Prostasomes as Diagnostic, Prognostic and Therapeutic Vesicles

LOUISE DUBOIS
Abstract

This thesis explores prostasomes and their ability to be used as a new diagnostic tool for prostate cancer. Alongside diagnosis, this thesis also suggests prostasomes as a tool for prognosis and therapeutic treatment in patients with prostate cancer. By further characterizing prostasomes we can identify a biomarker and also a method of visualizing and interpreting the information provided in order to conduct a correct and fast diagnosis for prostate cancer.

In Paper I, we show that the prostasomal bilayered membrane consists of lipid rafts, clusters that holds cholesterol, sphingolipids and gives receptors a rigid platform upon which to work. We compare the proteomic content of prostasome lipid rafts with the entire prostasome membrane in the search for a specific biomarker.

In Paper II, we show that purified lipid rafts from the prostasome membrane can re-vesiculate and create new bioengineered vesicles. These new vesicles can carry different agents inside them and we find that the method is also applicable to blood cells. This suggests a new method for cell-specific delivery of drugs and cancer therapy.

In Paper III, we further characterize the prostasome membrane, this time mapping purinergic receptors. This could be used in the development of prostate cancer treatment and to gain better understanding of how prostasomes interact with surrounding cells in their ambient environment.

In Paper IV, we investigate the difference in thymidine kinase 1 (TK1) enzyme activity between prostasomes and malignant exosomes. TK1 is considered to be a biomarker of cell proliferation and could therefore be used as a biomarker for prostate cancer diagnosis and progression.

In summary, this thesis contributes to the puzzle of how to better diagnose, prognose and treat prostate cancer. Although it is mainly pre-clinical research it opens up new possibilities for the diagnosis and treatment of prostate cancer.

Keywords: Exosomes, Prostasomes, Lipid rafts, Bioengineered vesicles, Prostate cancer, Purinergic receptors, Thymidine kinase 1

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Till min familj
“Everyone is a genius. But if you judge a fish by its ability to climb a tree, it will live its whole life believing that it is stupid.”

-Albert Einstein
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


III  **Dubois, L.**, Larsson, A. (2018) Occurrence of purinergic receptors in the prostasome (prostate epithelial cell-derived exosomes). *Purinergic Signalling, Submitted*

IV  **Dubois, L.**, Löf, L., Kamali-Moghaddam, M., Larsson, A^, Stålhandske P^ (2018) Increased levels of thymidine kinase 1 in malignant cell-derived exosomes. *In manuscript*

*Indicates shared first author
^Indicates shared senior author

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List of papers not included in this thesis


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## Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADT</td>
<td>Androgen deprivation therapy</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>ALIX</td>
<td>Programmed cell death 6-interacting protein</td>
</tr>
<tr>
<td>APL</td>
<td>Acute promyelocytic leukemia</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign prostate hyperplasia</td>
</tr>
<tr>
<td>CRPC</td>
<td>Castration-resistant prostate cancer</td>
</tr>
<tr>
<td>DRM</td>
<td>Detergent-resistant membrane</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-to-mesenchymal transition</td>
</tr>
<tr>
<td>ESCRT</td>
<td>Endosomal sorting complex required for transport</td>
</tr>
<tr>
<td>EVs</td>
<td>Extracellular vesicles</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>ILVs</td>
<td>Intraluminal vesicles</td>
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<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MVBs</td>
<td>Multivesicular bodies</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>PM</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate-specific antigen</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RP</td>
<td>Radical prostatectomy</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SP</td>
<td>Seminal plasma</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscope</td>
</tr>
<tr>
<td>TK</td>
<td>Thymidine kinase</td>
</tr>
<tr>
<td>TDP</td>
<td>(deoxy)Thymidine diphosphate</td>
</tr>
<tr>
<td>TMP</td>
<td>(deoxy)Thymidine monophosphate</td>
</tr>
<tr>
<td>TTP</td>
<td>(deoxy)Thymidine triphosphate</td>
</tr>
<tr>
<td>TSG 101</td>
<td>Tumor susceptibility gene 101 protein</td>
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Introduction

When I was a little girl my father told me that we are all made of stardust and this really sparked my interest in science. The fact that my grandfather was a professor at Chalmers University of Technology helped a bit too, of course.

I wanted to know more about the human body, what it is made of, how it works and what we can do when it doesn’t work. I started my studies in Biomedicine at Uppsala University in 2008 and I loved it from the start! This was what I wanted to do, although I’m not saying it was always easy. A few “omtentor” later and I was finished with my degree. So, what to do now? I knew I wanted to be a scientist; being at the forefront of knowledge intrigued me and in 2014 I contacted a group that was conducting research about pros- tasomes. I’d never heard about it, but it sounded interesting! This opened up the world of exosomes to me, how they act as nano-messengers in the body and how cancer can both spread and be contained with the help of exosomes.

Every type of cancer has six specific hallmarks: sustaining proliferative signaling, evading growth suppressors, activation of invasion and metastasis, enabling replicative immortality, inducing angiogenesis and resisting cell death [1]. Normal cells control their cell-cycle and a homeostasis of the number of cells is achieved. Cancer cells bind growth factors which help them sustain proliferative signaling [2]. Cancer cells are not only capable of upregulating growth factors, they can also circumvent the powerful tumor suppressor genes, such as the retinoblastoma gene and the p53 gene [3].

When prostate epithelial cells lose their polarity and adherence to each other, they undergo an epithelial-to-mesenchymal transition (EMT) and this helps the cancer cells migrate and invade other parts of the body. Thus, metastases are formed [4]. Unlike other cells, including stem cells, cancer cells have the ability to replicate forever and evidence points to the telomers which protect the ends of the chromosomes, as they are involved in unlimited proliferation [5]. Tumors need oxygen and nutrients just like normal cells and tissue, but in tumor progression, angiogenic sprouting is activated so that new blood vessels are formed as the tumor grows [6].

Programmed cell death, apoptosis, is something all cells holds as a natural way of resisting cancer development. Unfortunately cancer cells develop a variety of ways of limiting and avoiding apoptosis so they can continue to proliferate [7]. A proposal for another hallmark is evading immune destruction, meaning tumors somehow manage to go “under the radar” and avoid
detection from the body’s immune system, thereby evading eradication [8]. A further emerging hallmark is the reprogramming of energy metabolism. In the 1930s [9, 10], Otto Warburg discovered what is now known as the “Warburg effect”, where cancer cells produce their energy through aerobic glycolysis [11].

Being able to solve the puzzle of cancer, or at least help find one piece of the puzzle, has been the motivation for the duration of my PhD. Now, I get to present my thesis to you all. I am so happy and proud of what I have achieved. I hope whoever reads this thesis will find it inspiring.
The prostate

The male accessory glands consist of the seminal vesicles, the bulbourethral glands (also known as Cowper’s glands) and the prostate gland. When ejaculation occurs, sperm from the epididymis is transported by the vas deferens to the ejaculatory ducts within the prostate, where it mixes with fluids from the male accessory glands and forms semen [12]. The prostate is divided into two lobes (left and right) and three zones: the central zone, transition zone and peripheral zone. A large portion of the gland is also encapsulated [13].

The prostate gland continues to grow throughout a man’s life, from about 20 g in his thirties to about 30 g in his eighties [14]. If the prostate increases in size the urethra is obstructed and urinating becomes increasingly harder. This is not normally noticed until a tumor occurs or there is a benign hyperplasia in the prostate.

Due to its location in the body, see Figure 1, the prostate is difficult to examine. It is surrounded by many vulnerable organs, such as the urinary bladder, rectum and important blood vessels. Many nerves connected to male sexuality are also located around the prostate. An operation or other interventions may damage the surroundings of the prostate and cause severe side effects such as incontinence, impotence or rectal bleeding.

Figure 1. Location of the prostate in the male body. Image credit Wikipedia, CC-BY-SA-3.0.
Seminal plasma

Seminal plasma (SP) is ejaculate fluid, minus spermatozoa, which together constitute the semen. SP consists of a mix of fluids from the prostate and the male sex accessory glands. At each ejaculation about 3-4 mL is released and has a pH of 7.5-8 [15], which means the fluid is slightly alkaline. The largest amount of seminal plasma comes from the seminal vesicles (65-75%), the prostate contributes with around one half of this amount (25-30%) and the remainder comes from the bulbourethral glands and the epididymis (<1%).

Before 1935, SP was only regarded as a transportation medium for spermatozoa. However, SP is actually essential to the normal function of male fertility [16]. Seminal plasma comprises many proteins, sugars, ions and extracellular vesicles (prostasomes), all necessary for the correct function of spermatozoa [15].

Semenogelin and fibronectin are proteins that help create a coagulum which then traps the spermatozoa [17, 18]. Liquefaction starts when the semenogelins are degraded with the help of prostate-specific antigen (PSA), releasing spermatozoa [19, 20]. A vaginal pH level of around 4 cleaves the protein progastricsin into the active enzyme gastricsin, which degrades other proteins, including PSA. This degradation process may occur in order to prevent immune infertility from forming [21].

Seminal plasma acts as a buffer for the spermatozoa in the acidic environment of the vagina [22] and ions such as calcium, sodium, potassium, magnesium and zinc help the immunosuppressive and antibacterial functions [23, 24]. The monosaccharide fructose provides energy and nutrition for the spermatozoa [25] and the movement, acrosome reaction and capacitation are orchestrated by the influence of prostasomes and are necessary for the spermatozoa to mature and fertilize the ovum [26].
Prostate cancer

Diagnostics, Prognostics and Therapeutics

Prostate cancer is the most frequent type of cancer among men in the Western world [27]. The prognosis is often good and most men die of other causes than prostate cancer. However, it is still the third leading cause of death among men.

Prostate-specific antigen (PSA), which is widely used today for diagnosis, has clear limitations as a cancer biomarker. It is very prostate specific, but not very cancer specific, and a positive PSA test must always be followed by a prostate biopsy, which is an invasive operation that causes much pain for the patient. Also, PSA does not differentiate between aggressive tumors and less aggressive tumors, which means that many men undergo unnecessary prostatectomies and therefore have reduced quality of life due to the side effects associated with the surgical procedure, such as impotence and incontinence.

In Sweden alone, more than 10,000 new cases of prostate cancer are discovered every year (10,473 cases, National Board of Health and Welfare/Socialstyrelsen, 2016). With increasing age, the incidence of prostate cancer rises, but the mortality stays the same throughout. In fact, most prostate cancer patients die of other causes than cancer. This is because prostate cancer has many forms, ranging from highly aggressive to non-aggressive. The aggressive forms often cause metastases in either the skeleton or the brain, while slow, non-aggressive cancers can remain in the prostate for many years or be held back by hormone treatment. There is a clear genetic component in prostate cancer and the risk of being diagnosed with prostate cancer is three times higher if you have a first degree relative who had prostate cancer compared to same age controls [28, 29].

To diagnose prostate cancer today, a blood sample is taken and the level of PSA is measured. PSA is a small protein of 34kD, which means it can easily leak from the prostate cell into the blood stream. It is an exocrine serine protease and is important for seminal fluid where it cleaves the prostate derived protein seminogelin, which helps with semen liquefaction [30]. PSA is an organ-specific biomarker but is not a cancer-specific biomarker and an elevated level of PSA does not indicate cancer, but requires further investigation. High level PSA could for instance be due to benign prostate hyperplasia (BPH) [31]. The incidence and prevalence of BPH and prostate cancer both increase with age [14].
STHLM3 is a new model for the diagnosis of prostate cancer and Grönberg et al. have shown that it reduces the number of biopsies needed by one third as well as maintaining sensitivity to the Gleason Score in comparison with PSA tests [32]. However, STHLM3 has its flaws: it is very expensive compared to PSA tests and is primarily a risk predictor rather than a true cancer diagnosis test.

Commonly used forms of therapy for prostate cancer are drugs, hormones and cytostatics, radiation, prostatectomy or expectancy. Androgen deprivation therapy (ADT) is a form of treatment that uses hormones to reduce androgen levels. The male hormone group androgens can stimulate cancer growth. ADT aims to slow down or even stop cancer cell proliferation, and PSA also decreases [33]. To achieve this deprivation, there are two solutions: antiandrogen monotherapy or castration. Castration can then be achieved either surgically, by removing the testicles, or medically by estrogen, which lowers testosterone levels by affecting the hypothalamus. Gonadotropin-releasing hormone analog (GnRH) antagonists works by blocking androgen receptors [34]. The patient will need long-term hormone therapy that can cause side effects such as gynecomastia, anemia, hot flashes, fatigue, osteoporosis and decreased sexual function [35-38]. Unfortunately, over time castration-resistant prostate cancer (CRPC) will develop despite low levels of testosterone [39, 40].

If a patient show signs of an aggressive form of PC, a radical prostatectomy (RP) must be performed. Complications for the patient include incontinence and sexual dysfunction but can be reduced by nerve-sparing surgery. Nerve-sparing surgery cannot be used in all cases [41]. Often, RP is combined with radiation treatment, hormone treatment, or cytostatic drug treatment.
Extracellular vesicles

Exosomes, microvesicles and apoptotic bodies

Extracellular vesicles (EVs) are nanosized particles released by virtually every cell in the body [42]. Cells have always communicated with each other by either cell-cell contact or by transferring molecules secreted by them. Initially, researchers believed the vesicles exocytosed by cells were just cell debris, or garbage, but over the last 40 years these vesicles have emerged as a very important means of intercellular communication, the extracellular vesicles.

The vesicles that are shed from the cells originate either from the plasma membrane (PM) or they first fuse with the PM and are then exocytosed. The different particles have been given names such as microvesicles, microparticles, ectosomes, apoptotic bodies, shedding vesicles or exosomes [43, 44].

Exosomes are part of the extracellular vesicle family. They are formed inside the mother cell, which could be any cell from prostate cells, to cardiac cells to immunological cells etc., thus creating subtypes of different exosomes [45-47]. Each EV carries proteins, DNA and RNA representative of the mother cell and EVs can exchange proteins, lipids and nucleic acids with other healthy and diseased cells, as well as promote metastasis in malignant cells [48, 49].

Exosome biogenesis starts in the endosomal pathway [50], see Figure 2. Late endosomes are formed by acidification and changes in protein content [51]. After the formation of late endosomes they start inward budding, creating intraluminal vesicles (ILVs) and the late endosome forms into a multivesicular body (MVB) [52]. The MVB then fuses with the PM to release the exosomes into the extracellular space. The fusion of the MVB to the PM is mostly driven by Rab GTPases, myosins and SNAREs [53]. The sorting of proteins and budding of ILVs into MVB and subsequently exosomes is not yet fully understood, but the endosomal sorting complex required for transport (ESCRT) is thought to play an active role in the budding [54]. Membrane microdomains such as lipid rafts have been discovered to be a passive role of sorting [55].
Figure 2. The biogenesis of exosomes starts with the invagination of the plasma membrane at the location of a lipid raft area. This creates an early endosome, which starts to invaginate, creating intraluminal vesicles (ILVs) and a multivesicular body (MVB) is thus formed. The MVB migrates towards the plasma membrane (PM) and fuses with the PM to exocytose the exosomes into the extracellular space. Image from Waldenström, A. and Ronquist, G. [56].

The extracellular vesicle family sub-types are classified based on size, origin and biomarker proteins. Exosomes are normally classified as double membrane vesicles that contain the proteins ALIX, TSG101 and proteins from the tetraspanin family such as CD9, CD63, CD81 and CD82 [57]. They have a diameter of 30-100 nm, although the upper limit is debated and tends to increase [58].

Microvesicles (MVs), are vesicles released directly from the PM and are up to 1000 nm in diameter [48, 59]. The sorting of proteins into MVs and forming of MVs are still not very well defined, but the oligomerization of cytoplasmic proteins as well as myristoylation and palmitoylation appears to form part of both [60].

Exosomes have a bilayered membrane, as does the cell, but exosome membranes are enriched with cholesterol and sphingomyelin. They do not have an abundance of phosphatidylcholine and phosphatidylethanolamine. The fatty acids in exosome membranes are saturated or monosaturated. Exosomes also have a high ratio of cholesterol and this makes their PM more rigid and they can withstand mechanical force. The cell PM has a ratio of cholesterol to phospholipids close to 1:1, but the exosome (prostasome) membrane has a ratio of 2:1 [61, 62].
Malignant cells also release exosomes into the tumor microenvironment and evidence has emerged that these can contribute to tumor progression and metastasis [63]. The exosomal protein profile reflects the cells content [64], thus making the exosome a potentially perfect biomarker for liquid biopsies, helping to diagnose many diseases [65, 66].
Prostasomes were first reported in prostatic fluid in the 1970s by Ronquist and Hedström [67] and were later observed as extracellular organellar structures in vivo [68, 69], see Figures 3 and 4. Prostasomes are a sub class of exosomes which are derived and secreted from the acinar, epithelial cells of the prostate gland [70]. They are secreted by both benign and malignant cells, and in healthy males prostasomes can be found in both prostatic and seminal fluids, although in the case of cancer, they can also be found in increased amounts of blood because of the epithelial-to-mesenchymal transition that promotes leakage into bodily fluids [71].

![Prostasomes](image)

**Figure 3.** Scanning electron microscopy image of prostasomes. The bar size indicates 100 nm. Image taken by Kjell Hultenby, Karolinska Institute, Huddinge.

Prostasomes play a pivotal physiological role by interacting with sperm cells and enabling the transfer of molecules and proteins essential to fertilization [72, 73]. Prostasomes were first observed to fuse hydrophobically with spermatozoa in 1990 by Ronquist et al. [72] and the interaction was later discovered to be pH dependent, where a more acidic pH is to prefer [74]. Sperm cells gain important abilities from prostasomes so they can survive the hostile environment of the female genital tract in order to reach and penetrate the oocyte.
for fertilization [72]. Prostasomes promote sperm motility, capacitation and an acrosome reaction on their way to the oocyte [75, 76]. The motility promotion effect may come from the transfer of calcium signaling proteins from the prostasome to the sperm [77]. Sperm cells become less active in acidic surroundings, but since seminal plasma and prostasomes are slightly alkaline, they appear to have a protective effect in the vaginal area [78], and sperm motility is crucial to natural fertility [79]. The capacitation of the sperm cell occurs when the glycoprotein coat is removed. The capacitation process must be complete before the acrosome reaction takes place [80]. The acrosome reaction permits sperm cells to fuse with the egg but it cannot happen prematurely and it is believed that the cholesterol in the prostasome stabilizes the acrosomal cap [75].

Furthermore prostasomes show a protective ability against reactive oxygen species (ROS) and against bacteria [81, 82]. When prostasomes were incubated with *Bacillus megaterium* it was observed that the bacteria decreased or experienced a total withdrawal. Thus prostasomes are considered to be antibacterial agents [81]. When spermatozoa enters the female genital tract they are considered to be intruders and prostasomes act as immunosuppressants to protect the sperms [83].

Prostasomes make an important contribution to the normal fertilization process because of their antibiotic, antioxidant and immunosuppressive proper-

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*Figure 4.* Transmission electron microscope image of prostasomes, where the bilayered membrane is clearly visible. The bar size indicates 200 nm. Image taken by Kjell Hultenby, Karolinska Institute, Huddinge.
ties, as mentioned above. This leads us to believe that disruption to the prostasome function could cause infertility. Kravets et al. [84] suggested that medium containing prostasomes could be effective in assisted reproductive technologies.

The prostasomal membrane has a unique composition with a 2:1 ratio of cholesterol to phospholipids [85], where sphingomyelin is the most abundant. The high cholesterol makes the prostasomal membrane stable and resistant to detergents [86]. The composition is similar to that of lipid rafts and it is believed that the biogenesis of prostasomes (and exosomes) starts with these micro domains [55, 87].

DNA has been found inside prostasomes [88, 89] and was proved to be transferred into sperm cells [90]. This could be important for the normal development of the pregnancy [91].

The membrane attack complex (MAC) inhibitory protein CD59 is localized on the prostasomal surface, attached via a glycosylphosphatidylinositol (GPI) anchor [92]. Because of this the spermatozoa are saved from the female complement system. CD59 from prostasomes can be transferred to blood cells lacking this protein and this results in preserved function [93]. Prostasomes have a high coagulant activity and this is due to the protein tissue factor (CD142), which is located on the surface of the prostasomal membrane [94].

Tissue factor is known to promote cancer growth [95] and because prostasomes have such high levels they can be active players in the proliferation of cancer cells. Both prostasomes and malignant cell-derived exosomes are capable of producing adenosine triphosphate (ATP) but prostasomes have a higher ATPase activity compared to malignant exosomes, thus creating a higher net value for ATP production [96], which is essential for an active uptake into recipient cells, and in turn, making them active messengers in the spread of cancer.
Lipid rafts

In 1988 van Meer and Simons [97] put forward the first hypothesis of lipid rafts, domains in the membrane enriched with glycosphingolipids, proteins and cholesterol, see Figure 5. Glycosphingolipid clusters were insoluble in non-ionic detergents at 4°C, thus the term detergent-resistant membranes (DRMs) was created [98]. These domains play an important role in cell endocytosis and cell exocytosis, as well as cell signaling. Lipid rafts and DRMs have been designated as platforms for cellular signaling [99, 100] because of the many proteins localized to these domains, as well as for signal transduction [101] and membrane trafficking [102].

There are primarily two types of lipid rafts: caveolae, with the primary biomarker caveolin [103] and non-caveolae (planar), which is recognized by the protein flotillin instead [104, 105]. GPI-anchored proteins (glycosylphosphatidylinositol) have been shown to be associated with DRMs and primarily when it comes to signaling [106, 107].

Figure 5. Image of a planar lipid raft situated in the bilayered plasma membrane. Cholesterol and glycosphingolipids make the lipid raft more rigid compared to the surrounding membrane and hold transmembrane proteins and receptors. Image credit Wikipedia.
Plasma membranes are ordered structures where the DRMs are regions of high cholesterol content. The phospholipids in the lipid rafts fatty acid side chains have high saturation and this results in a denser packing together with the acyl chains of the sphingolipids. This leads to phase separation and a more rigid domain compared to the normal plasma membrane [108].

Exosomes could play an important role in biomarker studies for certain diagnoses due to their isolation from bodily fluids and the fact that they can provide a more specific insight into the protein composition of the micro domains derived from the respective host cells [109].
Purinergic receptors comprise of three classes: P1 receptors (adenosine), P2X receptors and P2Y receptors. Each class then has subtype receptors; A1-A3, P2X1-P2X7 and P2Y1-P2Y14 (see Figure 6) and together they are involved in many different biological processes [110]. Purinergic receptors are all membrane bound [111] and P1 is a G-protein coupled receptor, as well as P2Y. The P2X receptor belongs to the ligand gated ion-channel family.

![Diagram of Purinergic Receptors](image)

**Figure 6.** A schematic diagram of how purinergic receptors are divided into three classes: P1 receptors, P2X receptors and P2Y receptors as well as further division into subtype receptors. Image adapted from the manuscript *Occurrence of purinergic receptors in human prostasomes (prostate epithelial cell-derived exosomes)* by Dubois et.al., 2018.

There is an abundance of adenosine in seminal plasma [112] and previous work shows the production of ATP by human prostasomes [113]. Adenosine receptors are involved, for example, in cardiac rhythm, circulation [114, 115], the immune system [116], neurogenerative diseases [117] and angiogenesis [118]. Due to their wide range of biological functions, previous studies have pointed to P1 receptors as therapeutic targets [119, 120]. However, the only
drug on the market is an antagonist to the A2a receptor, Regadenoson (Lexiscan; Astellas Pharma) [121]. Caffeine is also an antagonist to the adenosine receptor [122].

Among the most investigated receptors in the P2X subgroup is the P2X7 receptor [123]. Studies show that the P2X7 receptor is a mediator of invasiveness and metastasis in PC [124, 125]. P2X7 has been shown to regulate the release of microvesicles [126-128] as it effects the composition of lipids and proteins in the PM. The receptor mediates trafficking in the PM as well as intracellular organelles [129].

The drug Clopidogrel, which reduces the risk of blood clots forming, is an antagonist to the receptor P2Y12 [130] and has antiplatelet activity. Thus it is often used in patients with cardiovascular diseases. The receptor P2Y12 is involved in platelet aggregation [131] and a way of reducing a tumor and stopping metastasis from forming is to inhibit platelet activity [132, 133]. This has been shown by Clopidogrel in both prostate and colon cancer [134].
Thymidine kinase 1

Thymidine kinase (TK) consists of two isoenzymes: TK1 (cytosolic) and TK2 (mitochondrial). Thymidine is salvaged from extracellular catabolic activity and TK converts salvaged thymidine from extracellular activities into its monophosphate counterpart (TMP) by the coupling of a phosphate group from ATP. After two additional phosphorylation steps, TMP is converted into deoxythymidine triphosphate (dTTP), which is subsequently incorporated into DNA [135].

The TK1 enzyme activity is upregulated during cell proliferation, through the S-G2 phase of the cell cycle, see Figure 7. During the G1 phase the levels of TK1 activity are low and activity peaks during the S phase. The levels decrease during the G2 phase and for the M phase TK1 can hardly be detected [136].

Figure 7. An image of the cell cycle where thymidine kinase expression is at its highest level during the S phase. Picture from Biovica International AB, Uppsala, Sweden.

Tumor aggressiveness have been shown to correlate with high levels of TK1 activity in serum. It has been suggested that this is indicative of the early events of carcinogenesis [137, 138]. Using both liquid and solid biopsies, TK1 activity can be detected [139]. TK1 has mostly been applied on hematological
malignancies as a tumor marker in routine health care and to a lesser extent applied to solid tumor diagnostic applications, such as PC [140, 141]. However, if we combine prostasome detection with a TK1 assay, the specific signal from TK1 could be detected more easily and help in the diagnosis of prostate cancer.
Methods

Purification of prostasomes
Seminal fluid was collected from the Reproduction center at Akademiska Hospital, Uppsala, Sweden. The pooled seminal fluid was then subjected to a centrifugation at 3000 g for 12 min. The supernatant was transferred to ultracentrifugation tubes and spun at 10,000 g for 30 min at 4°C using a 90Ti rotor (Beckman Coulter, Brea, CA, USA).

The supernatant was transferred again and another ultracentrifugation took place, at 100,000 g for 2h at 4°C. The pellet was resuspended in phosphate buffered saline (PBS) over night at 2-8°C. The suspension was then loaded on to a XK16/70 Superdex 200 gel column (GE Healthcare, Uppsala, Sweden) and equilibrated with PBS in order to exclude amorphous material [45].

At a flow rate of 5 mL/h fractions were collected in volumes of 1.3 mL. Peaks with elevated absorbance at 260 nm (nucleic acid) and 280 nm (proteins) were measured and the fractions containing prostasomes were pooled and ultracentrifuged at 100,000 g for 2 h at 4°C. The pellet was resuspended in PBS overnight at 2-8°C before loaded on to a sucrose density gradient of 1M, 1.5M and 2M sucrose. The gradient was ultracentrifuged at 85,000 g for 21 h at 4°C using a SW28.1 rotor (Beckman Coulter), see Figure 8.

The fraction at 1.5M contains prostasomes and is considered the main fraction (density range 1.13-1.19 g/ml). This fraction was ultracentrifuged at 100,000 g for 2 h at 4°C and then solved in PBS. The solution was adjusted to 2 mg/mL in accordance with a BCA protein assay kit (Merck, Darmstadt, Germany) and stored at -70°C until use.
Purification of lipid rafts

Prostasomes, purified as stated above, were pooled to contain approximately 8 mg and then top loaded on to a 1% Triton X-100 sucrose density gradient at 0.15 M, 0.8 M and 1.5 M. The gradient was ultracentrifuged at 256,000 g for 28 h at 4°C with a SW40Ti rotor (Beckman Coulter), see Figure 9.

The fraction at 0.8M (density of 1.10 g/mL) was collected and ultracentrifuged at 100,000 g for 2 h at 4°C. The pellet was resuspended in PBS and stored at -70°C until use.
Figure 9. Presenting the purification of lipid rafts. The final pellet from ultracentrifugation of prostasomes are top loaded onto a sucrose gradient of 1% Triton-X 100 and ultracentrifuged, creating a lipid raft fraction of 0.8M. Image from the article by Dubois et al. [142].

**SDS-PAGE**

Prostasomes and/or lipid rafts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a Novex® NuPage 4-12% Bis-Tris polyacrylamide gel (Invitrogen, Carlsbad, CA, USA) at 200 V for 35 min in accordance with the manufacturer’s instructions.

The molecular weight of the denatured proteins in the sample was visualized by Seeblue® pre-stained Standard (Invitrogen), see Figure 10. Analysis was performed on a Molecular Imager ChemiDoc™ XRS Imaging System using the software Image Lab 5.1 (Bio-Rad Laboratories, Hercules, CA).
Immunoblotting

The proteins from the SDS-PAGE gel were subsequently transferred to a 0.2 µm Novex® Nitrocellulose membrane (Invitrogen) using 25 V for 1 h. Bovine serum albumin (BSA 1%) in PBS was used to block any unspecific binding.

Primary antibodies (1:1000) directed against receptors and proteins of interest were incubated with the nitrocellulose membrane at room temperature (RT) for 1 h and then washed in PBS. The secondary biotin conjugated antibody (1:1000) was added and reacted with the membrane in RT for 1 h and washed in PBS. Streptavidin conjugated to alkaline phosphatase was added to the membrane and incubated at RT for 1 h. The binding of biotin and streptavidin is a very strong non-covalent interaction.

The nitrocellulose membrane was washed in PBS and the proteins were visualized using a BCIP/NBT kit (Invitrogen), see Figure 11, and analysis was then performed on a Molecular Imager ChemiDoc™ XRS Imaging System using the software Image Lab 5.1 (Bio-Rad Laboratories, Hercules, CA, USA).
Mass spectrometry

Prostasomal lipid rafts (20 µg) were denatured and reduced in SDS (1%), ammonium bicarbonate (0.1 M) and dithiothreitol (DTT) (10 mM) by heating for 5 min at 98 °C, and subsequent alkylation by iodoacetamide (20 mM) followed by incubation for 15–30 min at 20 °C in the dark. The sample was thereafter transferred to a spintube, 10 kDa cutoff (Pall Corporation, Port Washington, NY) [143].

Urea (8 M) and ammonium bicarbonate (50 mM) were added four times (4 x 450 µL) and centrifuged for 15 min at 14,000 g each time as previously described (40). Before the fifth centrifugation only 2 M urea and 50 mM ammonium bicarbonate were added and spun after which the solution was diluted to a final concentration of 1 M urea and 50 mM ammonium bicarbonate. The digestion was executed for 16 h at 20 °C by 0.5 µg trypsin, and stopped by acetic acid.

UV-absorbance was measured in a NanoDrop 2000 (Thermo Fischer Scientific Inc., Waltham, MA) to establish protein concentration. A C18 filter absorbed the digest for 24 h [144] before mass analysis was performed on a Thermo Velos (Thermo Fischer Scientific Inc.). The column was 100 mm long with an internal diameter of 75 µm, packed with 3 µm C18-Aq particles. The elution was carried out by a constructed gradient from 5–65% over 60 min starting with two solutions: 0.1% formic acid and 100% acetonitrile with 0.1% formic acid, respectively.

A maximum of 10 fragmentations were made for each MS-spectrum. Fragmentation was carried out by CID using standard settings. The list of peaks obtained was used to search the X! Tandem website.
Electron microscopy

Scanning electron microscopy
The prostasome pellet received after the final 100,000 g ultracentrifugation was fixated with PBS containing 2% glutaraldehyde. The fixated pellet was briefly rinsed in distilled water and dehydrated with 70% ethanol for 10 min, 95% ethanol for 10 min and 99.9% ethanol for 15 min.

All steps were conducted at 4 °C and then transferred to acetone. The pellet was dried in a critical point dryer (Balzer, CPD 010, Liechtenstein) using carbon dioxide. After drying, the pellet was mounted on an aluminum stub and coated with carbon (Bal-Tec MED 010, Liechtenstein).

Analysis was performed with an Ultra 55 field emission scanning electron microscope (Zeiss, Oberkochen, Germany) at 5 kV, see Figure 3.

Transmission electron microscopy
The prostasome pellet received after the final 100,000 g ultracentrifugation was treated with 2% glutaraldehyde for fixation. The fixated pellet was washed once in PBS and once in PBS containing 2% osmium tetroxide at 4 °C for 2 h. The pellet was then transferred into ethanol for dehydration followed by acetone and subsequently embedded in LX-112 (Ladd, Burlington, VT, USA). The embedded pellet was then cut into sections of around 50-60 nm using a Leica Ultracut UCT (Leica, Wien, Austria).

The sections were then contrast colored using uranyl acetate and lead citrate, then analyzed in a Tecnai 12 Spirit Bio TWIN transmission electron microscope (FEI Company, Eindhoven, The Netherlands) at 100 kV and a Veleta camera (Olympus Soft Imaging Solutions, Münster, Germany) was used for preparing the digital images, see Figure 4.

TK1 real-time assay
Prostasomes and malignant cell derived exosome samples, respectively, were mixed 1:1 with lysis buffer containing a protease inhibitor (Complete Lysis®, Roche GmbH), incubated at RT for 10 min and vortexed for 2 sec. Samples and TK1 activity calibrator samples (to create an activity standard curve) respectively were mixed with a reagent mix (Biovica International AB, Uppsala, Sweden) to a final sample concentration of 10 %.

Calibrator materials were from Diasorin S.r.l. Catalytic activity concentration values are expressed as TK-REA Units per liter. One TK-REA Unit is 1.2x10⁻¹² mole TMP converted per sec. A 20 µL reagent sample mix was transferred into 100 µL PCR tubes (Qiagen, Hilden, Germany) and loaded into
a Rotor-Gene Q (Qiagen) real-time PCR instrument. All samples were run in triplicate.

The incubation profile was 30 min at 37 °C followed by fluorescence capture every minute for a total of 60 min at 37 °C. A runfile was created from the RotorGene Q Series (Qiagen) software. From this file, fluorescence pattern images of developing normalized fluorescence as well as final activity data, based on the assigned values for the calibrators, were subsequently downloaded as an editable Excel file for further analysis.
Perspectives on Paper I:
Proteomic profiling of detergent resistant membranes (lipid rafts) of prostasomes

Introduction
Prostasomes consist of a bilayered membrane similar to normal cells, but with a higher composition of sphingomyelin and cholesterol. Lipid rafts are domains within the bilayered cell membrane that are more densely packed and it has been proposed that they act as a signaling center for active receptors.

We asked whether prostasomes had these lipid rafts in their membrane and, if so, what kind of proteins and receptors they hold as a way of further characterizing the prostasome in the search for a specific biomarker.

Aim and procedure
The aim of this paper was to characterize the proteins in the prostasomal membrane using mass spectrometry. By targeting proteins in the DRMs we hoped to identify proteins that could be good candidates for specific prostasome biomarkers, but also potential proteins responsible for interactions with the surrounding cells.

By treating the prostasomes with 1% Triton X-100 we were able to separate the lipid rafts, or DRMs, from the rest of the membrane using a density gradient. The DRMs were then subjected to liquid chromatography-tandem mass spectrometry (MS).

Summary
By examining the DRMs from prostasomes we can highlight some of the interesting proteins and biomarkers, many of which would be lost in the “background noise” of all the other proteins if whole cells, or just whole prostasomes, were subjected to homogenization.

Almost 400 specific proteins were identified as being part of the prostasomal lipid raft, many of them involved in intraluminal vesicle formation. The proteins with the highest Mascot score were CD13, CD26 and CD10, which
have long been associated with prostasomes. Both CD26 and CD10 associate with tetraspanins (CD9), which are used as a marker for exosomes.

The general markers for exosomes CD81 and CD82 were not found in prostasomal DRMs and this could mean they are not associated with lipid rafts in the prostasomal bilayered membrane.
Perspectives on Paper II: Human erythrocyte-derived nanovesicles can readily be loaded with doxorubicin and act as anticancer agents

Introduction

Lipid rafts, or DRMs, from prostasomes were found to re-vesiculate when subjected to osmotic pressure in a physiological buffer. From one vesicle we could engineer another, which could now be loaded with the desired cargo. Due to the immunologic response of the prostasomes if administered intravenously, we used erythrocytes in this method instead, as erythrocyte transfusions have been used for many years with few adverse events.

When the loaded vesicles were subjected to cells, they showed greater uptake into the cells, thus delivering the cargo (in this case, doxorubicin) into the target cells, and killing them.

Aim and procedure

The aim of this paper was to investigate if we could use these engineered vesicles to deliver cytostatic drugs more efficiently compared to cytostatic drugs free in solution the way they are administered today, in the blood of cancer patients.

Erythrocytes were emptied of hemoglobin, turning them into erythrocyte ghosts which were subjected to Triton X-100 and ultracentrifugation. The re-vesiculated lipid rafts from the erythrocyte ghosts were loaded with the desired cargo (i.e. hemoglobin, gold colloidal particles and cytostatic drugs) by osmotic pressure and administered into the media of PC3 cells.
Summary

This is a new approach for the on-point delivery of pharmaceuticals. Many cancer drugs have high pH values, making them weak bases. The microenvironment is acidic and thus the weak bases are protonated and neutralized when entering cells.

By using a biologically engineered vesicle for delivery, this problem is circumvented and, as shown in the outcome of this article, doxorubicin encapsulated in erythrocyte DRM vesicles can kill malignant cells more efficiently than when the doxorubicin is administered in the cell medium.
Perspectives on Paper III: Occurrence of purinergic receptors in human prostasomes (prostate epithelial cell-derived exosomes)

Introduction

Purine and pyrimidine receptor subtypes were cloned and characterized during the 1990s. P1, the adenosine receptor, includes four subtypes. P2X, the ion channel, has seven subtypes and P2Y, the G-protein coupled receptor, has eight subtypes.

Purinergic receptors are involved in many biological functions in the body and the idea of nucleotides as signaling molecules had already been proposed as early as 1972 [145]. The rapid action of purinergic signaling can be seen in secretion, neurotransmission and neuromodulation. The long-term action can be seen in, for example, proliferation, differentiation and migration, as well as regeneration [146].

Aim and procedure

In this project we investigated what kind of purinergic receptors are present in the prostasomal membrane.

Prostasomes were purified from human seminal plasma and then subjected to SDS-PAGE and immunoblotting using an array of antibodies directed against purinergic receptors.

Summary

We found that almost all purinergic receptors are represented in prostasomes.

Adenosine has been used clinically since the mid-1900s and prostate tumor cell proliferation is inhibited by adenosine and the drug Regadenoson is an A2a receptor antagonist [121]. P2X7 has been suggested to be an early marker for prostate cancer [147] and the receptor effects both the lipid and the protein
content of the plasma membrane [129]. P2Y12 receptor inhibitors, such as Clopidogrel, are used by many patients as an antiplatelet therapy [148, 149] and the drug was found to be able to reduce the risk of metastasis in PC [150].
Perspectives on Paper IV: 
Increased levels of thymidine kinase 1 in malignant cell-derived exosomes

Introduction

Thymidine kinase 1 has long been regarded as a marker of cancer. TK1 and TK2 are isoenzymes, where TK1 is found in the cytoplasm and is cell cycle dependent, whilst TK2 is cell cycle independent and is present in the mitochondria [151]. TK1 phosphorylates nucleosides into nucleotides which are needed for DNA synthesis during cell division.

Weagel et al. [152] showed that in Burkitt’s lymphoma, acute promyelocytic leukemia (APL), T cell leukemia and acute lymphoblastic leukemia (ALL), TK1 is upregulated and localized to the plasma membrane.

Aim and procedure

In this project, we compared non-malignant prostasomes with malignant exosomes derived from different PC cell lines. We aimed to investigate the relationship between TK1 and malignant exosomes from prostate cancer cell lines (PC3, LNCaP and Du145) and non-malignant prostasomes from healthy donors.

Prostasomes and exosomes were purified and subjected to the TK1 activity enzyme assay based on real-time determination of TK1 activity from Biovica International AB (Uppsala, Sweden). TK1 enzyme activity was measured over time tracing a fluorescence pattern.

Summary

We observed a difference in TK1 enzyme activity between malignant exosomes and prostasomes, where malignant exosomes showed higher enzyme activity. Thus, TK1 could prove useful for monitoring and prognosis of prostate cancer.
Conclusions and future perspectives

The work in this thesis has contributed to the pre-clinical research that is greatly needed to solve the prostate cancer puzzle.

- In Paper I we were able to purify lipid rafts from the prostasome membrane and through a proteomic analysis highlight some of the more abundant proteins that gathered in these microdomains.
- In Paper II we allowed the purified lipid rafts from prostasomes to re-vesiculate and create bioengineered vesicles capable of loading agents of choice. We used this method on erythrocytes and loaded them with doxorubicin. Malignant cells were subjected to these vesicles and the result was a better uptake of doxorubicin compared to free drug.
- In Paper III we mapped the existence of purinergic receptors on prostasomes in order to understand how current and future drugs might affect patients with prostate cancer.
- In Paper IV we looked at benign human prostasomes and malignant cell-derived exosomes and how TK1 enzyme activity varies using a real-time TK1 assay developed by Biovica International AB.

Further studies could be directed toward looking more closely at (malignant) prostasomes from prostate cancer patients. I would also suggest pursuing the creation of specific antibodies for prostasomes as a continuance of Paper I. This could be used to develop an assay for capturing prostasomes from blood samples.

In further studies for Paper II we could let prostasomes donate receptors, as in the papers by Babiker et al. [93, 153], to erythrocytes to be able to target recipient cells more specifically. This would give the bioengineered vesicles and address tag to better find their way to the correct recipient cells as a way for a new drug treatment for prostate cancer.

For Paper III it would be interesting to verify what the effects of drugs targeted to purinergic receptors have on PC patients and other diseases. Also I would suggest to repeat the Western blot experiments, this time using prostate cancer cell-derived exosomes.

For Paper IV, as a minimum, the next project would be to look at more exosomes from other type of cells and the TK1 enzyme activity they hold.
TK1 is mostly measured in plasma, but is it free or is it bound to other exosomes? By further investigating TK1 enzyme activity in exosomes a liquid biopsy assay could form, making it easier to monitor, or even diagnose prostate cancer.

In conclusion, this thesis provides new knowledge to the fight against prostate cancer and how to better diagnose and treat it more efficiently.

Förutom PSA består seminalvätskan av en mängd olika proteiner som har specifika roller för spermiernas funktion. Vår forskargrupp var den första att visa att det sker en aktiv utsöndring av små vesikler omgärdade av ett dubbelt lipidmembran till prostateavvätskan, så kallade prostasomer [70].


Huvuduppgiften för prostasomer är att skydda spermierna på deras väg upp till ägget genom att tillföra proteiner som stöd vid migrationen [74, 157].


Vi har kunnat visa att prostateceller som omvandlats till cancerceller behhåller förmågan att utsöndra prostasomer till omgivningen [158]. Funktionen hos prostasomen, att primärt skydda spermier, kommer då att flytta sig att gälla skyddande av prostatecancerceller, så tack vare egentillverkade prostasomer får cancercellen en överlevnadsfördel.

För att få fram lipidflottor används detergenten Triton X-100 i den lösning som man vid 4 °C låter provet (i detta fall prostasomer) centrifugera i vid väldigt högt g-tal, ca 256 000 g, under 28 h mha en swing out rotor (SW40) [163]. Vi har funnit att lipidflottor finns i prostasomernas membran och vi har gjort proteomik på dessa i jakten efter nya intressanta biomarkörer.


Vi laddade dessa med cytostatika och utsatte prostatacancerceller för dessa nya vesikler. Vi såg då att våra nya bio-vesikler upptogs av cellerna i större utsträckning än om man gav cytostatiska blandat i cellernas medium. Detta gör att våra nya bio-vesikler skulle kunna användas som terapi mot prostatacancer i framtiden.

Det finns tre typer av purinerga receptorer, P1, P2Y och P2X. P1 receptorn, som också kallas adenosinreceptor, delas in i fyra subtyper: A1, A2a, A2b, samt A3. Receptorn P2X har sju subtyper; P2X1-7 och denna receptor är en jonkanalreceptor, medan de två andra typerna (P1 och P2Y) är GPCR-kopplade receptorer. Slutligen har vi P2Y receptorn som består av åtta subtyper; P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 och P2Y14 [164].

P2 receptorn uttrycks av alla tumörer. Genom att inhibera specifika subtyper av P2 receptorn kan man framkalla antingen apoptos/nekros eller reglering av celltillväxten. P1 receptorn är inblandad i inhibering av aktiverade immunceller, vilket gör att cancercellerna kan växa och bli resistenta mot kemoterapi [165, 166]. Mikromiljön hos tumörer är hypoxisk vilket inducerar ackumuleringen av adenosin pga den accelererande hydrolysen av ATP [167]. Under många år har man sett att adenosin varit inblandad i tumörassocierad immunsuppression [168].

Vi har genom Western blot kunnat se att det finns flera subtyper av P1, P2Y och P2X receptorer på prostasomerna, vilket innebär en ökad förståelse hur prostasomer påverkas av olika läkemedel vid exempelvis terapi av prostata cancer.
Thymidinkinas 1 är en biomarkör för cancer [151]. Då en cell ska dela sig ökar enzymet TK1 för att kunna fosforylera thymidinmonofosfat (TMP) till först thymidindifosfat (TDP) och sedan till thymidintrifosfat (TTP), som då kan inkorporeras in i DNA [135].

Vi ville ta reda på om det finns en skillnad i thymidinkinas 1 aktivitet hos prostasomer från friska män jämfört med exosomer från maligna celler, och vi fann att det var en högre aktivitet hos de maligna exosomerna. Detta kan i förlängningen möjligtvis användas för att ställa diagnos vid misstänkt prostatacancer.
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References


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)