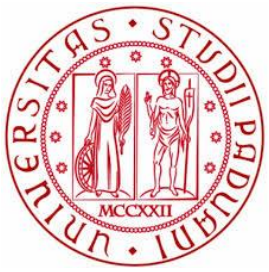

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DIPARTIMENTO DI SCIENZE DEL FARMACO

CORSO DI LAUREA MAGISTRALE IN PHARMACEUTICAL BIOTECHNOLOGIES

TESI DI LAUREA

Development of a novel exosome-based platform for anticancer drug delivery

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Breath, breath in the air. Don't be afraid to care.

Leave, but don't leave me. Look around, choose your own ground.

(Pink Floyd, Speak to me/Breath)

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ABBREVIATIONS

ABBREVIATIONS

BCA	Bicinchoninic Acid
DCC	Dicyclohexylcarbodiimide
DCU	Dicyclohexylurea
DDS	Drug Delivery System
DHPE	1,2-Dyhexadecanoyl-sn-glycarol-3-phosphoethanolamide
DLS	Dynamic Light Scattering
DMSO	Dimethyl Sulfoxide
DPBS	Dulbecco Phosphate Buffer Saline
DSPE	1,2-distearoil-sn-glycero-3-phosphoethanolamine
EPR	Enhanced Permeability and Retention
EV	Extracellular Vesicle
ESCRT	Endosomal Sorting Complex Required for Transport
FA	Folic Acid
FBS	Fetal Bovine Serum
FITC	Fluorescein
FR	Folate Receptor
H-NMR	Hydrogen Nuclear Magnetic Resonance
HPLC	High Pressure Liquid Chromatography
MALDI-TOF	Matrix Assisted Laser Desorption-Time of Flight
MVB	Multi Vesicular Bodies
MPS	Macrophage Phagocytic System
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium

ABBREVIATIONS

NTA	Nanoparticle Tracking Analysis
PBS	Phosphate Buffer Saline
PEG	Poly(ethylene) Glycol
PTX	Paclitaxel
RES	Reticulo-Endothelial System
RT	Room Temperature
SEC	Size Exclusion Chromatography
UV	Ultra Violet [light]
UV-Vis	Ultra Violet-Visible light

ABSTRACT

Cancer is currently one of the major cause of death and represent a worldwide challenge in healthcare. Traditional therapies are insufficient to meet the medical needs for patients and are rather unspecific, largely resulting in side effects. The research is focusing on innovative drug delivery systems, nanocarriers to be exploited in the administration of therapeutics with enhanced specificity and bioavailability.

In particular, these systems should be able to cross barriers and accumulate in the tumor tissue for the release of the payload, both for passive diffusion through leaky capillaries (EPR effect) and through targeted delivery inside malignant cells. Indeed, most of nanomedicines suffer from degradation or uptake by macrophages of the MPS.

In this project, we designed a drug delivery system based on nanosized extracellular vesicles called exosomes which are secreted by several cell types and are involved in intercellular communication. The interest comes from their roles in physiology and in disease conditions which render them ideal nanocarriers, equipped with a natural tendency to be endocytosed and a high biocompatibility. Since their discovery, extracellular vesicles have been characterized and isolated in different ways, while the urge for standardization is increasing in scientific community.

Attempts have been made to decorate the surface of exosomes with stabilizing agents and targeting agents to increase their circulation time and to confer them selectivity for the uptake.

We propose a method of decoration of EV surface by incubation with functional modules and targeting agents terminated with lipids to endow selectivity and ameliorated delivery of the chemotherapeutic drug paclitaxel. The surface of exosomes has been modified with FA-PEG$_{3.4kDa}$-DSPE, a PEG-lipid end tipped with folic acid as targeting agent, mPEG$_{2kDa}$-DSPE and the fluorescent label FITC-DHPE.

Before coating was performed, exosomes were harvested from PC-3 cells, a prostate cancer cell line that express folate receptor, by means of differential ultracentrifugation and density gradient centrifugation. They were characterized for size and zeta potential, respectively by Nanoparticle Tracking Analysis (NTA) and Dynamic Light Scattering (DLS). Upon formulations, size was preserved, while the negative zeta potential was decreased in terms of absolute value as a result of PEG shielding.

Formulation studies have been carried on to evaluate the effect of targeting agent density on cell uptake efficiency. We set up dedicated procedures to quantify the PEG coating efficiency. The uptake was evaluated on PC-3 cells using flow cytometry for the detection of cell associated exosomes: formulations with three different densities of FA-PEG$_{3.4kDa}$-DSPE were tested, along with negative controls modified either with FITC-DHPE alone or with FITC-DHPE and mPEG$_{2kDa}$-DSPE.

ABSTRACT

The best performing formulation was identified to be the one carrying an intermediate molarity of the targeting agent and a density of total PEGylated phospholipids (mPEG$_{2kDa}$-DSPE and FA-PEG$_{3.4kDa}$-DSPE) of 1.26 PEG chains/nm^2.

The selected formulations were used for loading studies with paclitaxel. The loading capacities of the exosome-based drug delivery system for paclitaxel were in the range of 20-33%. The ability to encapsulate the drug was not dependent on the density of the targeting agent. A MTT viability assay performed on PC-3 cells with paclitaxel loaded EVs at drug concentration corresponding to the IC$_{25}$ of the free drug did not show inhibition of cell growth which may be ascribed to limited endosomal escape and/or drug release.

The project demonstrated the feasibility of functionalization of exosomes surface and the possibility to develop strategies for the use of extracellular vesicles as novel carrier for cancer treatment.

RIASSUNTO

Attualmente, il cancro è un'emergenza sanitaria mondiale. I trattamenti tradizionali non sono sufficienti a sopperire alle necessità dei pazienti, risultando per lo più aspecifiche, con conseguenti effetti indesiderati. La ricerca è volta a sviluppare sistemi di delivery innovativi, veicoli nanotecnologici da somministrare in terapie direzionate con aumentata specificità e biodisponibilità.

In particolare, questi sistemi sarebbero capaci di superare barriere biologiche e accumularsi nel tumore per il rilascio del farmaco, sia per diffusione passiva attraverso i capillari (effetto EPR) sia mediante *delivery* direzionato all'interno delle singole cellule cancerose. Nella fattispecie, la maggior parte delle nanomedicine sono esposte a degradazione e a fagocitosi da parte del sistema MPS.

In questo progetto, abbiamo teorizzato un sistema di *drug delivery* basato su vescicole extracellulari di dimensioni nanometriche, note come esosomi, secrete da vari tipi cellulari e coinvolte nella comunicazione cellula-cellula. L'interesse deriva dal loro ruolo in aspetti fisiologici e patologici che li rendono un sistema trasportatore ideale, dotato di una naturale tendenza a essere internalizzato e un'elevata biocompatibilità. Sin dalla loro scoperta, le vescicole extracellulari sono state caratterizzate e isolate in diversi modi, mentre cresce nella comunità scientifica la necessità di una loro standardizzazione. Sono stati mostrati vari tentativi di decorare la superficie di esosomi per incrementare la loro emivita nel circolo sanguigno e per conferire selettività di *uptake*.

Si propone, qui, un metodo di post-inserzione di derivati fosfolipidici per realizzare una migliore specificità e conseguire un *delivery* del chemioterapico paclitaxel. A tale scopo, la superficie degli esosomi è stata modificata con FA-PEG$_{3.4kDa}$-DSPE, un PEG-lipide coniugato all'acido folico in qualità di agente direzionante, mPEG$_{2kDa}$-DSPE e il marcatore fluorescente FITC-DHPE.

Prima della modifica, gli esosomi sono stati estratti da cellule PC-3, una linea cellulare di cancro alla prostata, per mezzo della ultracentrifugazione differenziale e del gradiente di densità. Sono stati caratterizzati per dimensioni e carica superficiale, rispettivamente con Nanoparticle Tracking Analysis (NTA) e Dynamic Light Scattering (DLS). Conseguentemente alla formulazione, le dimensioni sono preservate, ma il potenziale zeta risulta significativamente aumentato in tutte le condizioni sperimentali.

Studi di formulazione sono stati condotti per valutare la densità ottimale di acido folico necessaria per l'internalizzazione, mentre una caratterizzazione del protocollo in termini di efficienza di distribuzione del PEG superficiale viene riportata.

L'*uptake* è stato valutato su cellule autologhe mediante citofluorimetria per misurare la quantità di FITC internalizzata: sono state testate formulazioni con tre diverse densità di FA-PEG$_{3.4kDa}$-DSPE, con controlli negativi di esosomi modificati con il solo marcatore fluorescente o con FITC-DHPE e mPEG$_{2kDa}$-DSPE.

RIASSUNTO

La migliore formulazione è risultata essere quella decorate con una quantità intermedia di fosfolipide direzionante e con una densità di PEG calcolata di 1.26 catene per nm^2.
Le formulazioni selezionate sono state usate per gli studi di caricamento con paclitaxel e la quantificazione è avvenuta tramite RP-HPLC. Le capacità di caricamento finali sono emerse essere intorno al 20-30%. L'abilità di incapsulare farmaco non si è dimostrata essere affetta dal grado di modifica superficiale del sistema. Con queste informazioni e avendo calcolato l'IC50 del farmaco per quanto riguarda le cellule PC-3, abbiamo condotto un saggio di vitalità MTT per sviluppare i profili di tossicità delle vescicole caricate e abbiamo settato le condizioni sperimentali per avere una concentrazione finale di PTX uguale alla concentrazione di farmaco libero che causa il 75% di vitalità.
Nel progetto, si è affrontato criticità nel lavorare con tale inesplorato *carrier*, ma si è mostrata la possibilità di funzionalizzare la superficie di esosomi per poter sviluppare strategie per l'impiego di vescicole extracellulari come trattamento innovativo anti-cancro.

AIM OF THE PROJECT

This thesis project aims at developing a surface functionalized exosome-based drug delivery system (DDS) in order to reprogram the targeting features of the extracellular vesicles.

1) We aimed to generate a cancer cell targeted delivery system by decorating the surface of extracellular vesicles from PC-3 (prostate cancer cell line) with synthetic targeting agent FA-PEG$_{3.4kDa}$-DSPE at different density.
2) We aimed to identify the folate density that provide for suitable selectivity and targeting of the EV
3) We aimed to set up procedures for loading EV with paclitaxel.

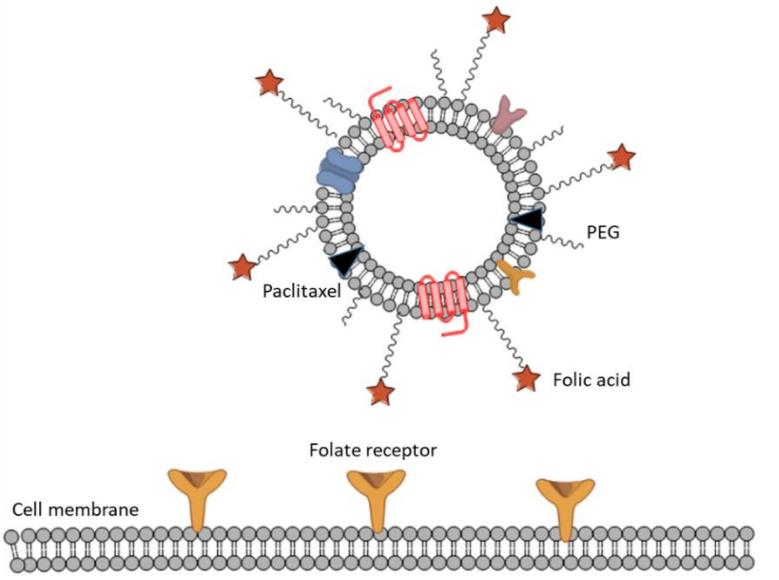

Figure 1 *- Schematic representation of targeted binding of exosome-based drug delivery system developed in the project.*

1. Introduction

1.1 Cancer definition

A limited number of pathologies involving the abnormal hypertrophy of cells and the enhanced tendency to migrate towards distal tissues is the current definition of cancer. According to WHO, cancer is the second world leading cause of death in humans, esteemed to be causing almost 10 million deaths per year which is 1 out of 6 deaths globally.

Cancer is a generic term under which more conditions' definitions rely: most common types in men are lung, prostate, colorectal, stomach and liver cancer; most common types in women are breast, colorectal, lung, cervix and thyroid cancer [1,2].

In general, cancer or cancerous tumors arise from a loss of differentiation state, one of the causes of uncontrolled cell division, as well as loss of ability to capture growth inhibition signals. Besides, cancer cells are able to condition the surrounding environment until, on a final stage, they are allowed to spread in other areas of the organism.

These changes are due to genetic mutations, both familiar and generated by external factors, that permit the escape of check-point mechanisms occurring normally in healthy cells: resistance to apoptosis, amplification of autocrine growth pathways, enhancement of metabolic hypertrophy and enhanced control on microangiogenesis or migration are only some of the hallmarks specific for this pathology, in different extent [3]. Hanahan and Weinberg defined six cornerstones which uniquely identify cancer as a pathology. Cells constituting a cancerous mass have to:

- Sustain proliferation,
- Elude growth suppression,
- Enable invasion,
- Activate immortalization pathways
- Trigger angiogenesis,
- Escape cell death mechanisms.

Contributing factors to the development of cancer remain uncertain, but socioeconomic and cultural factors are thought to have a role in the arising: NIH (US Health Institution) detailed both ethnic and environmental disparities which have a consistent impact in statistical evaluations of incidence, e.g. African Americans have more than double mortality from prostate and stomach cancer compared to white. This one and other disparities may reflect genetic variations which are not properly understood.

Otherwise emblematic is the financial burden represented by cancer in the world health systems. In the US, cancer expenditures are in increasing trend and, as of 2017, a medical

care of 144.4$ billion was calculated, with a "top three" most expensive conditions taken by breast, colorectal and prostate cancer respectively [4,5].

1.2 Cancer Treatment

1.2.1 Traditional treatment

Traditional therapeutic approach is constantly discussed and reviewed against the blooming technological field. Nevertheless, as of today, the vast majority of treatments remains the same depending on the stage of malignancy and age of patient.

In general, the simplest solution is the direct removal of the tumor via surgery intervention. Surgery suitability changes throughout the progression of the malignancy and in later stages is often coupled to radio- or chemo-therapy [6].

Mostly used in combination, traditional therapies have the advantage of eliciting a systemic effect, useful to reach all tissues of the body. Mechanisms of action of chemotherapeutic drugs involve the inhibition of cell cycle or DNA damage with consequent cell death.

Platinum-based drugs or alkylating agent act directly on DNA strands, antimetabolites interfere with synthesis of purine of pyrimidines, Topoisomerase I and II inhibitors harness the duplication of DNA, ionizing radiation creates chemical abducts on DNA molecules inducing apoptosis [7-9].

Each of the traditional treatments infers with cellular mechanisms related to cell division and growth which are extremely prone to develop resistance as most of oncogenic mutation occurs in those functional areas.

In addition, side effects are the main concerns for healthcare industry when it comes to cancer treatment: traditional therapies lack of specificity towards cancerous cells, resulting in a huge bystander effect versus surrounding tissues, as well as unpreventable systemic effect versus healthy proliferating cells.

Both effects limit the therapeutic window of most of the chemo drugs, making the research for effective dosage formulations even harder. Hence, the research for novel therapeutics such as targeted biologics and innovative drug delivery systems plays a key role to solve these issues.

1.2.2 Cancer nanomedicine

Nanomedicine is the branch of nanotechnology devoted to health and medicine. It is devoted to define biocompatible nanosystems, structures in the nanometer scale, with the

purpose to overcome biological barriers or achieve better therapeutic effects for the patients.

If we consider chemotherapeutics, the biggest complains derive from their poor pharmacodynamics and pharmacokinetics properties or from their poor biocompatibility. All of these features can be modulated using nanotechnology: sizes, surface/volume, release profile and possibility for surface modification to control targeting are important pros in these systems [10].

First successful innovations in the field were Doxorubicin-loaded liposomes, lipid bilayer based nano-vesicles representative of the most prolific class of nanomedicine. In spite of this, no liposomal formulation has exhibited a survival benefit yet, when compared to classical chemo drugs [11].

Abraxane, an albumin NP bound paclitaxel drug, symbol of the second class of nanomedicine ever marketed, entered the industry in 2005. Approved by FDA for treatment of breast cancer, Abraxane shows similar effect and PK profile to one-weekly-dosed paclitaxel without the need for dangerous excipients.

Polymeric NP and micelles followed, utilizing materials ranging from organic and inorganic constituents.

Nanoparticle field is in expansion while even biologic therapeutics like proteins, siRNA, mRNA or other oligonucleotides are being encapsulated in nanosystems. Chemical and genetic engineering facilitate the involvement of these systems in different applications from therapy to diagnosis.

The role of nanomedicine in the future of cancer is explicit: achievement of targeted, tolerated and tunable doses of APIs to reduce toxicity with the possibility to either amplify the therapeutic response for the patient, deliver diagnostic information or control the immune response.

In order to target the tumor site, nanotechnology exploits many properties, but the principal cause for accumulation of NP to tumor is the so-called Enhanced Permeation and Retention effect (EPR effect). Delivery to solid tumors is possible because of the leaky vasculature in the microenvironment created by the growing cell mass which allows NPs with a diameter of 20-220 nm to pass through and to be retained in the proximity as the reduced lymphatic drainage assures retention [12]. This concept has gained unanimous acceptance in the nanomedicine field, even though is strictly dependent on patients' differences and tumor subtype. In fact, many studies have shown the importance of preliminary screenings on cancer patients to evaluate the efficacy of nanotherapies in advance [13,14]. The need for additional targeting strategies is evident if we consider a possible toxicity to liver and spleen, containing fenestrated vessels, organs towards which NPs are hijacked with consequences to biodistribution.

For this purpose, research is focused on mechanism of active targeting. Opposed to the passive diffusion through vasculature, active targeting is the phenomenon to specifically increase and direct the uptake of NPs to cancerous cells. It is increasingly interesting to

direct biologics to the internalization pathways of cancer cells with natural implications regarding release from endosomal membranes [15,16].
In summary, we can sort three methods to achieve an active penetration of the tumor:

- Antibody-driven: the attachment of Ab or Ab fragments on the surface of targeted NP is the most exploited method to date. Immunoliposomes have entered clinical in recent times, examples are anti-EGFR-doxo IL or Her2 Ab scFv MM-302 liposomes. Evidence is present that variations in affinity and surface density are essential: around 10^5 receptors per cells is recommended for the benefit to be worth it, hence low affinity Ab are to be preferred in screenings respect to high affinity ones [17-19]. Alternative protein scaffolds for affinity belong to the same category [12].
- Receptor ligand-driven: this method exploits the recognition of a defined overexpressed membrane protein of tumor cells via the interaction with a specific protein, growth factor or small molecule. The most studied technologies are the coating with transferrin (Tf) to target the TfR, folic acid towards folate receptors, glutathione to target GSH transporters and carbohydrate chains to bind precise lectins [15,20]. Also synthetic ligands have been employed in clinical trials after screening of libraries of compounds as in the case of PSMA binding BIND-014 polymeric NP [21].
- Peptide-driven: in the form of linear or cyclic, peptides are coupled to NP surface to enhance internalization via specific pathways. RGD peptides are mostly employed to recognize αvβ3/5 integrins on both cancer cells and angiogenic endothelial cells [22]. CPPs, cell penetrating peptides, offer an alternative to transport to tumors. Notably, approaches are found where peptides are used as agonist or antagonist to achieve a better zip code for the NP to be delivered [12].

Examples in clinical trials exist for each strategy. Development is hurdled by the patient specifics required to validate the overall survival benefit. Indeed, individuals may differ from a passive targeting point of view, by reduction of EPR effect for particular architectures of the stromal and endothelial tissues surrounding the tumor. Importance in the tumor microenvironment in the progression of malignancy is established. Though, poorly predictable factors may hamper the active targeting approach: the protein corona found around NPs in variable extent is responsible of the loss of efficacy of many clinical cases submitted to trials. The field of knowledge around nano-bio surfaces interaction is expanding, but we can refer to the well-known fact of corona composed of opsonin proteins generate uptake of NPs from the monocyte phagocytic system, MPS or by tumor associated microphages, TAMs [23-25].

We invite the reader to treasure the explained concepts, since the aim and strategy of this project, which will be described in Section 1.5, was pursued relating to these important features regarding nanosized delivery system.

1.3 Extracellular vesicles (EVs) background

In the past twenty years, research around extracellular vesicles has gained much interest in the scientific community. The definition of EVs as membrane-enclosed macromolecules conveyors secreted by cells for intercellular communication is something that struggled to be accepted after discovery. When they were firstly discovered in the early 80s from TEM studies on transferrin receptor loss from reticulocytes, they were assumed to be involved in some excretion of waste material out of the cytoplasm [26,27].

Nowadays, clarification on nomenclature, biogenesis and features has allowed a sort of classification. In particular, we make a distinction between exosomes, 30-150 nm small vesicles derived from the endosomal pathways after the secretion from multi-vesicular bodies (MVB), and microvesicles, MVs, a less restricted category of up to 1 µm in diameter derived from the direct budding from the plasma membrane.

In addition, other EVs are categorized such as apoptotic or necrotic bodies ranging from few nanometers in diameter to few micrometer, or exosome-like vesicles and ectosomes, budding vesicles whose role is not understood yet [28].

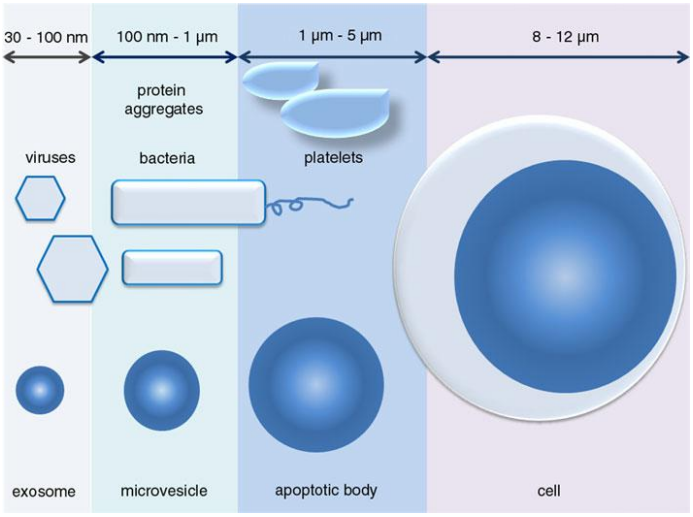

Figure 2 *– Adapted from Ref [28]. Scale comparison of EVs to other biological entities. Definition of EV classes based on size.*

Recent evidences confirmed that exosomes and MVs contain a large array of macromolecules which would be involved in their biological role: soluble and transmembrane proteins, lipids, ncRNA, miRNA, mRNA, DNA [27,29]. In Section 1.3.1.3, we will detail this content.

Several cell types have been shown to secrete EVs both in vivo and in vitro. B and T lymphocytes, platelets, monocytes, neutrophils, epithelial cells, neurons, endothelial cells and most of cancerous cells are some examples of the abundantly studied cell systems. In vivo detection is trickier, but vesicles have been found in specimens from many body

fluids, from sera to semen or urine, but also bronchoalveolar fluid, milk and amniotic fluid [29-31].

1.3.1. Physiology

1.3.1.1 EVs Biogenesis and Release

Exosomes were demonstrated to be part of the dynamic endocytic pathway needed by cells to internalize ligands or components from the environment or recycle/degrade macromolecules.

As described above, exosomes derive from the invagination of MVBs with the formation of intraluminal vesicles (ILVs) in the core of early endosomes. Specifically sorted cargo buds inward alongside the ILV membrane. The fate of the originated MVB is either devoted to degradation through the lysosomes or to exocytosis forming the EVs defined as exosomes. ILVs may form via the endosomal sorting complex required for transport (ESCRT), which is a protein machine consisting of almost thirty proteins divided into four complexes: ESCRT-0, -I, -II and –III [32]. Ubiquitinated transmembrane proteins are recognized by ESCRT-0 sorting them in the ILVs. ESCRT-0 recruits ESCRT-I protein TSG101 that allows the linkage to ESCRT-III via ESCRT-II or ALIX. This machinery works in stepwise manner to load cargo and perform the scission in the membrane after the invagination. There is evidence of a footprint of these proteins found in EVs from different sources. In spite of these findings, knock-out experiments directed to the genes revealed the existence of parallel ESCRT-independent pathways of exosome loading in vivo. It is still unclear how the whole cargo is loaded in the lumen, even if it was seen the delivery of ubiquitinated soluble proteins by the ESCRT complex to the core of the vesicles [27,33].

Once formed, MVBs are docked to the plasma membrane through intracellular trafficking: a pivotal role is given to Rab proteins a family of GTPases associated to specific intracellular compartment. RAB11, RAB27a, RAB35 have been selected in libraries from different systems to be responsible for the secretion of different extracellular vesicles: it remains difficult to discern the origin respect to the stage of the endosome, because current isolation protocols may co-purify vesicles with different destiny and hamper the interpretation of their physiology [27,34,35].

After tethering to the PM, multivesicular bodies are fused for the release via the soluble NSF-attachment protein receptors (SNAREs) in a Ca^{+2}-dependent mechanism with notable exceptions. Basically, v-SNAREs on the membrane of secreted lysosomes bind to t-SNAREs on the target compartment, namely the plasma membrane [36]. The fusion frees exosomes in the extracellular environment where they are thought to be very stable, according to the lipid composition, and they are able to permeate biological barriers including the blood brain barrier.

Finally, a different biogenesis is observed for the PM-budding microvesicles. The process can involve the ESCRT machinery and in this case it resembles the shedding of lysogenic viruses: it does not involve the action of ESCRT-0 to recognize ubiquitylation. It has been seen that also the action of enzymes responsible for membrane plasticity such as flippases or scramblases triggers the release of larger vesicles. The reason of this finding is that the symmetric disposition of phospholipids in the bilayer can destabilize the local surface tension, rendering the vesicle conformation thermodynamically more favored [37].

1.3.1.2 EVs Uptake on recipient cells

The fate of secreted EVs is to be taken up by recipient cells in specific manners which involve targeting mechanisms. Some ligand/receptor combinations have been observed: in such experimental approaches, vesicle-bound antigens are blocked by antibodies and this is translated in decreased capture by tested cells. In the case of phagocytic cells, i.e. DC and macrophages, these evidences are often misinterpreted because of their notable phagocytic capacity [38]. Genetic engineering of EVs to allow expression of tissue specific ligands has confirmed the possibility to direct the uptake to given cells in vitro [153].
Once adhered to cell surface, EVs are internalized in several ways: phagocytosis, micropinocytosis, receptor-mediated and clathrin-mediated endocytosis and so on. Also, the behavior of the same cell may differ depending on the dimension and subtype of vesicle approaching. For cytosolic delivery of cargo, a fusion step is then required. Fusion may occur at the level of the plasma membrane or with membranes of the endosomal milieu and is important to exert functions of gene silencing or expression [39,40].
In vivo, EVs from solid tissues have been found in liquid biopsies and in distal organs, except that is not clear how to assess their blood lifetime yet. Lack of sensitivity of labeling gives back poor data on biodistribution of EVs. Takafumi Imai et al calculated an approximated 2 minutes half-time in B16BL-6 derived exosomes injected in mice intravenously and showed the role of the MPS system in the sequestration from the circulation of EVs which are anyway accumulated in the liver still after 4 hours [41].

1.3.1.3 Macromolecular constituents and physiological role of EVs

The extensive study of the biochemical compositions of extracellular vesicles has led to the identification of several molecular biomarkers and helped in the definition of novel functionalities associated to exosomes and budding vesicles (see ***Figure 3***).
In the past decades, proteomic analysis on EVs has shown the exhibition of tissue specific proteins depending on the source, alongside conserved proteins from the endosomal compartment or from the PM and cytosol, with discrete absence of nuclear, RER, Golgi and mitochondria markers. Often considered EV markers are the tetraspanins (CD9,

CD63, CD81), transmembrane proteins spanning four times the plasmalemma involved in the formation of protein-protein complexes and having a role in ESCRT-independent sorting and in targeting to tissues. Furthermore, major histocompatibility complex (MHC), transport, cytosolic shock proteins and ESCRT derived proteins such as TSG101 or Alix are often found in samples. Post-translational modification like glycosylation of surface proteins were shown to direct the uptake towards specific cell types. Unconventional vesicular protein secretion has been found also for cytokines such as interleukin 1 beta (IL-1β). Of note both soluble and membrane bound matrix metalloproteases are found in EV proteomes, with a possible role in their permeability through the extracellular milieu [42-44].

Probably one of the most attractive discoveries in the EV field, made in 2007 by Valadi et al, was the presence of encapsulated, circulating nucleic acids in mast cell derived exosomes [45]. As the interest increased, mRNAs, mRNA fragments, lncRNA, miRNA, rRNA and many other RNA types have been found. RNase treatment of EV samples confirmed the enclosed nature of these RNA molecules, which could mean protection in the blood stream from degradation. There are evidences that RNAs are not passively loaded, but are indeed sorted to accomplish a function outside the producer cell. Less known is the function of DNA strands found in many cell types' EVs, which could mean a contamination of preparation with apoptotic bodies or actual transfer of mtDNA in certain conditions [46-48].

As for the lipid content, most of EVs are enriched with PS, sphingomyelin, cholesterol and ceramide: these ones are often organized in detergent-resistant raft like structures

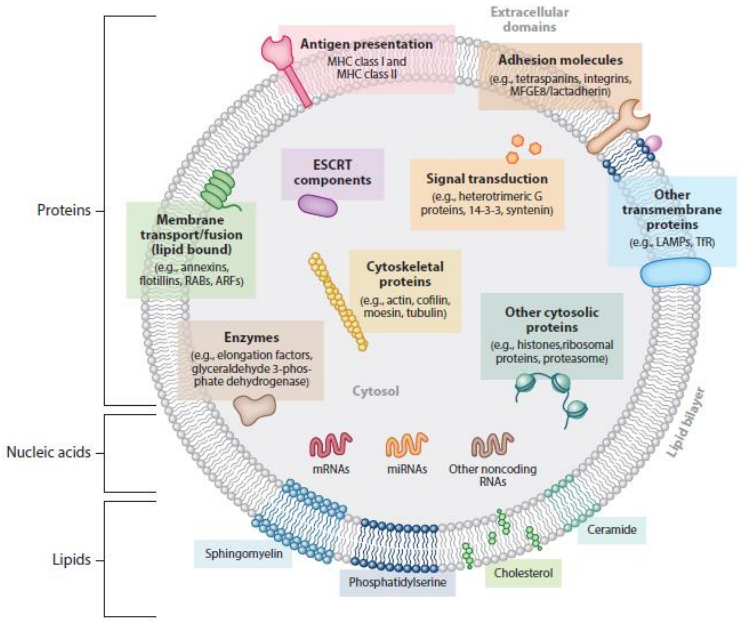

Figure 3 *– Adapted from ref [27]. Macromolecules found in EVs. In the picture it is clear the heterogeneity of soluble and membrane-bound constituents.*

which confer stability to the membrane. Lipidomics enrichment evaluations make suppose a kind of precise sorting of lipid molecule in the EVs to regulate given functions. Phuyal et al showed the role of treatment with ether lipid precursors in changing the whole lipidome and proteome of PC-3 derived EVs, hinting at the unexplored potential of lipid content [49,50].

In humans, EVs are thought to be part of several biological processes: we, hereby hint at few of the most established roles, so as to show the versatility of such horizontal transfer of biological information.

EVs play a role in both innate and acquired immunity. Macrophages secrete EVs upon activation in response to microbial infections. This behavior allows the spreading of proliferative signal such as miR-223 or pro-inflammatory cytokines: the action is thought as an immune-modulatory amplifier in the early phases of infections. Neutrophils release large EVs, called ectosomes, inducing the release of regulatory cytokines from DCs like TGFβ that decreases the inflammation. In the adaptive immunity, EVs role is to exchange antigen from cells to APCs to facilitate T cell activation: surface integrins and lectins would mediate the delivery and uptake from APCs, a phenomenon confirmed also in vivo. In another fashion, dendritic cells are able to secrete vesicles constitutively with a discrete increase triggered by maturation: EVs carry MHC class II molecules able to activate CD4+ T cells. DCs are prolific dispenser of EVs with the most different functions: they can block proliferation of T cells or NK cells and even suppress the stimulatory effect of antigen presenting cells. Tregs produce CD73 positive EVs involved in increased tolerability in murine model of kidney allograft [51-55].

Tissue regeneration is affected by EVs, too. Delivery of encapsulated RNA species via EVs was proposed as responsible for tissue repair. In stem cells microenvironment, for example in myocardial infarction, lung mesenchymal stem cells supernatants are sufficient to accelerate the recovery from trauma: this is associated to de novo synthesis of Argonaut proteins delivered with mRNA transfer [56,57].

Platelet-derived EVs are carriers of pro-angiogenic and endothelial protective factors such as miR-126. Research is focusing on understanding the cascade of vascular repair and the maintenance of vascular homeostasis through EV signaling [58].

The presence of EVs in seminal fluid are due to their potential in sustaining fertilization, in particular prostasomes induce changes in membrane composition of sperm cells to regulate the capacitation in the female reproductive tract and the acrosomal reaction in proximity of the oocyte. Yet the molecules participating in the process have to be detailed [59,60].

In embryonic development, EVs allow coordination of cell migration, creation of morphogen gradients and confer cell polarities: active transport by exosomes of Wnt protein and other lipid morphogens was shown in Drosophila [61].

At last, since we are reviewing cellular communication, we cannot exclude from the list the nervous system: both neurons and glial cells release EVs in vitro. Probably, neurons use synapsis microenvironment to secrete biomolecules. The presence of neurotransmitter receptors subunits reveals a possible role in the plasticity of the synapsis. Microglia derived EVs are instead associated to modulation of glutamatergic and GABA-ergic synapses. Myelination at the level of axons by oligodendrocytes and Schwann cells seems to be supported with EV-mediated transfer of neuroregenerative miRNAs, even peripherally coming from mesenchymal stem cells EVs crossing the BBB [62-65].

The above brief review on EVs functions aims to show how these biological entities are active in healthy conditions in the organism, a concept that evolves to question how they function during morbidity and whether they play a role in the progression of pathology.

1.3.2 Pathophysiology

Defined the areas where extracellular vesicles operate inside the body, it is important to establish what are the dysregulations and aberrant functions that may occur on the EV signaling and which may have relevant consequences in the perception of the pathology itself. Hopefully, by detecting the causes of these phenomena we could imagine to intervene pharmacologically on novel targets. To stay focused on the aim of this project we limit to describe EVs role in cancer with notable hints to inflammation and neurodegenerative diseases.

1.3.2.1 EVs in cancer development

Evidence that tumors produce EVs are spread in the literature. The exact proportion between MVB derived exosomes and PM-budding microvesicles is not determined yet, but the presence of tumor exosomes (TEX) and microvesicles is now clear [66].
Cancer cells exhibit an increased rate of EV secretion which is in accordance with the increased endocytic recycling of neoplastic cells. The altered remodeling of PM occurs in concert with sorting of specific pro-oncogenic and migration inducing biomolecular cargo. Many stimuli trigger the active secretion and receptor recycling. Hypoxia and pH are one of the most exploited ways to increase the EV release by overexpressing surface receptor [67,68].
Not only release, but also content is altered in tumor derived EVs: miRNAs pool modifications allow the spread of malignancy, e.g. breast cancer TEX containing miR-223 promote invasion in metastases, as well as protein constituents such as MMP-2 to cause matrix degradation, VEGF to enhance neoangiogenesis [69,70]. Exosomes are

physically associated to invadopodia, plasma membrane protrusions involved in the erosion of extracellular matrix in order for tumor cells to acquire metastatic potential [71]. Knocking down a gene upstream the tethering of exosomes to the bilayer, Rab27a, it decreases the matrix degradation together with the EV release. Several studies are devoted to demonstrate the capability of tumor EVs to perform the epithelial-to-mesenchymal (EMT) and mesenchymal-to-epithelial (MET) transition using specific miRNA regulation in the host [35].

Apart from migration, EVs influences the immune system recognition and activation toward producer cancerous cells. As discussed in Section 1.3.1.3, the presence of MHC molecules endows vesicles with the ability to interact with APC conditioning the process of antigen presentation. Thus, antigens from cancer can be internalized by DCs and exposed to the surface for the activation of T lymphocytes or tumor EVs may trigger release of APC-derived vesicles. Cross presentation of tumor antigen to CD8+ T cells induced by allogenic tumor exosomes was demonstrated in mice to affect the anti-tumor activity and cause rejection of the grafted mass [72].

More importantly, exosomes are characterized by features able to induce an activation or an inhibition of the immune system by themselves. In cancer, the immune-suppressant effect is enhanced to promote evasion: T cell apoptosis, NK cells suppression, phagocytosis and myeloid differentiation are all controlled via exosomes [73].

The release of FasL coated vesicles was found in murine melanoma to kill lymphocytes inducing apoptosis in activated T cells overexpressing Fas receptor. Significant reduction of NK cells cytotoxic activity is accomplished by NKG2D ligand expressing exosomes in breast cancer by decreasing the level of NK activating receptors. Exosomes from tumors are able to direct the differentiation of circulating monocytes out of the resident DC form, pushing the proliferation towards more quiescent cells types like MDCS (myeloid-derived suppressor cells), escaping in this way the action of phagocytes. This finding and a similar behavior on T cells of TGFβ secreting exosomes have been confirmed only in vitro [74-76].

It must not be forgotten the role of tumor EVs in eliciting chemotherapeutic resistance in treatments. Authors show that melanosomes are capable to sequester drugs from tumor cells and excrete them out. In ovarian cancer resistant to chemotherapy, it was found that exosomes contain high levels of cisplatin drug efflux proteins like MRP2, and cisplatin resistance is correlated to increased expression of trafficking and fusion protein of the exocytotic pathways of late endosomes where the drug is supposed to accumulate [66,77]. In addition, even resistance to biopharmaceuticals was found in breast cancer cell line where Trastuzumab activity is competitively inhibited by exosome-bound HER2 receptor [70].

1.3.2.2 EVs in inflammatory diseases

Considering the immunological activities found in EVs and described above, one of the most discussed topic is the capability of EVs to act as autoreactive triggers in chronic inflammatory diseases and autoimmune diseases [78].
Doubts originate from clinical evidences of autoantigens in EVs specimens from the most common autoimmune diseases. Therefore, autoantibodies and, rarely, autoreactive T cells have been demonstrated to bind such self-antigens [79].
In rheumatoid arthritis (RA), for example, some citrullinated epitopes were observed in synovial fluid EVs and notably these ones are an established biomarker of the pathology. Microparticles derived from platelet are significantly increased in patients' synovium and blood and they are responsible of some pathogenic features like formation of immune complexes, delivery of pro-inflammatory cytokines (IL-1β). The action of matrix eroding proteins such as MMP9 on the surface of fibroblast-derived EVs might have a role in the progression of the lesions on cartilage [80,81].
Additionally, even systemic autoimmune diseases are affected by MVs in the pathogenesis. An example is SLE, systemic Lupus erythematous, that is a condition of generalized autoimmune reaction against DNA molecule accompanied by the production of anti-DNA and anti-nucleosome antibodies: from certain studies, it may appear that the production of such antibodies would be correlated to IgG and IgM antibodies on the surface of MV and apoptotic bodies increased in amount in SLE patients. These specimens contain pathogenic determinants and may carry autoantigenic DNA to form immune-complexes [82]. It is still to be characterized if these evidences come from contamination of EV samples with immune-complexes or not, because of the limit of few techniques to distinguish EVs from aggregates. We will detail this limit in the Section 1.3.3.2.
Finally, we highlight the role of EVs in the pathogenesis of multiple sclerosis (MS) a demyelinating autoimmune disease of the CNS characterized by an accentuated dysfunction of the BBB with consequent infiltration of pro-inflammatory cell types. Endothelial originated EVs from activated EC in relapsing patients affect the permeability of blood brain barrier in vitro models [83]. Metalloproteases and cytokine containing EVs are investigated for the participation to the process while microglia derived EVs are thought to be responsible for the propagation of neuroinflammation in the parenchyma.

1.3.2.3 EVs in neurodegenerative diseases

Most of neurodegeneration is related to the detrimental effects of misfolded toxic proteins acting as templates for oligomerization of fibers interfering with the normal neuronal functions. EVs, for their involvement in non-contact cell-to-cell communication, have

been considered as shuttles of misfolded proteins in the CNS to represent seeds for the spreading of their own pathogenicity [84].
Prion-like peptides like amyloid β (Aβ) is recycled through the endosomal pathways and is sorted in MVBs to make possible the exocytosis of exosomes: specifically, in Alzheimer disease amyloid protein precursor APP on clathrin-associated vesicles is ultimately cleaved when it gets delivered to the endosomal membrane. A confirmation comes from the presence of flotilin-1 and Alix, important exosome markers, inside the plaques of AD patients. Plaques are considered dumps of amyloid fibers, while the action of small oligomers is recognized as fundamental in the progression of the degeneration. In this way, exosome could be the means by which amyloidosis is spread peripherally in longer distances [85,86].
Also cytosolic proteins associated to neurodegeneration have been found in EVs from several diseases specimens. One example is alpha-synuclein in Parkinson disease: it was shown that oligomers of alpha-syn are encapsulated in exosomes and both are found in TEM studies in the extracellular space. Furthermore, there is significant aided internalization of EV-oligomer complexes compared to free oligomers in proliferating cell lines and primary neuron cultures [87].

1.3.3 EVs in research: standardization

The sub micrometer size and the absence of complete biogenesis elucidation gives hampers to the rising research field on extracellular vesicles. On the following sections, we clarify how the current state-of-art knowledge around EVs is supported worldwide, underlining the limits at the basis of such investigation.
Being this a quite recent field, scientists have to define conventions about either isolation or characterization of these small cellular organelles. In the attempt to do so, in 2012 the International Society of Extracellular Vesicles (ISEV) was founded. The aim of the organization is to harmonize the scientific community, inform through events, coordinate positions worldwide in the matter of sample isolation, handling, characterization to put up guidelines for future applications of EVs in therapy and diagnosis [88].
Current methodologies are unable to differentiate all the EV subpopulations, which are co-purified and treated as such: information exchange helps to define regulatory positions to avoid data misinterpretation [89].
Even nomenclature is still under examination by the community, as different nouns are employed to describe EVs from specific tissue, pathology or cellular compartment source. According to one's purpose, researchers have defined "exosomes" depending on the endosomal origin definition, the broad dimensional definition or because of the centrifugal pelleting at high speed. For this reason, there is wide acceptance of the generic term "extracellular vesicle" to be preferred instead of more constrained words [90].

1. Introduction

As of today, in this field, scientists are encouraged to strictly define the sample origin, isolation procedure and characterization determinants, which is why a brief report about the present scientific tools to study EVs is important for this project.

1.3.3.1 EVs isolation and storage

Sources for EVs in research are the most different, from biofluids to cell culture conditioned media. Cell surface determinants should be assessed, as well as cell viability since apoptotic bodies from dying cells can interfere downstream [91]. Each sample type must be treated in a specified way and in a case by case approach. For our purpose we describe only the case of EVs derived from clear conditioned medium (CCM), a source indicated for pre-clinical and pre-formulation studies consisting in the supernatant medium from both primary cell culture and cell lines. Cells are cultured 24-48 hours in serum EV-depleted medium in appropriate vessels: regarding this, we report the use of Integra CELLine culture bioreactors used to achieve high cell density and allow better yields of EVs [92,93].

ISEV advises to proceed to isolation immediately after the collection of the source and, if needed, to validate storage in terms of EV adsorption on container material and temperature: there is consensus to store isolated EVs in – 80°C freezers and evidences are present in the literature demonstrating the preservation of ultrastructure and the relative insensibility to freeze/thaw cycles of the vesicles. Beyond that, samples should be frozen and thawed rapidly to avoid loss of structure and save the functional activity [94].

Methods to isolate EVs are several. Differential centrifugation (DC) by ultracentrifuge is the most common procedure to purify EVs. It allows to separate larger EVs (microvesicles) pelleted at 10,000-20,000 g from smaller EVs (exosomes) pelleted at 100,000-120,000 g [95,96]. Studies have now established the impurity of resuspended precipitates underlining the existence of different sub-population of particles and different protein and membrane aggregates at high speed centrifugation, besides clogging of the EV themselves [89]. To overcome this limit, it is preferred to add a density gradient in the centrifugation step: based on the different densities of sample's contaminants, this procedure would concentrate EVs in a density around 1.10-1.21 g/cm^3. Commonly, gradients are constituted by sucrose or iodixanol, used in increasing percentage form the top to the bottom of the tube, in order to fractionate the sample according to one's use. There is still the risk to co-isolate aggregates depending on the length and time of centrifugation, plus there is the addition of agents perturbing the osmotic pressure on the vesicles [97,98].

Size-dependent methods are often used with or without a precedent centrifugation. These methodologies include size exclusion chromatography (SEC), ultrafiltration, flow filed-flow fractionation (FFFF). The first offer the opportunity to separate EVs under low

pressure and physiological elution and eliminate up to 99% of plasma protein in blood samples [99]. The choice of the exclusion matrix depends on the empiric determination of size in characterized samples: components that may co-elute are always present such as viruses or large protein aggregates. Parameters like column dimensions or stacking and flow conditions have to be taken into account for reproducibility issues [100].

Ultrafiltration-based methods require a centrifugal force to concentrate EVs through a porous membrane. It is a way faster method than differential centrifugation that takes few hours to be completed, but it is being used mostly for concentrating samples rather than purifying them. Regardless, small centrifugal forces are recommended to avoid loss of sample sticking on the device or scission of the vesicles during the filtration [101].

A technology in recent blooming is flow field-flow fractionation or F4, consisting in the fine separation of EVs for their size subjecting the samples to a parabolic trajectory in a filtered chamber thanks to the action of a tangential flow removing the impurity below the cut-off and controlling the elution of the analytes. Asymmetric F4 has been interestingly used to separate distinct subpopulation of exosomes: there is few information on the scalability of this technology for the clinical application, but it has already captured the interest for its rapidity and relative reproducibility of the analysis [102].

Immunoaffinity capture related methods have been also exploited to achieve specific isolations in comprehensive EV samples. In this case, monoclonal antibodies directed to certain ligands on the surface of EVs are immobilized on a plate, column or chip. Ideally, the method requires an accurate choice of the ligand that must be validated for specific enrichment in the desired subtype of EV. CD63 tetraspanin protein, for instance, has been used for the development of immune-capture systems to enrich samples in exosomes. Extensive characterization on antibodies, important to avoid cross-reactivity, paved the way to the commercialization of some kits that managed to obtain high yields of purity coupling antibodies to magnetic beads to increase recovery [103].

Another approach being exploited by industry is the marketing of precipitation inducing polymers to incubate biofluids overnight at low centrifuge speed. Most of the times, the polymer is polyethylene glycol (PEG) which is notably a non-toxic nor denaturing solvent exclusion polymer. Contaminants are not eliminated as they may co-precipitate and in each case there are pre- and post-isolation handling to be done on the sample, respectively to remove larger membrane components or aggregates and to remove the polymer, e.g. using SEC [104].

Ultimately, microfluidics methodologies are available to handle small volume of sample and isolate EVs with a sieving approach or a physico-chemical specific approach [105].

These all methods are not to be thought as mutually exclusive, on the contrary orthogonality in using multiple isolation techniques is highly recommended by ISEV. Whilst ultracentrifugation is considered the gold standard in EVs isolation, it has a laborious and poorly scalable protocol with high percentage of co-pelleting contaminants: DC is often coupled to other techniques like a second ultracentrifugation with density

gradient or immunocapture to select a specific subpopulation of EV and remove contaminants [106]. In the same way, nanomembrane concentrators for ultrafiltration are employed after SEC to prevent clogging. In fact, SEC suffers from low scalability and typically needs enrichment of sample to exclude impurities [107]. On the other hand, precipitation is probably the easiest method, but it was shown that purity is compromised. Hyphenation of different techniques inevitably results in lower yield of recovery and high risk of human error in the manipulation. Also, since there is still uncertainty in the biogenesis, several population of different exosomes can be pulled-down by each of these methods. Downstream characterization should be ideally possible on-line to allow scalability and to meet the demands of the flourishing field.

1.3.3.2 EVs characterization

As a general concept, we intend as "characterization" each qualitative and quantitative assessment required to have a comprehensive knowledge of the system in use. For EVs many instrumentations are exploited to have such information. The first in history and still the most reliable for confirmation on morphology and size of single EVs is TEM, transmission electron microscopy. Typically, concentrated EVs samples are fixed in 4% paraformaldehyde and stained with uranyl acetate. Cryo-TEM is used to make more accurate assumptions on ultrastructure as the drying and the treatment of samples alters the shape of such entities. Immuno-TEM is used in many works not only to confirm the nature of the examined vesicles, but also to visualize ligand of interest which are recognized by colloidal gold immunoglobulins. Size estimations have to be carefully interpreted as different software may calculate a different value on the same sample, a reason why it is still preferred infer diameter information with different technique [108,109]. Nanoparticle tracking analysis (NTA) is an unavoidable technology to characterize EVs and gain a high load of information. It consists in hitting the sample with a focused laser beam and register the scattering of light by particles visualized with a CCD or a sCMOS camera. Size, concentration, refractive index, zeta potential, fluorescence of single EVs is calculated by the software in relation to the Brownian movement recorded and in agreement with the Stokes-Einstein equation. It is necessary a solid optimization of the capture parameters for the validation of data and reference materials should be used as standards to be compared. The inherent variability of NTA instruments hampers standardization and most of variables are meant to be individually fitted to one's system. General guidelines favor the image acquisition in many short videos to have a reliable standard deviation and avoid multiple particle tracking overestimation [110,111].
Another technology, less employed for polydisperse samples like EVs, is dynamic light scattering. It retrieves the z-average diameter of the hydrodynamic volume of the particle

and zeta potential values for surface characterization: useful in the definition of EV-based drug delivery system is not so suitable for general characterization of EV samples [112].
Much more interesting is tunable resistive pulse sensing (tRPS): the principle of functioning is the detection of altered ionic current by the passage of particles near a tunable nanopore on a polyurethane membrane. It allows measurement of concentrations in a range comparable to NTA, but it requires the use of different pore settings to enhance the recovery of the polydisperse EVs and it does not permit distinction with similarly sized contaminants with the additional limit of possible pore occlusion from sticky proteins in the sample [113].
Flow cytometry is a widely used technique for the detection and characterization of cells. Although, high resolution flow cytometers are being produced for the detection of nano-sized vesicles with a lower limit of 100 nm. Light scattering is a complex phenomenon involving diameter and refractive index of particles, thus the reliability of the polystirene bead standards to determine particle size is still under discussion. Besides, fluorescence with stains or immune-phenotyping antibodies should be controlled to avoid aspecific signals from protein aggregates, in fact detergent controls and dilutions of the sample must be provided to prove the actual presence of EVs and exclude possible signals from multiple events crossing the laser beam [108,114].
Novel methods to be explored for EVs determination include atomic force microscopy (AFM) and Raman spectroscopy. AFM would characterize the surface of EVs at the sub-nanometer resolution sensing the elastic properties and interaction forces with the scanning cantilever: still not so used for expertise demand for the interpretation of its biophysical outcomes. Raman spectroscopy measures the vibrational energy upon light interaction and is increasingly in use to dissect the biochemical composition of EVs in a label-free mode [108,115-117].
For a complete overview of EVs function, content must be characterized prior to further approval in therapy or diagnosis. In particular, we are mentioning protein and RNA evaluations with –omic approach.
Preliminary Western blot analysis is fundamental to dissect EVs sources or confirm sample purity. Tetraspanins CD63, CD9, CD81, CD82 or MHC molecules or cytosolic proteins like Tsg101, Alix or actin are all associated to EVs samples and are often used in immune-blot to evaluate the sample qualitatively. Proteomic data banks have been generated such as Vesiclepedia (www.microvesicles.org) or Exocarta (www.exocarta.org) associating almost 10,000 entries to EV source. Since EVs discovery, mass spectrometry and quantitative proteomic analysis resulted in enrichment data on the secretome, even though the risk of artifacts of peptides from serum protein contaminants is high and an effective control on isolation must be assessed [118-120].
RNA patterns recognition is highly dependent on cell source, method of isolation and sample handling, so it is not surprising that a series of ISEV guidelines for sequencing and characterization of circulating EV RNAs is needed. Next generation deep sequencing

analyses are under development, but correlation between miRNA or mRNA transcript and EV subtype is still impossible as an average RNA content can be measured from vesicles. Validation with PCR and Northern blot is necessary. Microarrays of RNAs are a useful tool to validate biomarkers for diagnosis, but they cannot be used for novel RNA discovery [121,122].

1.4 Extracellular vesicle-based drug delivery platforms

Regardless the advancements in the field of nanomedicine and nanotechnology manufacture, several issues hurdle the efficacy and the regulatory approval for many drug delivery systems. Synthetic nanoparticles suffer from inefficiency and toxicity based on the nature of the carrier and on less predictable behaviors of materials interacting with living tissues at the nano scale: the biophysical interaction with the cellular surface is dictated also by the adsorbed components on the carrier once it is injected in a biofluid and toxicity may occur from different properties from size to charge or even shape of the particle. The biodistribution to the target tissue is compromised by the uptake from undesired cell types such as the MPS or the RES, limiting the formulation of artificial nanomedicines [123-125].

That is why naturally occurring nanoparticles like exosomes offer a suggestive alternative to mask therapeutic moieties inside these vesicles. Additionally, exosomes might derive from ex vivo cultures of patient cells, carrying in such way a minimal immunogenicity and a high safety profile. EVs features and ubiquity in the body make assume a potentially beneficial biodistribution and clearance escape for clinical applications [126]. Being carriers of multiple biochemical signals for cellular communication, exosomes could deliver different therapeutic cargos, even at the same time. EVs, as described, have a physiological mediator function per se and might be exploited as unmodified vesicles depending on the parental cell source and target of the treatment (see Section 1.4.4).

Engineering exosomes to generate EV-based drug delivery platform is possible and has arisen interest from pharmaceutical research and industry. We will describe specifically the use of exosomes as they are referred as the most suitable for drug delivery application: the dimension below 200 nm in diameter makes of exosomes a perfect carrier to cross biological barriers and circulate undetected from phagocytes; membrane plasticity and stability are valuable characteristic for delivery applications as a gel state core is sustained through cytoskeleton proteins and a cholesterol rich membrane [127].

We discuss the current knowledge on how to insert a bioactive cargo, modify the surface and direct exosomes to a defined tissue and after we detail the aftermaths of such state of art for future medicine.

1.4.1 Loading of therapeutic cargo

Exosomes, as lipid sacks, are plausible vectors for a wide range of therapeutic drugs, in terms of hydrophilicity and dimension of the API. There are reports of encapsulation of small hydrophobic drugs, hydrophilic proteins and small molecules or nucleic acids in exosomal formulations [128]. Though, unlike liposomes which can be encapsulated during the synthesis, semi-synthetic exosomes must be loaded extrinsically, choosing one of the strategies described below:

- Prior to isolation from cell culture, exosomes can be loaded with transfection or incubation of the drug molecule in producer cells' media, taking advantage of the endogenous machinery.
- Ex vitro, post isolation methods in which exosomes are loaded by co-incubation, electroporation, sonication, membrane permeation or binding on the vesicle.

Passive, incubation based methods have the disadvantage of being cost ineffective as the loading capacity of exosomes without treatment is low. For this reason, both the strategies are under evaluation to find a way to enhance the encapsulation efficiency [129].
In general, hydrophobic drug compounds such as curcumin, cucurbitacin I, doxorubicin, paclitaxel, withaferin have been inserted via post isolation incubation with LC percentages in the range of 1-10%. For hydrophilic protein drugs like catalase, sonication or extrusion were seen to increase the capacity with retained activity of the formulation and for paclitaxel and doxorubicin, Kim et al showed increasing encapsulation with active loading with electroporation and sonication followed by in vitro reconstitution of the membrane [130,131].
Integrity of the membrane and dimensions consistency is the main issue for active loading strategies. Emblematic is the case of exosome membrane permeabilization with saponin: it was demonstrated that catalase loading is highly enhanced treating membranes with this agent, but it's still to be assessed if it results in a disruption of integrity with consequent immunogenicity of the EVs or if larger proteins may be delivered in the same conditions [132].
Electroporation and sonication both result in significant swelling of the EV size, hampering the standardization of the method. For hydrophilic cargos, the former method is the most suitable as they need to reach the cytosolic core of the EVs to be protected from diffusion or degradation, which is possible the reason why transfection and bioproduction are the gold standards to pack therapeutic proteins and nucleic acids in exosomes before the isolation.

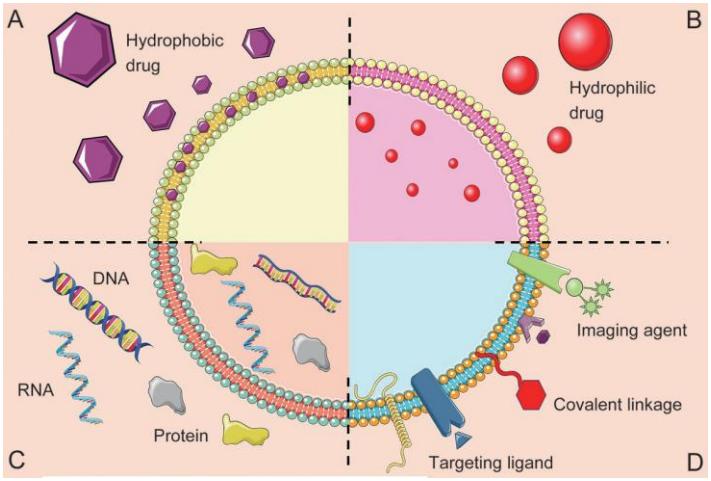

Figure 4 *- Adapted from ref [128]. Schematic representation of the different physico-chemical and biological properties of drugs for possible loading in extracellular vesicles delivery systems.*

1.4.2 EVs pharmacokinetic and surface modification

Being promoted as natural variant of liposomes, EVs offer great potentials from a pharmacokinetic point of view in terms of MPS escape and ameliorated permeation of biological tissues. Nevertheless, the promising pharmacological activity in vitro of unmodified EVs has yet to be confirmed in vivo as injected vesicles lack for sufficient circulation time to assure delivery and efficient intracellular release of cargo [133-135].

The eligibility of EVs as therapeutic agents relies on the ability of research to tune their surface composition in order to mask them from phagocytes and direct them preferentially to the uptake when injected in body fluids which, as mentioned before, are already enriched with similar vesicles. It is unclear why systematically administered EVs display such an unfavorable distribution similar to other synthetic nanoparticles. After injection in mice models, EVs are mainly found in liver, spleen, GI tract and lungs at time point of 24 hours, while dynamic analyses with reporter GLuc bound on EV membrane highlighted a window of 30 minutes to 6 hours for complete uptake in liver of perfused mice. In such way, spleen should be involved only in passive accumulation after liver macrophages saturation rather than active endocytosis of EVs in splenocytes [41].

Until further information about EVs in vivo behavior, it looks pivotal to explore a semi-synthetic approach of surface engineering to achieve a better therapeutic index. In a similar way as for liposomes, PEGylation has been shown as a candidate strategy to increase the circulation time of intravenously injected EVs: Kooijmans et al demonstrated the possibility to insert PEG-lipids in the bilayer of macrophage-derived exosomes with a sufficient surface density to ensure an extension of permanence in the blood up to 60 minutes compared to timings below 10 minutes of the controls [136].

Genetic manipulation allows to explore different strategies for example by overexpressing "self" proteins like CD47 or CD55 demonstrated to be responsible of a certain protection from clearance [137].
Even the addition of cell penetrating peptides CPPs on the surface of EVs could make the difference in the actual absorption of the drug from the recipient cell, or other peptides such as rabies virus glycoprotein RVG may facilitate the passage of the blood-brain barrier [138].
Together, these experiences give a hint to an important area of research for EVs applications which could dictate their future as possible competitors of traditional nanoparticles.

1.4.3 Targeting strategies

As mediators of extracellular communication, secreted vesicles are naturally equipped with biomolecular features that confer targeting to recipient cells. Surface lipids, proteins, lectins all concur to define the mysterious zip code responsible for the delivery of the sorted cargo to exert EVs functions.
Examples of molecules in EVs equipped with natural targeting capacity are tetraspanins and integrins: such proteins can form a web to promote membrane adhesion and enhance the efficiency of uptake. Being a well-known enriched protein subtype in exosomes, they have been hypothesized to recruit different protein counterparts to form specific nets of interaction resulting in various mechanisms of internalization depending on the cellular target [139].
Intrinsic capacity to deliver EVs to specific cells is strictly related to the source of isolation as a combination of targeting molecules can show preference to certain cell surfaces [140].
Nonetheless, EVs are engineered to express ligands and achieve better uptake or they are chemically modified on the exposed surface. Targeting peptides showed promises for this purpose, as they were successfully expressed downstream the EV protein lysosomal associated membrane protein 2b (Lamp2b): both RVG and iRGD chimeric Lamp2b genes significantly delivered nucleic acids to neuronal cells. Homing peptides have the advantage, compared to large protein ligands, to be less influenced by folding and orientation. Other moieties employed are GE-11 peptide directed to EGFR on breast cancer cells or C1C2 domain of Lactadherin used as scaffold for antigen cross-presentation [138,141].
Although, even small proteins like anti-EGFR nanobodies have been successfully loaded in the bilayer of exosomes for targeted delivery of chemotherapeutics to breast cancer [136].
Click chemistry is also used to attach targeting molecules to introduced azide function on EV surface: Tian et al conjugated an RGD derived peptide to activated proteins on the

surface of mesenchymal stem cells exosomes while Vandergriff et al generated a phospholipid conjugate of a homing peptide for cardiac tissue regeneration [142,143].
Surface could be modified by non-covalent chemistries: attempts have been made using positively charged polymers or phospholipids to be inserted in the membrane for an increased tethering toward cell surface [144]. As long as it's not possible to control the clearance of such nanoparticle, the development of novel targeting strategies is important to clinically test systems with an ameliorated and faster bioavailability.

1.4.4 Applications: from diagnosis to therapy

It is not straight-forward to sum up all the fields in which extracellular vesicles find their potential application in clinic. In general, for the characteristic abundance in biofluids and their unique content, a large part of the research is focused on realizing novel diagnostic tools to be used in liquid biopsies. The increasing understanding of the EVs subtype under the biochemical point of view has already revealed the possibility to consider the alteration of the number and composition of EVs in plasma as important for prevention of disease progression: prostasomes, prostate-derived microvesicles found in seminal fluid and secreted by cultured prostate cells, are under investigation as prognostic determinants in malignancies since the lipid vesicles may protect and spread tumorigenic cargos in distal areas. In particular, observations of in vitro promotion of tumor growth upon PCa EVs addition and the presence of prostate specific markers on prostasomes sustain the possibility of their use in diagnosis. For example, an increase of total EVs was shown to be significant in metastatic patients compared to non-cancer controls and benign conditions (below Gleason grade 7). Although different proteome and trascriptome studies have been carried out, there are still hurdles in the isolation of a genuinely enriched molecule and in the detection of such entity in blood samples of cancer patients or in benign hyperplasia cases [126,145,146].
Label-free detection of exosomes on SPR chips has been demonstrated an informative technology for the quantitative characterization of EVs from human samples and consistently speed up the research for biomarkers [147].
Another important factor affected by the action of EVs is the contraction of chemo resistance due to the efflux of drugs via exocytosis. Namely, docetaxel resistance in non-responder hormone refractive prostate cancer patients is attributed in part to the spreading of EVs carrying P glycoprotein [148]. This evidence links to a possible therapeutic intervention to selectively inhibit the secretion of such EVs and achieve a better prognosis in treatment.
Interestingly EVs themselves could be used in therapy after ex vivo isolation from autologous or even heterologous source. Several clinical trials are being applied and guidelines from ISEV ensure a consensus in the setting of limits and in the definition of

the pharmaceutical category of such cell-free drugs. There are reports on the use of plant or animal derived fluids' nanovesicles demonstrating the negligible toxicity and promising efficacy as drug delivery vehicles [149]. Unmodified EVs of human origin may serve the most disparate functions in clinic, such as anticancer immunotherapy: the first evidence of this possibility came more than 20 years ago from experiments of pulsing of dendritic cells with cancer antigens with subsequent isolation of exosomes and infusion in immunocompetent mice to reject grafted tumors. These proofs were translated in few clinical trials demonstrating the feasibility and safety of the administration. Tumor derived exosomes might be used in combination with adjuvants to inhibit their immune suppressant effect and allow their use as tumor antigen source for vaccination [150]. Otherwise, bioengineering of exosomes vehicles to boost the immune response might be a solution, using exosome-bound scaffold proteins to be overexpressed in recombination with an exposed antigenic peptide. Additional glycosylation sites are added on such protein chimeras to prevent lysosomal or proteolytic degradation of the carrier [151].
Exosomes miRNA content may have a therapeutic impact itself and EVs might work as immunostimulatory drug delivery vehicles: miR-155 in B cells' exosomes is responsible in vitro and in vivo for macrophage phenotypic activation towards cancer following a significant reduction of growth respect to controls [152].
In the regenerative field medicine, MSC-derived EVs serves as prospective tool to support anti-apoptotic activities and contribute to organ regeneration in the cases of liver, kidney, colon damages or myocardial infarction. There are many pre-clinical evidences of the benefit of MSC-exosomes therapies for immune modulation and tissue remodeling, while some of those led to clinical trials for various diseases [56,149].
Ultimately, EVs ability to cross biological membrane finds application in treatment of CNS pathologies: efforts have been made to build a delivery system for gene therapy in brain disorders such as Parkinson or Alzheimer and targeted electroporated exosomes seem to work in animal models and are currently under development [153].

1.5 Project strategy

Our strategy for this thesis requires the introduction of the molecular characters involved in the experimental section. In particular, PC-3 derived exosomes were isolated from culture media and a nanotechnological formulation was assessed inserting in the bilayer PEGylated phospholipid derivatives to avoid unspecific tethering and a synthetic PEG lipid functionalized with folic acid as targeting moiety. After formulation selection, the semi-synthetic exosomal vesicle was tested in vitro as drug delivery vehicle for anticancer paclitaxel.

1.5.1 Folic acid

Pteroylglutamic acid, also known as folic acid, is an important diet nutrient being part of the vitamins of group B. In ***Figure 5*** is reported the basic chemical structure of the glutamyl amide of the pteroic acid, respectively formed by a 6-mehylpterine aromatic ring linked to a *p*-aminobenzoic acid (PABA).
The carboxylic functions play a central role in the pharmacodynamics of folic acid as the orientation of the α- group is relevant for the correct recognition by the folate receptor, the class of proteins involved in the uptake of this vitamin.
Conversely, the γ- carboxyl function is exposed and prone to be chemically conjugated without affecting the activity significantly.
In its active form, folate is transformed enzymatically by DHFR, dihydrofolate reductase, into tetrahydrofolate (THF) in the liver, and then it can serve important functions in the synthesis of nucleic acids and aminoacids metabolism [154].

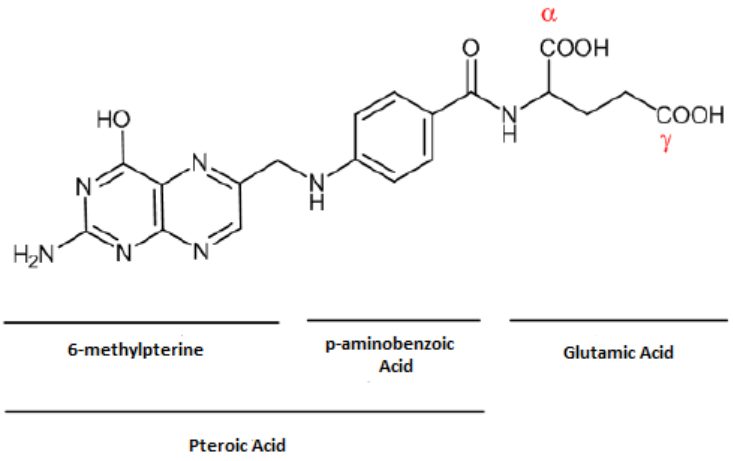

Figure 5 - *Chemical structure of folic acid*

Specifically, THF is involved as methyl group donors in the synthesis of purines and it has a central role during development and homeostasis to guarantee a correct DNA repair. For this reason, folic acid is a fundamental substance to be absorbed during pregnancy to prevent fetus malformations. In addition, it is involved in the synthesis of aminoacids like methionine, as a methylene donor through the action of vitamin B12. Deficiencies of folate in adult age may result in pathological conditions such as loss of appetite or weight, weakness and drowsiness or even diseases like a particular anemia with overproduction of large immature red blood cells, the megaloblasts.
Proton coupled folate transporter proteins are expressed in the intestine for dietary absorption of folic acid. Folate receptors are a family of GPI anchored proteins binding folate and its reduced derivatives and operating the uptake across cellular membranes. Folate receptor 1 or FRα (gene name FRA) is the most important isoform of folate

receptor family: it is expressed in healthy tissues like placenta, kidney or lung, but also in mutated cells where it is overexpressed, like in different cancers and it has affinity constants for folate and reduced species in the range 1-100 nM. It is not surprising, given folate function in DNA synthesis, that neoplastic cells benefit from an increased uptake of this vitamin by enhancing the production of folic receptor. It is established that some ovarian, lungs, breast and pancreatic cancers share this feature [155,156].

Folic acid is particularly feasible as targeting agent for the low abundance of the receptor in membranes of healthy cells, for the specificity and affinity of binding, for its well-known physico-chemical properties and minimal disruptive effect on the structure of the carrier [157].

As of today, many nanosystems have been theorized and tested to deliver a targeted payload exploiting the binding of such small molecule to the overexpressed receptor. There are examples in the literature of liposomal carriers, viral vectors, polymeric NPs or proteins delivered to cells naturally expressing the receptor like immortalized KB cells or stably transfected with a proper plasmid.

We decided to test the ability of cells carrying the FRα to uptake our targeted exosome drug delivery formulation.

1.5.2 Polyethylene glycol (PEG)

Polyethylene glycol, PEG, is a linear polymer obtained by ring opening polymerization triggered by anion radical formation into ethylene oxide. As shown in ***Figure 6***, after propagation and termination the polymer is stably constituted by repetition of ether bonds between ethylene units.

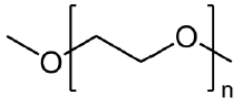

Figure 6 *- Polymeric structure of PEG.*

Depending on the termination step, PEG chains can be functionalized with different chemical groups and the reaction can be controlled to generate polymers with a wide range of molecular weights and a relatively low polydispersity. Such properties made the use of PEG derivatives profitable for the pharmaceutical industry. Being approved by FDA and EMA to be used for injectable formulations, PEG has minor undesired effects deriving from its poor biodegradation such as the accumulation in the liver for molecular weight above 30 kDa and the rare formation of anti-PEG antibodies [158].

Given its amphoteric nature, PEG is suitable to stabilize drug formulations in aqueous environment and in water solvent the polymer hydrodynamic volume is able to increase 5-fold. Polyethylene glycol is used in bioconjugation to confer enhanced solubility, prolonged circulation time, reduced antigenicity and immunogenicity. For this purpose, PEG must be functionalized with aldehyde, thiol, succinimide, N-hydroxysuccinimide and other functions.
There are products commercially available in healthcare which are bioconjugated to PEG chains for the reasons described: some examples are Adagen®, Pegintron® and Oncospar®, engineered biologics for the treatment of SCID, hepatitis C and acute lymphocytic leukemia respectively.
Also a large part of drug delivery systems available have undergone trials for inserting PEG polymers to stabilize the carriers and ameliorate their pharmacokinetics. Nano- and microparticles, hydrogels and liposomes belong to those carriers. Interestingly, the latter rely on the opportunity to conjugate phospholipids to PEG and form PEG-lipid derivatives [159].
In this project, PEG used was attached to DSPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine, a zwitterionic phospholipid to be inserted in the exosomal membrane. PEG chains were employed to decorate EVs and prevent unspecific binding. Polymers chosen were the non-reactive PEG methoxide and the reactive amino functionalized PEG for the synthesis of the targeted lipid conjugate with folate for folate receptor active targeting.

1.5.3 Paclitaxel

Paclitaxel (PTX) is a powerful chemotherapeutic molecule isolated from the bark of the Pacific Yew tree (Taxus brevifolia). In its pharmaceutical form, it has been used for the treatment of relapsing cancers such as ovarian, breast and lung cancer. Taxanes have a mechanism of action involving the complexation with tubulin in the cellular cytoskeleton preventing cell division by interfering with the formation of the so-called spindle to segregate chromosomes to the two cells. A programmed cell death, apoptosis, is generated upon microtubules destabilization from paclitaxel and anaphase blockage [160].
Paclitaxel, in ***Figure 7***, is characterized by a hydrophobic structure as it's poorly soluble in water and ionic solvents. It is soluble in methanol, dimethyl sulfoxide and ethanol. For

this reason, researchers are focused to develop formulations to increase the bioavailability of this drug in the blood stream.

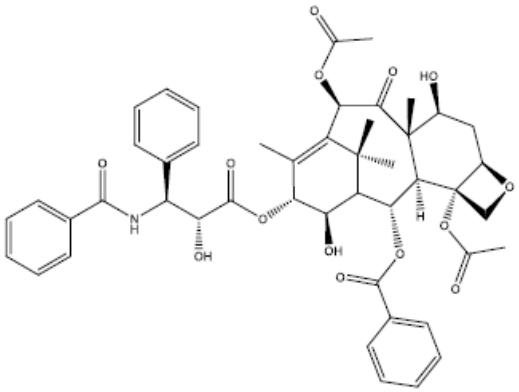

Figure 7 *- Chemical structure of paclitaxel.*

PTX has been commercialized as Taxol® in a formulation with polyethylated castor oil, Cremophor1 EL, and as Abraxane® in albumin-drug nanoparticles. The former shows a wide spectrum of adverse effects mainly due to Cremophor1 EL: this molecule was demonstrated to be responsible of several hypersensitivity reactions in patients, together with nephrotoxicity and neurotoxicity. A treatment with corticosteroids and anti-histaminic drugs is obligatory as a prevention when administering PTX [161].
Conversely albumin nanoparticles did not offer a justified increased in efficacy, but confirmed the source of medicine's side effects in the approved Taxol [162].
Scientific pressure is on developing a novel delivery system which could ensure the slow release in the tumor proximity, minimizing in such way the issues of solubility and promoting the constant inhibition of division in oncogenic foci. Besides, to reduce the systemic exposure of the drug, targeted nanosystems with enhanced permeation to the tumor are necessary. Attempts can be found in the literature where PTX was incorporated in liposomes, polymeric nanoparticles and micelles.
The hydrophobicity of the drug is ideal to direct it to the bilayer of extracellular vesicles and, given previous evidence, our exosome carrier is supposed to be a possible delivery system to fulfill this unmet medical need.

2. Materials and Methods

2.1 Materials

2.1.1 Reagents

- Exosome functionalization with amine-polyethylenglycol$_{3.4kDa}$-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (NH_2-PEG3.4kDa-DSPE) and mPEG$_{2kDa}$-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (mPEG$_{2kDa}$-DSPE) from LaysanBio (Arab, AL, USA), and labelling with Fluorescein-1,2-Dihexadecanoyl-sn-Glycerol-3-Phosphoethanolamine (FITC-DHPE) from Gibco, Thermo Fisher (Madison, WI, USA).
- Folic Acid dihydrate, N-hydroxysuccinimide (NHS), N,N-dicyclohexylcarbodiimide (DCC), anhydrous Pyridine from Sigma-Aldrich (St. Louis, MO, USA).
- Cell culture with Kaighn's Modification of Ham's Medium (F-12K Medium), Fetal Bovine Serum (FBS), L-Glutamine, Dulbecco's phosphate-buffered saline DPBS from Gibco, Thermo Fisher (Madison, WI, USA).
  D-(+)-Glucose from Sigma-Aldrich (St. Louis, MO, USA).
  All culture plates were provided by Falcon (Plymouth, England).
  CELLine AD1000 bioreactor flask for adherent cells was used for EVs harvesting and was purchased from Sigma-Aldrich (St. Louis, MO, USA).
- OptiPrep™ Density Gradient Medium for exosomes purification from Sigma-Aldrich (St. Louis, MO, USA).
- Paclitaxel from Selleckchem (Munich, Germany).
- MTT, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide from Sigma-Aldrich (St. Louis, MO, USA).
- Salts powders for buffer preparation from Fluka Analytical (Buchs SG, Switzerland) and Sigma-Aldrich (St. Louis, MO, USA).
- Organic solvents from Sigma-Aldrich (St. Louis, MO, USA).
- MilliQ water 18.2 MΩ obtained from Millipore MilliQ systems (MA, USA)

2.1.2. Instruments

- Spectra/Por 3 dialysis membranes with a cut off of 3.5 kDa from Spectrum Labs (Rancho Dominguez, CA, USA) used for Folic acid–PEG–DSPE purification
- MALDI mass spectrometry analysis with the 400 Plus MALDI TOF/TOF Analyzer provided by AB Sciex, (MA, USA) and H-NMR analysis with DPX400 MHz Ultrashield by Brucker (Fallanden, Switzerland).
- Spectrophotometer UV-Vis Evolution 201 provided by Thermo Scientific (Madison, WI, USA).
- For cell culture maintenance, an Airstream Class II Biological Safety Cabinet by ESCO Micro Pte. Ltd (Singapore) and HERA CO_2 incubators by Thermo Fisher (Madison, WI, USA).
- Bench centrifuges used: Eppendorf Centrifuge 5810R by Eppendorf (Hamburg, Germany) and Hermle Z300 from The Lab World Group (Woburn, MA, USA).
- Ultracentrifuge Optima L-0 XP from Beckman Coulter (Fullerton, CA, USA) to purify exosomes.
- Microplate readers iEMS 96 well Microplate Reader provided by MTX Lab Systems (Bradenton, FL, USA) and Varioskan LUX Multimode Microplate Reader provided by Thermo Scientific (Madison, WI, USA).
- CoolSafe Pro Freeze-drier from Labogene (Lillerød, Denmark) was used for PEG lyophilization before quantification.
- PEG density assessment retrieved from Varian Cary 100 provided by Agilent (Santa Clara, CA, USA).
- Exosome superficial charge characterization using Dynamic Light Scattering (DLS) Zetasizer NanoZS (Malvern Instrument Ltd, UK).
- Exosome concentration and size was estimated via Nanoparticle Tracking Analysis (NTA) with Nanosight LM14 (Malvern Instrument Ltd, UK).
- BD Accuri C6 Plus flow cytometer provided by BD Biosciences (San Jose, CA, USA) for flow cytometry analysis. Data were analyzed with FlowJo v10 software developed by BD Biosciences (San Jose, CA, USA).
- Omni Sonic Ruptor 250 ultrasonic homogenizer (Omni International) for drug loading.
- Ultrasonic bath from Branson Ultrasonics (Danbury, USA) to sonicate eluents before HPLC analysis.
- Jasco HPLC system used for paclitaxel quantification and FITC-DHPE quality control was equipped with two PU-2080 pumps, UV-2075 UV detector, a FP-2020 fluorescence detector and a RP column Phenomenex Luna C18 (2) 250x4.60 mm 100Å.

2.2 Methods

2.2.1 Iodine assay for PEG quantification

Protocol of polyethylene glycol quantification was derived from "Sims, G. E. & Snape, T. J. *Anal. Biochem.* **107,** 60–63 (1980)". In brief, 1 mL PEG samples were added with 250 µL of barium chloride solution, 5% w/v in HCl 1N, and 250 µL of iodine solution, 1.27% w/v I_2 in 2% w/v KI.
After 15 minutes of incubation protected from lights, absorbance at 535 nm was measured with UV-Vis spectrophotometer. Standard calibration curves were prepared with known concentrations of polymer to calibrate each instrument used in the project (***Figure 8***).

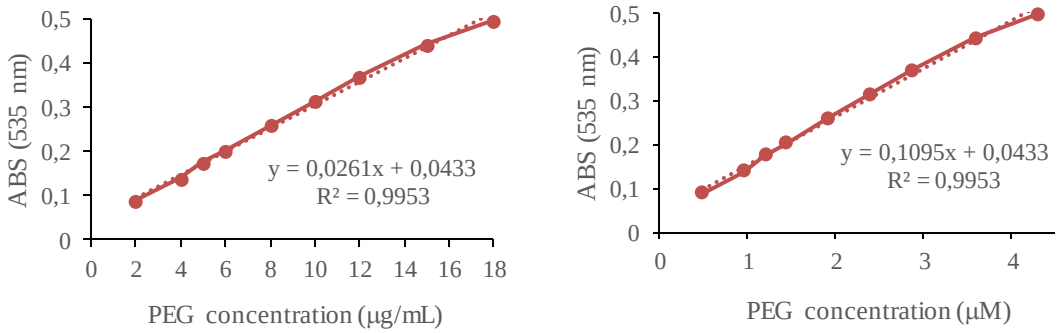

Figure 8 *- Calibration curve for iodine assay on NH2-PEG3.4kDa-DSPE. Serial concentrations are expressed in µg/mL (left) and µM (right). n=3*

2.2.2 Synthesis of FA-PEG$_{3.4kDa}$-DSPE conjugate

FA-PEG$_{3.4kDa}$-DSPE synthesis was performed following previous experience (Gabizon et al., *Bioconjugate Chemistry*, 1999). In brief, folic acid (26.3 mg, 59.7 µmol) was dissolved in 450 µL DMSO. Amino-PEG$_{3.4kDa}$-DSPE (50 mg, 12 µmol) and dicyclo-hexylcarbodiimide (12.3 mg, 59.7 µL) with the addition of pyridine (170 µL) were mixed to the solution. The mixture was left to react 48 hours at room temperature protected from light. Dicyclohexylurea (DCU) by-product was present as a visible white powder. After centrifugation to remove DCU, the solution was dropwise added to 50 mL of diethyl ether under stirring. Then, the precipitate was dried first with nitrogen flow and then under vacuum for at least 5-6 hours.
Powder was dissolved in NaCl 50 mM pH 9 and put in a 3.5 kDa cutoff membrane for dialysis, conducted against 4 L of NaCl 50 mM solution pH 8.5 for 4 days and then for one day in MilliQ water ensuring sink condition by changing dialysis medium two/three

times per day. Degree of conjugation was calculated from ratios of folate and PEG concentrations in aqueous solution: the latter was deduced by UV-Vis analysis in PBS, pH 7.4, at 363 nm (molar extinction coefficient 6197 M^{-1} cm^{-1}), the former via Iodine assay (see Section 2.2.1).
After the complete removal of folate and salts, the conjugate was lyophilized and stored at −20 °C for further use.
Molecular weight of the conjugate was evaluated with MALDI mass spectrometry: powder was dissolved in water with TFA 0.05% and 2,5-dihydroxybenzoic acid was used as matrix. Chemical structure was confirmed by ^{1}H-NMR (400 MHz, DMSO-d_6).

2.2.3 Cell culture

PC-3 cells from prostatic adenocarcinoma cell line were purchased from ATCC (American Type Culture Collection, Virginia, USA).
For EVs harvesting cells were amplified in T-175 culture flasks in antibiotic-free Ham's F-12K (Kaighn's) Medium supplemented with 10% vesicle-depleted FBS incubated at 37 °C and 5% CO_2 atmosphere under controlled conditions. EVs free FBS was obtained by ultracentrifugation of FBS at 150,000 g and cleared supernatant was filtered with sterile 0.22 μm Steritop filters (Millipore).
CELLine AD1000 bioreactor flask was used for mass isolation of EVs following the instructions provided by the manufacturer. In brief, cells were grown in six T-175 flasks as previously described, detached with Trypsin, centrifuged and re-suspended in 15 mL of inner chamber medium containing serum-free F-12K Medium supplemented with L-glutamine. The outer compartment was filled with 750 mL of F-12K Medium with the addition of a 4.5 g/L glucose solution and 10% FBS. Medias were filtered with sterile 0.22 μm Steritop filters (Millipore).
For in vitro testing, T-75 flasks (Corning Costar, Sigma-Aldrich) were used to culture cells. Seeded cells were grown in complete F-12K medium containing 10% EV-free serum and sub-cultrured when confluence was about 80%. Cells were incubated at 37 °C and 5% CO_2 atmosphere.
For viability assay, cells were cultured in T-75 flasks in RPMI-1640 medium supplemented with 2% (v/v) L-glutamine and 10% (v/v) FBS and seeded on 96-well plates.
All cell cultures were monitored routinely for contamination.

2.2.4 Exosomes isolation and purification

EVs isolation from culture medium was performed as previously described (Saari et al., *Journal of Controlled Release*, 2015). To sum up, differential centrifugation protocol was

adopted by pelleting DPBS cleared conditioned medium first at 2,500g in a desktop centrifuge 25 minutes at +4 °C to remove cell debris and apoptotic vesicles.
After supernatant collection, a second centrifugal step was conducted in Optima L-0 XP ultracentrifuge equipped with SW32Ti rotor at 20,000g for 1 hour at 4 °C in order to pellet microvesicles, which were discarded. The last step was performed with the same equipment at 110,000g for 2 hours at 4 °C to obtain a pellet of exosomes.
Pellets were added of 200 μL DPBS and left to detach overnight by flask contact with ice at 4 °C. Re-suspended exosomes were stored in –80 °C until use.
To purify exosomes from co-precipitating protein impurities, a previously adopted protocol with OptiPrep™ density gradient was used.
Briefly, in Ultra-Clear ultracentrifuge tubes 14x89mm from Beckman Coulter (Fullerton, CA, USA) a continuous density gradient was prepared as follows: 6 mL of 30% (v/v) iodixanol solution in DPBS were added in the bottom of the tube, then DPBS was poured dropwise to set a discontinuous gradient. Filled tubes were sealed and disposed horizontally in stationary conditions for 30 minutes until the formation of the continuous gradient. Tubes were gently put back in vertical position ready to be used. For sample loading, 400 μL were removed from the top of the gradient and exosomes were gently injected in the bottom with a glass pipette. Gradients were centrifuged 200,000g for 3 hours at 4 °C using a SW42Ti rotor.
One mL fractions of the fractionation medium were pooled and the validated method was confirmed with NTA: PC-3 exosomes were found in fractions 4-8 corresponding to densities of 1.08-1.15 g/mL. Those fractions were pooled and transferred in Amicon Ultra-15 MWCO 10kDa (Merck Millipore, MA, USA) filters for ultrafiltration in order to remove the iodixanol from the vesicles: in particular, three washing steps in excess DPBS were performed at 5,000g 60 minutes in 5810R Eppendorf centrifuge at +4 °C. Concentrated exosomes in 200-250 μL were left overnight in ice to let them detach from the cellulose filter and, the next day, they were aspirated and stocked in deep freezer at –80 °C.

2.2.5 Exosomes characterization

As a colloidal system, both purified and modified exosomes were characterized for size, concentration and surface charge by means of Nanoparticle Tracking Analysis (Nanosight LM14, Malvern Instruments Ltd) and Dynamic Light Scattering (ZetaSizer Nano ZS instrument, Malvern Instruments Ltd).
NTA Nanosight LM14 equipped with blue (404 nm, 70 mW) laser and SCMOS camera was used to gain information on size and concentration of EVs particles. The samples were diluted in MilliQ water depending on their source and three videos of 60 seconds

each were recorded using camera level 15. The data were processed using NTA software v3.1 with the detection threshold optimized for each sample and the screen gain fixed. Zetasizer NanoZS was mainly used to assess the zeta potential (ζ) of exosomes. Dilutions in MilliQ water (at least 1:50 to remove PBS salts) were measured in disposable folded micro-capillary cuvettes (DTS1070) from Malvern Instruments.

2.2.6 Exosomes functionalization

In this project, exosomes' surface was modified according to a previously investigated protocol by inserting the following phospholipid derivatives on the lipid bilayer:

- FITC-DHPE as a fluorescent labeling agent
- $mPEG_{2kDa}$-DSPE for shielding the protein embedded on the EV bilayer and reduce unspecific uptake
- FA-$PEG_{3.4kDa}$-DSPE, as targeting agent

In particular, stock solutions were prepared according to the following method: FITC-DHPE was dissolved in DMSO in a stock solution at 5 mg/mL before being diluted in DPBS, while for $mPEG_{2kDa}$-DSPE and FA-$PEG_{3.4kDa}$-DSPE powders were directly solubilized in DPBS at 6 mg/mL.

Modifying agents in a final volume of 510-530 µL were added in 15 mL falcon tubes after a 5×10^{10} exosomes suspension had been transferred in the tube. The final concentrations used in the modification protocol considering a final volume of 510 µL are reported in ***Table 1***.

	Label names of formulations				
Components	Exo FITC	Exo PEG	Exo FA 1	Exo FA 6	Exo FA 30
FITC-DHPE	19.5 µM (9.9 nmol)	7.8 µM (4 nmol)	7.8 µM (4 nmol)	7.8 µM (4 nmol)	7.8 µM (4 nmol)
$mPEG_{2kDa}$-DSPE	-	11.8 µM (6 nmol)	5.9 µM (3 nmol)	5.9 µM (3 nmol)	5.9 µM (3 nmol)
FA-$PEG_{3.4kDa}$-DSPE	-	-	1.2 µM (0.6 nmol)	5.9 µM (3 nmol)	29.4 µM (15 nmol)

Table 1 *- Concentrations and moles of reagents used to modify EVs.*

Mixtures were vortexed few seconds and incubated 30 minutes at room temperature protected from light. After incubation time, modified exosomes were purified from reagents in excess either with OptiPrep™ density gradient or through a Sephadex® G-50 Superfine size exclusion chromatography resin (GE Healthcare, IL, USA). In the first case gradient was set as described above, mixtures were added with 1.75 mL of iodixanol stock 60% (v/v) and samples injected gently in the bottom of ultracentrifuge tubes after the removal of 2.5 mL volume from the top of the gradient. Fractions collection and

purification from iodixanol was performed as described in Section 2.2.4. The selection of the fractions containing modified exosomes was done by measuring the absorbance at 496 nm and the emission at 521 nm of the fluorescent label in 96-well plate using Varioskan LUX Multimode Microplate Reader.
When EV were purified by SEC Sephadex G-50, DPBS was used as mobile phase and at least 24 fractions with volumes of 1 mL were collected and analyzed in an analogue way at the microplate reader.
Fractions were tested with NTA to detect the presence of early eluting vesicles. Once modified exosomes were stored at –80 °C.

2.2.7 PEG coating assessment

Purified exosomes were modified as described above and were eluted in a SEC column loaded with a Sephadex® G-50 stationary phase and a PBS mobile phase with a flow rate of about 1mL/min. After fraction collection and analysis of FITC fluorescence at 521 nm, a pool of fractions eluted at 10th-13th mL of the run was chosen as corresponding to unbound PEG-DSPE and FITC-DHPE micelles. Method was validated by eluting FITC-DHPE and PEG-DSPE alone. These fractions were frozen in liquid nitrogen, lyophilized and finally re-dissolved in MilliQ water to have a 10-fold concentration of PEG. Iodine assay for PEG quantification was performed on the exosome free volumes fractionated from the different formulations with Agilent Varian Cary 100 spectrophotometer. Efficiency of modification was estimated by subtracting the weight of unbound recovered PEG to the weight of total PEG loaded in the mixtures. The experiment was repeated in triplicate for each formulation.

2.2.8 Flow cytometry uptake analysis

1.5×10^5 PC-3 cells were seeded in 6-well plates (Corning Costar, Sigma-Aldrich) in 1 mL of vesicles-depleted complete culture media and were grown for 24 hours. On the following day, cells were washed with DPBS and were conditioned with 1 mL of 3×10^9 exosomes suspension in normal medium for 6 hours. After the incubation time, medium was removed and plates were washed extensively with DPBS four times to ensure the removal of exosomes which did not associate with cells. Trypsin was added to detach cells. Then, all wells were added of 1 mL of DPBS to deactivate trypsin and recover the samples in flow cytometer tubes. Cells were pelleted for 10 minutes at 800 g and re-suspended in about 600 µL of DPBS. BD Accuri Plus B6 flow cytometer was set to record 10,000 events per sample and FITC fluorescence was detected with a 488 nm laser. Data was handled with FlowJo v10 software: MFI values and percentages of FITC positive cells were determined and data were normalized considering the relative fluorescence of

the different formulations previously measured with Varioskan LUX multiplate reader (n=3). Statistical determinations were performed using one-way-Anova and t-student to confront each data set independently.

2.2.9 Paclitaxel loading

Protocol adjustment was studied in the attempt to show the efficiency of loading of paclitaxel (PTX) in EVs. Starting from previous evidence, 5 mL or 1 mL of 7.5 μM Paclitaxel solution in DPBS were used and incubation with 2×10^8 exosomes/mL at RT for 1 hour. After initial attempts, protocol was modified and 10^9 exosomes/mL at 37 °C for 1 hour was used.

At first, paclitaxel solubility in DPBS was assessed via RP-HPLC (conditions in Section 2.2.10) after a sample was prepared as follows: a 10 mg/mL suspension by dispersing paclitaxel powder and agitation overnight; on the day after, non-dissolved compound was removed from supernatant by centrifugation at 10,000g for 5 minutes; this solution was centrifuged again at the same speed and time and supernatant was analyzed by HPLC. Thus, paclitaxel was solubilized in DMSO in a concentration of 10 mM and diluted in DPBS to have a final concentration of DMSO below 0.1% (v/v) and paclitaxel 7.5 μM.

To sum up, 4 different protocols were tried:

1. Incubation of 2×10^8 exosomes/mL with 7.5 μM PTX either at RT or 37 °C for 1 hour. Purification method: ultrafiltration with Amicon U15 10 kDa MWCO spinned for 30 minutes at 5000 g at 4 °C.
2. Incubation of 10^9 exosomes/mL with 7.5 μM PTX at RT for 1 hour. Purification method: ultrafiltration with Amicon U15 10 kDa MWCO spinned for 30 minutes at 5000 g at 4 °C.
3. Incubation at 37°C/Sonication at RT of 10^{11} exosomes/mL with 7.5 μM PTX. Protocol of sonication derived from Kim et al[131]: 6 sonication cycles of 30 seconds ON/OFF for 3 minutes per cycle, followed by 2 minutes cooling on ice. Omni Sonic Ruptor 250 ultrasonic homogenizer (Omni International) was equipped with 5/32" Stepped Micro Processing Tip and set at 20% amplitude.
   Purification method: SEC was used for removal of non-loaded paclitaxel; a column of 140 mm×20 mm was packed with PBS. Sephadex G-25 medium was used and eluted fractions were tested spectrophotometrically at 227 nm to detect PTX. Fractions containing exosomes (4-7) were pooled and concentrated by ultrafiltration.
4. Incubation of 10^9 exosomes/mL with 7.5 μM PTX at 37 °C for 1 hour. Purification method: ultrafiltration with CentriCon YM-10 Centrifugal Filter (Millipore), spinned with a Hermle Z300 centrifuge set at 2,500 rpm for at least three washing steps of 15-20 minutes in DPBS.

Protocol 4 was adopted to load exosomes for the cell viability studies.
After purification of non-associated PTX, 50 μL samples of loaded exosomes were analyzed by RP-HPLC to detect paclitaxel (see section 2.2.10).

2.2.10 RP-HPLC analysis

A Jasco RP-HPLC system equipped with a Luna C18(2) 250×4.6mm 100 Å reverse phase column by Phenomenex was used for two applications:

- FITC-DHPE quality control
- Paclitaxel (PTX) quantification to derive loading efficiency of EVs.

The quality control of FITC-DHPE and possible presence of free FITC was derived according to this procedure: 1 mg/mL of FITC-DHPE was dissolved in DMSO and an equimolar solution of 6-aminofluorescin in methanol was used as control. HPLC system flow was set at 1 mL/min and injected samples were eluted with mobile phase of H_2O/ACN both added with 0.05% trifluoroacetic acid (TFA) used with the gradient condition in ***Table 2***.

H_2O%	ACN%	Gradient time (min)
95	5	2
5	95	30

Table 2 *- Gradient liquid phase method for elution of FITC-DHPE.*

Fluorescein isothiocyanate was detected with a UV-2075 UV detector and a FP-2020 fluorescence detector measuring absorbance at 496 nm and emission at 521 nm.
Purity of the conjugate was comparatively derived from the peak areas of FITC-DHPE chromatogram to the peak of 6-aminofluorescein. The relative percentage of area was considered as the grade of purity. PTX was quantified by eluting the HPLC system in isocratic mode of H_20 + 0.05% (v/v) TFA and ACN + 0.05% (v/v) respectively at 45/55 in composition and 1 mL/min in flow. Elution was monitored with UV detection at λ=227 nm and a calibration curve was prepared from 1 mg/mL PTX stock in methanol with serial dilutions from 0.1 μg/mL to 100 μg/mL (***Figure 9***). Samples of exosome loaded with paclitaxel (exoPTX) in the different formulations were diluted 1:1 with acetonitrile and centrifuged 10 minutes at 10,000g before injection in the instrument.

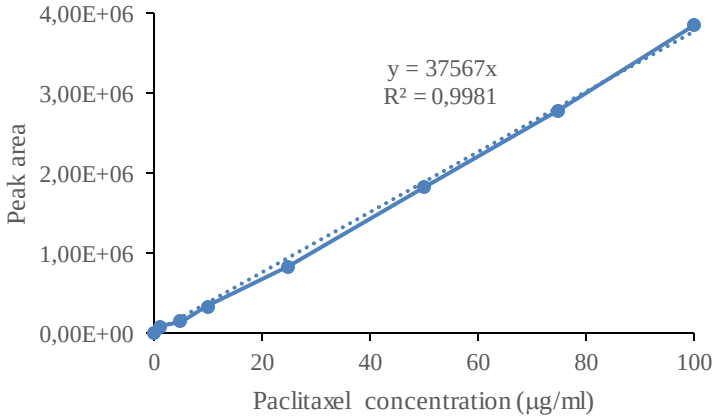

Figure 9 *- Paclitaxel calibration curve by RP-HPLC.*

2.2.11 Cell viability assay

To assess the cytotoxicity of EV formulations, MTT assay was used. The IC50 of free paclitaxel was assessed with PC-3 cells. 10^4 cells/well were seeded in 96-well plates and grown overnight in RPMI medium supplemented with L-glutamine, Penicillin/Streptomycin and 10% fetal bovine serum. On the following day, cells were washed twice with PBS w/o calcium and magnesium and treated 24 hours with free paclitaxel at ten different concentrations in a range of 1-250 nM and incubated at 37 °C in controlled atmosphere.

exoPTX toxicity evaluation was conducted by choosing a dose equivalent to the IC25 of paclitaxel. Data were normalized considering the toxic effect of exosomes alone (no paclitaxel), EVs spiked with equimolar amount of paclitaxel encapsulated in paclitaxel loaded EVs or free paclitaxel.

After incubation time, medium was removed and cells were added of to 180 µL/well of serum-free medium and 20 µL of MTT reagent (from 5 mg/mL in PBS). Solutions were kept protected from light at 37 °C for 3 hours. Later, the formation of formazan crystals was observed on the bottom of the wells: supernatant was removed and crystals re-suspended in 200 µL DMSO. Plates were put under agitation for 15 minutes before recording the absorbance at 570 nm with iEMS Microplate Reader by MTX Lab Systems (Bradenton, FL, USA).

3. Results and Discussion

3.1 FA-PEG-DSPE conjugate characterization

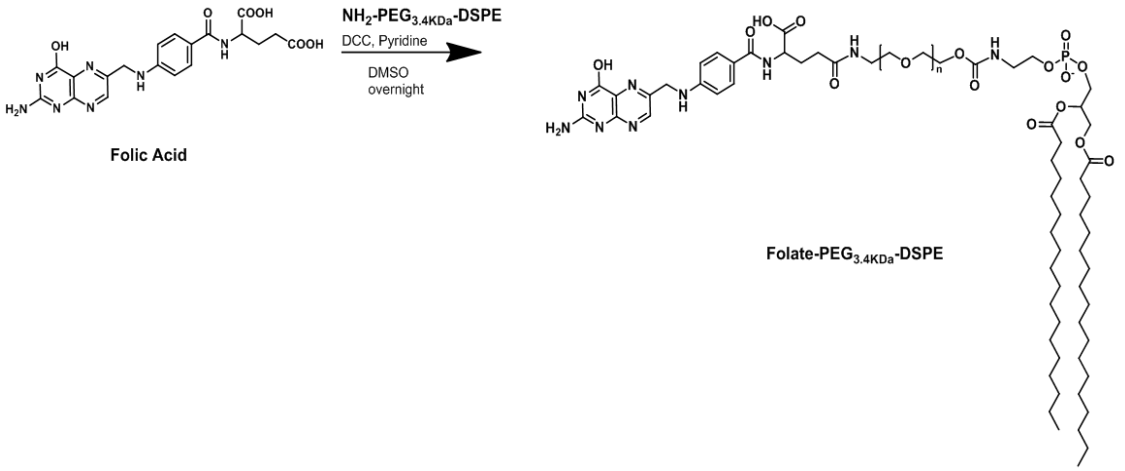

Figure 10 *- Conjugation of folic acid to NH_2-PEG-DSPE: reaction condition and catalysts.*

The synthesis of the conjugate used as targeting agent is described schematically in ***Figure 10***. In particular, dicyclohexylcarbodiimide (DCC) was used as condensing agent as it is involved in the catalysis of nucleophilic attachment of carboxylic acids to amino groups. It forms an isoureic intermediate that activates the COOH group providing for a leaving group with high reactivity towards nucleofiles. The presence of pyridine ensures a basic environment to favor the reaction of DCC with the carboxyl group.

The issue with the use of DCC for folic acid activation is that the reaction is desirable to involve only the γ carboxyl group of the glutamic acid as the α carboxyl group is important for receptor recognition. For this purpose, we used equimolar ratio of Folate and DCC for activation. Folate:DCC:NH_2-PEG-DSPE were reacted at a 5:5:1 molar ratio. After removal of DCU by-product with ether precipitation, conjugate was purified by dialysis and the conjugation yield was assessed from the ratio of folic acid/PEG (%) measured by UV-Vis spectrophotometry and Iodine test: 99%.

The conjugate recovered after purification resulted to have a molecular weight 4655 Da as shown by the mass analysis (***Figure 11***).

The identity of the conjugate was assessed by 1H NMR in deuterated DMSO after freeze-drying and powder re-suspension. Indeed, ^{1}H-NMR (400 MHz) revealed DSPE and PEG peaks at 0.83 and 1.22 ppm and at 3.4 – 3.6 ppm, respectively, while folic acid aromatic system signals were identified at 6.5 – 8.5 ppm as expected.

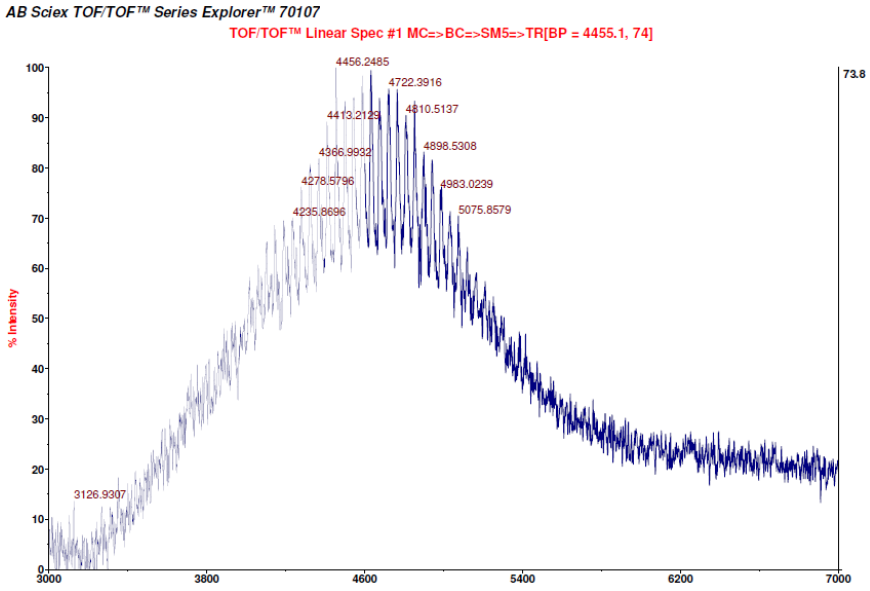

Figure 11 *- MALDI-TOF spectra of FA-PEG-DSPE conjugate.*

3.2 Exosomes modification approach and efficiency

The molar ratio of agents used for decoration of EV surface was established in a previous work. Nevertheless, an accurate characterization of the system to assess the decoration efficacy with the PEG-lipids was still missing. Thus, the decoration procedure was performed using FA-PEG-DSPE in concentrations 5-fold lower and 5-fold higher than the one used in previous studies (5.9 μM) while the number of modified EVs was kept constant to 5×10^{10} exosomes.

This investigation had two aims: define the optimal density of targeting agent which could be stably inserted in the bilayer of exosomes and correlate it to the cell targeting efficacy and cellular uptake.

The composition of the formulations used are described in Section 2.2.6 and they will be referred as:

- exoFITC: exosomes labelled with FITC-DHPE, no PEG-lipid (control)
- exoPEG: exosomes labelled with FITC-DHPE and decorated with $mPEG_{2kDa}$-DSPE

- exoFA1: exosomes labelled with FITC-DHPE, and decorated with $mPEG_{2kDa}$-DSPE and FA-$PEG_{3.4kDa}$-DSPE in lower amount
- exoFA6: exosomes labelled with FITC-DHPE, and decorated with $mPEG_{2kDa}$-DSPE and FA-$PEG_{3.4kDa}$-DSPE in medium amount
- exoFA30: exosomes labelled with FITC-DHPE, and decorated with $mPEG_{2kDa}$-DSPE and FA-$PEG_{3.4kDa}$-DSPE in higher amount

The control exoPEG was formulated with the double amount of $mPEG_{2kDa}$-DSPE compared to the targeted formulations in order to compensate for the contribution of PEG provided by the FA-PEG-DSPE.

After initial attempt to remove iodixanol from exosomes modified using the density gradient fractionation, we decided to perform the quantification of PEG association to EV after Sephadex G-50 SEC purification. Indeed, iodixanol is a large and viscous compound which would interfere with the iodine assay for the assessment of PEG. OptiPrep gradient was anyway used to produce modified exosomes in large scale for uptake or loading studies.

In this case, decorated exosomes were obtained after DGU (density gradient ultracentrifugation) purification as reported in ***Figure 12***. The purification process allowed to fractionate the FITC labelled EVs (in fractions 4-7) from late eluted fractions containing FITC-DHPE micelles. The large ratio of non-bound FITC-DHPE to EV with respect to the associated one was possibly due to dense FITC-DHPE micelles that can migrate to the bottom of the gradient. Furthermore, we analyzed FITC-DHPE by RP-HPLC, as described in Section 2.2.10. We noticed that the purchased FITC-DHPE contains 28% of free NH_2-fluorescein which may result in further instability.

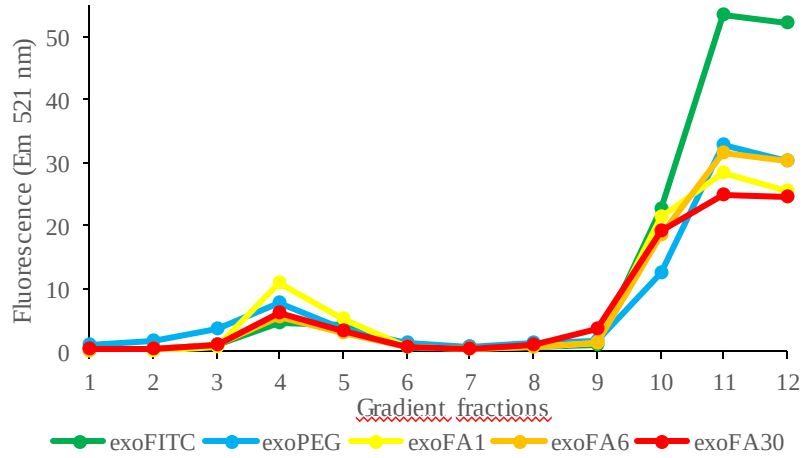

Figure 12 *- OptiPrep density gradient fractions (1 mL) analyzed by fluorescence of EV loaded after modification with functional agents. EVs were always identified in fractions 4-7. The bottom fractions represent micellar aggregates containing FITC.*

Figure 13 shows the chromatographic profiles obtained by running formulations of extracellular vesicles through a Sephadex G-50 prepacked column by detecting the fluorescence. The first fluorescent peak appears in fractions 5-8 and it is related to the labeled EVs which are not retained by the resin and excluded at early time due to their hydrodynamic size, as confirmed by NTA. FITC-DHPE self-associated and associated to micelles of PEG-lipids are eluted from fraction 13 until fraction 32.

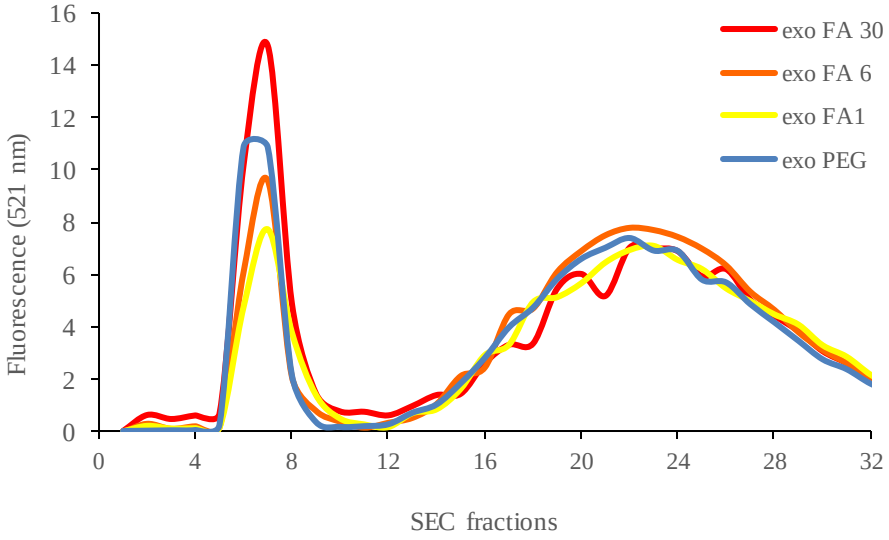

Figure 13 *– Sephadex G-50 chromatograms of FITC-DHPE labelled EV formulations detected by fluorescence (Abs λ=496 nm Em λ=521 nm). Fractions of 1 mL were collected. Separation of EVs from FITC-DHPE micelles and FITC-DHPE/PEG-DSPE micelles is evident and reproducible (n=3)*

The PEG-DSPE alone and FITC-DHPE alone were loaded in the column as a control to validate the method (***Figure 14***). They are eluted after faction 15 and 20 respectively which confirmed that the purification method to remove these component non associated to the EV is efficient.

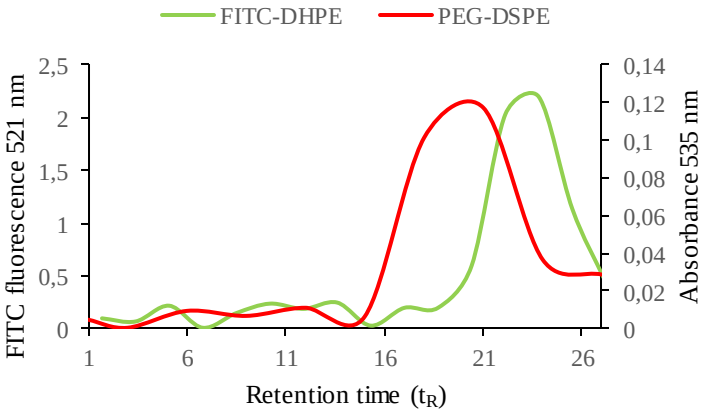

Figure 14 *- Sephadex G-50 chromatograms of 20 μM FITC-DHPE and 500 μg/mL PEG-DSPE. Fractions were analyzed by fluorescence to detect FITC-DHPE and with Iodine test for PEG detection.*

Thus, the fractions that contain PEG-DSPE micelles that did not associate with exosomes were tested by iodine assay after being concentrated by lyophilization. Samples were analyzed and concentration of PEG was derived from a calibration curve. obtained with serial concentrations of $mPEG_{2kDa}$-DSPE from 1 µg/mL to 18 µg/mL.

The ***Figure 15*** reports both the quantities in weight of PEG non bound to the formulations and the efficiency of surface decoration with respect to initial moles of PEG-lipid added to the mixture. A comparable efficiency of modification was observed regardless the amount of PEG added.

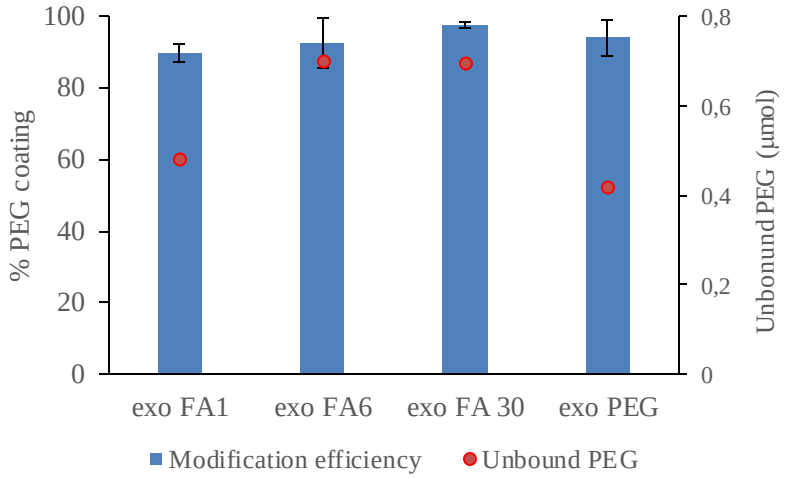

Figure 13 *- PEG coating efficiency (blue bars) and non-associated PEG (uMols) (red dots) to exosome formulations after choromatographic fractionation with SEC. (n=3)*

On ***Table 3***, we estimated the density of PEG chains per exosomes based on the area of exosome surface by approximating their shape to a perfect sphere and a size of 129 nm.

	nmoles FA PEG	Total nmoles PEG	Total PEG chains	PEG chains/exosome	**PEG chains/nm^2**
exoFA 1	0.53	3.22	1.9×10^{15}	38,851	**0.73**
exoFA 6	2.77	5.55	3.3×10^{15}	66,838	**1.26**
exoFA 30	14.68	17.62	1.1×10^{16}	212,206	**3.99**
exoPEG	-	5.64	3.4×10^{15}	67,904	**1.28**

Table 3 *- Approximation of PEG density on exosomes surface, considering the efficiency of decoration shown in Figure 15. Exosome average surface was derived by average diameter measures by NTA.*

We expected that a density in PEG on a drug delivery system surface should be around 1 PEG chain/nm^2 (Rabanel, J.-M. et al., *J of Control Release* 185, 71–87, 2014). Thus the density of PEG in the EV formulations we prepared is compatible with this request with the exception of exoFA30 which has a density of PEG that is almost 4 times higher. This

may be due to the quite high ratio of PEG-DSPE used to coat the ExoFA30 formulation. However, the purification method to remove unbound PEG-DSPE may suffer from a bias due to material loss with adsorption on the column matrix which cause an underestimation of non-associate PEG-DSPE to EVs.

3.3 Exosomes characterization

Exosomes were characterized after DGU (density gradient ultracentrifugation) purification and after each surface modification step, to assess the average size and the zeta potential. Nanoparticle Tracking Analysis was used to measure particle size distribution, while zeta potential was assessed by Dynamic Light Scattering (***Figure 16***).

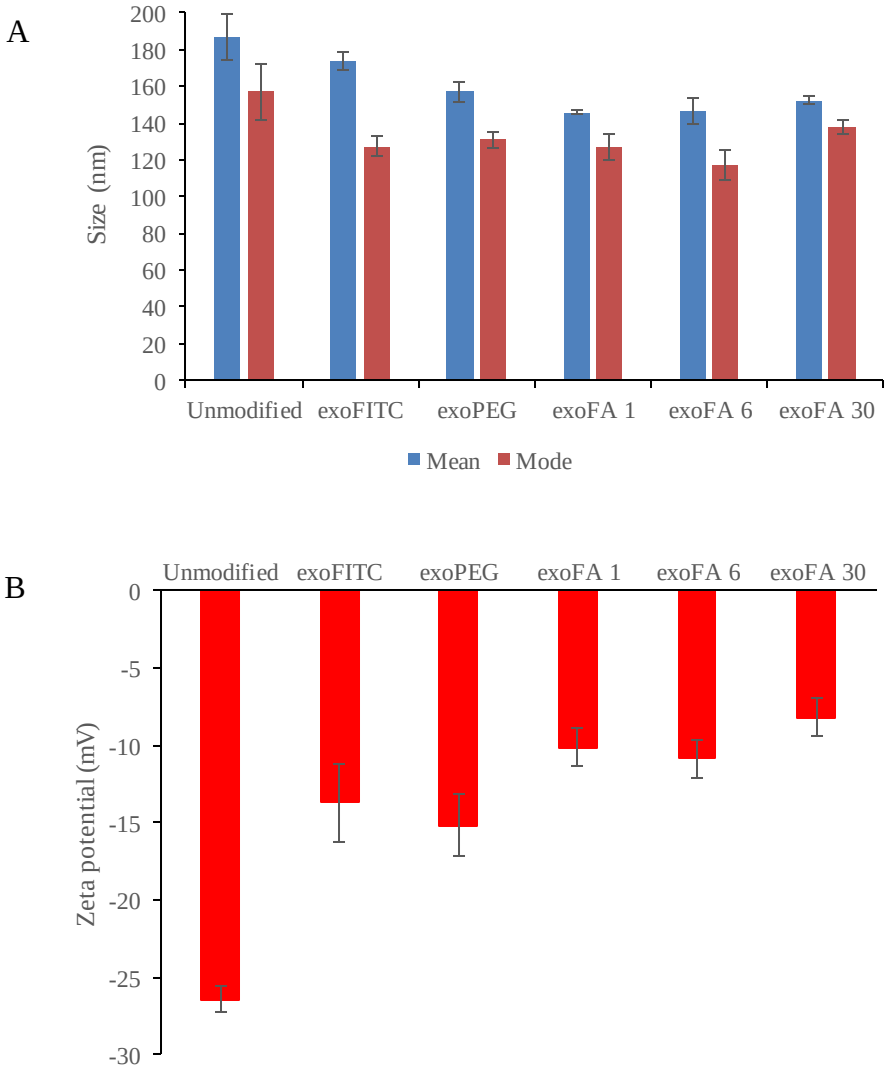

Figure 14 *- Average size of exosomes before and after surface modification as determined by NTA (A). Zeta potential of the same formulations (B). (n=4)*

Data from NTA are expressed as mean and mode of particle diameter as calculated by the instrument according to the Stokes-Einstein equation. Figure 15 shows that, after anchoring to the lipid bilayer of FITC-DHPE and PEG-lipid, the sizes of exosomes do not change significantly and result in an average diameter in the range of 120-150 nm, which is suitable to exploit EPR effect for tumor biodistribution. The modal distribution of polydisperse vesicles, such as exosomes, is usually a more reliable way to report average size and shows a slight decrease of diameter from unmodified samples to modified ones. This can be attributed to a partial rearrangement of lipid bilayer during the two steps of gradient purification to remove EVs from unbound reagents resulting in size contraction. In general, PEG coating is not expected to increase remarkably the size of a drug delivery system. Also we expect phospholipid-linked polymer to be flexible and most likely close to the surface to exert the shielding effect.
Concerning zeta potential, this feature provides information about the overall charge of a colloidal system. Since the EV are closed containers full of water, the zeta potential is mostly dictated by the bilayer associated molecules, in particular proteins and polysaccharides. We observed a significant decrease of the absolute value of the negative zeta potential that in naked EVs is −26.4 mV. After mPEG-DSPE coating, negative charges of glycoproteins, polysaccharides or proteins are shielded resulting in a zeta potential of −15.2 mV. Similar decrease was also observed in targeted exosome with FA-PEG-DSPE that showed a zeta potential of −10.9 mV which confirm that a comparable shielding is provided. The decrease of the absolute value of the negative zeta potential appears also in EVs labelled with FITC-DHPE alone, which is responsible for redistribution of charge consistent with a previous work of our group.

3.4 Flow cytometry uptake studies

The selection of the EV formulation with better selectivity to target PC-3 cancer cells, flow cytometry analysis was used to detect the fluorescently labelled exosomes. The incubation time was chosen from previous work of our laboratory and collaborators. We hypnotized that the folic acid on EV surface should increase the association of the vesicles with cells due to biorecognition of the folate receptor of PC-3.
Figure 17 shows the EV association profile to PC3 cells based on the values of mean fluorescence intensity (MFI) derived from the median of the distribution of intensity in the gated cell population and the percentage of FITC-positive cells with respect to the autofluorescence of cells. MFI has been correlated to the relative fluorescence intensity as measured on the formulation beforehand by Varioskan LUX microplate reader.
We report that statistical analysis of the results revealed a not significant difference between data. However, the data showed an increase of EV association to cells as the density of FA increase up to that of exoFA6 but at the highest density tested (exoFA30),

a decrease of EV association to cells was observed. Thus density of FA on the surface of EV is relevant to reprogram their biorecognition of cells, but a threshold of density exsist above which the folate may compromise the association of EV to cells or their uptake. Indeed, the exoFA30 have a lower cell uptake even than the PEGylated negative control

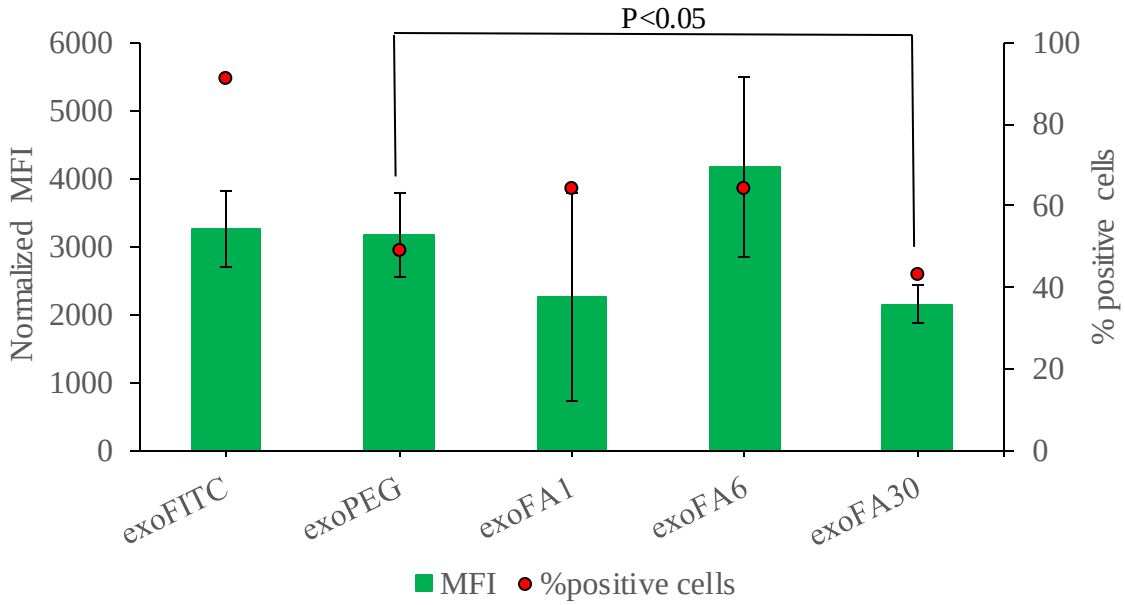

***Figure 15** - MFI profiles and percentage of FITC positive cells incubated with different EV formulations . PC3 cell samples were analyzed by flow cytometry. Statistical analysis performed with t-student (n=3).*

(no FA) and the difference is the only with statistical significance (P value below 0.05). The slight reduction of cell association of EV exoFA1 can be explainde by their lower stability in culture medium, given the fact that density of PEG is lower than the one in exoPEG formulation.

Future studies will be focused on further investigations on the stability of the formulation and the option to increase the density of mPEG-DSPE on targeted EV, while keeping constant the density of targeting agent.

The final targeted formulation selected for further drug loading studies and cell viability was exoFA6, which will be now referred as exoFA.

3.5 Paclitaxel loading

Aiming at loading EV with anticancer drugs for delivery to the tumor, several attempts were tried to encapsulate the chemotherapeutic drug paclitaxel by inclusion in the exosomes membrane. The protocol was taken from previous studies of our group and collaborators and modifications of the process were introduced according to evidences from the literature [131].

Initially, EV were incubated for 1 hour at room temperature with a 7.5 μM PTX solution in DPBS with a concentration of 2×10^8 exosome particles per milliliter. We also performed the same loading procedure by increasing incubation temperature to 37 °C to favor the fluidity of the lipid bilayer and thus the packing of paclitaxel. Under both conditions, after removing non associated PTX through ultrafiltration, we did not obtain the expected efficiencies of loading compared to the previous experience of our group. Despite ***Figure 18*** shows a lower loading of PTX at 37 °C with respect to room temperature, which can be attributed to the fact that, while EV lipid bilayer becomes more

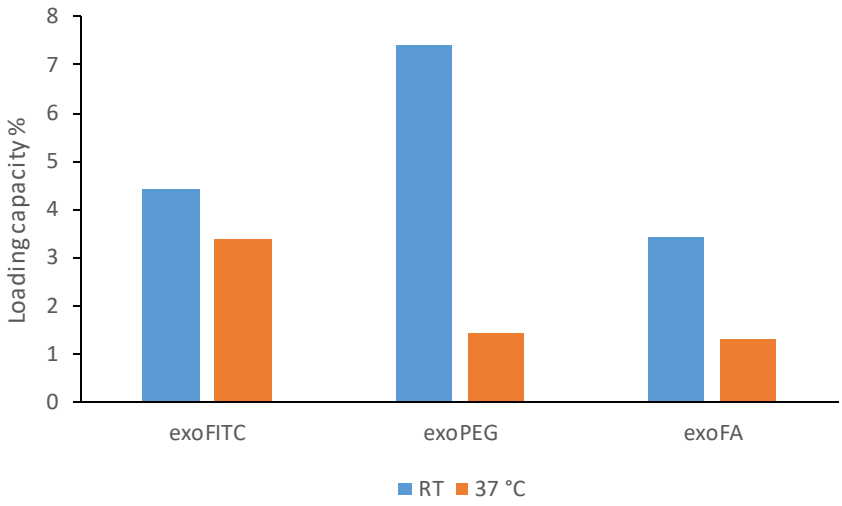

Figure 16 – *Loading capacity of EVs for PTX. Incubation of EV with PTX was performed at room temperature (in blue) and 37 °C (in orange). Preliminary information from measurements near the limit of detection of the instrument.*

fluid at 37 °C, it is also more leaky toward the associated hydrophobic molecules which may desorb from EV after initial association.

Since precipitation of PTX was noticed after drug solubilization, we treated the samples by centrifugation before the purification step and managed to isolate the EVs, yet the amount of loaded PTX was still very low and comparable to the one provided with no centrifugation for all the formulations. In addition, the resulting vesicles showed a lower quality of size distribution compared to the starting EV batch as measured by the NTA (***Figure 19***).

3. Results and Discussion

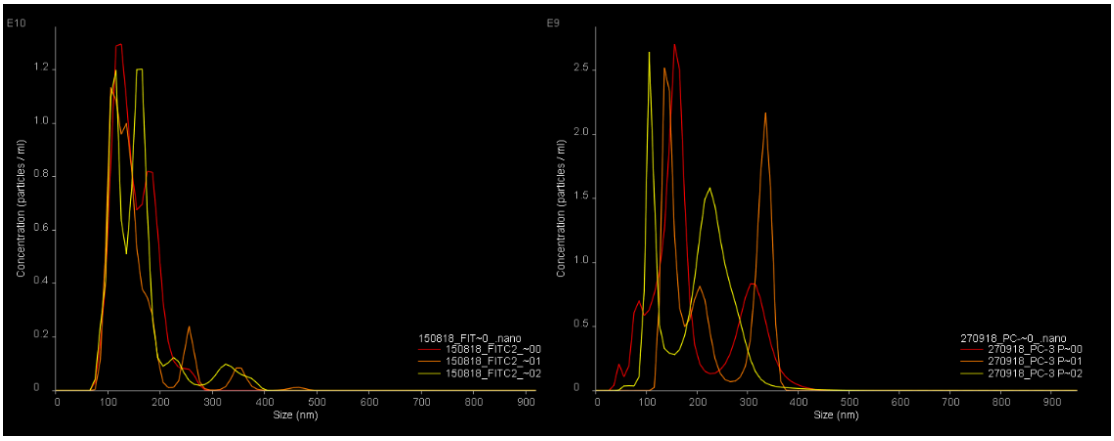

Figure 17 *- Size profiles from light scattering data processed by NTA before (left) and after (right) incubation of the exoPTX solution with the addition of a centrifugal step 5000g 20' of the mixture before purification by ultrafiltration.*

Due to the phase separation of PTX during the loading process, we decided to in assess the solubility of PTX in the buffer used for the loading process. The concentration of a saturated mixture of PTX in DPBS was assessed by HPLC analysis and corresponds to 8.57 ± 0.7 μM. It should be pointed out that the procedure for PTX loading (as stated in previous protocols) used a drug concentration of 7.5 μM. This may have triggered phase separation of PTX and destabilization of the EVs.

Exosomes at higher concentrations (10^9 particles/1 mL of 7.5 μM PTX solution) and higher loading yields were not achieved. Afterwards, a method reported in the literature was attempted according to Kim et al[131]. In brief, we used 10^{11} unmodified exosomes and incubated them at 37 °C with the PTX at a 7.5 μM concentration or treated them under mild sonication condition before purification to remove non loaded PTX with size exclusion chromatography using Sephadex G-25 resin. Since non labelled EV were used for loading, it was difficult to detect them in the collected fractions and they were spotted by BCA assay for protein detection and by Molybdenum blue dye to detect phospholipids; however, this strategy was not efficient due to the quite high detection limits with respect to the EVs concentration. The elution of paclitaxel was monitored by UV absorbance at λ=227 nm (***Figure 20***). EVs were analyzed qualitatively before and after the SEC by DLS: zeta potential was increased to – 17.2 mV which may be attributed to a certain destabilization of membrane properties upon treatment.

3. Results and Discussion

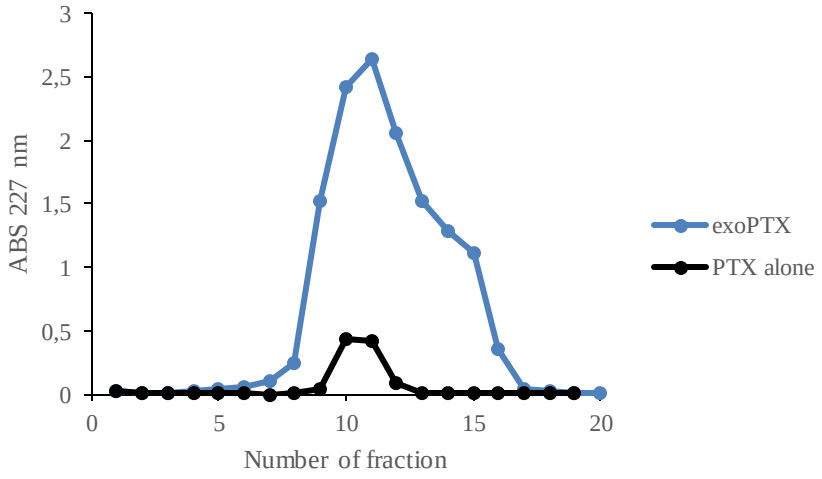

Figure 20 *– Chromatographic profile of EVs incubated with PTX and loaded on a column prepacked with Sephadex G-25 column. In blue the chromatogram of exoPTX samples and in black the elution profile of PTX alone. Fraction were tested by UV-Vis spectrometry at 227 nm to detct PTX.*

After the SEC, early eluting EVs (fractions 1-6) were either concentrated in Millipore Centricon device or analyzed as such. In both cases, results of encapsulation were poor respect to expectations probably because of adsorption of paclitaxel to G-25 column.
Thus, in order to minimize the interaction with the resin of the column used for size exclusion separation, we purified non loaded paclitaxel from EVs through ultrafiltration with Centricon YM-10 from Millipore. We cannot exclude that PTX might adsorb to cellulose membrane of the ultrafiltration device which would limit the applicability of this procedure to remove non-loaded PTX.
However, in ***Table 4***, we report our final formulations used in the viability assay and their loading capacity (LC). As shown in the literature[131], the LC was referred to the weight of drug divided by the weight of exosomal protein as derived by BCA assay. LC values were between 20-33% which would represent an ideal loading for drug delivery purposes with nanocarriers, although better investigations should be performed to validate the removal of the non-loaded PTX. The loading capacity was slightly higher for PEGylated EV probably because the flexibility of PEG and the insertion of the lipid of PEG-DSPE promote fluidity of the bilayer and thus the disposition of the PTX.

	Measured [drug]	Amount of drug (ng)	Weight of exo protein (ng)	Loading capacity
exoPTX FITC	0.32 μM	109.3	540	20%
exoPTX PEG	0.52 μM	178	540	33%
exoPTX FA	0.38 μM	130	540	24%

Table 4 *- Final formulations of PTX loaded EV used for biological MTT assay*

3.6 Cell viability assays

The toxicity of the exoPTX formulations were evaluated by MTT assay, a colorimetric method based on the formation of formazan crystal in living cells. Before administering loaded exosomes to PC-3 cells, a profile of toxicity of the free drug was evaluated with the aim to assessing the IC_{50} of paclitaxel for the specific cell line used in this study. Paclitaxel was dissolved in complete culture medium in the following increasing concentrations: 2, 5, 10, 50, 75, 100, 150, 200, 250 nM. ***Figure 21*** shows the fitted curve in logarithm scale of drug concentration versus the percentage of viable cells after 24 hours incubation with drug respect to the untreated controls. Previous testes and literature

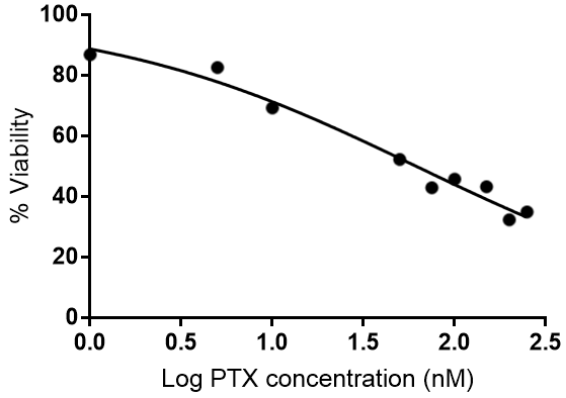

Figure 18 - *Dose-response logarithmic curve of PC3 viability after 24 hours from the incubation with paclitaxel. Incubation time is set at 24 hours*

evidences showed that the non-sigmoidal shape of the logarithmic viability profile is due to some efflux pump activities responsible for drug resistance (Takeda, M. et al. *The Prostate* 67, 955–967, 2007). The IC_{50} derived corresponds to 61.88 nM, which is in agreement with literature for PC-3 cells. Using non-linear regression, we calculated the dose of free drug that causes 75% vitality, i.e. 6.89 nM and we decided to incubate PC3 cells with the different EVs formulation by using the equimolar dose of PTX that provide 25% of cell killing (IC_{25}).

The cytotoxicity provided by PTX alone 6.89 nM, exosomes alone, exosomes spiked with PTX at 6.89 nM or exosomes loaded with PTX at drug equimolar concentration (6.89 nM) in complete medium was tested by MTT assay.

The concentrations of EVs to be used was set in order to provide concentrations of exoPTX particles that provide for 6.89 nM PTX concentration. The number of EV per mL was different among various surface-modified EVs because of their different loading capacity. In detail, the amount of exosomes per well was, 5.38×10^7 exoPTX FITC /100

µl for sample, 3.32×10^7 exoPTX PEG/100 µl for sample, 4.52×10^7 exoPTX FA /100 µl.

We used empty vesicles at the same concentration as a control and we also spiked them with free PTX in order to validate the efficacy of active delivery.

The cytotoxicity profile in ***Figure 22*** shows that Paclitaxel as a free drug in the medium is confirmed to be responsible for 25% cell viability decrease respect to the control.

Interestingly, we noticed a decrease of viability when cells were treated with PTX added as free drug to non-loaded exosomes. However, this was not remarkable with respect to cells treated with free PTX. We speculated that likely the EV can enhance the diffusion of free PTX across the cell membrane which may not take place efficiently when the bilayer of the EVs is altered by the presence of embedded PTX. On the contrary, drug loaded exoPTX did not show a significant toxicity toward cells which may be due to a limited release of the drug by vesicles after internalization.

Future studies will be undertaken to assess the stability of the delivery system and investigate the kinetics of release. Additionally, further studies are required to assess the effect of the time of exposure to exoPTX in order to better profile their therapeutic window and have additional insights on their toxicology.

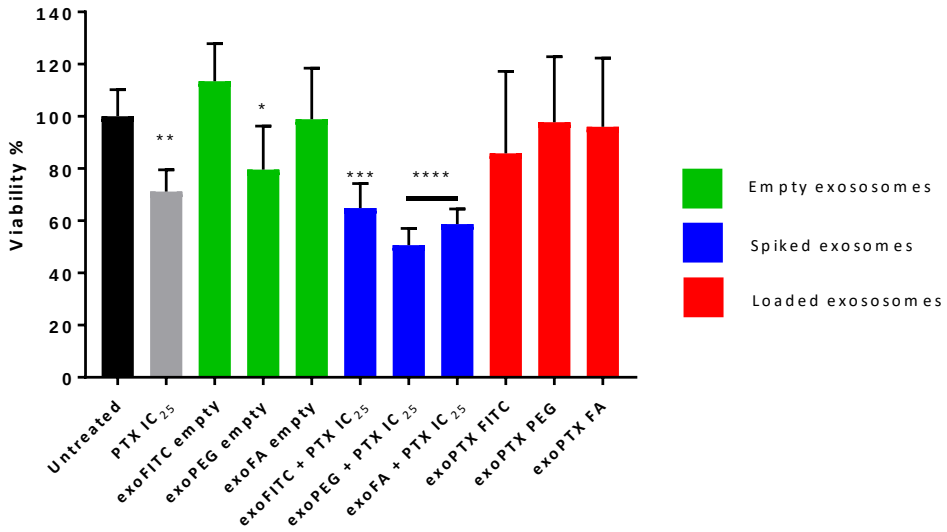

Figure 19 - *Cell viability tests with PC-3 cells. Statistics: t-student vs control, P<0.05 *, P<0.01 **, P<0.001 ***, P<0,0001 ****. (n=1)*

4. Conclusions

This thesis project was aimed at defining the feasibility of developing exosomes as efficient drug delivery system to encapsulate paclitaxel. Furthermore, we sought for the first time to reprogram the targeting abilities of EV by decorating them with folic acid through a flexible PEG chain.

A lot of efforts were dedicated to develop methods for coating EV with functional components, to remove unbound components, to quantify the surface associated modules, to load the vesicles with drug.

We have successfully extracted exosomes-sized extracellular vesicles from a population of cancer cells and we were able to manipulate them after isolation in the attempt to modify their surface. We managed to identify a density of targeting agent that can increase the biorecognition of folate receptor expressing cancer cells while not compromising the exosome stability. Cell uptake is a very complex phenomenon which is further affected by the composition, size, conformation of a semi-synthetic colloidal system. It is still to be defined which pathways are involved in the uptake of semisynthetic targeted EVs and how the synthetic functional components combined on their surface can affect the biodistribution in vivo.

Paclitaxel, despite being a highly hydrophobic drug with a significant tendency to phase separate in aqueous medium, was loaded in EVs although additional investigations to improve loading and remove non loaded PTX are required.

The efficacy of loaded exosomes in terms of cytotoxicity should be further discussed providing information on the mechanism of action of the carrier. Beyond that, we were witnesses of the facilitating effect of EVs to deliver PTX when combined with non-loaded EVs.

This work has shown that exosomes can be coated with different functional agents without compromising their colloidal properties. however, we also learned that it is not trivial to standardize the experimental formulative procedures to reprogram the homing capacity of EVs.

The evidences provided in the project are of uttermost relevance to exploit exosomes as potential future tool in the fight against malignancies.

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