of PSMA in the cells lines but such expression was inconclusive for exosomes. Specific capture protocols require further adjustments, namely in blocking. Once these are made, future experiments will include biological samples as blood and urine and testing of proprietary matrixes for specific capture. The future liquid biopsy modular platform will enable pre-symptomatic screening and early detection of cancer, using low amounts of samples and reagents.

Key-words: diagnostics, exosomes, extracellular vesicles, isolation, microfluidics, prostate cancer, PSMA

II. Introduction

Exosomes: These membranous extracellular vesicles (40-150 nm) are released by virtually all cells and are abundantly present in body fluids (Figure 1). At first discovered to take part in sheep reticulocyte maturation and mostly studied for their roles in immunomodulation (1), it is known that these vesicles have a more general role in intercellular communication and regulation of the cellular niche (2), and not just as initially thought of, as cellular waste disposal vesicles.

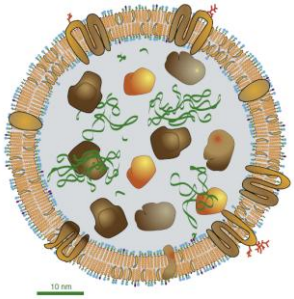

Figure 1. Representation of the average exosome structure: Lipid bilayer enclosing cytosol with RNA (green ribbons) and proteins (blobs); proportionally drawn **(3)**

Moreover, these nanovesicles are known to represent their tissue of origin, since they contain cytosol encapsulated by a cholesterol-rich phospholipid membrane. Exosomes are thought to origin as the intraluminal vesicles bud inwards, forming an early endosome which then matures into a multivesicular endosome and releases the exosomes it contains by fusion with the plasma membrane or with lysosomes for content degradation. During this process exosomes are enriched in proteins, bioactive lipids and nucleic acids such as mRNA and miRNA that can be translated into proteins when transferred to target cells. This makes not only exosomes, but extracellular vesicles in general, holders of valuable biomarkers in the case of altered characteristics in pathological states. Hence, exosomes have a great potential to

be used for non-invasive diagnostics, liquid biopsies and therapeutics. (4) (5)

Despite the high clinical value of these vesicles, detection and isolation are still a challenge due to insufficient differences in physical properties such as size, morphology and buoyant density between exosomes and microvesicles (6). Several methods are used to isolate and purify exosome solutions from fluid samples, with variable levels of purity.

The most generally used method is ultracentrifugation, in which the culture medium is filtered by microporous membranes, then differentially centrifuged and finally centrifuged at very high centrifugal forces (70.000 to 100.000 × *g*). The resulting pellet, resuspended in PBS, can be again ultracentrifuged at the same speed (5). However, this method needs large-scale instruments which are not usually available at point of care locations, is time consuming and demanding in terms of personnel and reagents, and is not selective enough to discriminate exosomes from different cellular origins or from other vesicles or large protein aggregates (7) (8).

Exosomes can also be isolated based on their buoyant density, of 1.08 to 1.22 g/cm^3, by a discontinuous iodixanol gradient or by precipitation solutions that are easy to use but often fail to distinguish between differently sized vesicles and membrane-free macromolecular aggregates (6). For specific selection, immunoaffinity capture will make use of antibodies against antigens of interest, immobilized onto a matrix or magnetic beads, for instance. For visualization, electron microscopy is the most suitable technique, with a maximum resolution of 0.1 nm (9).

Quantification of exosomes is a complicated process, due to the fact that the exosomes' size overlaps with that of compounds in commonly used solutions, such as PBS buffer, and due to the limited resolution of common devices such as FACS (Fluorescence-Activated Cell Sorting), that can be partially overcome by fluorescently labeling and binding the exosomes to microspheres, although not being able to count exactly how many exosomes are bound to each bead, but providing approximate values for further comparisons.

Biomarkers: A biomarker, as defined by the National Cancer Institute, is "a biological molecule found in the blood, other body fluids, or tissues that is a sign of a normal or abnormal process or of a condition or disease" (10).

The ideal biomarker should screen for a disease and its consequent progression and response to treatment, identify high-risk individuals and predict recurrence. There are no specific markers for exosomes *per se*, so, for detection and isolation, general enriched surface proteins are used such as CD9, CD68 or CD81, or proteins essential for multivesicular formation such as TSG101 and Alix. Flotilin and HSP70 are also commonly detected. EpCAM is also present in exosomes, in different levels according to their cellular origin. (11)

Prostate Cancer: PCa is the most common cancer in Europe, ranking second worldwide, in men. Regarding both sexes, both in Europe and Worldwide, it is the fourth most common type of cancer and the third leading cause of death in Europe, fifth worldwide, in men (12).

PCa is very heterogeneous in terms of biological, hormonal and molecular characteristics. So, finding a good biomarker in body fluids is no easy task. There are many reports of different promising biomarkers, but a lack of strategies to determine which candidate is worth long-term investment for laboratorial and clinical studies (13). Exosomes can then be a source of diagnostic and prognostic markers, which can be retrieved from plasma, urine and semen (14).

PCa Biomarkers: Regarding PCa, two biomarkers are considered: PSMA (Prostate Specific Membrane Antigen) and PSA (Prostate Specific Antigen).

PSMA is a transmembrane-carboxypeptidase produced in the prostate gland, up-regulated at least 10-fold in PCa and in its metastasis. This overexpression of PSMA in PCa is correlated with prognostic factors, which makes it a clinically useful biomarker for diagnostics, imaging and therapy, being considered the gold standard for the detection of PCa (15). PSMA can be detected by anti-PSMA antibodies or by the small molecule PSMA-617 (Glu-CO-Lys). This molecule has shown high binding affinity to PSMA and a highly efficient internalization by PCa cells, which makes it a good candidate for diagnostics, by PET imaging, and therapeutics since it can also be conjugated with radionuclides. (16) Free PSA, also known as human kallikrein 3, hK3, is the biomarker currently used for screening. However, screening for total PSA blood levels should not be prescribed for a general population screening, but for monitoring PCa patients after treatment (17) since PSA-based screening leads to a decrease in the prevalence of advanced PCa and a reduction of PCa-related mortality by 20%. Despite its good sensitivity, PSA screening lacks specificity, being consequently associated with a high risk of overdiagnosis and overtreatment. Therefore, new biomarkers are needed to prevent unnecessary biopsies and monitor and improve the overall quality of treatment.

Lateral Flow assay: This immunochromatographic method relies on capillary forces to transport a liquid along the surface of a porous membrane, and the result of the test is visible without any reading device and is generally a yes/no value. The most known application of these assays is perhaps the home pregnancy test but there are other applications for detection of toxins, pathogens, nucleic acids or drugs. The sample is applied in the sample pad and as it is absorbed, will be put in contact with the conjugate pad that contains dried particles conjugated with marker molecules - one type of marker for specific isolation and another marker for detection. The liquid will flow through the nitrocellulose membrane into the analysis area, where the targeted molecules in the sample

will bind to both markers and to the test line, whereas the unbound particles, will bind to the control line, in a sandwich-type assay (Figure 2). Competitive assays are mostly meant for smaller molecules that lack the ability to bind two antibodies simultaneously. In these assays, the test line has pre-immobilized antibodies that bind specifically to labeled antigens in solution. Antigens from the sample and the labeled-antigens compete to bind with the antibodies at the test line. In case the target molecule is present, it will displace or prevent the binding of the labeled antigens in solution, therefore the color of that line disappears, and only the control line will be seen. If there is no target in the sample, there is no displacement of the labeled antigen in the test line and both lines will show color (20).

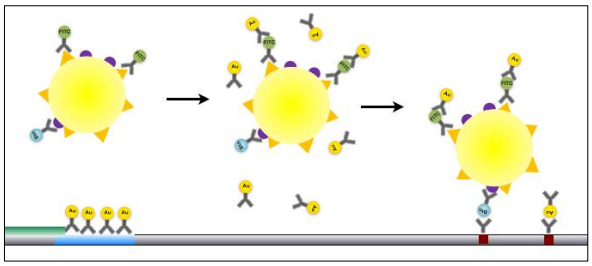

Figure 2. Lateral flow strip schematics – sandwich format with biotin-labeled anti-target antibody for isolation and anti-FITC gold labeled antibody for detection. Green: sample pad; blue: conjugate pad with gold labeled antibodies; gray: lateral flow strip membrane; red: test and control lines.

III. State-of-the-art

Considering the above mentioned methods and their limitations, a need for a faster, high-throughput and selective method arises, to which microfluidic platforms show great promise.

What most of these platforms have in common is the use of microscale volumes and the specificity provided by the binding of antibodies that can lead to an integration of other functions such as nucleic acid or protein analysis of the content of the vesicles. Generally, these devices can fall in two, non-exclusive, categories: Immunological separation, making use of antibodies for selection and detection; and physical methods that use sieving, where samples are filtered by pressure or electrophoresis, or that trap the exosomes in porous matrixes.

Immunological separation: These platforms use antibody covered magnetic beads (7) or channel surfaces to capture exosomes. These can then be characterized *in situ* by fluorescence or lysed for nucleic acid or protein content extraction and further analysis (21).

Samples can vary from cell culture medium to plasma or ascetic fluid and its mixing is many times enhanced by changing the structure of the channels (4). Other analytical methods can be applied, such as surface plasmon resonance (22) or qPCR (23) and the signal can be amplified by labeling the exosomes with gold nanoparticles (22).

Physical methods: Sieving methods are done by flowing blood through membranes (microporous silicon nano-wire structures (24) or silicon nanoscale lateral displacement arrays (25)) and filtering it by pressure or by electrophoretic forces. These methods are non-selective and yield a low recovery of exosomes, performing however well in terms of running time and RNA yields, which can be higher than ultracentrifugation in some cases (26). In order to obtain a pure exosome population, immunological methods are so far the only suitable ones. Other methods relying on physical properties (size, density, surface charge) lead to higher percentages of contaminants (4).

IV. Materials and Methods

Cell lines: PC-3 (ATCC CRL-1435) and HeLa (ATCC® CCL-2™) PSMA negative, LNCaP (ATCC CRL-1740) PSMA positive were cultured according to provider instructions in media supplemented with 1% Penicillin/Streptomycin at 10.000 U/mL / 10.000 µg/mL (Biochrom) and 10% FBS (Fetal Bovine Serum), that when stated, was vesicle depleted (Invitrogen). The incubator was set to 37° C with a 5% CO_2 atmosphere.

Fluorescent exosomes: Cells were transfected with the plasmid pEGFP-C1 (Clontech), with Lipofectamine® 3000 Transfection Reagent (Thermo Fisher, L3000015), according to the instructions provided. For selection, the antibiotic Geneticin® G-418 (ThermoFisher) was used (50-100 µg/mL). Cells were also incubated with 10 µM CellTracker™ Green CMFDA Dye (CTG) (ThermoFisher, C7025), for 45 minutes.

Purification: exosomes were initially purified from conditioned cell media that were centrifuged for 5 min at 300 × *g*, 4° C. After this, the supernatant was centrifuged for 30 minutes at 10.000 × *g*, 4° C. Then the supernatant was strained by a 1 µm membrane (pluriSelect, 43-50001-03) and centrifuged in an ultracentrifuge (Sorval Discovery UZ) for 90 minutes at 70.000 × *g*, 4°C. Pellets were resuspended in 1 mL of 0.22 µm filtered PBS and stored at -20°C. To avoid contamination by smaller cellular debris, this method was changed, based on (27), by replacing the 1 µm strainer by a 0.22 µm flask-top filter and the ultracentrifugation speed was increased to 100.000 × *g* and the pellets resuspended in half of the volume, 0.5 mL and stored at -80°C.

Vesicle analysis: The iQUE screener (IntelliCyt®) is a FACS-like device that was used to analyze cell, exosome and bead-bound exosome samples. In general, the method consists of three main steps: the binding of exosomes to beads, whether directly via adsorption of the vesicles or via binders, the anti-PSMA antibody or the small molecule PSMA-617. Secondly,

the blocking, as it is necessary to block the surface of the beads to avoid nonspecific binding by the labeling molecules, generally by 1,5% BSA and 50 mM glycine in PBS (30 minutes at (RT) room temperature). In between incubations, washing the beads is necessary to remove unbound molecules, by resuspending in PBS and centrifuging at 9.200 × *g* for 2-5 minutes. So it is necessary to optimize the binding, blocking, labeling and washing. 15-30 µg worth of exosomal protein, as measured by BCA assay (Pierce) (28) were used directly or bound to 4 µm aldehyde/sulfate latex beads (4% w/v, molecular probes). Detection of exosomes was done by anti-EpCAM-FITC (Life Technologies), minimum of 3 µg/mL, by Cy5-labeled secondary antibody (1:2500 dilution) targeting anti-EpCAM or anti-PSMA (6 µg/mL) primary antibodies, or by CTG self-fluorescent exosomes. *Centrifugation duration test* – beads and LNCaP vesicles were incubated for 1 h at RT. Then centrifuged at 9.200 × *g* for 2, 4, 6, 12 and 24 minutes. After blocking and incubation with anti-EpCAM-FITC antibody (3 µg/mL, 45 min) samples were again centrifuged for the same time intervals and washed once. Supernatants were kept in all steps for measuring. *Low bind tubes test* – beads and CTG-LNCaP vesicles were incubated in low binding tubes (Sigma Aldrich, Z666505-100EA) and in standard tubes (Sigma Aldrich, T9661-1000EA), then blocked and incubated with anti-PSMA-antibody (30 minutes, RT). After washing 3 times, the secondary antibody labeled with Cy5 was added (30 minutes, RT). Beads were pelleted, the supernatants collected, and the beads were washed 3 times with PBS. *Blocking tests* – beads were blocked with 1% BSA, 3% BSA, glycine at 33.3 mM and 100 mM or 50 mM glycine and 1,5% BSA, in PBS; and then incubated with anti-EpCAM-FITC antibody. Beads were also incubated with anti-EpCAM antibody, blocked with 3% BSA, 100 mM glycine or 1,5% BSA and 50 mM glycine, in PBS, and incubated with anti-mouse-IgG-Cy5 labeled antibody dilution. Other set of beads were blocked with 3% BSA, 100 mM glycine, 1.5% BSA and 50 mM glycine, or polyethylene glycol PEG-4000 (Roth) at 50 nM, 200 nM and 500 nM (45 minutes, RT), and incubated with CTG-PC-3 vesicles. *Surface marker detection via Cy5-labeled secondary antibody* – Beads with exosomes or cells from the three cell lines were blocked and incubated with anti-PSMA or anti-EpCAM antibodies. The secondary anti-mouse IgG-Cy5 conjugated antibody is then incubated in the same conditions. *Specific capture via anti-PSMA antibody and small-molecule PSMA-617* - beads were incubated (30 minutes, RT) with the binders anti-PSMA antibody (6 µg/mL) or PSMA-617 (8 µg/mL). Then the samples are blocked and exosomes from GFP LNCaP, CTG PC-3 and CTG HeLa were incubated with the beads.

Carboxyl-activated glass slide: A functionalized glass slide (PolyAn) was covered with spotted PCR film (Roche), and the spots were activated with a 1:1 mixture of EDC/NHS (both at 50 mg/mL), in MES (pH 6, 30 minutes) and the binders (anti-EpCAM antibody (6 µg/mL), anti-PSMA-antibody (6 µg/mL) or small molecule PSMA-617 (8 or 80 µg/mL)) or the vesicles directly, are incubated for 2 h at RT. Afterwards, the slide is deactivated by 0,5 M ethanolamine (pH 8,5, 30 minutes) and blocked (1,5% BSA and 50 mM glycine in PBS, 30 minutes). At this stage, a volume of purified exosomes equivalent to 30 µg of exosomal protein (assessed by BCA assay) is incubated for 45 minutes. For detection, the samples are again incubated for 30 minutes at RT with anti-EpCAM-FITC labeled antibody or via primary and secondary antibody labeled with Cy5, each incubated for 45 minutes, in the dark. It is also possible to detect the signal by using vesicles from cells incubated with CTG (Figure 3). After this, the samples were washed, the foil removed and scanned with the Genepix reader (Molecular Devices).

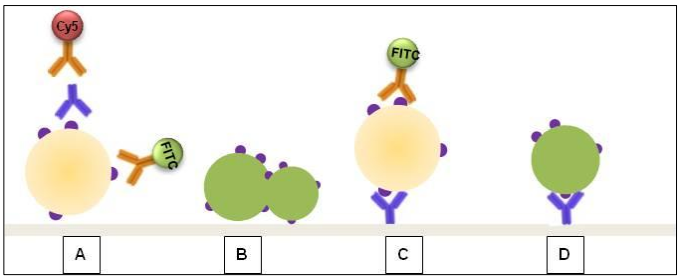

Figure 3. Possible latex bead or glass slide setups: Direct adsorption of vesicles (yellow circles) and detection by FITC-labeled antibody or Cy5-labeled secondary antibody (A) or detection by self-fluorescent exosomes (B, green circles); Specific capture via antibodies or PSMA-617 and detection by FITC-labeled antibodies (C) or self-fluorescent exosomes (D)

Scanning Electron Microscopy: exosomes from the three cell lines were incubated on silica wafers (hydrophobic (Fraunhofer Institute für Siliziumtechnologie) and hydrophilic wafers (Austrian Institute of Technology and SIEGERT WAFER GmbH)) either directly, via binders or latex bead-bound. Samples are fixed with 3,7% glutaraldehyde in PBS and washed with increasing ethanol concentrations of 40, 60, 80 and 98% and left to dry before sputtering a 10 nm gold layer. Imaging is then performed by SEM (GeminiSEM) at beam energies of 5.0 kV at the IOM, Leipzig.

Zetasizer: This device was used to measure exosome size range and zeta potential. The Zetasizer Nano ZSP (Malvern) is set to measure backscatter at 4°C, in triplicate, of 200 runs of 3 seconds each.

Lateral flow assay: As an alternative to specifically isolate and detect PSMA-positive PCa exosomes, a new lateral flow assay was done using a sample containing LNCaP exosomes incubated with biotinylated anti-PSMA antibody (MA110335, Thermo Fisher) (Mix-n-Stain Biotin Antibody Labeling Kit, Sigma Aldrich) and with anti-EpCAM FITC labeled antibody, both antibody concentrations either at 15 µg/mL or 5 µg/mL, with enough PBS to make a final 100 µL volume.

V. RESULTS AND DISCUSSION

Vesicles: *Centrifugation times* - The total of beads lost in the supernatants tends to decrease as the centrifugation duration was increased. However, despite this overall decrease, still 58.51% ± 8.24 of the beads lost in the supernatant had a positive signal for FITC, which is a significant loss of signal (Figure 4). This fluorescent signal can be due to labeled exosomes but also to free antibody bound to the beads, however more unlikely since the beads are previously blocked.

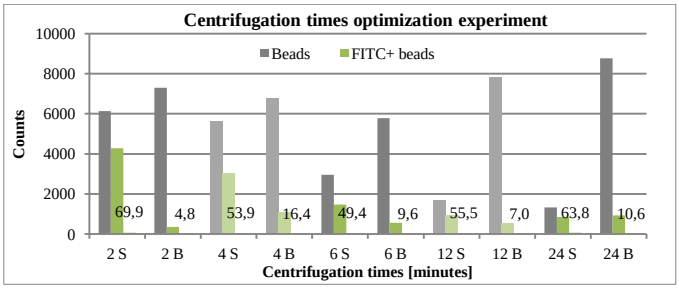

Figure 4. Centrifugation times experiment. *S:* sum of counts from the three measured supernatants; *B:* final sample. Next to the bars, the percentage of FITC-positive beads is shown.

Based on these results it was decided to set the centrifugation time to 5 minutes, since a bead loss between 37,38%, for 4 minutes centrifugation time, and 19,72%, for 6 minutes, was considered acceptable, in order to balance good results with experimental practicality.

Tubes - The amount of counted beads is approximately 11 times higher in the final sample in low binding tubes than in its correspondent supernatant, and approximately 28 times higher when compared to the final sample in standard tubes (Figure 5). The use of these standard tubes can then explain bead losses noticed throughout experiments.

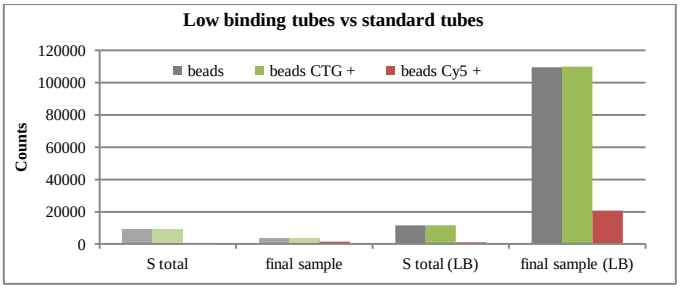

Figure 5. Event counts for standard tubes and low binding tubes – *S:* sum of the supernatants, *LB*: Low Bind tubes

Blocking tests - 1,5% BSA and 50 mM glycine in PBS was the most effective blocking solution for both primary or secondary labeled antibodies, since about 99% of the beads for both tests do not fluoresce. However, none of the tested solutions are effective for CTG-exosomes, since even the best blocking solution, 3% BSA in PBS, still allows for 53% of nonspecific binding.

Surface marker detection via Cy5-labeled secondary antibody - According to literature, LNCaP cells are PCa cells known to highly express PSMA, whereas HeLa and PC-3, this last one also a PCa cell line, do not (29). The expected expression pattern was obtained and is shown in Figure 6.

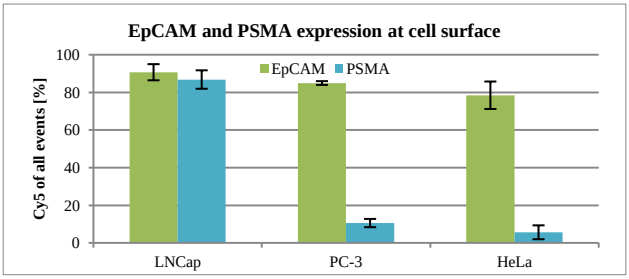

Figure 6. EpCAM and PSMA expression assessed by secondary antibody labeled with Cy5 in LNCaP, PC-3 and HeLa cells

Cells expressed EpCAM in high levels (HeLa 78,4% of the cells expressed the marker, PC-3 84,9% and LNCaP 90,7%) and PSMA in the expected pattern (HeLa 5,6 %, PC-3 10,5% and LNCaP 86,8%). HeLa cells appear positive for PSMA, possibly due to nonspecific binding of the Cy5 labeled antibody, since HeLa cells are not derived from prostate. According to literature (29), LNCaP and PC-3 derived exosomes should follow the same PSMA expression pattern of their parent cells. However, it was not the obtained result in this experiment (Figure 12), since fluorescence from Cy5 labeled secondary antibodies was in general low, EpCAM/PSMA (HeLa 3,62%/2,79% of the exosomes expressed the markers, PC-3 16,85%/5,51% and LNCaP 7,88%/3,70%).

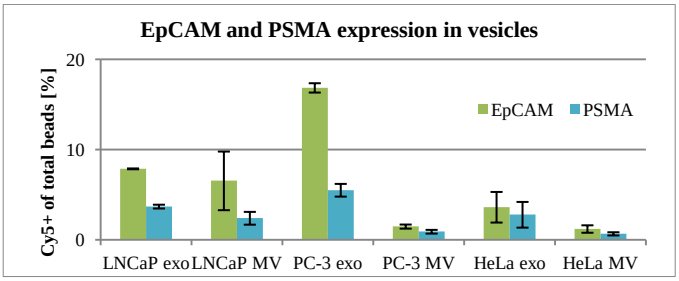

Figure 7. EpCAM and PSMA expression assessed by Cy5-labeled secondary antibody - exosomes (exo) and microvesicles (MV) for the respective cell lines

Specific capture via anti-PSMA antibody and small-molecule PSMA-617 - Higher signals (from anti-EpCAM-FITC antibody or fluorescent-exosomes) were expected for LNCaP and lower for PC-3 and HeLa exosomes (29). When testing fluorescent-exosomes the signal is overall low (<20 % of the beads fluoresce) and the LNCaP positive-control accounts for only 2,1% fluorescent beads, maybe due to the fact that GFP does not seem to be packed into exosomes (Figure 13).

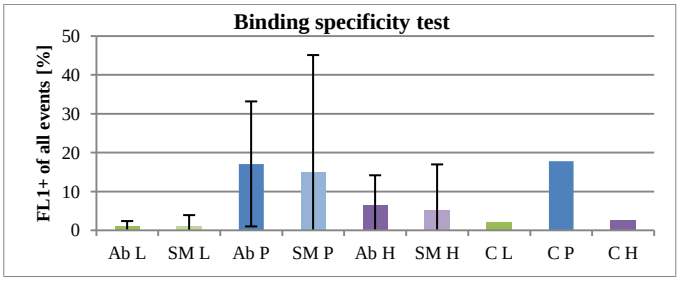

Figure 8. Binding specificity test - *Ab*: anti-PSMA antibody; *SM*: small-molecule PSMA-617; *C*: blocking control; *L*: GFP LNCaP vesicles; *P*: CTG PC-3 vesicles; *H*: CTG HeLa vesicles

PC-3 vesicles showed the highest fluorescence (17,1 % for antibody binding) but the standard deviation was also very high (±16,08%), in the same magnitude of the average fluorescence value. The blocking controls are also in this same fluorescence range as the binder samples, so it is not possible to know if the beads have indeed captured these exosomes with their binders of if these just adsorbed to the surface.

Regarding samples detected by FITC (Figure 9), most fluorescence was found in the supernatant and LNCaP and PC-3 were FITC-negative for both binders.

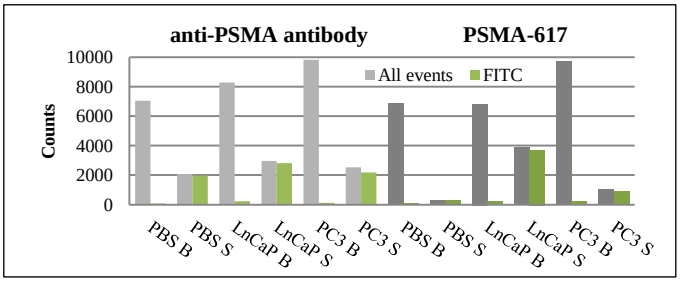

Figure 9. Specific capture of exosomes via anti-PSMA antibody (lighter colors) or small-molecule PSMA-617 (darker colors) detected by FITC-labeled anti-EpCAM antibody. *S*: Supernatant; *B*: Beads

This can be due to inadequate concentration of the binders, to an insufficient availability of PSMA in LNCaP exosomes for stable binding to the beads or to a low concentration of the FITC-labeled antibody due to the losses in the supernatant. In any case it was not possible to assess the specific binding of the exosomes with respect to PSMA with this protocol and not possible to assess the binding capacity of the small molecule against the antibody.

Carboxyl-activated glass slide: *PSMA and EpCAM detection* – In Figure 10, a higher presence of Cy5 correlates with a higher presence of the marker in question, EpCAM, which is consistent with literature since EpCAM is ubiquitously expressed in virtually all simple epithelia (30). Regarding the samples incubated with anti-PSMA, the intensity of the signal was lower and there was no visible distinction between the PSMA positive sample, LNCaP, and the PSMA negative samples, PC-3 and HeLa.

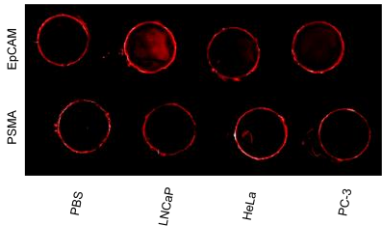

Figure 10.Glass slide for PSMA and EpCAM detection by Cy5 read at 635 nm

Specific capture – It was not possible to verify the specific capture via PSMA since the blocking failed to prevent nonspecific binding of the exosomes, as in Figure 11 – *Blocking test* row all spots are fluorescent.

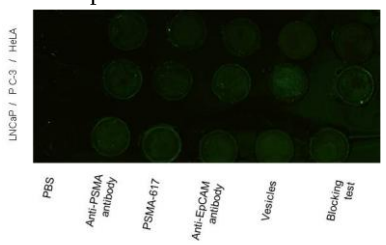

Figure 11. Specific capture of CTG exosomes, read at 532 nm

Scanning Electron Microscopy: The hydrophobic surface can lead to the agglomeration of vesicles, during fixation steps, as droplets dry and gradually cluster the exosomes (Figure 12B). This effect might be problematic in a microfluidic channels and chambers context, since it can promote obstruction of these structures. In terms of size, the vesicles seem to be in the expected range (30-200 nm).

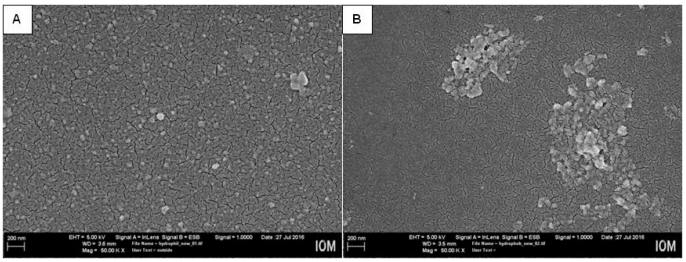

Figure 12. SEM image of LNCaP vesicles on an hydrophilic (A) and hydrophobic silica wafer (B). Scale bar 200 nm

Figure 13 shows close-ups of the beads with, what appears to be, bound exosomes, based on size and morphology and in 13B some fibrils that could not be removed, even when using a 0,22 μm filter during purification.

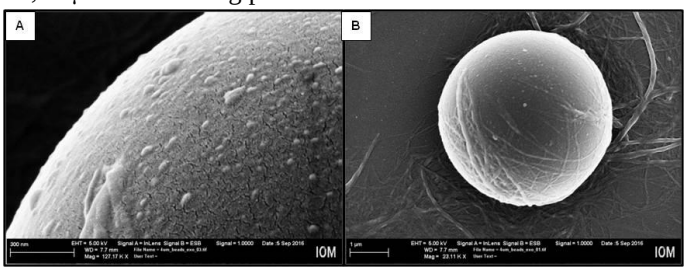

Figure 13. SEM image of beads with exosomes. Scale bars: 300nm(A), 1μm(B)

Zetasizer: The optimized purification method rendered more homogenous exosome purifications form the three cell lines (40 nm-1 µm). Although a 0.22 µm was used, vesicles or aggregates bigger than that were detected, perhaps due to conformation changes that allow these structures to pass through the filter pores. Nevertheless, the highest intensity corresponds to vesicles around 200 nm, the expected size for bigger exosomes. Regarding zeta potential measurements of LNCaP purified vesicles in PBS the obtained value was, on average, -12,6 mV. Comparing this value to those from Figure 14, it falls on a category between the expected value for exosomes, -17 mV, and microvesicles, -7 mV, which can be interpreted as the exosome sample also containing some microvesicles that lower the sample‘s potential, which was consistent with the size measurements, and also considering the inevitable biological variability. These -12,6 mV promote the beginning of agglomeration (31).

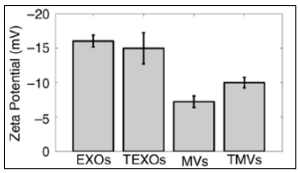

Figure 14. Literature results for zeta potential for LNCaP extracellular vesicles: microvesicles (MVs) and exosomes (EXOs) and trypsinized microvesicles (TMVs) and trypsinized exosomes (TEXOs) (32)

Lateral Flow assay: In these experiments, it was expected to detect LNCaP exosomes by the development of the two bands in the strips, which did not happen. It can be due to the fact that the exosomes and vesicles might agglomerate and not even get past the sample or conjugation pads or non-specifically bind to the nitrocellulose membrane.

This was, to our knowledge, the first experiment using exosomes as targets for lateral flow assays, so further experiments are needed in this area, such as investigating possible interactions of the membrane with vesicles, optimizing the concentrations of the antibodies and trying different membranes with different pore sizes.

Final remarks: *Cell culture and exosome collection standardization is required:* A higher yield of exosomes was obtained after a 48 h incubation, rather than longer periods, since exosomes are communication vesicles that can be up-taken by neighboring cells. Protein amount is an easy but not the ideal measurement to estimate exosome quantity since protein cargo can be highly variable amongst exosomes (33), therefore this standardization of culture conditions ensures higher uniformity of exosome samples and higher reproducibility between experiments.

High centrifuge forces (100.000 × g) and filtering (0.22 µm filter) are required to obtain homogenous exosome purifications: The optimized centrifugation exosome purification from culture media protocol at higher centrifuge forces (100.000 × *g*) and including filtering the medium by a 0.22 µm filter, renders a more homogenous purification of vesicles, as assessed by SEM or Zetasizer. This protocol promotes then reduced intra-sample variability.

Low binding tubes avoid sample losses: Using special low binding 1.5 mL microcentrifuge tubes instead of standard tubes has proven to greatly reduce the amount of beads lost while performing experimental protocols.

CTG is a viable alternative to labeled antibodies: Incubating cells with the fluorescent dye CellTracker Green proved to be an excellent source of fluorescent vesicles and an alternative to the conventional use of labeled antibodies. In general, these vesicles were fluorescent over the course of the experiments, not being susceptible to fluorescence loss after freezing at -80ºC or storage at 4ºC. Using these vesicles allows for shorter laboratory procedures at the time of the experiments, since there is no need for 45 min-1 h incubation with the labeled antibody and further washing steps to remove excess antibody, which can promote loss of exosomes, and beads when it is the case, in the supernatants.

Zeta potential of purified exosomes promotes agglomeration: The Zetasizer results suggest possible agglomeration of LNCaP exosomes, which could explain the negative results in the lateral flow assay. Would be valuable to measure the changes in the zeta potential of exosomes while resuspended in other fluids, such as PBS/0,05%Tween, the previously tested blocking solutions or PBS with different pH that could reduce the zeta potential to levels were agglomeration is less likely.

Literature described surface blocking is efficient for antibodies but not for exosomes: Blocking solutions such as 1%-3% BSA or 50-100 mM glycine have been described to efficiently block surfaces against antibody nonspecific binding, but these solutions are not valid for exosome blocking.

Improvement of the iQUE method and complementing exosome characterization with other methods is necessary: It was possible to optimize the method by adjusting the centrifugation duration of 5 minutes and using low binding tubes to perform the experiments. These alterations yielded higher amounts of beads in the final sample. It was also possible to validate the use of 1,5% BSA and 50 mM glycine as a blocking buffer against antibody nonspecific binding. For further experiments, other types of beads can be tested, with different surface groups or made of other materials. Other complementary methods such as Western blot can be used to assess protein expression, since the goal of the project is not optimizing exosome analysis on the iQUE screener, but to use

it as a control for the future results obtained in the microfluidic platform. Western blot is however more time consuming, taking 1-2 days of laboratory work, and it also requires time investment in optimizations, regarding the amount of loaded protein and concentrations and incubation times of the antibodies, primary and secondary, if it is the case of indirect detection.

LNCaP cells highly express PSMA whereas PC-3 and HeLa do not; exosome protein expression pattern was inconclusive: As verified with the iQUE, the cells' PSMA expression behavior is consistent with literature, however, it is not possible to know if the exosomes also follow this pattern. Literature is also inclusive in this matter since there is a source reporting PSMA-negative LNCaP exosomes (34).

Specific capture of exosomes with anti-PSMA antibody or small molecule PSMA-617 was inconclusive: Despite all efforts to reproduce protocols already described in literature that use antibody-coated latex beads to selectively capture exosomes, there are still problems with the specific capture of exosomes on the glass slide and most importantly on the latex beads. The major problem that needs to be addressed is the unspecific binding, so new blocking solutions or surface modification techniques of relevance regarding the microfluidic platform should be researched and tested. After this adjustment, it will be necessary to optimize the capture molecule concentration, to maximize vesicle capture.

VI. CONCLUSIONS

This work was integrated in the Fraunhofer IZI's NanoCapture in-house project that ultimate aims to develop an integrated platform for prostate cancer diagnosis. This diagnosis will be based on exosomes' protein expression. Exosomes and microvesicles show great promise in the diagnostics and therapeutic fields due to their valuable content in nucleic acids, protein and bioactive lipids that directly correlate to their parent cell, which allows to assess otherwise inaccessible tissues' health state.

Despite clinical potential, to date there are no well-defined, reproducible protocols for exosome isolation. The available protocols rely on time consuming ultracentrifugation steps and the retrieved pellets are not a homogenous collection of exosomes, but rather heterogeneous populations of exosomes, microvesicles and bigger protein aggregates. Size exclusion methods do not only concentrate exosomes and can cause pressure-related structural damage. The extracellular vesicle analysis relies on Western blot, ELISA and mass spectroscopy methods which are again time consuming and not sensitive enough for small sample volumes. These are all factors that slow and limit the progress on exosome research and therefore delay its application on diagnostic and therapeutic use. These limitations can however be overcome by making use of microfluidic technologies due to high throughput, single molecule sensitivity and automation with minimal sample consumption, thus facilitating quantification in biological and medical fields. And despite current advances on microfluidic devices applied to diagnostics and more specifically to exosomes, there is still no commercially available platform for PCa exosome analysis.

As a preliminary step towards this device, characterization of exosomes from two PCa cells lines, LNCaP PSMA positive and PC-3 PSMA negative, and negative control HeLa cells was attempted. This was done by using a FACS-like device, the iQUE screener, to measure exosome-coated latex beads' fluorescence emitted either by CTG self-fluorescent exosomes for specific capture experiments or Cy5 labeled secondary antibody for surface protein expression. This method was optimized in terms of centrifugation duration (5 minutes), tubes to use (low binding tubes), bead number (260.000 beads per minute of measurement in a total volume of 100 μL), blocking solution for antibodies (1,5% BSA and 50 mM glycine in PBS) and self-fluorescent vesicles (CTG dye). It was possible to confirm the expression of PSMA in the cells lines but not possible to establish a parallel with the exosomes these cells release due to iQUE's malfunction. Specific capture with anti-PSMA antibody or small-molecule PSMA-671 protocols still require further adjustments particularly in terms of blocking. Simultaneously, two other setups were tested: a carboxyl activated glass slide, for the same experiments as the beads but on a planar surface and analysis on a more qualitative basis, that suffered from the same blocking problems as the beads; and a lateral flow strip, to detect exosomes in this standardized method, by capture with anti-PSMA antibodies. This test was negative possibly due working concentrations of exosomes out of the detection range of the strip, agglomeration of these vesicles, inadequate antibody concentrations, amongst other factors.

So there is still a long path to cover in terms of vesicle characterization. It will be however a road worth taking, since novel diagnostic platforms will benefit PCa patients by allowing their doctors to closely follow the course of the disease, personalize the treatment to the patient specific needs and overall to avoid late and inappropriate treatment options.

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