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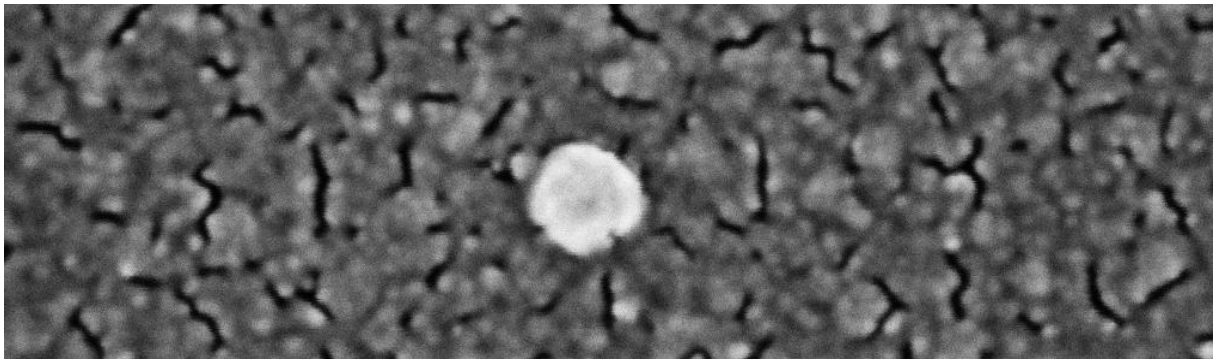

Characterization and specific isolation strategies of prostate cancer exosomes

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

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Biomedical Engineering

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I. Acknowledgements

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(cover image: SEM image of a single exosome bound by anti-PSMA antibodies to a carboxyl-activated glass slide; courtesy of IOM Leipzig)

II. 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 their size and limited resolution of the commonly used analysis methods, which are also highly manual and time consuming. For future development of a microfluidic device for prostate cancer (PCa) diagnosis at the Fraunhofer IZI, Leipzig, 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 nitrocellulose 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

III. Resumo

Os exossomas são vesículas lipídicas secretadas por células que representam o seu tecido de origem, ao expressar marcadores de superfície e por terem conteúdo proteico e de ácidos nucleicos semelhantes à célula-mãe. Devido a esta correlação, à alta disponibilidade e acessibilidade em fluidos corporais, os exossomas são uma grande promessa no ramo de Diagnósticos. Analisá-los é no entanto complexo devido ao tamanho e resolução dos métodos usualmente usados, que por sua vez são pouco automatizados e demorados. Antes do desenvolvimento do dispositivo de microfluídica no Instituto Fraunhofer de Leipzig, para diagnóstico do cancro da próstata, os exossomas de linhas celulares deste cancro, LNCaP e PC-3, são caracterizados. A expressão proteica foi avaliada por anticorpos secundários fluorescentes anti-*PSMA* (sigla em inglês, Antigénio de Membrana Específico da Próstata) e por exossomas fluorescentes para experiências de captura específica. Estas experiências foram efectuadas num aparelho tipo FACS, o *iQUE screener*, medindo exossomas conjugados com contas de látex, e exossomas livres em lâminas de vidro activadas com grupos carboxilo ou em tiras de teste por fluxo lateral. A morfologia das vesículas foi confirmada por *SEM* (sigla em inglês, microscopia electrónica por varrimento). O método do iQUE foi optimizado em termos de duração de centrifugação, tubos, concentração das contas de látex e soluções de bloqueio para os anticorpos. Já os protocolos de captura específica requerem mais optimizações, especialmente com soluções de bloqueio válidas para vesículas. Depois disto, experiências futuras incluirão amostras biológicas mais complexas como sangue e urina e teste de matrizes poliméricas para captura específica. A futura plataforma modular de biopsias líquidas irá possibilitar testes pré-sintomáticos e detecção precoce de cancro, usando pequenas quantidades de amostra e reagentes.

Palavras-chave: cancro da próstata, diagnóstico, exossomas, isolamento, microfluídica, PSMA, vesículas extracelulares

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VII. List of abbreviations

BSA Bovine Serum Albumin

CMFDA 5-Chloromethylfluorescein Diacetate

CTG	CellTracker™ Green
EGFP	Enhanced Green Fluorescent Protein
EpCAM	Epithelial Cell Adhesion Molecule
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
FSC-H	Forward Scatter - Height
MEM	Minimum Essential Medium
MHC	Major Histocompatibility Complex
MVE	Multivesicular Endosomes
NEAA	Non-Essential Amino Acids
nPLEX	Nano Plasmonic Exosome Sensor
PBS	Phosphate-Buffered Saline
PCa	Prostate Cancer
PDMS	Polydimethylsiloxane
PE	Phycoerythrin
PEG	Polyethylene Glycol
PSA	Prostate Specific Antigen
PSMA	Prostate Specific Membrane Antigen
P/S	Penicillin/Streptomycin
PTEN	Phosphatase and Tensin Homolog
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
SEM	Scanning Electron Microscopy
SPR	Surface Plasmon Resonance
SSC-H	Sideward Scatter - Height
WHO	World Health Organization

VIII. Goal and motivation

The aim of this work, developed at the Nanotechnology unit of the Diagnostics department at the Fraunhofer Institute for Cell Therapy and Immunology, IZI, (Leipzig, Germany), is to characterize

exosomes in terms of morphology and protein marker expression by optimizing exosome isolation and analysis by known methods such as FACS and SEM. Exosomes are abundantly present in all body fluids which facilitates the minimally invasive access to these promising, biomarker rich vesicles. These nanosized vesicles have been shown to have specialized functions which can have potential for diagnostics and treatment. However, despite this growing interest in the medical research field, current isolation methods are not suitable in a clinical context due to the high sample volume required and long processing times, therefore the need for a new method for efficient separation and enrichment from body samples becomes a reality (1).

Vesicles from prostate cancer (PCa) cell lines will be used for proof of concept since PCa has the highest incidence in Europe, second worldwide, in men. It is also the third ranking cause of death in men in Europe, fifth worldwide.

Circulating exosomes can allow for an earlier detection of PCa cases, avoiding painful and expensive biopsies and detecting the cancer in earlier stages, where treatment can be more effective.

1. Introduction

1.1. Exosomes and other vesicles

Exosomes are membranous extracellular vesicles with diameters in the size range of 40 to 200 nm released by virtually all cells and abundantly present in body fluids (Figure 1).

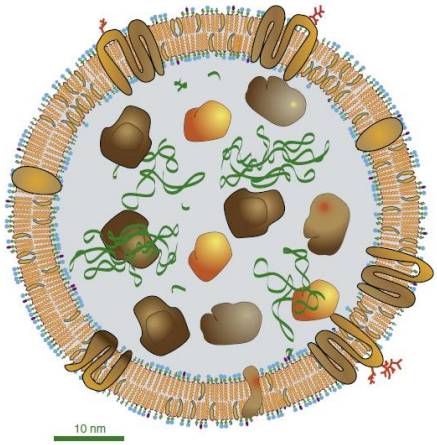

Figure 1. Representation of the average exosome structure: Lipid bilayer enclosing cytosol with RNA (green ribbons) and proteins (blobs); proportionally drawn (2)

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 both endogenous and exogenous intercellular communication (3), and not just as initially thought of as cellular waste disposal vesicles, making them important cellular niche regulators. 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 be formed as depicted in Figure 2: whereas microvesicles bud directly from the plasma membrane, exosomes form as the intraluminal vesicles bud inwards, forming an early endosome which then matures into a multivesicular endosome and releases the exosomes it contains by fusing with the plasma membrane or fuses 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).

This recent interest in exosomes as a source of biomarkers has led to the creation of compendiums of molecular data found in different classes of extracellular vesicles (ectosomes or shedding microvesicles, exosomes and apoptotic bodies), such as Vesiclepedia or ExoCarta. Vesiclepedia collected to date (September 18th, 2016) data from 538 articles, of which 92.897 resulted in protein entries, 27.642 in mRNA entries, 4,934 miRNA entries and 584 for lipids.

However, exosomes can differ up to 5-fold in size and 10^4-fold in concentration in different biological samples, which makes accurate isolation and measurement of concentration challenging (6).

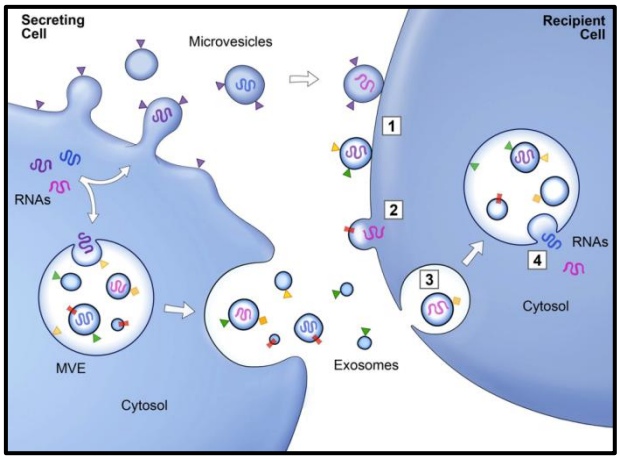

Figure 2. Exosome and microvesicle biogenesis. Proteins (triangles and rectangles) and RNA molecules are selectively incorporated into MVEs or into microvesicles budding from the plasma membrane. MVEs fuse with the plasma membrane to release exosomes and these may dock at the plasma membrane of a target cell [1]. From there, vesicles may either fuse directly with the plasma membrane [2] or be endocytosed [3]. Endocytosed vesicles may then fuse with the membrane of an endocytic compartment [4] resulting in the delivery of proteins and RNA to the target cell **(7)**.

1.1.1. Exosome isolation and enrichment methods

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 (7). Several methods are used to isolate and purify exosome solutions from fluid samples, with variable levels of purity. Regarding exosome purification, two main methods can be considered: ultracentrifugation and immunoaffinity capture.

The most generally used method is ultracentrifugation, in which the culture medium is filtered by microporous membranes and then differentially centrifuged and suffers a final centrifugation at very high centrifugal forces (70.000 to 100.000 × *g*) and the resulting pellet, resuspended in PBS, can be again ultracentrifuged at the same speed (5).

There are some downsides to this method, since large-scale instruments and centrifuges are needed, which are not usually available at Hospitals, point of care locations or at lower resources settings. Also, it makes use of very large culture volumes (higher than 200 mL) and overall the process is time consuming and demanding in terms of personnel and reagents. Moreover, centrifugation procedures are not selective enough to discriminate exosomes from different cellular origins or from other vesicles or large protein aggregates. (6) (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. Carefully deposited layers of different dilutions of aqueous iodixanol

60% (w/v) with 0.25 M sucrose and 10 mM Tris are then used to separate the exosomes from a solution, after being subject to very high centrifugal forces. The final step would be to collect different fractions of the column, assess its density and compare it to that of the exosomes (9).

Other than physical methods, precipitation solutions are also commercially available which make use of polymers that precipitate exosomes while rendering them suitable for further molecular analysis, having the advantages of being easy to use, with a only one or two step procedure and do not require any expensive equipment or technical expertise, being however expensive and often fail to distinguish between differently sized vesicles and membrane-free macromolecular aggregates (10) (11).

For a more specific selection, as the name indicates, immunoaffinity capture will make use of antibodies against antigens of interest, immobilized onto a matrix or magnetic beads, for instance. Needless to say, the application of this method relies on the previous knowledge of the surface antigens to target.

1.1.2. Exosome visualization and quantification

Due to the nanoscale of the sample, electron microscopy is the most suitable technique to assess the morphology of the exosomes, with a resolution ranging from 20 µm to as far as 0.1 nm (12).

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.

1.1.3.Exosomes and 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” (13).
The ideal biomarker should screen for a disease presence or absence and its consequent progression and response to treatment, identify high-risk individuals and predict recurrence.
A new relevant biomarker needs to provide information that cannot be acquired in a more simple and cost-effective way and over all needs to answer a clinical question in a consistent, non-invasive, quantifiable, faster and more economical way than existing methods.

As previously stated, there are no specific markers for exosomes *per se*, so, for detection and isolation, general enriched surface proteins are used. These common proteins are from the tetraspanin family, such as CD9, CD68 or CD81, or proteins essential for multivesicular formation such as TSG 101 and Alix. Other than these, Flotilin and HSP70 are also commonly detected. And since exosome content is related to cellular origin, some more specific markers can be used, as MHC class II for detecting antigen presenting cells, A33, for intestinal epithelial cells and CD3 for T cells. (14). EpCAM is also present in exosomes, in different expression levels according to their cellular origin. (15)

1.2. Prostate Cancer and biomarkers

Prostate Cancer 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 (Figure 3).

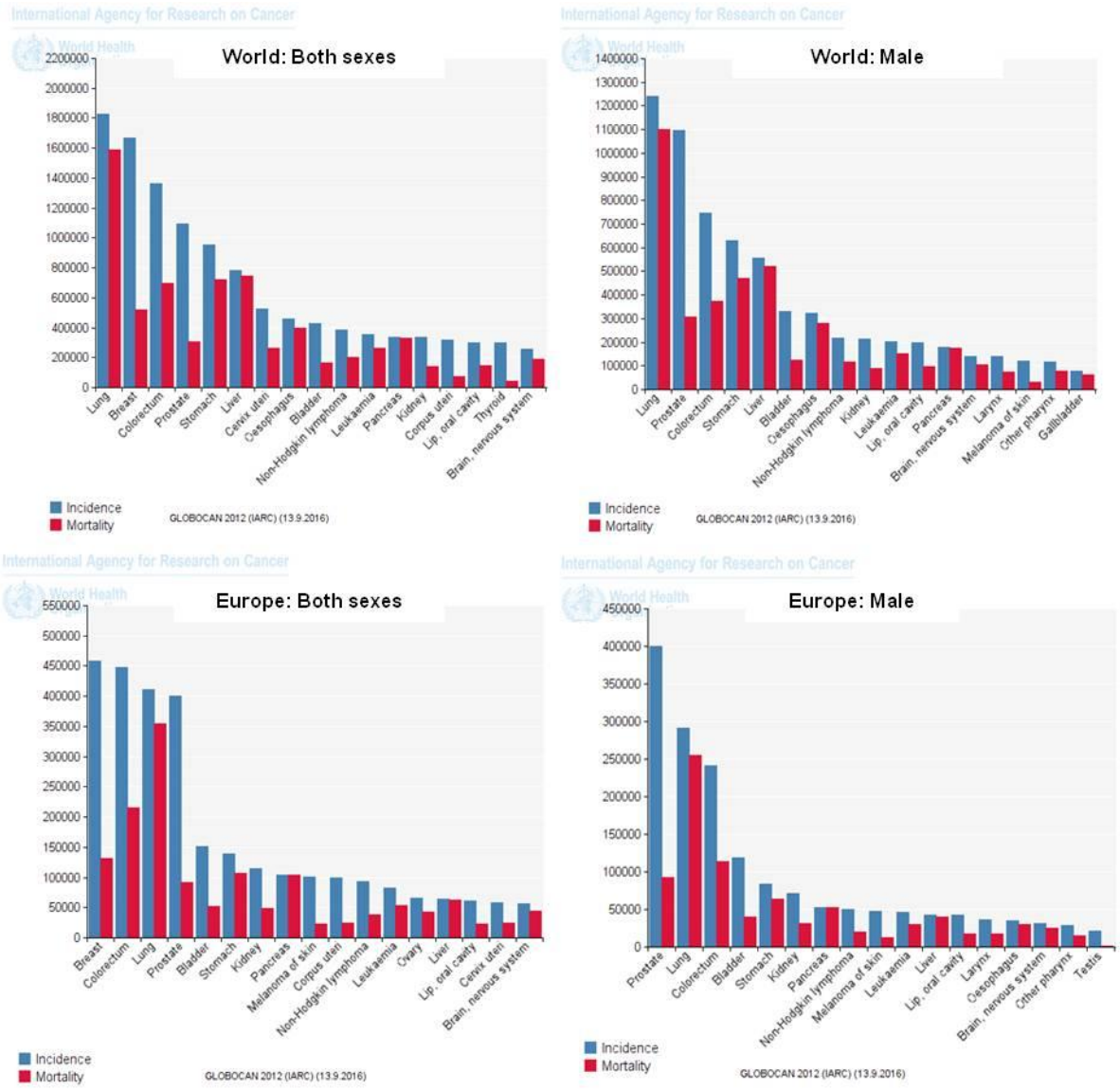

Figure 3. Prostate cancer Incidence and Mortality statistics, in the World and in Europe in both sexes and male context (adapted from GLOBOCAN 2012 IARC)

Approximately 1,1 million men were diagnosed worldwide with PCa in 2012, which represents 15% of the total of cancers diagnosed in men, with almost 70% of the cases occurring in more developed regions.

The highest incidence occurs in the developed areas of Australia/New Zealand and Northern America and in Western and Northern Europe, due to the generalized application of the diagnostic Prostate Specific Antigen (PSA) test in these regions, and therefore higher positive diagnostic rates. Incidence is also relatively high in some less developed regions such as the Caribbean, Southern Africa or South

America, but remains low in Asian populations. PCa is the third leading cause of death in Europe, fifth worldwide, in men.

With the expected increase in the life expectancy of European men and the subsequent rise in the incidence of PCa, the disease's economic burden in Europe is also expected to increase. (16)

PCa is very difficult to define in terms of biological, hormonal and molecular characteristics, since it is a very heterogeneous disease in terms of grade and oncogene/tumor suppressor gene expression.

So, finding a good biomarker in body fluids is no easy task, mostly due to the high concentration of general proteins such as immunoglobulins, albumin or transferrin in blood, and the fact that proteins in lower abundance, sometimes on the ratio of 1:7.500.000 in the case of PSA, the most promising biomarker candidates, are for this reason difficult to identify and quantify. This "needle in a haystack" problem can be partially solved by depleting the sample from these abundant proteins (by chromatography or precipitation, for instance) or by specific enrichment, with the added problems of longer process times and the markers of interest for enrichment being unknown. So one path to follow is to make use of the fact that circulating microvesicles and exosomes contain a large variety of proteins and RNA molecules and express membrane proteins representative of their origin tissue, which can in turn be used for tissue-specific exosome isolation from more complex fluids and then processed on for further biomolecular analysis. (1)

Other than inherent intra- and inter-variability between patients, analytical and regulatory barriers are also to be expected. These comprise barriers related to patents and intellectual property, to the complexity of the assays and clinical trials and the application of quality control methods for reproducibility and accuracy. There are many reports of different promising biomarkers, but a lack of strategies to determine which candidate is worth long-term investment for further laboratorial and clinical studies (17).

Concerning exosomes that can serve as source of diagnostic and prognostic markers, these can be found in three types of fluids. Plasma contains a high number of exosomes from several different cellular origins and is minimally invasive to collect. Urine can be non-invasively collected in large volumes at low cost, and provides a narrower source of exosomes with respect to the prostate when comparing to blood, although in low concentration and subject to variability between patients. Disadvantages present when using semen, which is also minimally invasive to collect and a direct source of prostate exosomes (18).

1.2.1.PSMA

PSMA is a transmembrane-carboxypeptidase produced in the prostate gland, up-regulated 10-fold or more 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, being considered the gold standard for the detection of PCa. In addition, PSMA is a commonly used biomarker for imaging, to track the progress of treatment, or for treatment itself, as PSMA is internalized after binding, it can be a specific target for radionuclide therapy (19). The most common way to detect PSMA resorts to anti-PSMA antibodies, there is however interest in finding an alternative binder due to the fact that this generalized immunoaffinity capture method has its downfalls as antibodies are unstable, need special careful handling and require complex and costly production procedures. By using the small molecule PSMA-617 (Figure 4) these complications are avoided. 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 such as ^{68}Ga, ^{111}In, ^{177}Lu, and ^{90}Y (20).

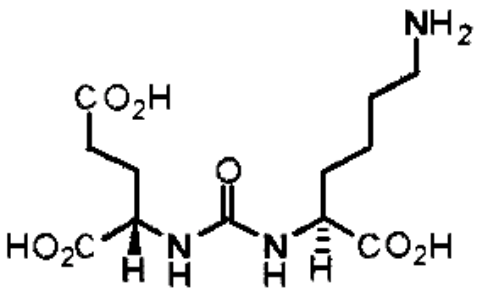

Figure 4. Small-molecule PSMA ligand, PSMA-617 (Glu-CO-Lys), chemical structure

1.2.2.PSA

Free PSA, also known as human kallikrein 3, hK3, is the biomarker currently used for screening. However, according to the 060/2011 norm from Direção-Geral da Saúde (21) (organization part of the Portuguese Ministry of Health in charge of coordinating healthcare related activities), screening for total PSA blood levels should not be prescribed for a general population screening, but for monitoring PCa patients after treatment since, overall, PSA-based screening leads to a decrease in the prevalence of advanced PCa and a reduction of PCa-related mortality by 20%. However, despite its good sensitivity, PSA screening lacks the specificity for discriminating between inflammation, benign prostate hyperplasia, indolent or aggressive PCa, being consequently associated with a high risk of overdiagnosis and overtreatment based on findings on complementary diagnostic prostate biopsies. Therefore, new biomarkers are needed to prevent unnecessary biopsies and monitor and improve the overall quality of treatment.

1.3. 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, whether the target is present or not. The most known application of these assays is perhaps the home pregnancy test, which detects human chorionic gonadotropin hormone in urine, but there are several other applications regarding detection of toxins and pathogens, RNA and DNA, pesticides or metal ions, or pharmaceuticals and drugs.

As far as the setup is concerned, the sample is applied in the denominated sample pad, which promotes a controlled distribution of the fluid onto the conjugate pad and can also be used to pre-treat the sample, such as blocking to avoid non-specific binding downstream. As it is absorbed, the sample will be put in contact with the conjugate pad which contains dried particles, usually gold but can also be latex or magnetic beads or other luminescent materials, conjugated with marker molecules - one type of marker for specific isolation and another marker for detection (Figure 5). The liquid will dissolve these particles out of this pad and will flow through the commonly used nitrocellulose membrane, flow which is partially controlled by the absorbent pad that has the main functions of increasing the total volume of sample entering the test strip and preventing the liquid from returning to the analysis area. The targeted molecules in the sample will bind to both markers, whereas the unbound particles, will bind to the control line, if the strip has not been corrupted in any way (24).

1.3.1. Sandwich assays

In the case of sandwich assays (Figure 5), there is a test line with anti-biotin (or other analyte) antibodies, which will capture the complex and, due to the optical properties of the gold particles, a red color will appear. The excess labeled antibody will be captured at the control line by another secondary antibody. Possible results are schematized in Figure 6.

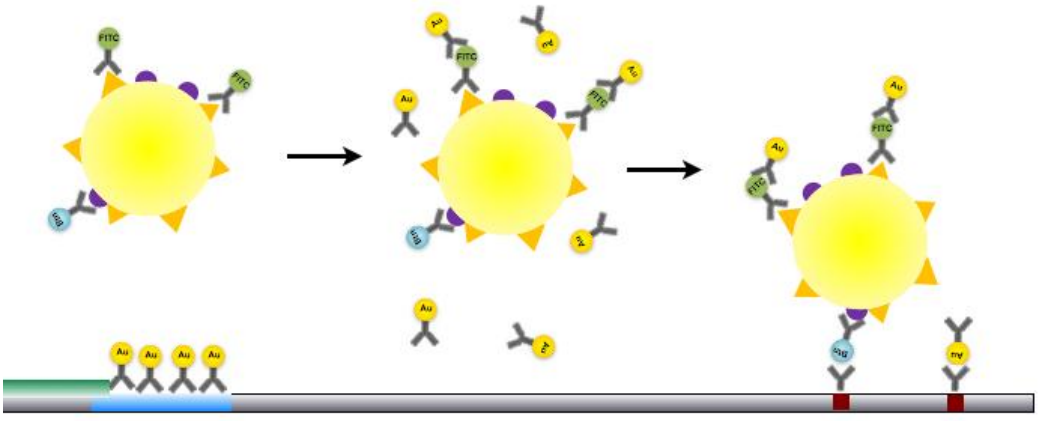

Figure 5. Lateral flow strip schematics – sandwich format with biotin-labeled anti-target antibody for isolation and anti-FITC gold labeled antibody for detection. Green: sample pad; blue: conjugate pad with gold labeled antibodies; gray: lateral flow strip membrane; red: test (left) and control (right) lines.

1.3.2.Competitive assays

Competitive assays are especially 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 (unlabeled) 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 (Figure 6).

Figure 6. Possible results in a lateral flow strip – sandwich assay: positive (left) and negative (right); competitive assay: negative (left) and positive (right); color code as in Figure 5

1.3.3.Multiplex detection format

It is also possible to detect more than one target, by placing more test lines, according to 1.3.1 or 1.3.2. Adjustments have to be made to the amounts of labeled antibodies and other reagents so that all the targets have enough detecting labels.

2. State-of-the-art

2.1. Isolation methods

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.

According to a 2013 report by McKinsey & Company on Personalized Medicine (25), tests for screening and risk-identification will grow the next years, since these will become less invasive and have a greater clinical relevance, with a market share for high-value diagnostics in oncology expected to reach 3 billion dollars by the end of 2018. Even though some screening tests, like the PSMA blood test, can have a questionable benefit, medical professionals will still welcome all the available information about a disease. Ultimately, the establishment of a link between genomic and/or proteomic

markers and a certain pathology will drive the development and common practice adoption of these tests.

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.

2.1.1. Immunological separation

Mei He *et al.* developed a microfluidic continuous-flow mixing platform for exosome immunomagnetic isolation and *in situ* immunoassay, the ExoSearch. The sample is injected into this polydimethylsiloxane (PDMS) device by a Y-shaped injector, flows through a serpentine channel so that fluorescently labeled antibodies and antibody-covered magnetic beads bind exosomes, and these are finally collected in a microchamber by a removable magnet. Full analysis is achieved with as low as 20 µL plasma samples in about 40 minutes. The quantitative detection of intact exosomes was achieved with a limit of detection of 7.5×10^5 particles per mL (6).

Immuno-chip, developed by Chen *et al.* also makes use of proteins on the outer membrane of the exosome for specific capture by anti-CD63 antibodies immobilized on the surface of the herringbone structure. Bound exosomes can then be characterized *in situ* or lysed for nucleic acids extraction (26).

Similarly designed but with the addition of on-chip fluorescence quantification by a standard plate-reader, Kanwar *et al.* developed ExoChip (**Figure 7**). Sample mixing is enhanced by making it flow through circular wells alternated with narrow channels, increasing the retention time and overall strengthening the interaction with the functionalized surface, again with anti-CD63 antibody. One inconvenient of this device is that it uses serum, so sample preparation is required (4).

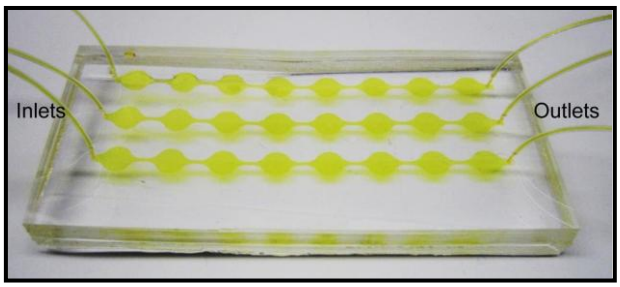

Figure 7. Prototype of the ExoChip (three channel) depicting the flow of serum.

He *et al.* developed an integrated platform (Figure 8) to study plasma non-small-cell lung cancer exosomes. This platform isolates and enriches the sample in exosomes, lysating them next for protein capture by immunomagnetic beads, which is followed by an immunoassay with chemifluorescence detection. This device was shown to capture exosomes in a smaller size range and in their majority, intact exosomes, unlike the heterogeneous vesicular populations obtained by ultracentrifugation (27).

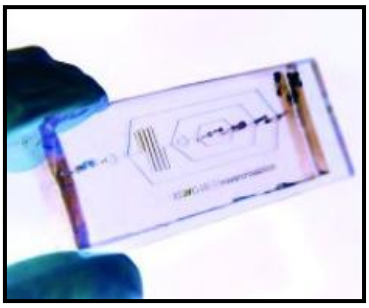

Figure 8. Image of the prototype PDMS chip containing a cascading microchannel network.

Other analytical methods can be applied, such as surface plasmon resonance (SPR), leading to the development of nPLEX by Im *et al.*. SPR is sensitive to the point of allowing real-time display of the binding intensity, between the functionalized nanohole array surface (Figure 9) with different affinity ligands and the surface exosome markers, amplifying the signal by labelling the exosomes with gold nanoparticles. The authors used ascitic fluid from ovarian cancer patients, and directly applied it on the device after filtration through a 0.2 μm membrane, not being necessary further sample treatment (28).

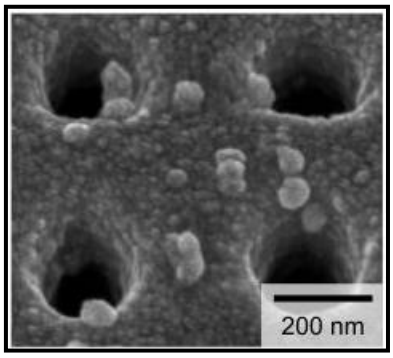

Figure 9. SEM of specifically captured exosomes by the nanohole array of the nPLEX.

iMER platform is a microfluidic chip (Figure 10) developed by Weissleder *et al.* which aims to analyze mRNA levels in enriched tumor exosomes obtained from blood. This integrated device comprises a chamber for exosome enrichment by immunomagnetic capture and another one for RNA isolation and elution by glass beads, RNA elute which then proceeds to a chamber for reverse transcription and preamplification of rare targets. Finally, multiple qPCR sites are present to detect the target mRNA (29).

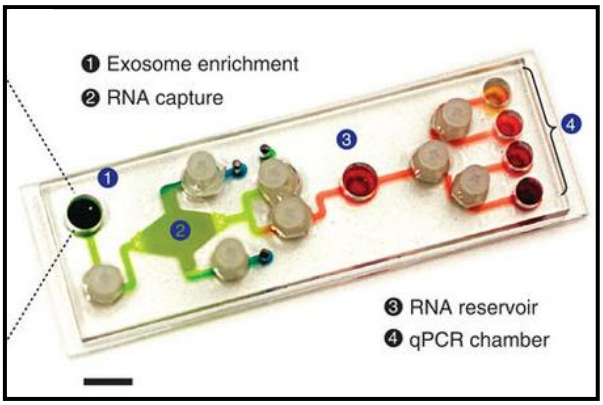

Figure 10. The microfluidic iMER prototype. Scale bar: 1 cm.

2.1.2.Physical methods

Sieving methods rely on physical properties of the extracellular vesicles, which are directly extracted from blood by being passed through a membrane and filtrated by pressure or by electrophoretic forces. This method is by nature non-selective and yields a low recovery of exosomes, performing however well in terms of running time and with higher RNA yields than ultracentrifugation: the electro-driven filtration yields about 79 ng of RNA *per* 100 µg of protein from a 100 µL sample, whereas ultracentrifugation yields 187 ng of RNA *per* 100 µg of proteins however from a much higher volume of 5 mL (30).

A microporous silicon nano-wire structure, by Wang *et al.*, is capable of selectively collecting intact phospholipidic exosome-like vesicles, of sizes between 40 and 100 nm, in a relatively fast time (around 10 minutes), while filtering out proteins and cell debris. Volumes of 30 µL are used, more than that, the retention rate of the bigger vesicles decreases, possibly due to saturation of the micropillars (31).

A recent paper by Wunsch *et al.*, from the IBM T.J. Watson Research Center (32), showed promising results in the separation of exosomes down to 20 nm of size by using manufacturable silicon processes to produce nanoscale lateral displacement arrays with gaps ranging from 25 to 235 nm. Samples are injected into the array by a hydrodynamically focused jet, and according to their size, their interaction with this array will differ, which promotes separation. Moreover, in the collecting outlet, the exosome fractions can be channeled out of the array, for further biochemical assays.

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)

3. Materials and Methods

3.1. Cell lines

For this work, a binary distinction in terms of PSMA expression was necessary, for which PC-3 (ATCC CRL-1435) and LNCaP (ATCC CRL-1740) metastatic site derived cell lines were used. PC-3 is a PSMA negative, androgen-independent cell line, while LNCaP is androgen-sensitive and PSMA-positive. These cells are cultured in RPMI 1640 Medium (Gibco) for LNCaP and F-12K Nut Mix (1X) Nutrient Mixture Kaighn's Modification (gibco) in the case of PC-3, both supplemented with 1% Penicillin/Streptomycin (P/S) at 10.000 U/mL / 10.000 µg/mL (Biochrom) and 10% FBS (fetal bovine serum), that when stated, was vesicle depleted (Invitrogen) (33).For some experiments, as a negative control, HeLa (ATCC® CCL-2™) cells were used along with PC-3, incubated in MEM supplemented with 10% vesicle free-FBS, 1% P/S + 5% Sodium Pyruvate + 5% NEAA. The incubator was set at 37° C with a 5% CO_2 atmosphere.

3.2. Fluorescent staining of exosomes

To obtain PSMA-positive self-fluorescent exosomes, two methods were tested. One was the transfection of the LNCaP cells with the plasmid pEGFP-C1 (Clontech). Transfection was done with Lipofectamine® 3000 Transfection Reagent (Thermo Fisher, L3000015), according to the instructions provided. For selection of the transfected cells, the antibiotic Geneticin® G-418 (ThermoFisher) was added to the culture medium at least two days after transfection, initially at 50 µg/mL and, according to cell response as assessed by fluorescence microscopy, increased to 100 µg/mL. The other method consisted of incubating the cells for 45 minutes with 10 µM CellTracker™ Green CMFDA Dye (CTG) (ThermoFisher, C7025), according to the manual provided with the kit. This procedure renders cells fluorescent for over 72 h, or the equivalent to 3 to 6 generations.

3.3. Exosome Isolation

3.3.1. Initial protocol

Exosomes were purified from conditioned cell media, collected three days after the cells grew in their respective media supplemented with 10% exosome-depleted FBS and 1% P/S, as described in section 3.1. In case the media was frozen, it was left to thaw overnight at 4°C before the procedure. To pellet cells, the collected medium was centrifuged for 5 min at 300 × *g*, 4°C. After this, the supernatant was centrifuged again for 30 minutes at a higher speed of 10.000 × *g*, 4°C, to remove dead cells and debris. Then the supernatant was transferred to special ultracentrifuge tubes (seton scientific tubes) by straining through a 1 µm membrane (pluriSelect, 43-50001-03). It was then centrifuged in an ultracentrifuge (Sorval Discovery UZ) for 90 minutes at 70.000 × *g*, 4°C. Each pellet was resuspended in 1 mL of 0.22 µm filtered PBS and aliquoted in 100 µL to store at -20°C.

3.3.2. Optimized protocol

To avoid contamination by smaller cellular debris, the method described in 3.3.1 was changed, based on (14), in the following way: samples are no longer strained by 1 µm membranes but, before the ultracentrifugation, are filtered using a 0.22 µm flask-top filter with a vacuum pump, to remove bigger vesicles and smaller debris. Moreover, the ultracentrifugation speed was increased to 100.000 × *g* and the pellets resuspend in half of the previous volume, 0.5 mL and stored at -80°C. These alterations theoretically ensure a higher concentration of more homogenous exosomes.

3.4. Vesicle Analysis

The iQUE screener is an integrated cytometry platform from IntelliCyt®, to perform cell or bead suspensions multiplexed analysis by the ForeCyt software and as such, it was used to analyze and characterize cells and vesicles from PCa cell lines.

Before the first use of the day, the iQUE Screener goes through a quality control routine, and according to these results, can be put through different cleaning or unclogging protocols, if necessary.

The samples can be placed in multiple well plates or in single tubes, in a minimum volume of 100 µL, which are then pumped into the device through plastic tubes that lead the sample to the optical analysis area. The iQUE Screener is equipped with two lasers: blue at 488 nm and red at 633 nm. These lasers excite the fluorophores in the samples, which in turn emit radiation, that can be detected by four different channels: FL1, with the filter 533/30, for fluorophores such as FITC (excitation maximum at 490 nm / emission maximum at 525 nm), GFP (488/510 nm) or CTG (492/517 nm); FL2, with the filter 585/40 for PE (excitation maxima at 496, 546, 565 nm, emission maximum at 578 nm); FL3 with the filter 670LP, for Propidium Iodide (535/617 nm) and finally FL4, with the filter 675/25, for Cy5 (649/666 nm). In this optical analysis area, the samples will scatter the incident laser light that will hit the detector in different angles according to the sample's size and granularity. (34)(35)

The graphical information can be treated with the ForeCyt software and displayed as dot plots in which the horizontal axis is usually, in this document, set to be the FSC-H (Forward Scatter - Height), which

is related to size and the vertical axis can display SSC-H (Sideward Scatter - Height), related to granularity, or the fluorescent filters FL1-H, FL2-H or FL4-H.

The following experiments were made in order to attempt a quantification of the vesicles in the samples and to assess the PSMA and EpCAM expression by PCa vesicles.

3.4.1. Outlook on the general protocol

In general, the iQUE screener method consists of three main steps: the binding of exosomes to beads, whether directly via adsorption of the vesicles or specifically via binders, the anti-PSMA antibody or the small molecule PSMA-617. Secondly, it is necessary to block the surface of the beads to avoid nonspecific binding by the labeling molecules, which finally are added, targeting the surface proteins of interest. In between these steps, washing of the beads (resuspending in PBS and pelleting again) is necessary to remove unbound molecules. So it is necessary to optimize these four points: binding, blocking, labeling and washing, in order to establish a reproducible method to estimate exosome concentration and PSMA expression.

3.4.2. Settings

3.4.2.1. Direct measurement of exosomes

To assess the behavior of the exosome suspensions on the iQUE, two 100 µL samples were measured: PBS (Gibco) as a negative control and LNCaP undiluted exosomes purified according to section 3.3.1. The software was limited to measure for one minute and a threshold for FSC-H of 10 was set.

3.4.2.2. Beads

Due to the detection limit of around 0,5 µm of the iQUE, vesicles were coupled to 4 µm aldehyde/sulfate latex beads (4% w/v, molecular probes, approximately 1.3 x 10^9 beads/mL), as suggested in literature (36). The detection maximum of the device is 10.000 events/s and considering a measurement time set to 60 seconds, 260.000 beads will be used per sample, to remain on a reasonable range.

3.4.2.3. Vesicles

The amount of vesicles to use in each experiment was set to 15-30 µg worth of exosomal protein, measured by BCA assay (Pierce)(37). A range is given, since the amount to use depends on the availability of the vesicle samples at the moment of each experiment.

3.4.2.4. Controls - Establishing thresholds and gating plots

For optimal results, the FSC-H threshold should be set to a value between 10.000 and 50.000 before each measurement. This way, most counts relative only to the dispersion medium are ignored, and more relevant counts are attributed to the bead-bound exosomes. Unless stated otherwise, all FSC-H thresholds were set to 10^4.

In order to retrieve important and relevant information of the plots, these have to be gated, which means, defining an area (gate) enclosing the points of interest. To do so, beads in PBS, the dispersion medium in these experiments, are measured. With these measurements it is possible to separate background fluorescence caused by PBS from the sample bead signal and is possible to limit the area where most of the beads will be found, as seen in Figure 11.

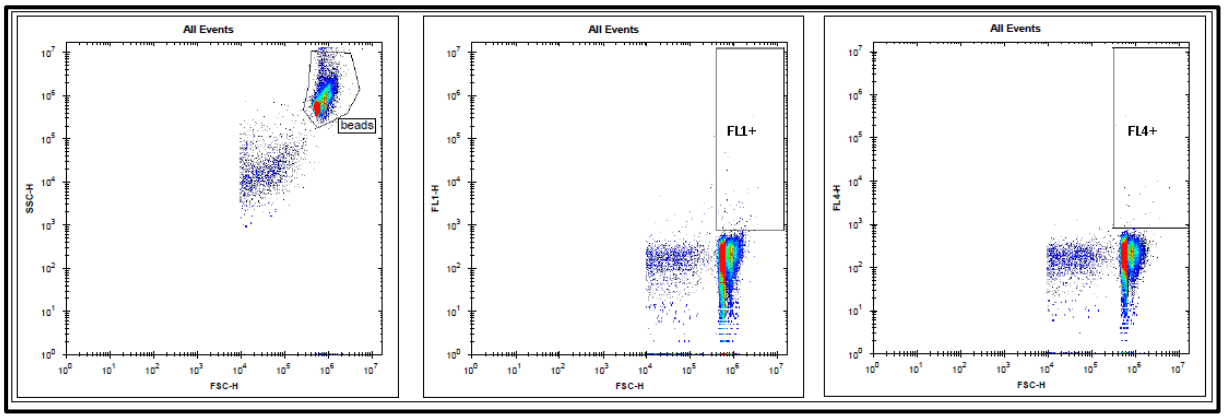

Figure 11. Gating in ForeCyt plots, in a sample of beads in PBS

It also important to set a Noise filter, enclosing the area with most of the beads (Figure 12). Anything outside of these boundaries will be considered noise, and will not be displayed in the main plots.

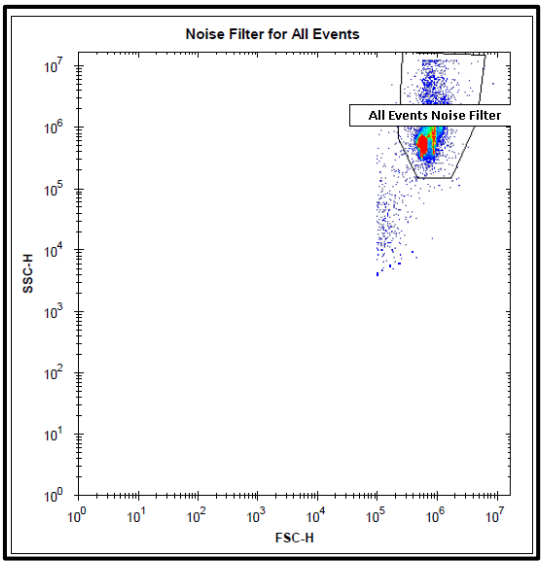

Figure 12. Noise filter for a beads-in-PBS sample

3.4.3. Optimizations

3.4.3.1. Detection

3.4.3.1.1. Anti-EpCAM FITC-labeled antibody titration

With vesicles from the PC-3 and LNCaP cell lines, purified as described in section 3.3.1, an anti-EpCAM-FITC (Life Technologies) antibody titration was performed. 4 µm aldehyde/sulfate latex beads (4% w/v, molecular probes) are incubated with the vesicles for 2 hours, in a total volume of 1 mL. Then, the beads are blocked with 100 mM glycine in PBS for 45 minutes at RT and then incubated with anti-EpCAM-FITC in increasing concentrations from 0,75 µg/mL to 24,00 µg/mL. Finally, the BCA assay was repeated with the supernatant, before washing the beads with PBS.

3.4.3.1.2. Self-fluorescent vesicles

Another way to detect vesicles other than surface labeling with EpCAM-FITC is by having an intravesicular fluorescence source. Vesicles can be collected and purified from the growth media of cells expressing GFP or from cells that have up taken CellTracker™ Green.

This self-fluorescence is beneficial when compared to antibody staining as it allows for a more specific signal, since the detection is no longer dependent on the concentration and epitope availability of a surface marker, which cannot reflect so well the number of exosomes present in a sample.

To confirm if the exosomes exhibited the same fluorescent properties as their mother-cells, purified exosomes from CTG LNCaP cell medium were directly measured or incubated with beads in PBS, during 45 minutes, at RT, and then measured with the iQUE.

3.4.3.2. Centrifugation

3.4.3.2.1. Duration

Between each incubation step, samples can be centrifuged for bead pelleting or for washing steps in PBS to eliminate unbound molecules from the previous incubation. In general, a washing step consists of resuspending a bead pellet in PBS, centrifuging and discarding the supernatant. In some protocols, up until ten centrifuge cycles can be executed, which can lead to a great loss of beads in the supernatant, so it is critical to optimize this procedure. In order to reach a centrifugation time that minimizes bead loss, beads were incubated with LNCaP vesicles for 1 h at RT. Then centrifuged at 9.200 × *g* for 2, 4, 6, 12 and 24 minutes and this supernatant kept. After blocking for 45 minutes with 50 mM glycine & 1.5% BSA in PBS and incubated also during 45 minutes with anti-EpCAM-FITC antibody (3 µg/mL) samples were again centrifuged for the same time intervals, washed once and their supernatants kept. Finally, the bead samples are resuspended in PBS and measured individually with the iQUE Screener, as are the collected supernatants.

3.4.3.2.2. Tubes

To test whether low binding polypropylene tubes (Eppendorf® Protein LoBind microcentrifuge tubes, Sigma Aldrich, Z666505-100EA) would contribute better to the reduction of bead losses in the supernatant when compared to standard tubes (Eppendorf® Safe-Lock microcentrifuge tubes, Sigma Aldrich, T9661-1000EA), 280.000 beads/reaction were initially centrifuged for 5 minutes at 4.500 × *g* and washed once with PBS. These beads were incubated with 30 µg of CTG LNCaP vesicles, then blocked with 100 µL 1.5% BSA & 50 mM glycine in PBS, and after this, incubated with anti-PSMA-antibody (6 µg/mL). All incubations are 30 minutes long at RT, and followed by a centrifugation step, in the same conditions as before and the supernatant kept. After this last incubation with the antibody, the beads were washed 3 times with PBS and the secondary antibody, anti-mouse IgG-Cy5 conjugate, was added in the dilution of 1:40.000, for 30 minutes at RT. Beads were pelleted and the supernatant collected and washed 3 times with PBS. As controls, PBS alone and beads suspended in PBS were also measured, after going through one centrifugation cycle and resuspended in PBS. When this protocol was ran in standard tubes, PBS was used, and when ran in low binding tubes, PBS was supplemented with 0,05% Tween-20, also in an attempt to minimize the interactions between exosomes and tube walls.

3.4.4.Blocking

3.4.4.1. FITC-EpCAM bead blocking test

Since nonspecific binding of the antibody coupled fluorescent dyes to the bead surface was detected, bead blocking needed to be optimized.

For this five different blocking solutions were tested: 1% BSA, 3% BSA, 33.3 mM Glycine, 100 mM Glycine and 50 mM glycine + 1,5% BSA, in PBS.

Beads in PBS were firstly centrifuged and the supernatant discarded, and then separately incubated for 45 minutes at RT, shaking, with the above mentioned blocking solutions. Afterwards, anti-EpCAM-FITC antibody was added, to a final concentration of 3 µg/mL, incubated in the same conditions as before. To remove the unbound antibody, samples were then centrifuged at 9.200 × *g*, for 2 minutes, and washed once with PBS. The supernatant was kept for measuring and the pellets were resuspended in 100 µL of PBS and measured.

3.4.4.2. Cy5-labeled secondary antibody bead blocking test

The necessary amount of beads was pelleted from the stock solution in low binding tubes (Eppendorf® Protein LoBind microcentrifuge tubes, Sigma Aldrich, Z666505-100EA) and washed once in PBS, to continue then with 45 minutes incubation steps. Primary antibody anti-EpCAM-FITC (6 µg/mL) was directly incubated with the beads and washed once with PBS/0,05% Tween20. Then blocked with three different solutions: 3% BSA, 100 mM glycine or 1,5% BSA and 50 mM glycine, in PBS, washed twice and finally incubated in a 1:1000 anti-mouse-IgG-Cy5 labeled antibody dilution. As

a control, beads were directly incubated with a 1:1000 dilution of the Cy5 labeled antibody. In the end, the samples were washed three times and resuspended in 100 µL of PBS/0,05% Tween for measurement (all samples were made in triplicate for statistical purposes).

3.4.4.3. CTG vesicles bead blocking test

To test whether the blocking solutions for antibodies are also valid for exosome samples, 3% BSA, 100 mM glycine, 1.5% BSA and 50 mM glycine, and polyethylene glycol PEG-4000 (Roth) at 50 nM, 200 nM and 500 nM concentrations were tested. Beads were pelleted and washed once in PBS, some samples incubated with anti-EpCAM antibody (ThermoFisher), 6 µg/mL, and blocked with the previously named solutions for 45 minutes at RT, washed twice and incubated with PC-3 CTG vesicles, purified according to section 3.3.2. As a control, beads were directly incubated with the same amount of CTG vesicles, 45 minutes at RT. Finally, the samples are washed three times and resuspended as described above.

3.4.5.Final Protocols

3.4.5.1. Surface marker detection via Cy5-labeled secondary antibody - cells

To know the expression profile of EpCAM and PSMA in the cell lines in use, the PSMA-positive LNCaP, and PSMA-negative PC-3 and HeLa, these were incubated with the respective antibodies which were then detected by Cy5 labeled anti-mouse IgG.

Cells were harvested, washed and centrifuged for 5 minutes at 300 × *g* and resuspended in ice cold PBS supplemented with 1% BSA for counting in a Neubauer counting chamber. For PSMA testing, $3x10^5$ cells per 100 µL were used, and $2x10^5$ cells for the EpCAM expression test. These were then blocked for 30 minutes at RT with 1,5% BSA and 50 mM glycine in PBS and afterwards, centrifuged for 8 minutes at 350 × *g* so that the primary antibodies could be added, the anti-PSMA (6 µg/mL) or anti-EpCAM (6 µg/mL). After 30 minutes incubation at RT, the sample is centrifuged again and washed three times with PBS/0,05% Tween20. The secondary anti-mouse IgG-Cy5 conjugated antibody is then added at a 1:2500 dilution and incubated in the same conditions. Samples are centrifuged at 400 × *g* for 8 minutes, and washed three times with PBS/Tween20, and finally resuspended in 100 µL for measurement in the iQUE. Protocol adapted from (38)

3.4.5.2. Surface marker detection via Cy5-labeled secondary antibody - vesicles

To compare the expression profile of the surface markers EpCAM and PSMA of the cells to their respectively purified vesicles, exosomes and microvesicles from the PSMA-positive LNCaP, and negative controls PC-3 and HeLa, were incubated with the respective antibodies which were then detected by Cy5 labeled anti-mouse IgG antibody.

Exosomes and microvesicles were purified according to section 3.3.2 with the only difference being the filters used: for the exosomes, a 0.22 µm filter was used and for the purification of microvesicles, a 1 µm strainer was used. Beads were washed once in PBS and incubated with exosomes or microvesicles for 45 minutes at RT. Then, the beads were blocked with 1,5% BSA and 50 mM glycine in PBS for 45 minutes and then the primary antibody anti-PSMA or anti-EpCAM was added, at a concentration of 6 µg/mL, in the same incubation conditions. After three washing cycles, the beads are incubated with the secondary antibody anti-mouse IgG-Cy5 labeled, in a 1:2500 dilution. The samples are measured after going through three washing steps.

3.4.5.3. Specific capture via anti-PSMA antibody and small-molecule PSMA-617

3.4.5.3.1. Detection by anti-EpCAM-FITC labeled antibody

Beads were first centrifuged for 2 minutes at 9200 × *g* and their supernatant discarded, and then incubated overnight at 4ºC with anti-PSMA-antibody, 6 µg/mL, or small molecule PSMA-617, 8 µg/mL. The next day, beads were pelleted under the same conditions and blocked for 45 minutes with 1,5% BSA & 50 mM glycine in PBS. After another centrifugation step, beads were incubated for 2h at RT with vesicles from LNCaP, positive control, and PC-3 vesicles and PBS as negative controls. For vesicle detection, beads were incubated with anti-EpCAM-FITC-labeled antibody for 45 minutes, at a concentration of 3 µg/mL. Before measuring, beads were centrifuged and the supernatant of each sample was also kept for analysis.

3.4.5.3.2. Detection by self-fluorescent exosomes

With fluorescent exosomes purified from the three cell lines in use, GFP LNCaP, CTG PC-3 and CTG HeLa, according to section 3.3.2, binding to beads via anti-PSMA antibody or via small molecule PSMA-617 was tested.

Latex beads were incubated for 30 minutes with the binders anti-PSMA antibody (6 µg/mL) or PSMA-617 (8 µg/mL), in low binding tubes. As controls, another set of beads had no binders. Then, these samples were centrifuged for 5 minutes at 9200 × *g* and resuspended in blocking solution, 50 mM glycine & 1,5% BSA in PBS, and incubated for 45 minutes, shaking. Once again, samples were centrifuged for 5 minutes at 9200 × *g* and the supernatant discarded, washed three times in PBS-0,05% Tween20 and then the samples were incubated with GFP-LNCaP, CTG-HeLa or CTG-PC-3 exosomes, for 1 h at RT with agitation. Finally, the beads are centrifuged in the previous conditions and washed three times with PBS before measuring.

3.5. Carboxyl-activated glass slide

In a first attempt to move one step closer to the microfluidic device, specific isolation of exosomes on a functionalized glass surface was tested.

The rationale is similar to that of the beads measured in the iQUE screener: a surface is functionalized with capture molecules, blocked, incubated with exosomes and the intended signal detected by fluorescence.

All slide measurements are made with the GenePix (Molecular Devices) reader which is equipped with two filters, optimized for Cy3 (550 nm excitation peak / 570 nm emission peak) and Cy5 dyes (650/670 nm)(39), however FITC (495/519 nm) labeled antibodies were used due to the proximity of the wavelengths to the filters and their immediate availability at the laboratory. Vesicles from cells incubated with CTG (492/517 nm) and Cy5-labeled secondary antibodies were also used for detection.

A carboxyl-activated glass slide (2D-Carboxyl, PolyAn) is firstly covered with LightCycler film (Roche), with holes cut into it, so each sample remains in place and does not contaminate the others, as seen in Figure 13.

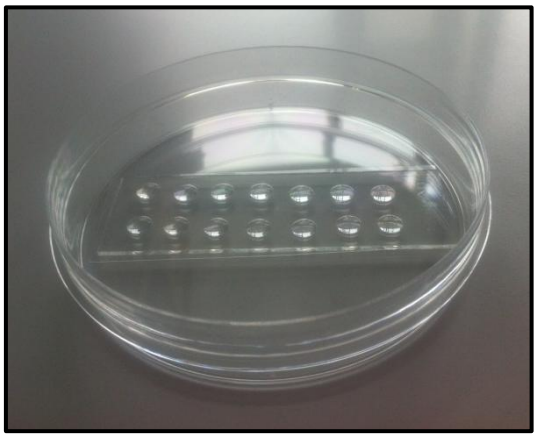

Figure 13. Glass slide covered with punctured PCR film with 20 µL PBS per sample

In all experiments the slide is activated with a 1:1 mixture of EDC/NHS (both at a concentration of 50 mg/mL), in MES pH 6 for 30 minutes and, after washing twice with PBS, the binders or the vesicles directly, are incubated for 2 h at RT. These binders can target EpCAM via anti-EpCAM antibody (6 µg/mL) or PSMA via anti-PSMA-antibody (6 µg/mL) or small molecule PSMA-617 (in variable concentrations). After this, the slide is deactivated with 0,5 M ethanolamine pH 8,5, for 30 minutes and blocked to avoid nonspecific binding to the surface with 1,5% BSA and 50 mM glycine in PBS, for 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. After this, the samples need to be washed with PBS to remove unbound antibodies, dried very well so there is no contamination when removing the foil (it is fluorescent, therefore needs to be removed, see Figure 14) and scanned with the Genepix reader.

Figure 14. Glass slide covered with PCR film, read with Genepix, at 532 nm

3.5.1. Blocking test

For this test, two columns of spots in the glass slide were blocked with two different solutions and a third one served as negative control, only treated with PBS instead of any blocking solution, as pictured in Figure 15.

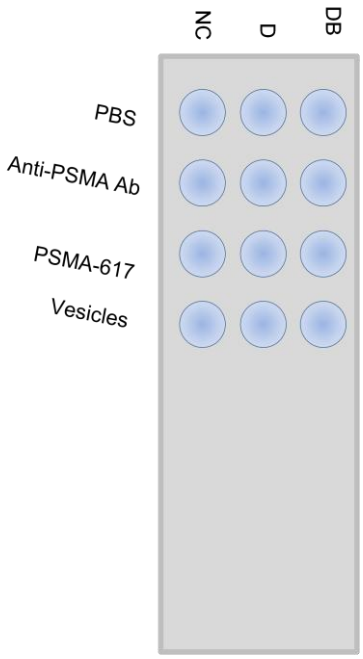

Figure 15. Setup of the blocking experiment (*NC*: negative control; *D* deactivation; *B* blocking; *Ab* antibody)

The protocol goes along the general lines previously stated: the surface was activated by a 1:1 mixture of EDC/NHS and after washing twice with PBS the binders were added as indicated in Figure 15: anti-PSMA antibody at a concentration of 6 µg/mL, the small molecule PSMA-617 at 80 µg/mL, and the PSMA-positive LNCaP exosomes were incubated directly on the activated surface. PBS was added as a control. After a 2h incubation period at RT, the respective columns as indicated in Figure

15 – *D; DB* were deactivated by 0,5 M ethanolamine in PBS, pH 8.5, for 30 minutes at RT. Afterwards, the indicated column in Figure 15 - *DB* was blocked with a 3% BSA solution in PBS. The slide was incubated overnight at 4º C, with slight agitation. The following day, it was washed three times with PBS and the exosomes were incubated in the rows with the binders (Figure 15: Anti-PSMA Ab and PSMA-617 rows) and the detection antibody, anti-EpCAM-FITC, 6 µg/mL, was added to the other two rows (Figure 15: PBS and Vesicles rows), for 30 minutes at RT. Then, the exosome samples (Figure 15: Anti-PSMA Ab and PSMA-617 rows) were washed and incubated with the labeled antibody, as in the previous step. The slide was dried and the foil removed before measurement.

3.5.2.EpCAM and PSMA detection by Cy5 labeled secondary antibody

As previously tested with vesicles immobilized on latex beads, EpCAM and PSMA surface expression was assessed by Cy5 secondary antibody detection.

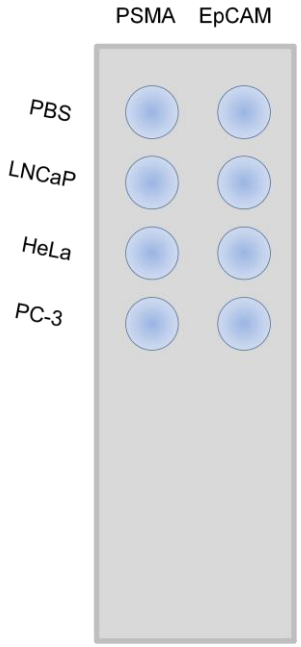

Figure 16. Setup of the surface marker detection via Cy5

As before, the glass slide was activated by EDC/NHS and the vesicles were incubated for 2 h at RT as represented in Figure 16. After that, the slide was deactivated and blocked. After three washing steps with PBS/0,05% Tween20, the primary antibody was added at a concentration of 6 µg/mL, in its respective column (see Figure 16) and incubated for 30 minutes. The secondary antibody anti-mouse IgG-Cy5 conjugated was incubated for 30 minutes at RT, in a 1:1000 dilution. To avoid sample contamination when removing the foil, since it also presents fluorescence at 635 nm, it is important to previously dry the slide very well.

3.5.3. Specific isolation of fluorescent vesicles

In an attempt to specifically capture the vesicles according to their surface protein expression, the slide was activated as before and the binders were added as indicated in Figure 17: anti-PSMA and anti-EpCAM antibodies (6 µg/mL), small molecule PSMA-617 (8 µg/mL) or the exosomes directly in one of the rows. After deactivating and blocking the surface, as before, exosomes purified from cells incubated with CTG are added to all spots (excluding the PBS control or the spot already containing vesicles).

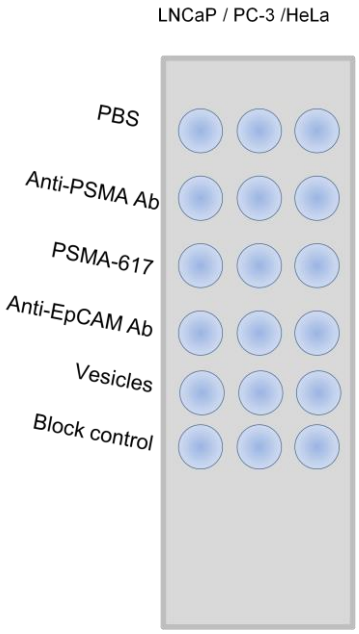

Figure 17. Setup for specific isolation experiment (*Ab*: antibody)

3.6. Scanning Electron Microscopy

Conventional optical microscopy does not offer enough resolution to visualize exosomes, due to their size range of 30-200 nm of diameter, so SEM was kindly performed by Mr. Hirsch at the IOM (Leibniz Institute of Surface Modification), Leipzig.

3.6.1. Hydrophobic and hydrophilic wafers

For morphological characterization and assessment of exosome behavior in surfaces with different water affinities by SEM, LNCaP exosomes were bound to hydrophobic (from Fraunhofer Institute für Siliziumtechnologie, Itzehoe) and hydrophilic wafers (Austrian Institute of Technology, Vienna). In brief, the surface is activated by EDC/NHS chemistry, and then the exosomes are directly adsorbed to the surface. After a 45 minute incubation period, the surface is deactivated by 0.5 M Ethanolamine at a pH of 8.5 and blocked by 1,5% BSA and 50 mM glycine in PBS. Finally the vesicles are added to the two fields with the binders and incubated overnight at 4°C. The next day the samples are fixed in 3,7% glutaraldehyde in PBS and washed with increasing ethanol concentrations from 40, 60, 80 to 98% and left to dry so the sample can be made conductive by sputtering an approximately 10 nm gold layer. Imaging is then performed by SEM (GeminiSEM) at low beam energies of 5.0 kV.

3.6.2. Untreated silica wafer

In an attempt to improve image quality by reducing the crackling of the surface after applying the gold layer, an untreated silica wafer was used (L14017, SIEGERT WAFER GmbH) as a base for exosomes directly or beads with bound exosomes.

The wafer was cut into the appropriate size and covered with PCR foil (Roche) with punctured holes. Approximately 1.000 beads (4 µm aldehyde/sulfate latex beads 4% w/v, molecular probes) were incubated with LNCaP vesicles for 30 minutes at RT with agitation and after this, centrifuged for 5 minutes at 4.500 × *g*, so the pellet could be resuspended in 15 µL of PBS/0.05%Tween20 and applied in one of the spots of the chip. To the other spot, the same amount of exosomes in PBS was directly added and the chips were incubated for 45 minutes at RT. The fixation steps went according to 3.6.1.

3.6.3. Exosomes and microvesicles on untreated silica wafer

With new purifications from LNCaP vesicles, that aimed to collect only exosomes (by filtering with a 0.22 µm filter) or to collect the entire set of microvesicles (by straining the culture medium with a 1 µm strainer), new SEM images were taken, with sample preparation according to 3.6.1, on an untreated silica wafer (L14017, SIEGERT WAFER GmbH).

3.6.4. Specific binding to carboxyl-activated glass slide

In this experiment, LNCaP exosomes were specifically bound to carboxyl-activated glass slides. The protocol follows the same steps and in 3.6.1 with the only difference that after surface activation, binders are added: anti-PSMA antibody (6 µg/mL) and small-molecule PSMA-617 (8 µg/mL).

3.7. Zetasizer

The Zetasizer Nano ZSP (Malvern) is a device used to measure particle size (size range 0.3 nm to 10 µm), zeta potential (of particles with diameters in the range of 3.8 nm to 100 µm), molecular weight (in the range of 342 Da to $2x10^7$ Da), microrheological properties or protein mobility.

The Zetasizer determines a particle's size by first measuring the Brownian motion of the particles, which is the random motion of such particles suspended in a fluid due to collisions with the molecules in the fluid (40), by making use of Dynamic Light Scattering and then interprets a size by using established theories, which correlate a particle's speed with its size.

When a charged particle is suspended in a liquid, ions will be attracted to the particle's surface and form layers around it (Figure 18). Ions of the opposite charge of the particle and close to its surface will form a strongly bound layer, and as distances increases, ions will be more loosely bound, forming a diffuse layer. In this layer, a plane splitting the ions that move along with the particle and those that do not, can be defined - the slipping plane. The potential measured at this plane is then the zeta potential.

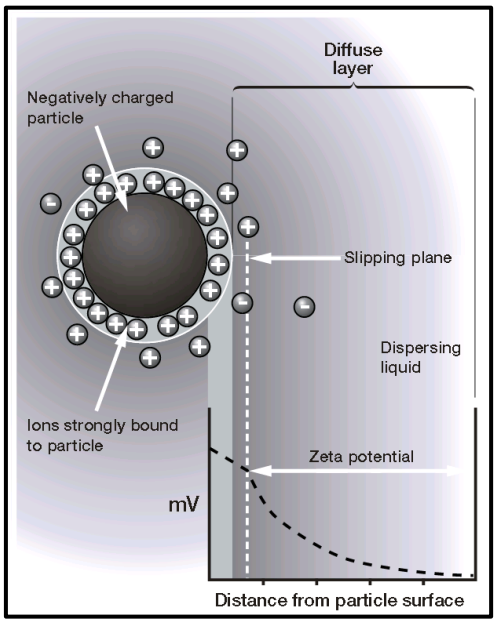

Figure 18. Ionic interaction of a particle in fluid and Zeta Potential

Zeta potential is measured by combining electrophoresis and laser Doppler velocimetry, to measure the particle's speed when an electrical field is applied. Once this velocity is known, along with known

properties of the solvent - viscosity and dielectric constant - the zeta potential can be calculated. Overall, this potential can aid to predict whether particles will tend to aggregate or not (Figure 19) (41).

Assessment of stability	Zeta-potential (mV)
Maximal agglomeration and precipitation	0 . . . + 3
Region of strong agglomeration and precipitation	+ 5 . . . − 5
Beginning of agglomeration	− 10 . . . − 15
Beginning of peptization (disperging)	− 16 . . . − 30
Medium stability	− 31 . . . − 40
Good stability	− 41 . . . − 60
Very good stability	− 61 . . . − 80
Extremely good stability	− 81 . . . − 100

Figure 19. Suspension stability depending on the zeta potential (42)

3.7.1.Zetasizer protocol

Vesicle samples purified from conditioned culture media are diluted in PBS (0.2 µm filtered) with 1:10 and 1:100 ratios.

The device is set to measure the backscatter at 4°C, in triplicate, of 200 runs of 3 seconds each.

3.8. Lateral Flow Assay

As an alternative attempt to specifically isolate and detect PSMA-positive PCa exosomes, a lateral flow assay was done using a sample containing vesicles that to our knowledge, is not described in literature or has never been tried before, since this method is most commonly used for smaller targets such as free proteins.

A volume containing around 25.000 LNCaP exosomes, as estimated by the iQUE method, was 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.

4. Results and discussion

4.1. Cell culture

4.1.1. Cells transfected with GFP

In this section, transmission and fluorescence microscopy images of LNCaP cells transfected with GFP are shown.

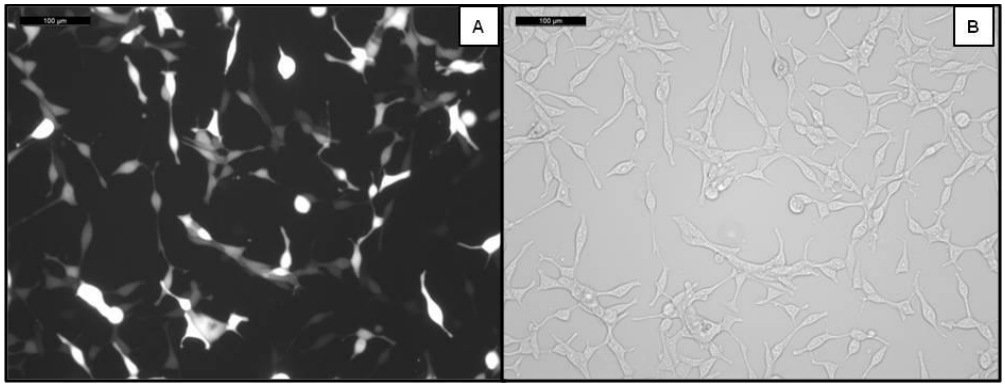

Figure 20. Microscopy images (Leica DMI 4 000 B) from LNCaP cells transfected with GFP, one week after transfection. Scale bar: 100 μm

On the following picture, plots from the iQUE screener are shown, of a sample of beads incubated with exosomes purified from GFP transfected cells.

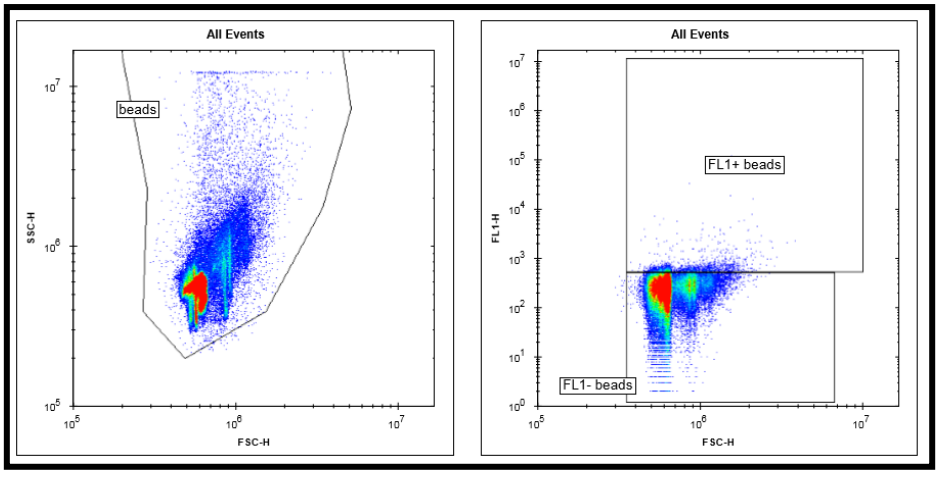

Figure 21. ForeCyt plots for beads incubated directly with vesicles purified from LNCaP cells transfected with GFP

Although LNCaP cells show a high transfection rate, over 80% (Figure 20), it is not a feature passed on to the vesicles since nearly the totality of beads are negative in the fluorescence channel that detects the fluorescent protein GFP (Figure 21 - *FL1+ beads*).

4.1.2.Cells incubated with CTG

As an alternative fluorescence source to GFP, cells were incubated with CTG and the results are shown in the following figures:

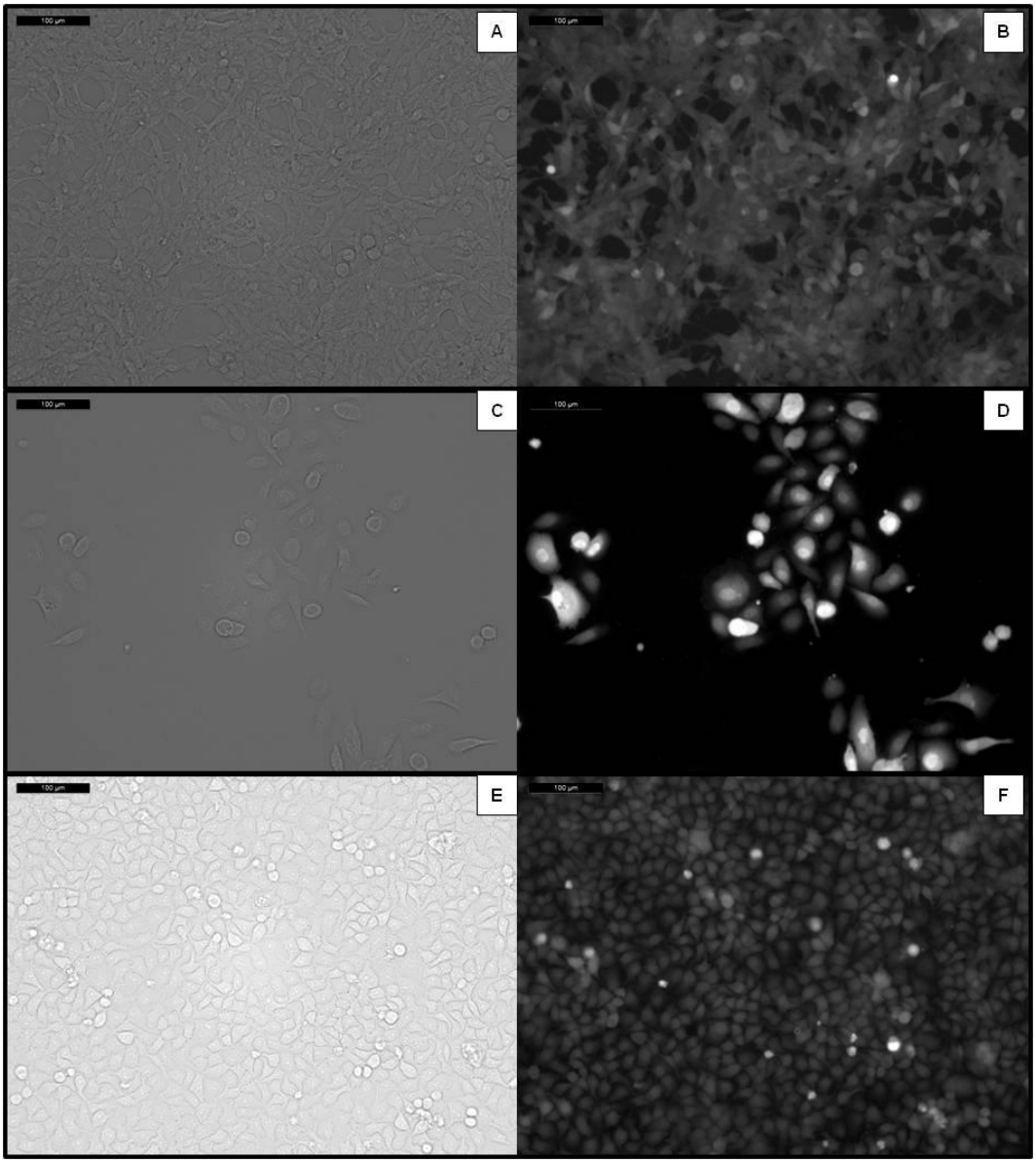

Figure 22. Microscopy images (Leica DMI 4 000 B) from LNCaP (A,B), PC-3 (C,D) and HeLa (E,F) cells 72 h after incubation with CTG, after culture medium removal. A,C,E: transmission; B,D,F: fluorescence

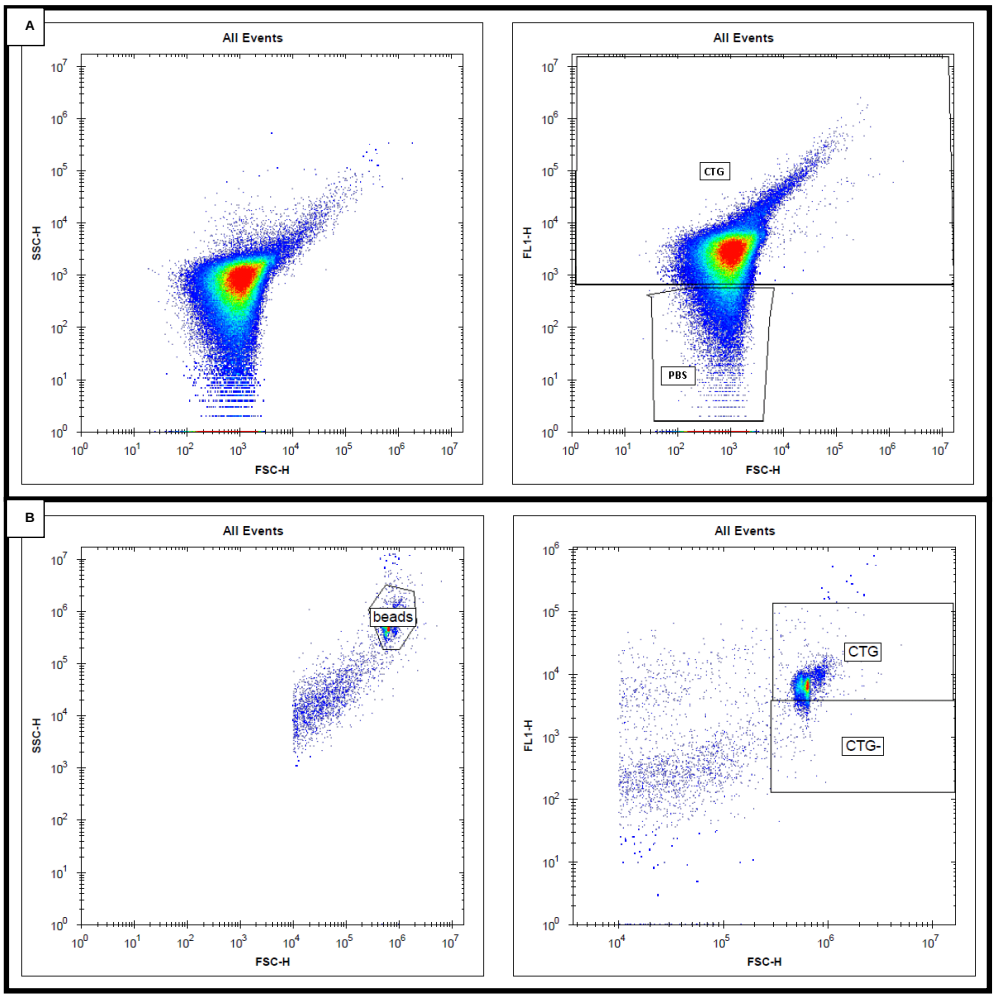

Figure 23. ForeCyt plots for CTG+ LNCaP purified vesicles in PBS (A), 80,41% CTG+ and CTG+ LNCaP bead bound vesicles in PBS (B), 80,48% CTG+

Cells incubated with CTG also show high levels of fluorescence (Figure 22), comparable to the standard GFP transfection method; however, unlike cells transfected with GFP, cells incubated with CTG pass this dye on to their exosomes. In Figure 23, it is seen that the CTG LNCaP exosomes show fluorescent signal against the PBS background (Figure 23A) and when bound to beads, there is also a clear fluorescence shift to higher values (Figure 23B).

Using self fluorescent exosomes when comparing to labeling via fluorescent antibodies is advantageous in a way that it removes one source of variability, since there are no false positives due to possible nonspecific binding of the label and the signal read it is then known to originate from the vesicles. Although the cell cultures need to go through extra steps before purifying the vesicles, it becomes an advantage to have self glowing exosomes at the time of the intended experiment, avoiding an extra incubation step with the label.

4.2. Vesicle characterization

4.2.1. Direct measurement of exosomes

The following figure shows ForeCyt plots of the PBS buffer, used to suspend the vesicles, to set the background values and the vesicles in PBS measured directly, without the addition of fluorophores or incubation with beads.

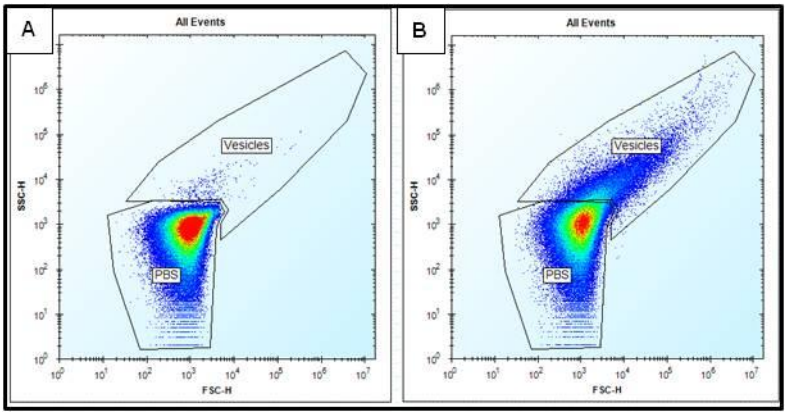

Figure 24. FSC-H vs SSC-H ForeCyt plots for PBS (A); LNCaP vesicles (B)

The size and granularity of the vesicles obtained by gating the plot as seen in Figure 24B, span across a 5- and 3-fold range, however these account for only approximately 10% of the total counts for the LNCaP derived vesicles, with the PBS accounting for the rest.

PBS shows a cloud on the lower range of the FSC-H scale (Figure 24A) which can overlap with the readings from other samples with equally small structures, such as exosomes.

So there is a need to distinguish the signal from vesicles from that of PBS, by shifting the exosome signal in terms of size, by bead coupling, and/or fluorescence.

4.2.2.Anti-EpCAM FITC-labeled antibody titration

This experiment was made to know in what concentration range the anti-EpCAM-FITC antibody would give optimal staining results, in other words, maximum fluorescence with minimum background noise.

Cell line	Concentration after purification [µg/mL]	210 µg of exosomes were used	Supernatant [µg/mL]	Amount bound to the beads [µg]
LNCaP	475		207	3 (1.43 %)
PC-3	263		205	5 (2.38 %)

Table 1. Bead and exosome incubation

Before the titration experiment itself, LNCaP and PC-3 vesicle samples' protein content was measured by BCA assay and the obtained concentrations are presented in Table 1 – *Concentration after purification*. 210 µg worth of exosomal protein, of each cell line, were incubated with latex beads, however only a small amount was bound to beads (Table 1 – *Amount bound to the beads*), 1,43% for the LNCaP and 2,38% for the PC-3 vesicles, having the rest been lost in the supernatants discarded during the washing steps (Table 1 – *Supernatant*). To note, this was one of the first experiments, so standard centrifuge tubes were used to perform the incubations.

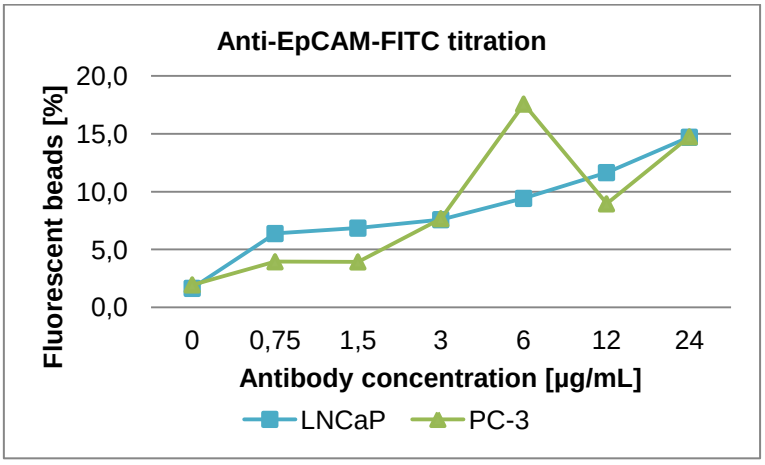

Figure 25. Anti-EpCAM-FITC antibody titration - percentage of FITC-positive beads

Overall, there is an increase in fluorescence proportional to the FITC labeled antibody concentration, for both cell lines, displayed in Figure 25. It is also seen that the percentage of fluorescent beads is fairly low, not even reaching 20%, which is not surprising due to the low amount of bead-bound exosomes (Table 1). As a control, beads were directly incubated with 6 µg/mL of anti-EpCAM-FITC and nearly 100% were considered to be FITC-positive. Regarding each cell type, 8,3 % ± 4,2 for LNCaP and 8,4% ± 5,9 for PC-3 show a positive fluorescent signal possibly due to the low amount of exosomes bound to the beads, and good blocking of the surface avoiding nonspecific binding of antibodies. With this experiment it is seen that any chosen concentration starting at a minimum of 3 µg/mL provides a fairly good signal and can therefore be adjusted according to the necessities of each experiment.

4.2.3.Centrifugation

4.2.3.1. Duration

The resulting bar chart compiling the information retrieved from the centrifugation times experiment is presented in the following figure:

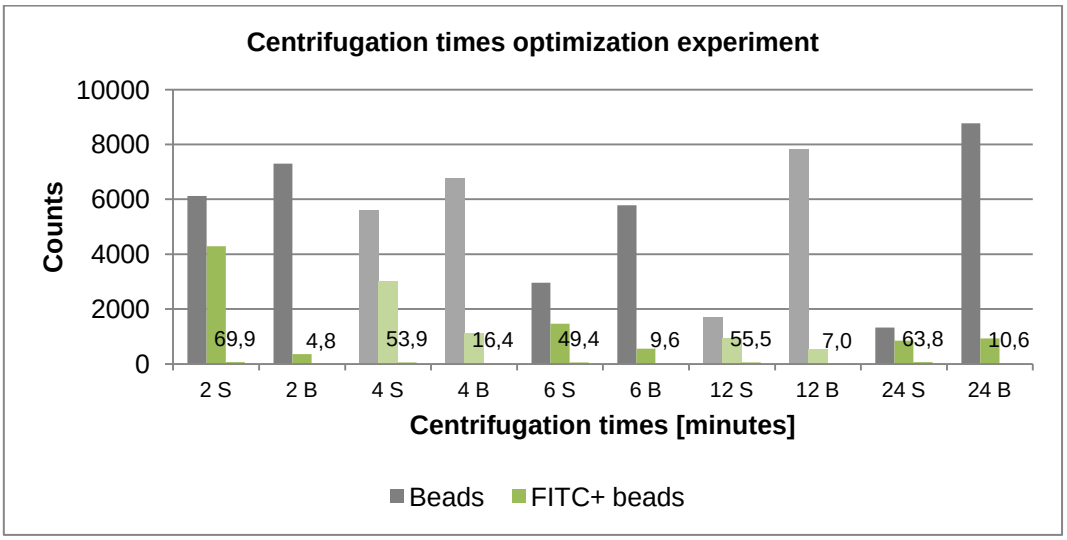

Figure 26. Centrifugation times experiment. *S:* sum of counts from the three measured supernatants; *B:* final sample. Next to the bars, the percentage of FITC-positive beads is shown.

From Figure 26, we see that the total of beads lost in the supernatants tends to decrease as the centrifugation duration is increased. However, despite this overall decrease, still 58.51% ± 8.24 of the beads lost in the supernatant have a positive signal for FITC, which is a significant loss of signal. 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.

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.

4.2.3.2. Tubes

ForeCyt plots, relating size with granularity or fluorescence, for standard tubes and low binding tubes, and the respective bar chart quantifying the results are shown in the following images:

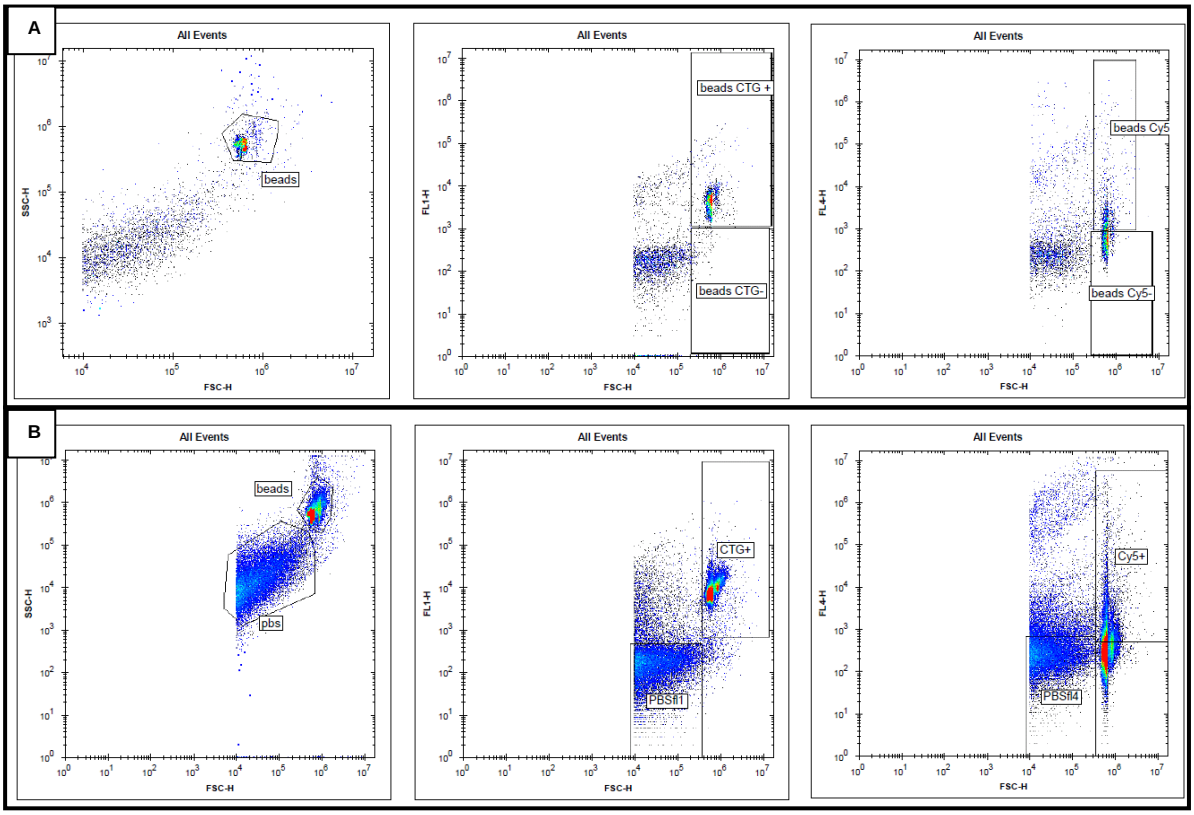

Figure 27. ForeCyt plots of the final sample in Normal tubes (A) or Low Binding tubes (B). From left to right: FSC-H vs SSC-H; FSC-H vs FL1-H, for CTG detection; FSC-H vs FL4-H, for Cy5

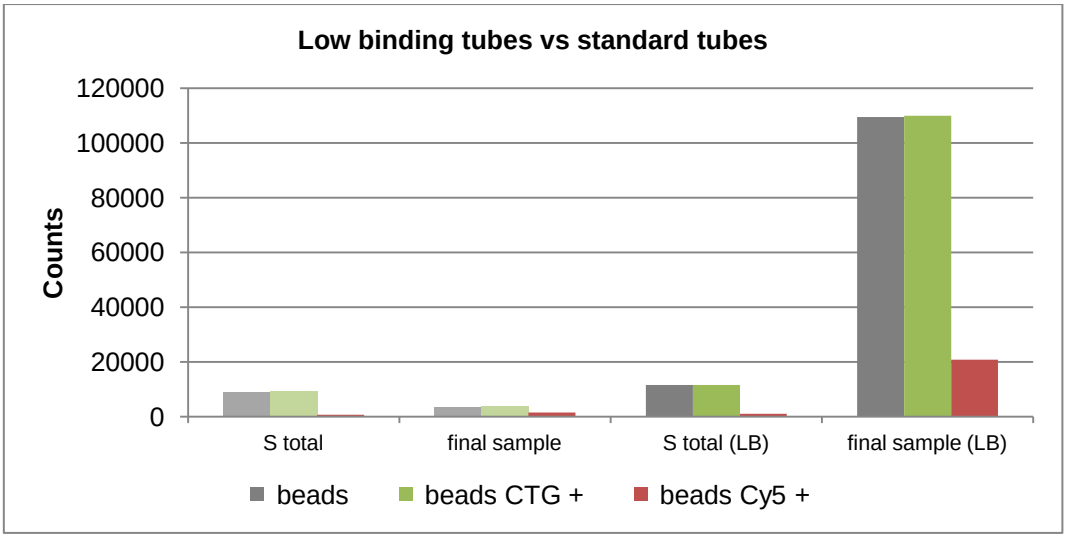

Figure 28. Event counts for standard tubes and low binding tubes – *S:* sum of the supernatants, *LB*: Low Bind tubes

Starting with the same number of beads and same sample volume, on Figure 27 there is a clear higher density in the sample corresponding to the low binding tubes, which is translated into numbers

in Figure 28, where it is seen that the amount of counted beads is approximately 11 times higher in the final sample than in its correspondent supernatant, and approximately 28 times higher when compared to the final sample in standard tubes. The use of these standard tubes can then explain why so very few beads were accounted for in the antibody titration experiment, Figure 25, and especially in Figure 26, for the centrifugation times experiment, where the error was not entirely due to the centrifugation duration but probably mostly due to the tubes used to perform the experiment. Therefore, the use of low binding tubes is recommended when working with exosomes and latex beads.

4.2.4.Blocking

4.2.4.1. Anti-EpCAM-FITC bead blocking test

A bar chart summarizing the effectiveness of different blocking solutions against a primary labeled antibody is shown:

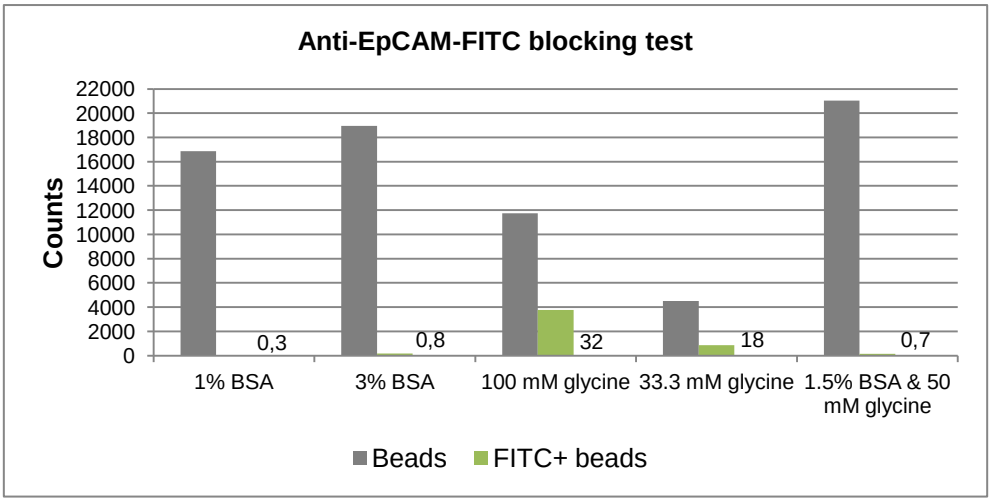

Figure 29. Primary antibody blocking test - numbers on the chart represent the percentage of FITC+ beads for the respective blocking solution

BSA, as a common blocking solution, is as expected providing good blocking results in both tested concentrations, since less than 0,8% of the beads in the final sample show fluorescence, as seen in Figure 29. A slightly higher bead recovery in the final sample is however possible when in presence of both BSA and glycine, as a slightly higher amount of beads is accounted for and only 0,7% show fluorescence.

4.2.4.2. Cy5-labeled secondary antibody bead blocking test

A bar chart summarizing the effectiveness of different blocking solutions against a labeled secondary antibody is presented in the next figure:

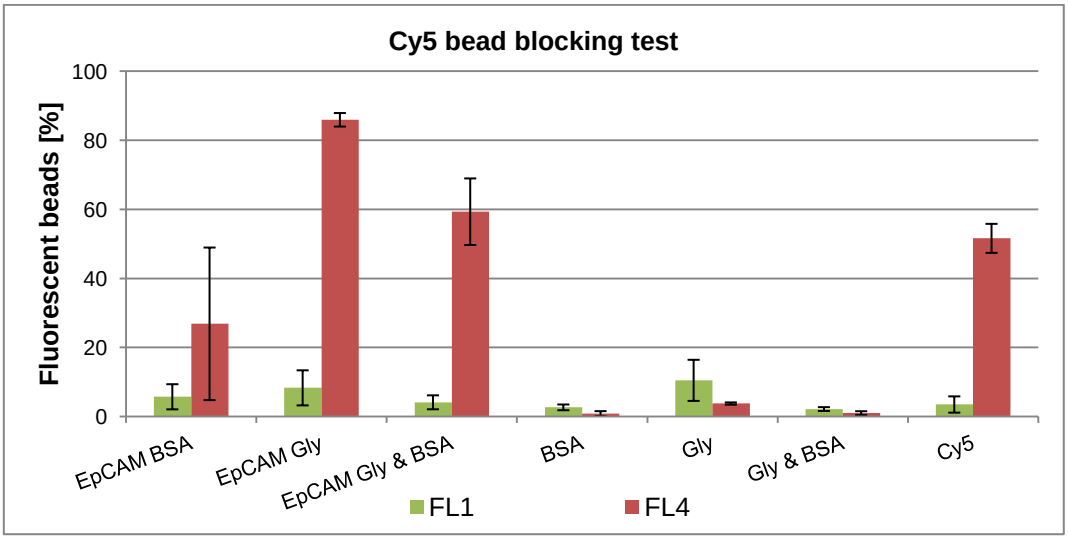

Figure 30. Cy5-labeled secondary antibody bead blocking test - *BSA*: 3% BSA; *Gly*: 100 mM Glycine; *Gly & BSA*: 50 mM glycine and 1,5% BSA in PBS; *Cy5*: 1:1000 Cy5-labeled secondary antibody dilution in PBS/0,05%Tween

Regarding the beads functionalized with EpCAM, blocked and then detected by Cy5 (Figure 30), even though the beads are blocked, the Cy5-labeled IgG will bind to EpCAM, since this binding has higher affinity than the blocking molecules. The direct blocking of the beads followed by IgG-Cy5 antibody incubation shows a maximum of 3,76% FL4 signal for 100 mM glycine, which shows high blocking by any of the three blocking solutions, which greatly avoid the nonspecific binding of IgG-Cy5 to the bead surface. As in section 4.2.4.1, blocking with 1,5% BSA and 50 mM glycine in PBS (0,98% of FL4+ beads) and 3% BSA (0,86% of FL4+ beads) show the best results. It is also worth of mention the relatively high background signal in the FL1 channel, since no fluorescent dyes for this channel were added in this experiment and yet the percentage of detected beads in this channel ranges from 2,16 % to 10,48%.

4.2.4.3. CTG vesicles bead blocking test

Finally in blocking matters, the results for the blocking experiment against nonspecific binding of fluorescent exosomes are presented:

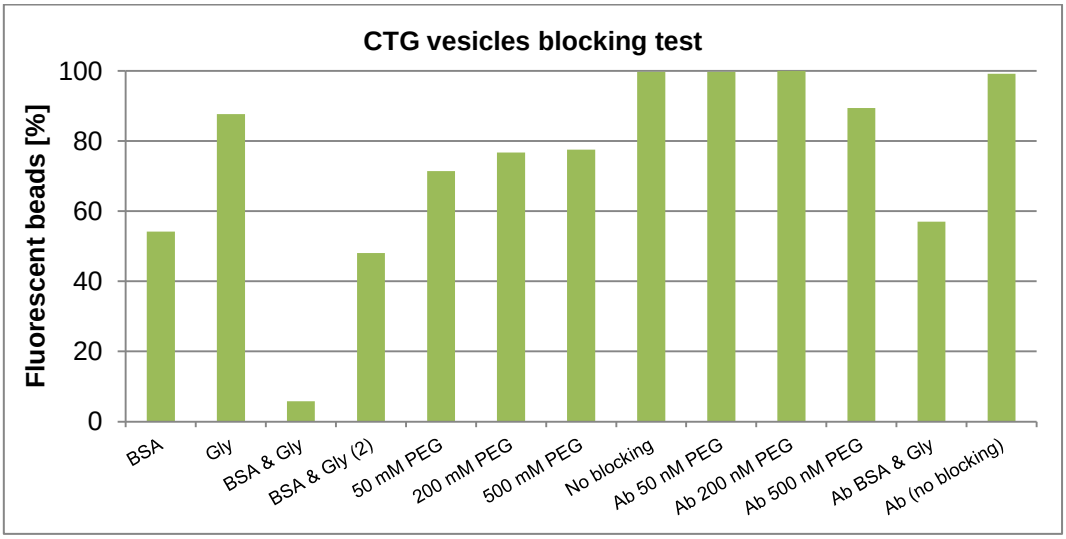

Figure 31. CTG PC-3 exosomes bead blocking test - *BSA*: 3% BSA; *Gly*: 100 mM Glycine; *BSA & Gly*: 1,5% BSA and 50 mM glycine in PBS; *Ab*: anti-EpCAM antibody

Regarding the blocking test for the binding of CTG self-fluorescent PC-3 purified vesicles, it is seen in Figure 31 that even though the beads were previously blocked and then incubated with the exosomes, there are still high levels of nonspecific binding. Apparently, 1,5% BSA and 50 mM glycine (*BSA & Gly* in Figure 31) seemed to provide good bead blocking, however when the same incubation was repeated in another set of experiments (*BSA & Gly (2)* in Figure 31) the nonspecific binding was again high. So there is a high variability regarding these measurements. PEG4000 was also tested, since it is also reported as a blocking agent (43), having however also not been effective for bead blocking regarding exosomes, since the best blocking PEG solution, at 50mM PEG4000, still allows for 71% of nonspecific binding. As a control, nearly all the beads directly incubated with CTG PC-3 exosomes or incubated with anti-EpCAM antibody show FL1 fluorescence. Overall, the best blocking solution is 3% BSA in PBS but it still allows for 54,18% of nonspecific binding.

So far, none of the tested blocking solutions provide the necessary blocking of latex beads to avoid nonspecific binding of exosomes.

4.2.5.Final Protocols

4.2.5.1. Surface marker detection via Cy5-labeled secondary antibody - cells

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 (36). The same expression pattern was obtained in this experiment and is displayed in Figure 32.

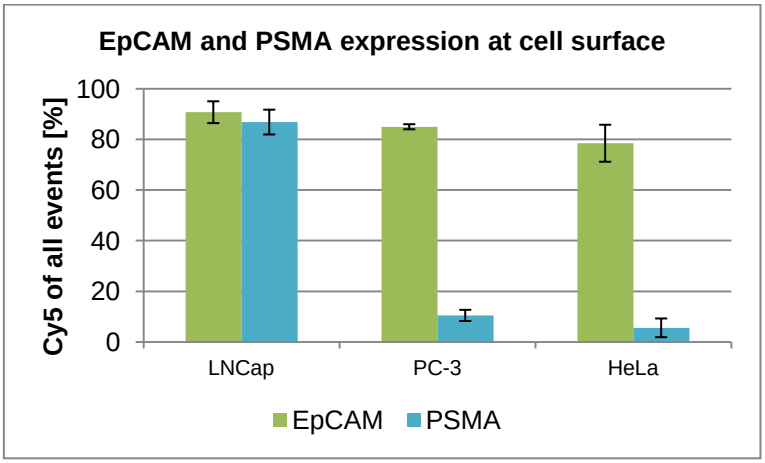

Figure 32. EpCAM and PSMA expression assessed by secondary antibody labeled with Cy5 in LNCaP, PC-3 and HeLa cells

As expected, due to their epithelial origin, the three cell lines express the surface marker EpCAM, in variable levels, with 78,4% of HeLa, 84,9% PC-3 and 90,7% LNCaP cells expressing the marker. Regarding surface expression of PSMA, 5,6 % of HeLa cells show PSMA expression, possibly due to nonspecific binding of the Cy5 labeled antibody, since HeLa cells are not derived from prostate. As stated throughout this document, PC-3 cells do not express PSMA whereas LNCaP do, which is coincident with the results in Figure 32, as the expression levels are respectively, 10,5% and 86,8%.

4.2.5.2. Surface marker detection via Cy5-labeled secondary antibody - vesicles

According to literature (36), 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.

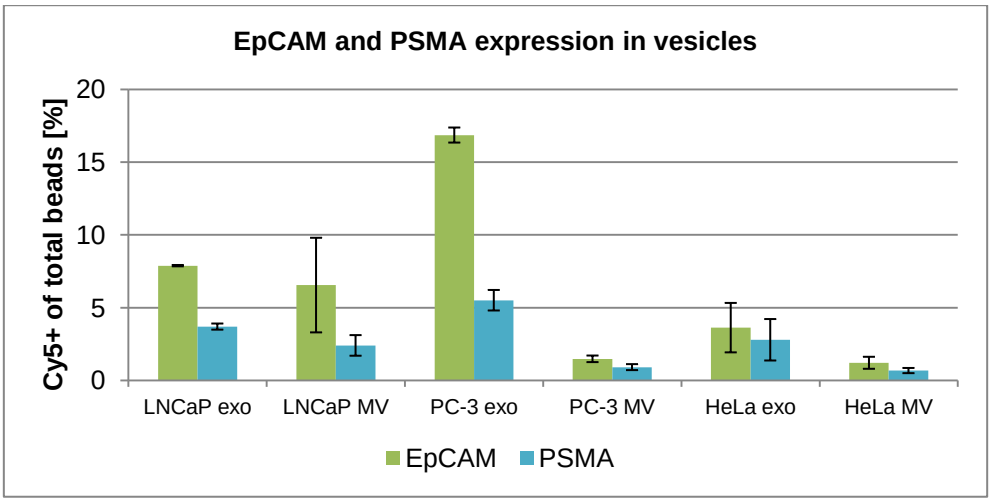

Figure 33. EpCAM and PSMA expression assessed by Cy5-labeled secondary antibody - exosomes (exo) and microvesicles (MV) for the respective cell lines

Fluorescent signal originating from Cy5 labeled secondary antibodies was in general low, not even reaching 20% of Cy5 positive exosomes. Comparing to cellular expression, values over 70% for EpCAM would be expected (section 4.2.5.1). Regarding PSMA, the highest value measured was 5,51% for PC-3 exosomes. LNCaP exosomes' and microvesicles' detected Cy5 values were much lower than expected and compared to cell obtained values. In these particular samples, exosomes appear to be richer in EpCAM and PSMA when compared to the entire set of microvesicles, confirming the importance of exosomes as biomarker holders.

However, the experiments need to be repeated to confirm the obtained data and performing a Western Blot with lysates from the exosome samples would be another way to assess surface protein expression. In this case, it would be possible to confirm if the vesicles purified from our cell cultures express or not PSMA and compare that result with the iQUE data.

4.2.5.3. Specific capture via anti-PSMA antibody and small-molecule PSMA-617

4.2.5.3.1. Detection by anti-EpCAM-FITC labeled antibody

In this experiment, high fluorescence was expected for LNCaP vesicles, since these are described to highly express PSMA, that is expected to bind to the anti-PSMA antibody or small-molecule PSMA-617 bound to latex beads. Conversely, low fluorescence was expected for samples with PC-3 vesicles, due to absence of PSMA (36).

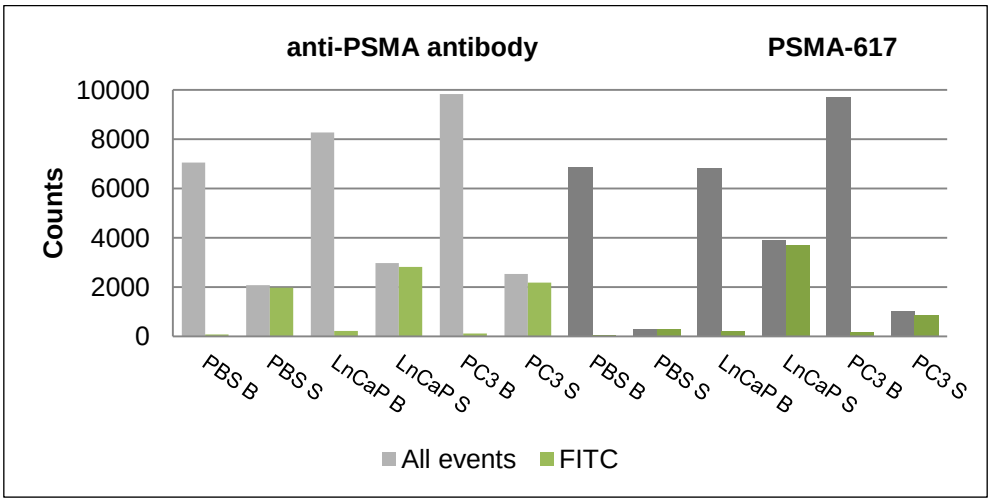

Figure 34. Specific capture of exosomes via anti-PSMA antibody (lighter colors) or small-molecule PSMA-617 (darker colors) detected by FITC-labeled anti-EpCAM antibody.
S: Supernatant; *B*: Beads (Final samples)

Regardless of the sample, nearly all beads lost in the supernatants were fluorescent and also outnumbered the detected fluorescent beads in the final samples (Figure 34). This can be due to the fact that exosomes do not bind to the beads but the anti-EpCAM-FITC antibody does, due to poor blocking of the surface or due to the use of standard centrifuge tubes that adsorb the exosomes and beads; or the exosomes bind but centrifuging for 2 minutes at 9200 × *g* is not enough to pellet the beads.

The final samples, marked with *B*, are all FITC-negative, regardless of the binder or the vesicle PSMA expression pattern. These results would be expected for PBS and PC-3 samples, but not for LNCaP vesicles, that should be captured by the functionalized beads. This can be due to an inadequate concentration of the binders, to a present but low expression of PSMA by LNCaP exosomes, insufficient for a stable binding to the beads or to a low concentration of the FITC-labeled antibody, inadequate for detection.

In any case, considering the high proportion of fluorescent beads lost in supernatants, it is not possible to assess the specific binding of the exosomes with respect to PSMA with this protocol. To improve it, low binding tubes should be used and the centrifugation time should be increased to 5 minutes. Then, after these adjustments, different concentrations of binders and detection antibodies can be tested.

4.2.5.3.2. Detection by CTG

To avoid bead loss, the previous experiment was repeated in low binding tubes and with self-fluorescent exosomes. By using these exosomes, is possible to reduce the number of washing steps since there is no need to eliminate unbound labels.

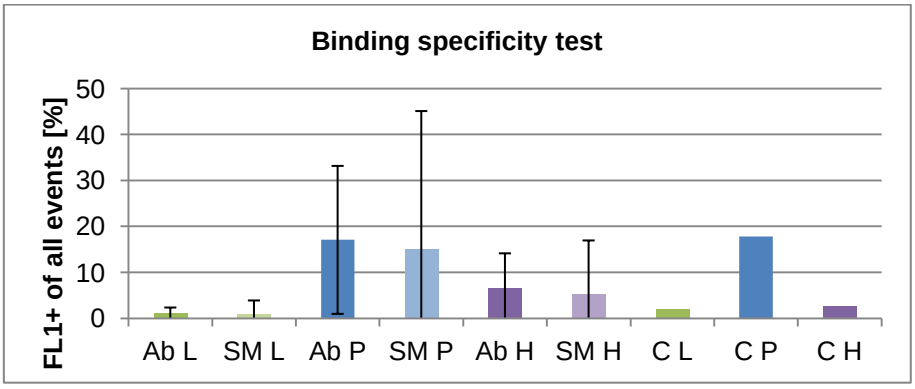

Figure 35. Binding specificity test - *Ab*: anti-PSMA antibody; *SM*: small-molecule PSMA-617; *C*: blocking control; *L*: GFP LNCaP vesicles; *P*: CTG PC-3 vesicles; *H*: CTG HeLa vesicles

As in the previous experiment, the signal itself is low; the highest not even reaching 20% of FL1+ beads, for PC-3 vesicles (Figure 35). And it would be expected that this highest signal corresponded to LNCaP. LNCaP GFP vesicles have the lowest signal of the three cell types, 1% of fluorescent beads for both binders. The control accounts for 2,1% fluorescent beads, due to either excellent blocking, which is unlikely based on the results from 4.2.4.3, or due to the fact that GFP does not seem to be packed into exosomes (section 4.1.1). PC-3 vesicles show the highest fluorescence, 17,1% for antibody binding but the standard deviation is also very high, in the same magnitude of the average fluorescence value. The PC-3 blocking control is 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. HeLa blocking control, can be, once again working well (unlikely, see section 4.1.1) only showing 2,7% of fluorescence or the CTG lost intensity over time, with freezing and thawing cycles. 6,6% and 5,2% of the beads are positive for the CTG, for capture with the anti-PSMA antibody and the small molecule, respectively. These values can be considered reasonable since HeLa do not express PSMA and this fluorescence can be due to some nonspecific binding of the vesicles.

In this experiment, a high fluorescent signal for the LNCaP vesicles was expected and low values for both PC-3 and HeLa. It was then not possible to verify the selective capture LNCaP exosomes due to their intrinsic low fluorescence. Was also a goal to assess the binding capacity of the small molecule against the antibody, which was not possible, for the previous reason.

In the future, this experiment could be repeated with some alterations in the protocol: exosomes should be collected from cells in the same culture conditions, such as adopting the same fluorescent dye and method, and keeping the number of incubation days in exosome depleted medium fixed for the three cell lines and for other experiments, for higher consistency of the results.

4.3. Carboxyl-activated glass slide

4.3.1. Blocking test

A critical step to obtain valid results in this set of experiments is surface blocking. And, as the glass slide was activated via EDC/NHS chemistry, it is also important to know how the deactivation step contributes to avoiding nonspecific binding. In this experiment, high fluorescence is expected for the negative control, since the surface will be highly reactive and is expected to bind exosomes and the FITC-labeled anti-EpCAM antibody. Lower fluorescence is expected for the column that has been deactivated since this process is expected to eliminate available esters (activated carboxyl groups) where the proteins would bind, whether exosomal surface proteins or antibodies and therefore would bind fewer labeled antibodies. Ideally, the deactivated and blocked column would show some signal in the specifically captured vesicles and those applied directly in the glass spots.

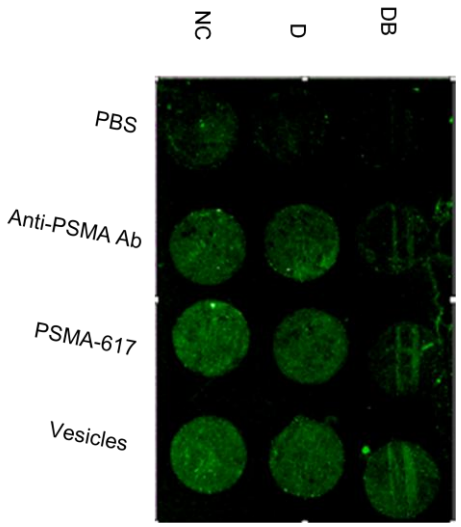

Figure 36. Blocking experiment glass slide, read at 532 nm, *NC*: Negative control; *D* deactivation, *DB* deactivation and blocking

Considering the negative control and the non-blocked spots in Figure 36 – *NC and D columns*, it is clear that no blocking nor deactivation (*NC*) or just deactivation (*D*) of the surface lead to a higher fluorescence signal when comparing to the deactivated and blocked spots in Figure 36 – *DB column*. This third column (Figure 36 – *DB column*), which was deactivated by 0,5 M ethanolamine in PBS, pH 8.5, and blocked with 3 % BSA in PBS, also has differences in the amount of fluorescence between the specifically captured vesicles and the directly bound vesicles. The specifically bound vesicles via PSMA marker show a lower signal, possibly because not all the vesicles in the sample express enough PSMA to promote the binding, as opposed to the indiscriminate adsorption of the vesicles to the glass surface.

4.3.2.EpCAM and PSMA detection by Cy5-labeled secondary antibody

PSMA and EpCAM expression pattern was assessed by Cy5-labeled secondary antibody, and as described throughout this document, fluorescence should be higher in the EpCAM spots for all the cell lines and in the LNCaP spot for PSMA detection.

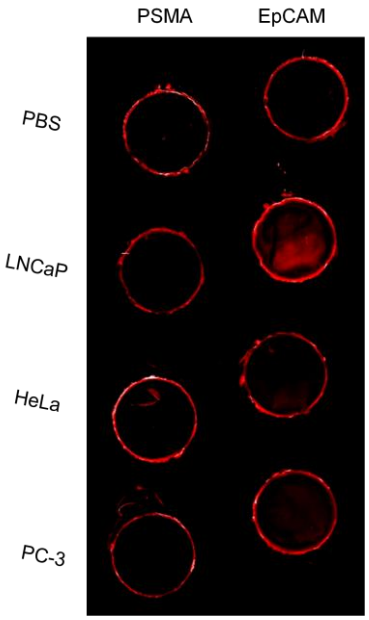

Figure 37. Glass slide for PSMA and EpCAM detection by Cy5 read at 635 nm

In this experiment, the control samples with PBS appear to be negative, showing low fluorescence, indicating an efficient deactivation and blocking of the surface, as seen in Figure 36. Regarding the samples incubated with anti-EpCAM antibody (Figure 37), these show overall higher fluorescence than their anti-PSMA counterparts. 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 (44). Regarding the samples incubated with anti-PSMA, the intensity of the signal is lower and there is no visible distinction between the PSMA positive sample, LNCaP, and the PSMA negative samples, PC-3 and HeLa. According to literature (15) (36), PSMA is enriched in LNCaP cell line exosomes and is therefore widely used for specific capture of these exosomes, so a higher intensity signal could have been expected for this spot. However, the detection is done by an optical method with less sensitivity when compared to other methods, making it difficult to observe minor differences. This low intensity can also be due to a very weak signal which cannot contrast with the background, or that there are lower concentrations of PSMA when compared to EpCAM and the reader does not allow for a resolution of such low values when both markers are measured simultaneously.

4.3.3.Specific capture of fluorescent vesicles

At last, as done with the latex beads in section 3.4.5.3, specific capture of exosomes by PSMA or EpCAM targeting was attempted and the results are discussed in this section.

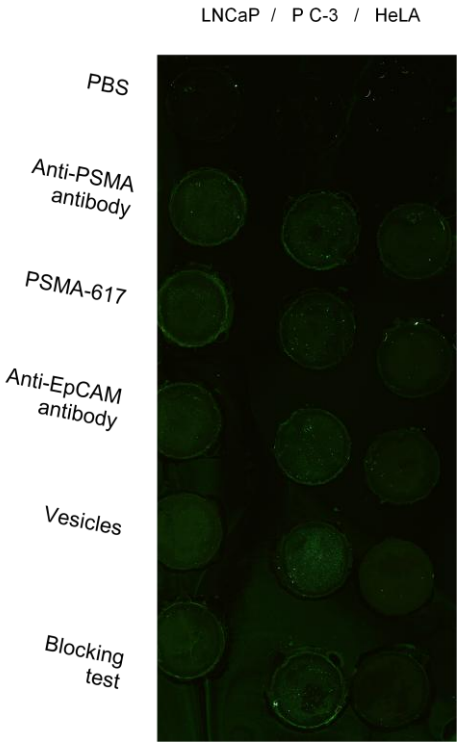

Figure 38. Specific capture of CTG exosomes, read at 532 nm

In this experiment, considering again that LNCaP exosomes are PSMA-positive and PC-3 and HeLa's are not, it was expected to see higher fluorescence in the LNCaP column when incubating the samples in the binder spot targeting PSMA and no fluorescence for PC-3 and HeLa cells. This does not happen, since all spots, apart from the untreated control PBS, are fluorescent (Figure 38). The main problem in this experiment is the blocking failure to prevent the nonspecific binding of the exosomes. Many publications recommend blocking reactive surfaces with BSA and/or glycine, even for prevention of nonspecific binding of exosomes and not only antibodies or labeling molecules to surfaces (45; 14; 3; 46) and although it was also seemingly successfully tested in section 4.3.1, it did not present the expected results in this experiment. All blocked surfaces which were then incubated with CTG vesicles showed fluorescence (Figure 38 – *Blocking test*), invalidating the specific capture results. Most importantly, it can be seen in Figure 39 that the spectra from CTG and Cy3 are not as coincident as initially thought and the Genepix's laser line at 532 nm barely excites the fluorophore.

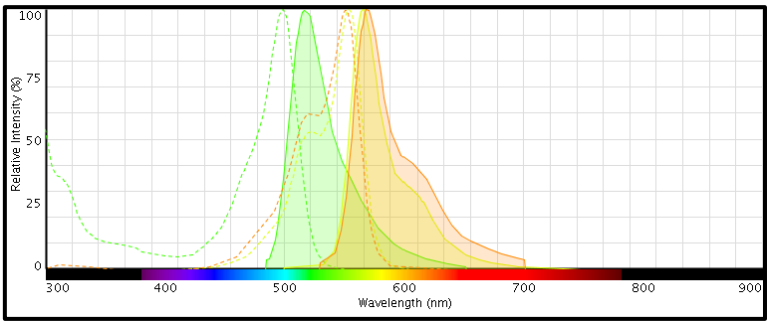

Figure 39. Absorption (dotted line) and emission (full line) spectra for CTG (green), Cy3 (yellow) and DiI (red) (34)

CellTracker™ Green was used due to immediate availability at the laboratory but ideally, to work with the Genepix device, cells could be for instance incubated with the same type of dye but of a different color such as CellTracker™ CM-DiI Dye (Thermo Fisher, C7000) with maxima at 553/570 nm instead of CTG (492/517 nm), since its spectrum is practically coincident to that of Cy3 (Figure 39). Any other cell absorbable dye on this wavelength range would also be appropriate, as long as the dye would be capable of being packed in the vesicles.

4.4. Scanning Electron Microscopy

4.4.1. Hydrophobic and hydrophilic wafers

SEM images for the two opposite water affinity surfaces are shown:

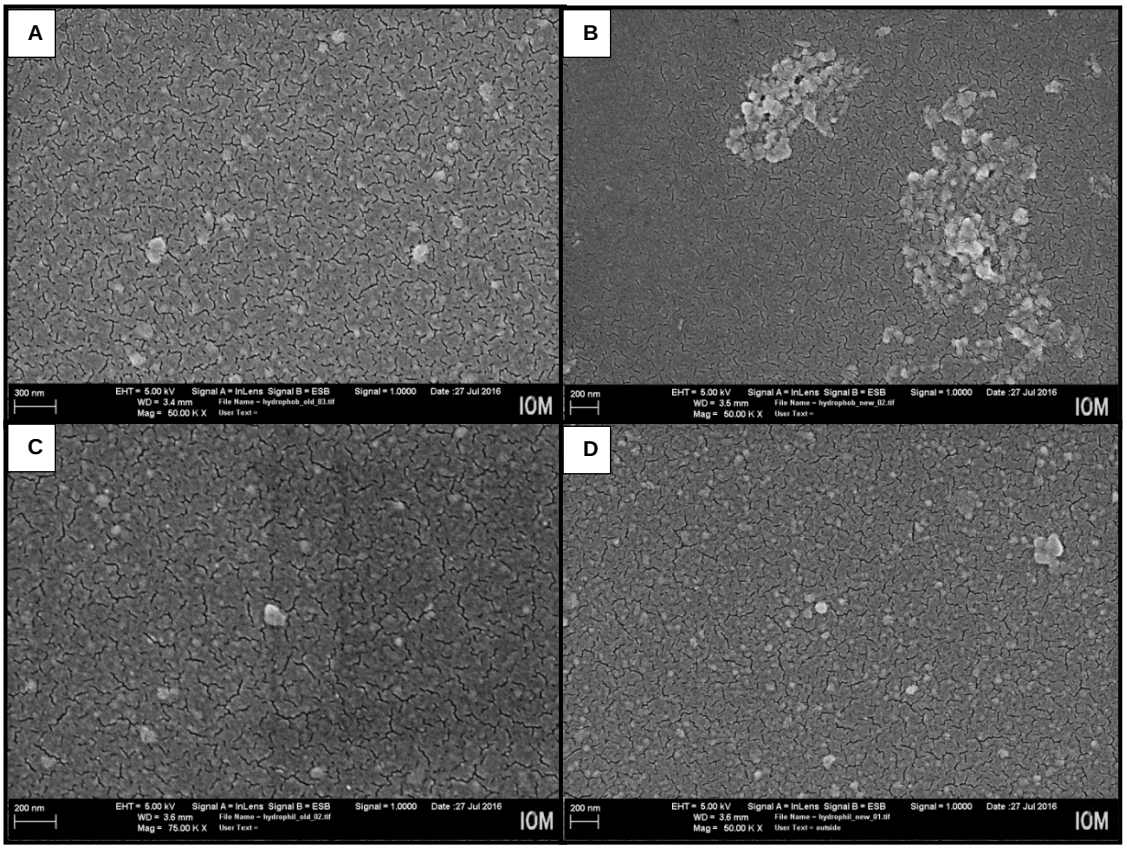

Figure 40. SEM image of LNCaP vesicles purified according to 3.3.1 (A, C) or 3.3.2 (B, D), on an hydrophobic silica wafer (A, B) or hydrophilic silica wafer (C, D). Scale bar 300 nm (A) or 200 nm (B-D)

The hydrophobic surface can lead to the agglomeration of vesicles (Figure 40 A, B), due to the formation of droplets, during fixation and ethanol dehydration steps, which when drying reduce their volume and gradually move the exosomes closer together, as seen in Figure 40B. This effect might be problematic in a microfluidic channels and chambers context, since it can promote obstruction of these structures.

Figure 40C and D refer to the hydrophilic surface where the exosomes seem evenly distributed and do not cluster together. In terms of size, the vesicles seem to be in the expected range, for both purifications.

4.4.2.Untreated silica wafer

The SEM images for exosomes or bead-bound exosomes fixated to a silica surface that was not modified prior to fixation, are presented in this section.

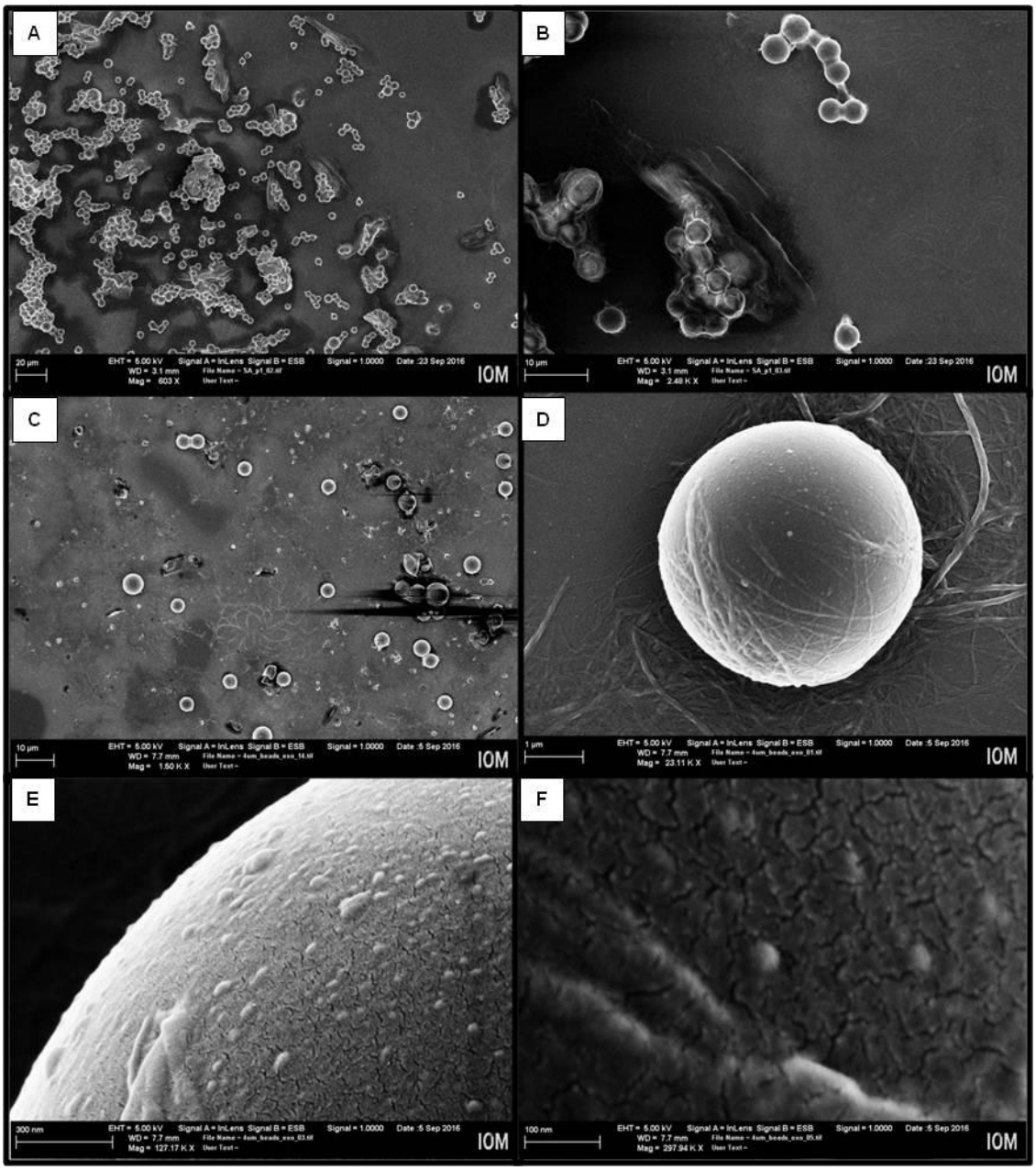

Figure 41. SEM image of beads (A,B) and beads after incubation with exosomes (C-F) Scale bars: 20 μm (A), 10 μm (B, C), 1 μm (D), 300 nm (E),100 nm (F)

Beads were directly imaged, in Figure 41A and B, and appear to form agglomerates which can be an imaging artifact due to the fixation protocol. Figure 41C shows an overview perspective of the beads after incubation with the exosomes. This sample, due to its biological nature, is more complex which is translated into larger debris and fibrils throughout the sample. Figure 41D-E are close-ups of the beads with, what appears to be, bound exosomes, based on size and morphology. In F, a detail of vesicles on bead surface is shown along with some fibrils, seen in other magnifications (Figure 41C-

F). Since these fibrils could not be removed by a 1 μm filter during purification, the centrifugation protocol was optimized from the one described in section 3.3.1 to the one in section 3.3.2, where a 0,2 μm filter is used.

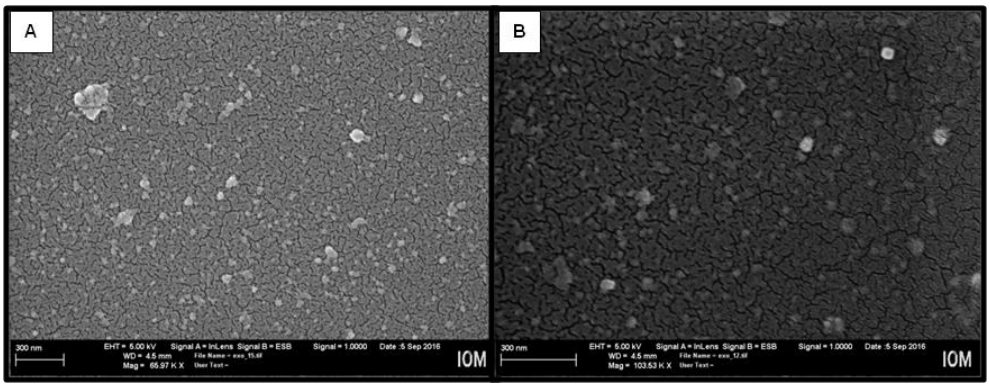

Figure 42. SEM images of LNCaP exosomes directly bound to the slide. Scale bars: 300 nm

Figure 42 shows, similarly to other purifications in Figure 40, the exosomes adsorbed directly on the chip, with no apparent visual difference from the samples in Figure 40C and D, showing the hydrophilic silica surface. On a first approach, it appears then, that the vesicle samples react well to the untreated and hydrophilic surfaces, but not to the hydrophobic surface, which promotes clustering.

4.4.3.Exosomes and microvesicles on untreated silica

Two different purifications were visualized by SEM: exosomes (< 0,2 μm) and microvesicles (<1 μm):

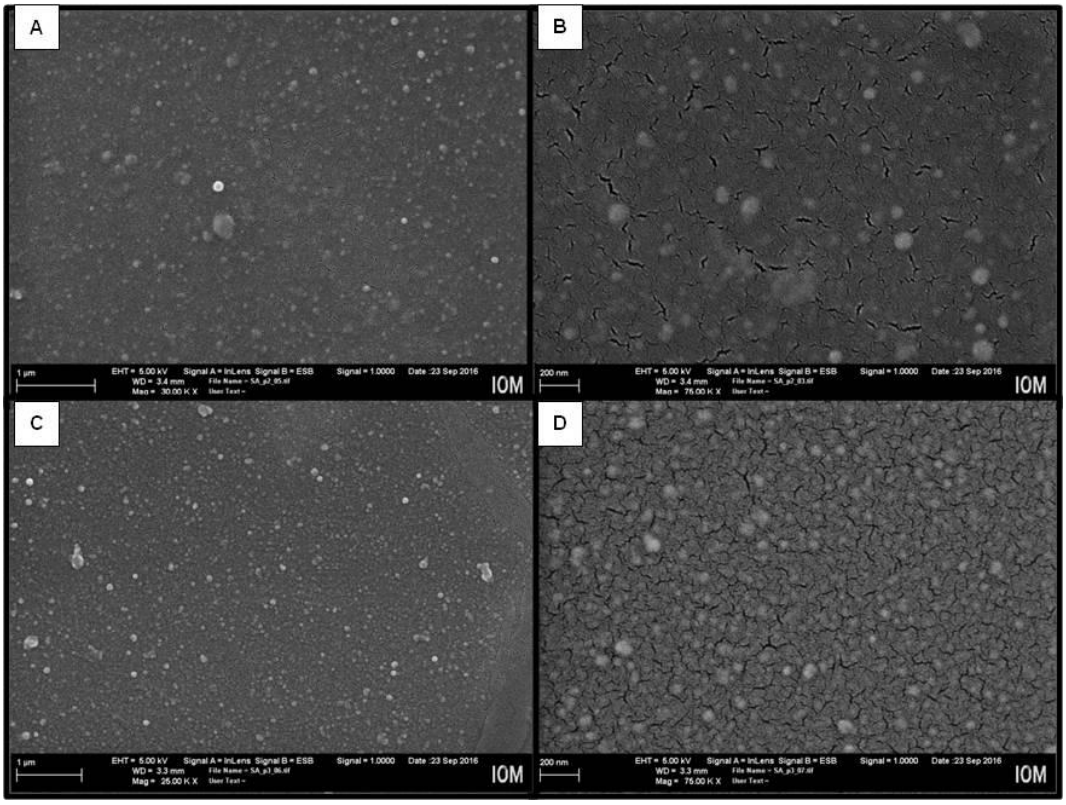

Figure 43. SEM images of LNCaP exosomes (A,B) and LNCaP microvesicles (C,D).

Scale bars: 1 μm (A,C); 200 nm (B,D)

In Figure 43 there is no significant visual difference between the exosomes sample and the microvesicles sample, apart from a seemingly higher density in Figure 43D when compared to the same magnification in Figure 43B. Bigger vesicles would be expected in the microvesicles sample, however, as the totality of the sample was not scanned, some areas with bigger vesicles could have been missed. There are other methods and devices more specialized in assessing size, such as the Zetasizer (section 3.7) that were used for more accurate measurements.

4.4.4.Specific capture by functionalized carboxyl-activated glass slide

Exosomes were specifically captured on anti-PSMA antibody or small-molecule PSMA-617 functionalized glass slide and imaged by SEM.

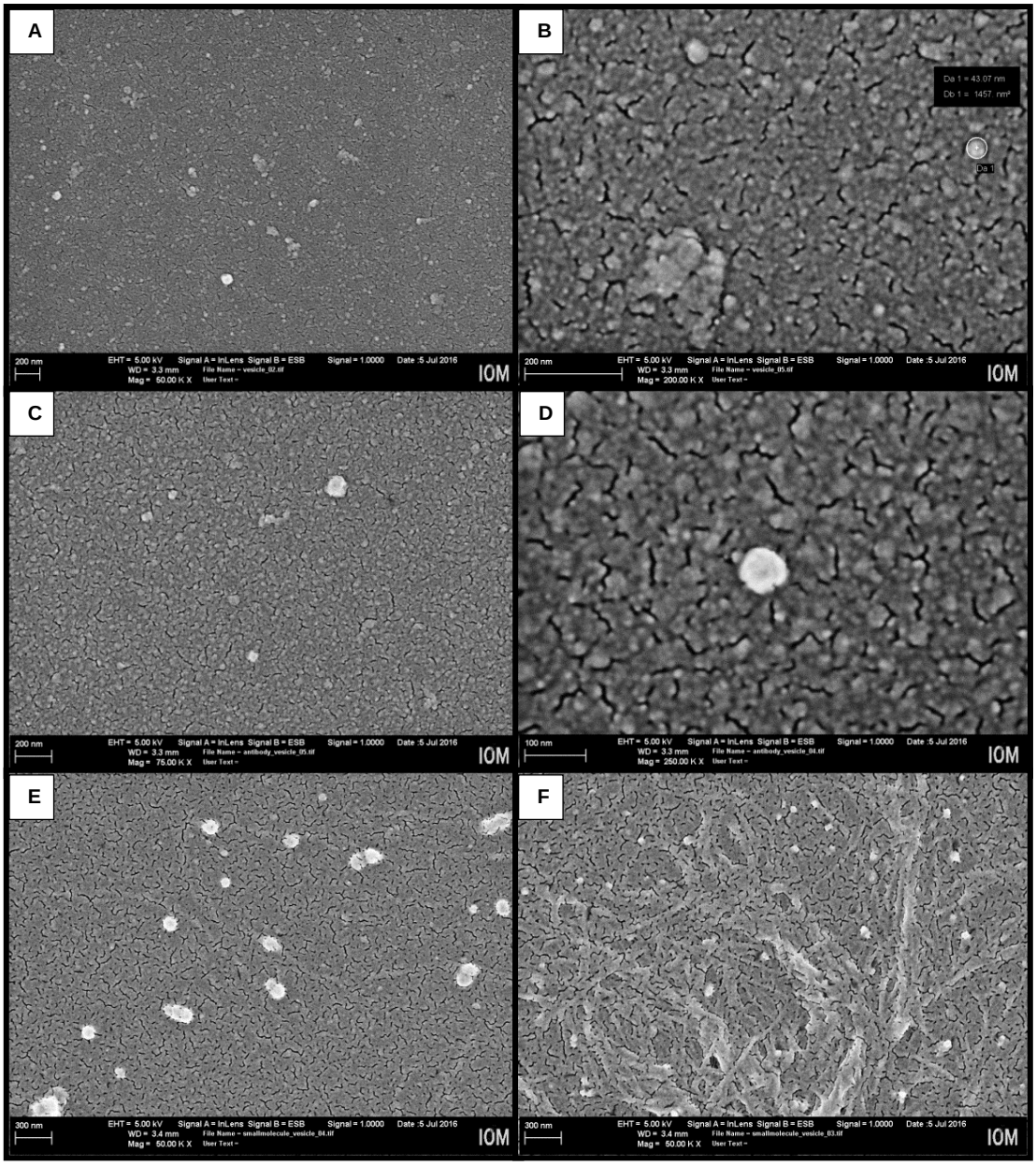

Figure 44. SEM images of LNCaP vesicles directly bound to the slide (A, B), via anti-PSMA antibody (C,D) or via PSMA-617 (E,F). Scale bars: 200 nm (A-C), 100 nm (D), 300 nm (E,F)

Regarding morphology, the obtained images presented the expected round, nanometric sized vesicles. The fact that the slide is activated does not seem to influence direct binding of the exosomes, since these samples in Figure 44A and B look similar to those in Figure 42 Figure 43. There was however some contamination by what appears to be intracellular cellular debris, such as cytoskeleton fibrils, as seen in great numbers in Figure 44F and a crackling of the surface due to the reaction of the sputtered gold in the carboxyl activated surface. Visually, the exosome capture seems equally

effective either for the antibody or the small molecule targeting PSMA. Nevertheless, when comparing these results to the LNCaP column of the glass slide in Figure 38, displaying invalid results due to poor blocking of the surface, and to other SEM images such as Figure 40Figure 42, the exosomes might in fact not even having been captured by the binders, but yet adhered directly to the slide.

Testing surfaces with different water affinities and functionalizations contributes to understanding how the exosome samples might react in a microfluidic device. With this visual data, hydrophilicity and, if necessary, carboxyl-functionalization for specific binding seem to provide good surfaces for exosome handling.

4.5. Zetasizer

4.5.1. Vesicle size

Plots of the percentages of particles with certain diameters measured by the Zetasizer are presented in this section.

Firstly, PBS was measured:

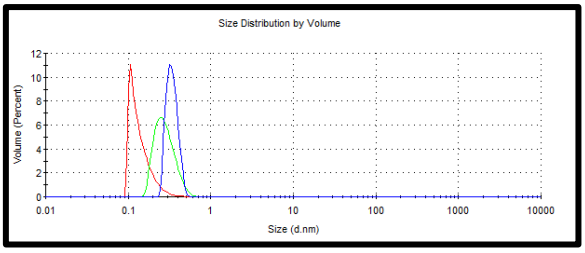

Figure 45. Size distribution by volume of a PBS sample

Commercial PBS, used to dilute the samples, contains structures under 1 nm of diameter (Figure 45), which are in a 10-fold lower size range than the vesicles and therefore should not interfere with the exosome samples measurement.

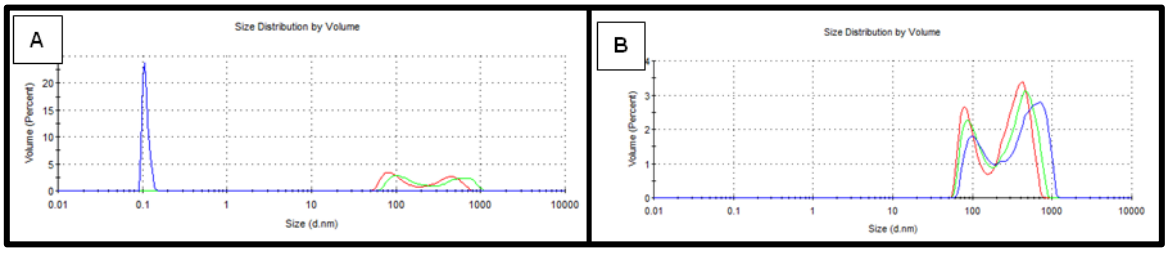

Figure 46. Size distribution by volume of vesicles from LNCaP (A) and PC-3 (B), 1:10 dilutions, purified according to 3.3.1.

In Figure 46 it is seen that the vesicles measured range from 40 nm to 1 µm, which is consistent with the described size of exosomes in literature and with the filter used in the purification of these

samples. The size distribution does not differ between cell lines, despite the peak at 0.1 nm in Figure 46A that could be due to PBS.

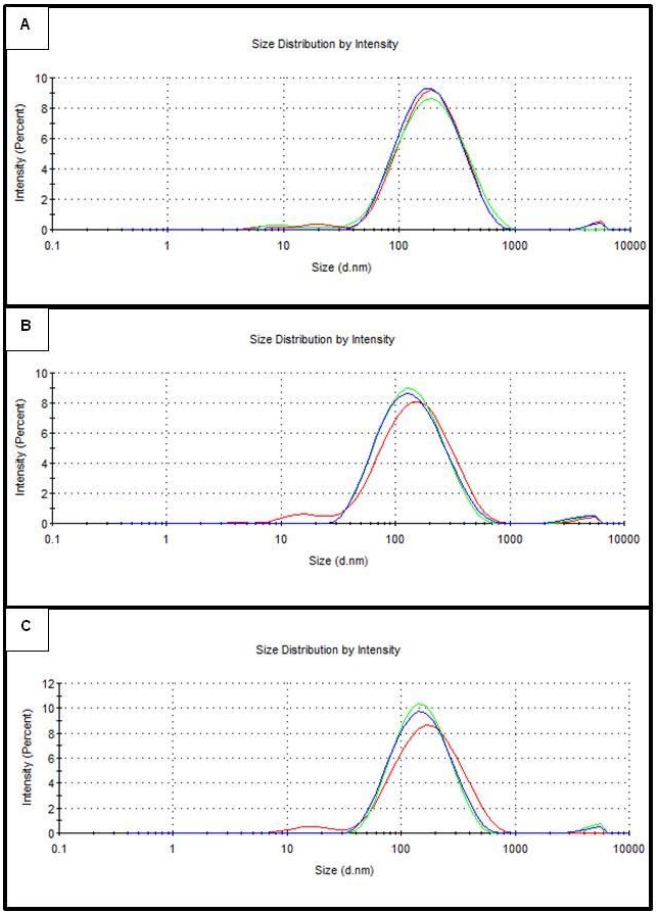

Figure 47. Size distribution by intensity of vesicles purified according to 3.3.2 from HeLa (A), LNCaP (B) and CTG-LNCaP (C)

The optimized purification method, described in section 3.3.2, renders more homogenous exosome samples as seen in Figure 47, with structures ranging from 40 nm to 1 µm. For this purification, a 0.22 µm was used, but in the obtained distribution, there are vesicles or aggregates bigger than that, perhaps due to conformation changes that allow these structures to pass through the filter pores. In any case, the highest intensity corresponds to vesicles around 200 nm, the expected size for bigger exosomes, which are present in larger numbers.

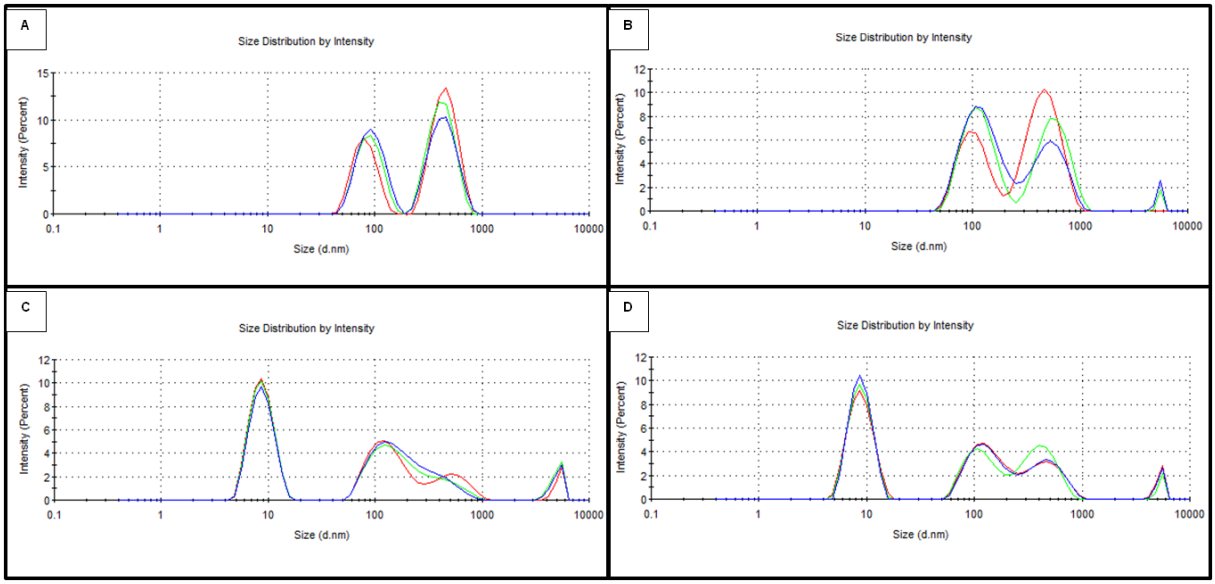

Figure 48. Size distribution by intensity of samples in PBS (A, B) or PBS/0.05%Tween20 (C,D) from LNCaP transfected with GFP (A,C) or PC-3 (B,D)

Samples of GFP transfected LNCaP and PC-3 purified exosomes are shown in Figure 48, and these are also in the expected size range of 40 nm-1 µm (Figure 48A and B), when measured in standard PBS buffer. However, PBS/0.05%Tween20 is not as innocuous, since it causes a sharp peak at 10 nm, perhaps due to the formation of structures promoted by the tensioactive properties of the surfactant Tween20 (Figure 48C and D).

4.5.2.Vesicle zeta potential

Regarding zeta potential measurements of LNCaP purified vesicles in PBS according to the optimized procedure (Section 3.3.2) the obtained value was, on average, -12,6 mV. Comparing this value to those from Figure 49, 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 in the previous section 4.5.1, and also considering the inevitable biological variability. These -12,6 mV fall in the category, in Figure 19, of ‘’beginning of agglomeration’’.

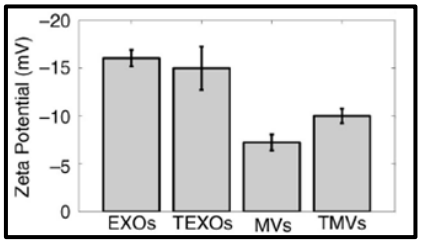

Figure 49. Literature results for zeta potential for LNCaP extracellular vesicles: microvesicles (MVs) and exosomes (EXOs) and trypsinized microvesicles (TMVs) and trypsinized exosomes (TEXOs) (3)

4.6. Lateral Flow Assay

Pictures of the lateral flow assay nitrocellulose strips, 20 minutes after being put in contact with 100 µL of LNCaP vesicles prepared sample are presented in the following figure:

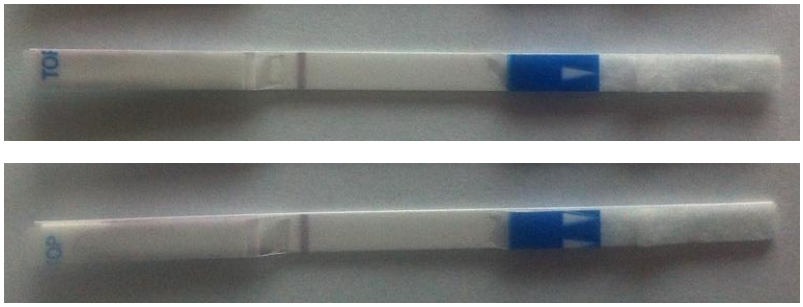

Figure 50. Lateral Flow assay with LNCaP exosomes – antibodies concentration at 15 µg/mL (top) and 5 µg/mL (bottom). Strip dimensions: 5,5 cm × 0,4 cm.

In these experiments, it was expected to detect the exosomes by the development of the two bands in the strips, which did not happen since both results are negative (Figure 50).

This can be due to the fact that, as seen in section 4.5.2, the exosomes and vesicles might agglomerate and not even get past the sample or conjugation pads or non-specifically bind to the nitrocellulose membrane.

As stated before, this was 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.

4.7. Final remarks

4.7.1.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 (47), therefore this standardization of culture conditions ensures higher uniformity of exosome samples and higher reproducibility between experiments.

4.7.2. 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, described in section 3.3.2, 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 seen in SEM images in Figure 42 or zetasizer size results in Figure 47. This protocol allows then for a reduced intra-sample variability.

4.7.3.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, as seen in section 3.4.3.2.2.

4.7.4. 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 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.

4.7.5.Zeta potential of purified exosomes promotes agglomeration

The results obtained in section 4.5.2, that the vesicle sample of LNCaP exosomes can potentially agglomerate, could explain the negative results in the lateral flow test (section 4.6). 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.

4.7.6.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, which was also seen in sections 3.4.4 and 3.5.1, but these solutions are not valid for exosome blocking. According to the results obtained for bead blocking, in section 3.4.4.3, and for the glass slide experiments, in section 4.3.3, none of these solutions nor a first attempt to block with PEG4000, provide the necessary blocking for vesicles.

4.7.7.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 in PBS/0,05%Tween20 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.

4.7.8. LNCaP cells highly express PSMA whereas PC-3 and HeLa do not; exosome protein expression pattern was inconclusive

As verified with the iQUE in 4.2.5.1, the cells' PSMA expression behavior is consistent with literature, however, in 4.2.5.2, it is not possible to know, based on this experiment, if the exosomes also follow this pattern. Literature is also inclusive in this matter since there is a source reporting PSMA-negative LNCaP exosomes (48).

4.7.9. 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.

5. Conclusions

This Master thesis 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 its 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 before the development of the NanoCapture device itself. It will be however a road worth taking, since this novel platform 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. This device will aim to bring personalized medicine one step closer to day-to-day clinical practice.

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