Interaction of mesenchymal stem/stromal cell-derived exosomes with human cell lines for different biomedical applications

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Thesis to obtain the Master of Science Degree in

Biotechnology

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October 2019

Preface

The work presented in this thesis was performed at the Stem Cell Engineering (SCERG) and Biospectroscopy and Interfaces (BSIRG) groups of institute for Bioengineering and Biosciences (iBB) at Instituto Superior Técnico (Lisbon, Portugal), during the period of October 2018 to September 2019, under the supervision of Dr. Nuno Bernardes and Prof. Fábio Fernandes.

Declaration

I declare that this document is an original work of my own authorship and that it fulfils all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Acknowledgements

First, I would like to thank my supervisor Doctor Nuno Bernardes for all the guidance, motivation and patience, and for trusting me with this work. Professor Fábio Fernandes for the availability and counselling throughout this project. I am also grateful to Professor Cláudia Lobato da Silva for introducing me to this work and for the support.

I would also like to thank Miguel Fuzeta for the constant input and for being so welcoming and helpful whenever I needed. To Tiago Dias who gave me support in the spectroscopy work and all the people in the lab who were always ready to help and advise me, a big thanks!

Thank you to the lunch crew for the fun times, to Miguel Ambrósio for being my judgmental writing buddy and to all of the friends I made during the master's, for sharing this experience with me.

I would also like to acknowledge João Pinto, Tânia Rodrigues and Mariana São Pedro, the best dysfunctional support group I could have asked for, thank you for being the best Morangoskas in the world. Also, a big thanks to Marta Reis, who deserves her own special shout out for being my sister, soulmate and mom away from home.

Finally, the sincerest thank you to my mom and dad who have always supported and believed in me no matter what, my brother for being annoying yet amazing and my grandma for the endless belief in my abilities.

Thank you all!

Abstract

Mesenchymal stem/stromal cells (MSCs) are multipotent cells characterized by their unique biological properties, already explored for the treatment of several conditions. However, accumulating evidence indicates that most MSC therapeutic properties are related to their secretome, amongst which are exosomes, small membrane-vesicles that have been shown to play an important role in intercellular communication and numerous biological processes. The mechanisms underlying exosome function are still mostly unknown, as is also their potential in the treatment of pathologies. The main objective of this work was to perform a preliminary characterization of the biological function of exosomes obtained from MSCs of different tissues and healthy donors expanded in bioreactors. In a first approach, the effects of these vesicles on the proliferation of two adenocarcinoma cell lines (MCF-7 and A549) and one representative of the blood-brain barrier (hBMEC) were assessed and a response pattern was noted for both MCF-7 and A549. In addition, A549 invasion through Matrigel was also evaluated and the presence of the MSC-exosomes was seen to be stimulating. Furthermore, control exosomes were isolated, for comparison with the functional activity of MSC-derived exosomes, and were characterized using BCA, NTA and Western Blot analyses. For the tested samples, size distribution and markers characteristic of exosomes were seen. Finally, the labelling of the vesicles was attempted with an anti-CD63 antibody conjugated with FITC and three protocols were tested and compared. These results highlight the possibility for the application of MSC-exosomes for therapeutic purposes, however further assessment of their mechanisms of action is still necessary.

Keywords: *Cell-therapy; Mesenchymal Stem/Stromal cells; Exosomes*

Resumo

Células Mesenquimais Estaminais/Estromais (MSCs) são células multipotentes caracterizadas pelas suas propriedades biológicas únicas, já exploradas para o tratamento de diversas patologias. No entanto, a maioria das propriedades terapêuticas das MSCs têm vindo a ser relacionadas com o seu secretoma, dentro do qual se incluem os exossomas, pequenas vesiculas membranares que demonstram ter um papel importante na comunicação intercelular e outros processos biológicos. Os mecanismos responsáveis pela função dos exossomas são ainda desconhecidos, assim como o seu potencial para o tratamento de patologias. O principal objectivo deste trabalho foi realizar uma caracterização preliminar da função biológica de exossomas obtidos de MSCs de diferentes tecidos e dadores saudáveis expandidas em bioreatores. Numa primeira abordagem, os efeitos destas vesículas sobre a proliferação de duas linhas celulares de adenocarcinoma (MCF-7 e A549) e uma representante da barreira hematoencefálica (hBMEC) foram determinados e um padrão de resposta foi observado para MCF-7 e A549. Adicionalmente, a invasão de A549 através de Matrigel foi também avaliada e a presença de exossomas de MSCs demonstrou ser estimulante. Inclusivamente exossomas de controlo foram isolados, para comparação com a actividade funcional de exossomas-MSC, e caracterizados através de análises BCA, NTA e Western Blot. Nas amostras testadas, foram detetadas distribuições de tamanhos e marcadores característicos de exossomas. Por último, a marcação das vesiculas foi experimentada com um anticorpo anti-CD63 conjugado com FITC e três protocolos foram testados e comparados. Estes resultados realçam a possível aplicação de exossomas de MSCs para fins terapêuticos, porém, ainda é necessária uma avaliação dos seus mecanismos de ação mais aprofundada.

Palavras-Chave: *Terapia Celular; Células Mesenquimais Estaminais/Estromais; Exossomas*

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List of Abbreviations

AT – Adipose Tissue

BBB – Blood-Brain Barrier

BM – Bone Marrow

CM – Conditioned Medium

DMEM – Dulbecco's Modified Eagle's Medium

EVs – Extracellular Vesicles

FBS – Fetal Bovine Serum

FITC – Fluorescein Isothiocyanate

ILVs – Intraluminal Vesicles

MSCs – Mesenchymal Stem/Stromal Cells

MVBs – Multivesicular Bodies

MVs – Microvesicles

NTA – Nanoparticle Tracking Analysis

PM – Plasma Membrane

UC – Ultracentrifugation

UCM – Umbilical Cord Matrix

1. Introduction

1.1. Extracellular vesicles

Extracellular Vesicles (EVs) are secreted membrane-derived vesicles that naturally originate from most cell types in an evolutionarily conserved process. This process includes not only their formation mechanisms but also the overall functions of EVs, which have been proven to be substantially similar in organisms extending from prokaryotes to higher eukaryotes, such as humans and plants[1–3]. The term extracellular vesicles is a generic one, used to describe all of the secreted membrane vesicles, however these are extremely diverse, being distinguished by specific membrane markers, biogenesis and size, which allows for their division into different subtypes[4], including microvesicles (MVs), apoptotic bodies and exosomes.

Microvesicles, also referred to as ectosomes or shedding vesicles, are mostly characterised by presenting diameters between 50 and 1,000 nm and by their distinct biogenesis process, as they are formed through direct outward budding and fission of the plasma membrane, which is a result of the activation of various internal and external stimuli. Shortly, the biogenesis of MVs involves a redistribution of phospholipids between the inner and outer membrane leaflets and is completed through contraction of cytoskeletal structures. The release of these vesicles occurs naturally and is a result of normal cell processes. In contrast, apoptotic bodies are heterogeneous fragments (500-2,000 nm) of cells formed only during programmed cell death. These vesicles are a result of apoptosis which occurs in several stages and culminates in the generation of membrane blebs containing organelles and other remnants of cell degradation processes[5].

Nevertheless, among all EVs, exosomes have raised great interest over the past years either for their biomedical application[6,7] or simply for the better understanding of their characteristics and *in vivo* functions[9,10], and thus are the main focus of this work and will be further discussed in more detail.

1.1.1. Exosomes: Biogenesis, Contents and Biological Properties

Ranging in sizes from 40 to 100 nm[11], exosomes are nanovesicles lined by a lipid bilayer and secreted by most cells upon fusion of multivesicular bodies (MVBs) with the plasma membrane (PM). MVBs are the outcome of the maturation of early endosomes into late endosomes, during which occurs the inward budding of the endosomal membrane and the consequent accumulation of intraluminal vesicles (ILVs). As shown in **Figure 1**, MVBs can have different fates depending on their biochemical properties: they can traffic to lysosomes, acidic compartments where the degradation of their contents will occur, or to the PM, where, upon fusion, they'll release the ILVs into the extracellular space. These ILVs contain proteins, lipids and other cytosolic components, entrapped at the time of their formation and, once released into the extracellular space, become known as "exosomes"[11,12].

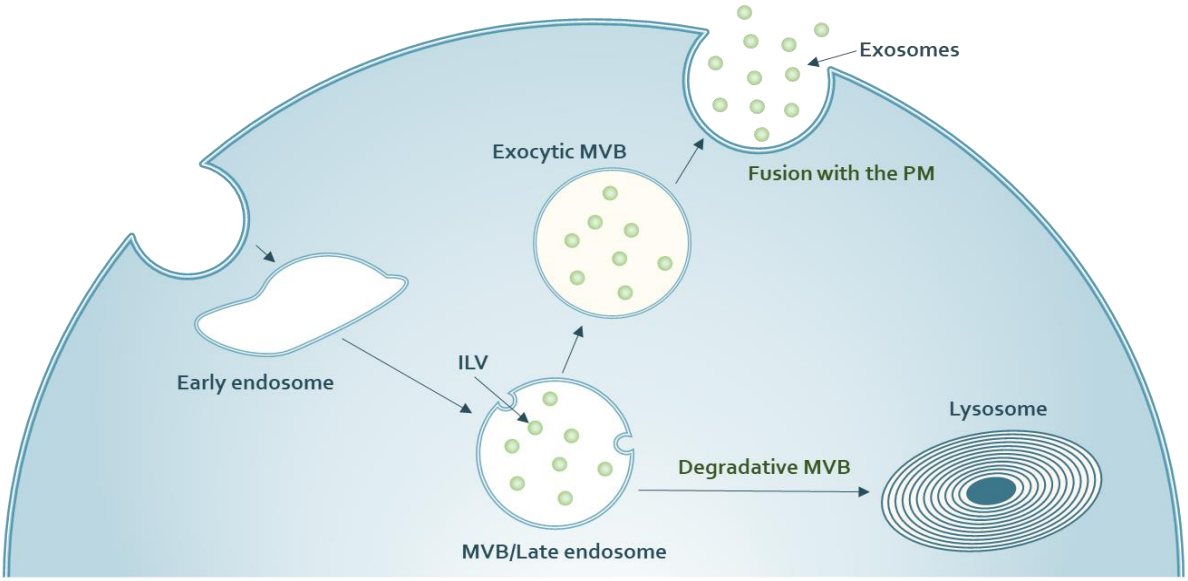


Figure 1 - Schematic representation of the release of exosomes into the extracellular space. Exosomes are produced in the course of the endocytic pathway, starting with the creation of multivesicular bodies (MVBs) during which occurs the accumulation of intraluminal vesicles (ILVs), formed by inward budding of the limiting membrane. These MVBs can either be exocytic (i.e. fuse with the PM, releasing their contents into the extracellular space – exosomes) or degradative (evolving into lysosomes).

This process was first described in the 1980's and it was thought to only have the function of cellular waste disposal[13], however more biological functions have been attributed to exosomes over the past years and several studies have confirmed that these vesicles have an important role in intercellular communication, as will be discussed further on. This interaction between exosomes and targeted cells can lead to the transfer of a wide range of molecules such as proteins, nucleic acids and lipids which might regulate various pathways and alter the phenotype of the recipient cells [14].

In fact, there are already thousands of proteins, lipids and RNA (including mRNAs and miRNAs) molecules registered in different online databases, such as Exocarta[15] and Vesiclepedia[16], that were identified as present in exosomes and other vesicles derived from different cell types, revealing that vesicle composition varies depending on cell/tissue origin. However, and regardless of cellular origin, a conserved set of proteins has been identified as possible exosome markers, including proteins from endosomes (such as Alix and TSG101), the PM (such as the tetraspanins CD63, CD81 and CD9) and the cytosol (such as HSP70). These molecules are a result of their specificity of formation and highlight the fact that exosomes represent a specific subcellular compartment, unlike other EVs. Also by acting as markers of these specific vesicles, they allow for the confirmation of the presence of exosomes in an isolated sample through analytical approaches, such as Western blots, flow cytometry and global proteomic analysis using mass spectrometry techniques[17]. Other identified exosomal proteins include certain metabolic enzymes and transmembrane, signal transduction, adhesion and cytoskeletal proteins, as well as some cell type-specific proteins as is the example of major histocompatibility complexes (MHC) class-I and class-II, which are a characteristic of antigen-presenting cells[11,12].

Regarding their lipid composition, studies show that exosomes differ from their parent cells and are generally enriched in sphingomyelin, phosphatidylserine (PS), cholesterol and ceramide or its derivatives. Also, there has been evidence of a mechanism for the sorting of these specific lipid molecules into the vesicles[12]. In addition, the cargo of exosomes also includes functionally active molecules of mRNA and miRNA, which have been evidenced to partake in numerous biological processes, such as angiogenesis, metastasis and tumorigenesis and also stem cell differentiation, organogenesis and hematopoiesis[18]. This described composition of exosomes has been summarized and is represented in **Figure 2**.

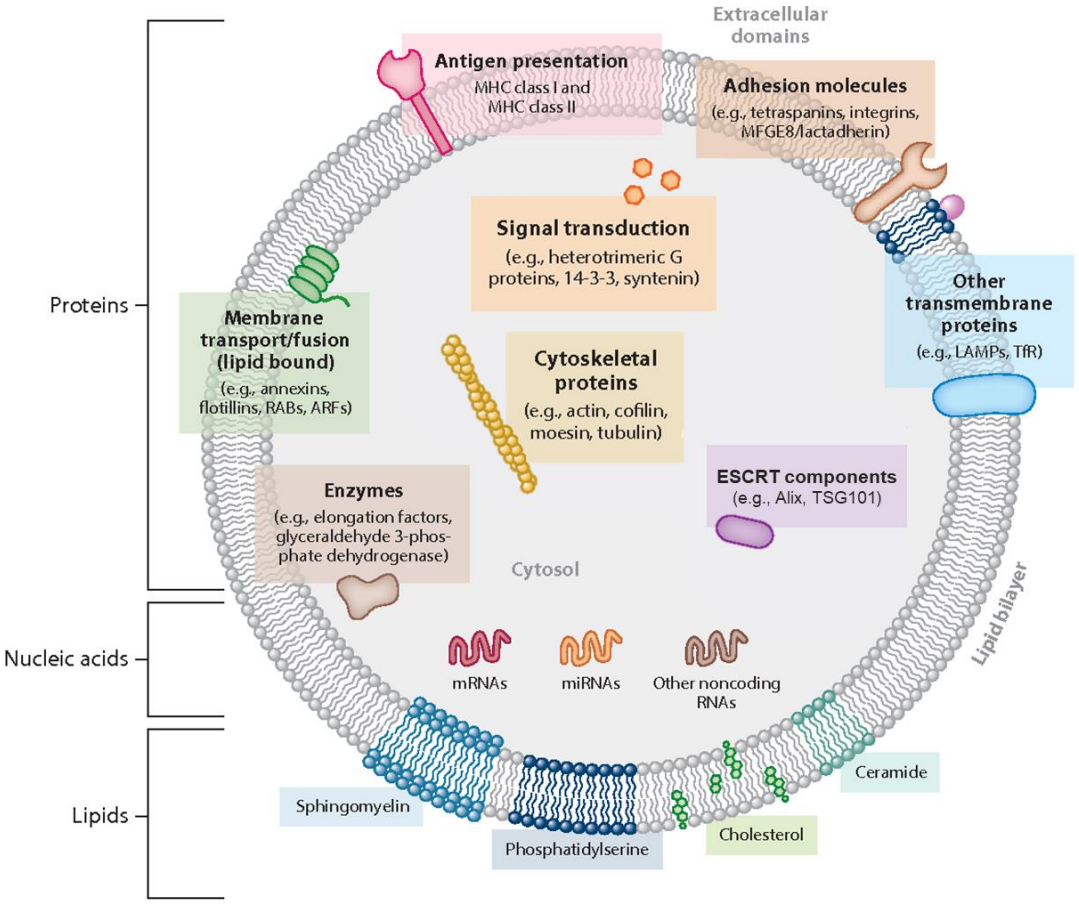


Figure 2 - **Overall composition of exosomes**. Schematic representation of the composition (families of proteins, lipids, and nucleic acids) and membrane orientation of exosomes. Abbreviations - ARF: ADP ribosylation factor; ESCRT: endosomal sorting complex required for transport; LAMP: lysosome-associated membrane protein; MHC: major histocompatibility complex; MFGE8: milk fat globule–epidermal growth factor-factor VIII; RAB: Ras-related proteins in brain; TfR; transferrin receptor. Adapted from Colombo *et al.*[12].

As aforementioned, exosomes can be released by most types of cells, from immune, to stem and tumour cells, and once they have been released, they can directly interact with cells in their proximity or shift to further locations through most biological fluids. The fate of the vesicles will be dependent on their source as well as the targeted cells, which determine the specificity of the binding of the exosomes through specific ligand/receptor pairs[12]. The physiological response elicited by the exosomes can be the result of direct activation of cell surface receptors or the delivery of effector molecules/particles and transfer of membrane contents into the PM of recipient cells. In this way, exosomes, as well as other EVs, participate in the maintenance of normal physiology by being involved in biological functions, like

stem cell maintenance, tissue repair and immune regulation. On the other hand, they can also have a pivotal role in pathogenicity, as observed in tumour biology[19].

As already mentioned, one of the main identified biological functions of exosomes is their role in immune regulation, which was first described in 1996 when Raposo et *al.*[20] discovered that exosomes secreted by Epstein-Barr virus-transformed B lymphocytes contain peptide bound MHC class II molecules and are able to present these MHC-peptides to T-cells, starting an immune response. After several studies, it was possible to postulate a general mode of action of exosomes in antigen-specific immune responses. These vesicles are able to directly activate memory T-cells through their MHC-antigen complexes, and spread the antigens as well as the MHC class II molecules in their surface to dendritic cells (DCs), thereby increasing the number of antigen presenting DCs and, consequently, the activation of naïve T-cells[21].

In contrast, exosomes have also been reported to have an important role in tumorigenesis, tumour angiogenesis, and metastasis, thus being important factors in the development of tumours. Tumorigenesis is the process through which normal cells turn into cancer cells and has been linked to the trafficking of oncogenic proteins and miRNAs by exosomes, who act as mediators in this transformation and modulate the balance between the heterogeneous cancer cell populations. Angiogenesis is the formation of new blood vessels and is one of the most important processes for the progression and growth of tumours. Several different studies have shown that cancer cell-derived exosomes are significantly involved in tumour angiogenesis by delivering angiogenic proteins and modulating the angiogenic function of endothelial cells. Additionally, it has also been found that the intercellular communication by exosomes contributes to tumour metastasis by enhancing tumour cell migration and invasion and assisting in the establishment of a pre-metastatic niche[22].

1.1.2. Methods for Exosome Isolation

Given their intrinsic properties, there has been a growing interest in the optimization of the isolation and purification of exosomes, in order to enable a more in-depth study of their characteristics as well as their application in different contexts. Exosomes can be isolated from several bodily fluids like blood, urine and cerebrospinal fluid, or from conditioned cell culture media, by making use of several techniques. These can be separated into five main different types: Ultracentrifugation-based, Size-based, immunoaffinity-based, precipitation and microfluidics-based [23].

Ultracentrifugation-based techniques are the most commonly used methods for the isolation of exosomes and are characterized by the high centrifugal forces at which they are carried (up to 1,000,000×g [23]). These can be further separated into two different types – Differential ultracentrifugation (UC) and density gradient ultracentrifugation. The first consists in the separation of the exosomes based on their density and size through a series of centrifugal cycles of different centrifugal forces and durations. On the other hand, in the case of density gradient UC, the exosomes are separated based on their size, mass and density in a medium with progressively decreased density (from bottom to top). For this, the sample is carefully layered on the top of the medium and exposed to subsequent rounds of UC, which allows for the particles to move through the density gradient medium, and consequently being

separated into individual zones, which facilitates their recovery. In general, UC is quite advantageous as it is easy to use and yields high amounts of exosomes, however, it is very time consuming and requires expensive specific equipment. Also, the heterogeneity of exosomes and the existing overlap in sizes of EVs may lead to contaminations and loss of exosomes when applying this technique. To overcome this, the two types of UC can be coupled in order to allow for a more effective purification of the vesicles [23].

As the term indicates, in **size-based** isolation techniques exosomes are isolated based on their size or molecular weights. Several strategies can be used for this purpose, two of the most common ones are ultrafiltration (UF) and size exclusion chromatography (SEC). UF makes use of a membrane filter with a defined molecular weight or size cutoff through which the sample is run, consequently resulting in the separation of suspended particles. This method is significantly faster and much more cost effective than UC, however the force used to perform this isolation can have an impact on the vesicles' integrity and therefore compromise the sample[23]. In the case of SEC, the separation occurs in a column containing a porous stationary phase, mostly composed of spherical beads. The pores of these beads are of a specific size, and the smaller components of the solution will pass through the matrix while the bigger ones will not be able to and will therefore be eluted faster, thus allowing for the purification of the desired vesicles. This strategy has been shown to result in a good recovery of target vesicles at a faster pace than UC. Also the integrity of the vesicles is kept as no shear stress is enforced[24].

In contrast to these last methods, **immunoaffinity-based purification** of exosomes, does not depend on their physical characteristics as it makes use of the known exosome markers in order to selectively capture these vesicles through their interaction with specific antibodies[25]. Different approaches have been developed for this type of purification, such as the utilization of antibody-coated beads and microfluidic circuits, and good results have been achieved as highly purified exosomes have been isolated. However, the isolation of the vesicles is done at a very small scale and its success depends on the quality of the initial sample. Therefore, these protocols are not of great interest when it comes to the clinical application of the exosomes[23,25].

Another strategy for isolation is the **precipitation** of exosomes, which is achieved by the alteration of their solubility when some polymers, such as polyethylene glycol (PEG) are employed. These polymers will successfully force the exosomes to precipitate by tying up water molecules as these are the less soluble components of the solution. Currently there are several commercially available kits developed for the effective purification of exosomes and other vesicles, that are compatible with the various complex body fluids, making this a convenient strategy to use as it is very straight-forward. Yet, these kits can result in varied yields and the purity of the isolated exosomes may be questionable as there is the risk of co-precipitation of other components like proteins and polymeric materials[23,26]. Therefore, there is a need for optimization of these methods depending on the desired application of the exosomes.

Still in this context, as has been mentioned, several **microfluidic** approaches have also been employed for the separation of exosomes from other EVs based on a variety of exosomal properties

(immunoaffinity, size and density). For example, in their work, Liu *et al.* presented a viscoelastic microfluidic system for this purpose, in which EVs are fractioned according to their sizes in a low concentration poly(oxyethylene) (PEO) solution. The results were promising, showing high separation purity and recovery achieved with a simple and fast process. Furthermore, this method shows great versatility and potential to be applied in several different fields[27]. Nevertheless, these techniques are still recent and present issues concerning scalability, validation and standardization, hence further testing is still needed in order to enhance their application[23].

All mentioned advantages and disadvantages of these isolation techniques are summarized in **Table 1.** It is possible to see that all of them come with some types of drawbacks and therefore the choice of which method to apply will depend mostly on the desired application of the isolated vesicles. The coupling of different techniques has also been shown to enhance their productivity and assist in overcoming some of the issues. However, this may result in higher costs, longer durations of purification and the increase in error rates. In this way, the development of highly efficient exosome isolation techniques, or the optimization of existing ones, are still necessary, particularly to achieve greater scalability, reproducibility and yield.

Table 1 - Comparison of exosome isolation techniques. Abbreviations - UF: Ultrafiltration; SEC: Size Exclusion Chromatography. Adapted from Li *et al.*[23].

Technique	Isolation Principle	Main Advantages	Main Disadvantages
Ultracentrifugation-based	Density, size, and shape based sequential separations of particulate constituents and solutes	• Large sample capacity and yields large amounts of exosomes.	• High equipment cost; • Long run time; • May damage exosomes.
Size-based	Isolation exclusively based on the size difference between exosomes and other particulate constituents	**UF:** Fast and does not require special equipment **SEC:** High-purity exosomes, preserves the integrity of vesicles.	**UF:** Shear stress induced deterioration of samples. **SEC:** Moderate equipment cost and longer run time than UF.
Immunoaffinity-based	Isolation based on specific interaction between membrane-bound antigens of exosomes and immobilized antibodies	• Allows for isolation of specific exosomes; • Highly purified exosomes.	• Low capacity and yields; • Only works with cell-free samples.
Precipitation	Alteration of the solubility or dispersibility or exosomes using water-excluding polymers	• Easy to use; • Does not require specialized equipment	• Co-precipitation of other non-exosomal contaminants (e.g. proteins and polymeric materials)
Microfluidics-based	Microscale isolation based on a variety of properties of exosomes like immunoaffinity, size, and density	• Fast; • Low cost; • Versatile.	• Lack of scalability, validation and standardization

1.1.3. Using Exosomes as Carriers for Drug Delivery

As aforementioned, exosomes have an important role in the communication and information transfer between cells. This is an important functionality and one that has fuelled great interest into the possibility for their application as drug delivery agents. Exosomes, as well as other extracellular vesicles, have intrinsic features that make them ideal carrier systems, such as their ability to overcome natural barriers (e.g.: Blood-Brain Barrier[28]), cell-targeting properties, low immunogenicity[29] and immunomodulatory effects, which have been shown in autologous settings [30]. Furthermore, due to the fact that they are lined by a lipid bilayer, the cargo within these vesicles is naturally protected from

degradation in the circulation[25], facilitating a higher stability and concentration in the blood of these molecules.

However, the presence of this membrane makes it more challenging to effectively load exogenous cargo (such as therapeutic molecules) onto the vesicles and therefore, several methods to load exosomes have been developed and described. In general, for this, there are two main approaches that can be used: exogenous and endogenous loading.

1.1.3. a) Strategies for loading of exosomes

Exogenous loading is the loading of therapeutic cargo within the vesicles once they have been isolated from the parent cells. It can be further subdivided into passive (i.e. incubation of the exosomes with the cargo molecules so that these are passively incorporated into the vesicles) and active (i.e. disruption of the vesicle membrane to facilitate the packing) loading. On the other hand, **endogenous loading** involves the action of the parent cells, who will deposit the therapeutic cargo directly into the exosomes preceding their release [31].

Passive exogenous loading approaches have been demonstrated by several groups to be significantly effective for the loading of hydrophobic drugs, such as curcumin, doxorubicin and paclitaxel, into exosomes[31]. Furthermore, passive loading of drugs into exosomes can also be achieved by cholesterol conjugation, which is characterized by the enhancement of the loading by covalently binding cholesterol to the cargo, as this molecule will convey increased hydrophobicity. This method is usually applied for the packing of RNA molecules, as these are quite hydrophilic [31].

On the other hand, active loading as already mentioned, involves some type of disruption of the vesicle's membrane and can be achieved through several different techniques, amongst which the most common are **electroporation** and the addition of surfactants. The first is based on the spontaneous formation of pores in the membrane of the exosomes caused by stimulation with an electrical signal, by which time the cargo is incorporated [31]. As for the addition of surfactants, the most widespread method is **saponin permeabilization**, which again involves cargo loading through permeabilization of the membrane of the exosome, as saponin is a detergent-like molecule that leads to the formation of pores by complexing with cholesterol. This method has been shown to be highly effective in exosome loading by Haney *et al.*[32] in their study towards the application of exosomes in Parkinson's Disease therapy. Besides high loading efficiency, persistent release and preservation of the tested molecule's (Catalase) activity, the loaded exosomes obtained by saponin permeabilization showed superior therapeutic effects than those obtained by other methods. These include **sonication** (cargo and exosomes are mixed and later sonicated using a homogenizer probe, membrane integrity is compromised by the mechanical shear force of the probe [33]), **freeze-thaw cycles** (co-incubation of therapeutics and exosomes, followed by fast freezing at -80ºC and subsequent thawing at room temperature. This cycle is repeated at least 3 times [32]) and **extrusion** (the exosome-drug mixture is loaded into and extruded through a syringe-based lipid extruder with a porous membrane, disrupting the membrane and promoting loading [33]) and

also demonstrated high efficiency in loading. All these exogenous loading methods are summarized and represented schematically in **Figure 3.**

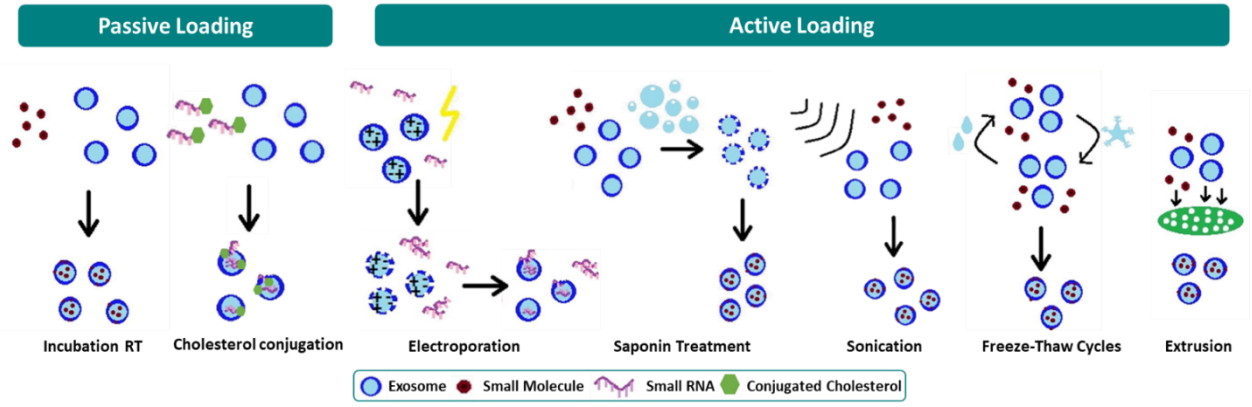


Figure 3 – Schematic representation of exogenous loading of exosomes. This approach can be subdivided in two types of loading: Active and Passive loading. Active loading involves the disruption of vesicle membrane and includes different approaches, such as electroporation, saponin treatment, sonication, freeze-thaw cycles and extrusion. Passive loading is based on the co-incubation of exosomes and the target cargo at room temperature (RT) and can be facilitated by cholesterol conjugation, in the case of hydrophilic molecules (e.g. RNA molecules). Adapted from Vader *et al.*[25].

When it comes to the endogenous loading of exosomes, this is mainly achieved by transfection-based approaches and applied for the loading of small RNA molecules. For this strategy the oligonucleotides (mRNAs/miRNAs/siRNAs), or a plasmid expressing those oligonucleotides, are transfected into the exosome producing cells, leading to an overexpression of the RNA molecule within the cells and the consequent loading of the cargo into the vesicles. This technique has been demonstrated by Wang *et al.* [34], who used lentiviral self-inactivating constructs expressing human miR-let7c (a molecule whose down-regulation has been linked to kidney fibrosis) to overexpress this miRNA in MSCs. After isolation of the exosomes released by the modified cells these were assayed and shown to present increased miR-let7c expression, thus suggesting that the induced overexpression of this miRNA in the MSCs leads to its increased loading into secreted exosomes. Furthermore, the loading of other types of therapeutic cargo has also been achieved through endogenous methods, as is the example of the simple co-incubation of parent cells with cargo. This was demonstrated to be effective by Pascucci *et al.* [35], through incubation of MCSs with paclitaxel (a small molecule anticancer drug) for 24 hours, with successful loading of released exosomes confirmed after isolation.

These data indicate that treatment of donor cells with the target cargo can in fact lead to its efficient loading into exosomes. However, this method shows low drug loading efficiency and may cause cytotoxicity to the donor cells [33]. In the same context, the overexpression of small RNAs may also induce adverse effects *in vivo*, like gene expression changes which could alter the contents inside the vesicles[31]. Additionally, the active exogenous loading of exosomes can come with its disadvantages as well, as some of these methods can induce aggregation of the vesicles, making their purification more difficult. Moreover, the use of surfactants such as saponin, may be toxic for the application of exosomes in *in vivo* situations [33], and it is still unclear whether the disruption of the vesicle's integrity affects their immunogenicity [25]. Therefore, it is safe to conclude that there are several available effective approaches for the loading of exosomes, and it is yet not entirely clear which is the most suitable for clinical applications.

1.1.3. b) Uptake of exosomes by recipient cells

Moreover, in order to facilitate the use of these vesicles as delivery agents it is important to understand the molecular mechanisms by which they and/or their cargo are taken into the cell, as it can improve and simplify the design of the delivery systems. Despite not being fully understood, the uptake process of exosomes can easily be divided into three distinct steps (**Figure 4**), starting with the targeting of the acceptor cell, followed by the internalization of the vesicles and, finally, the delivery of their content [36].

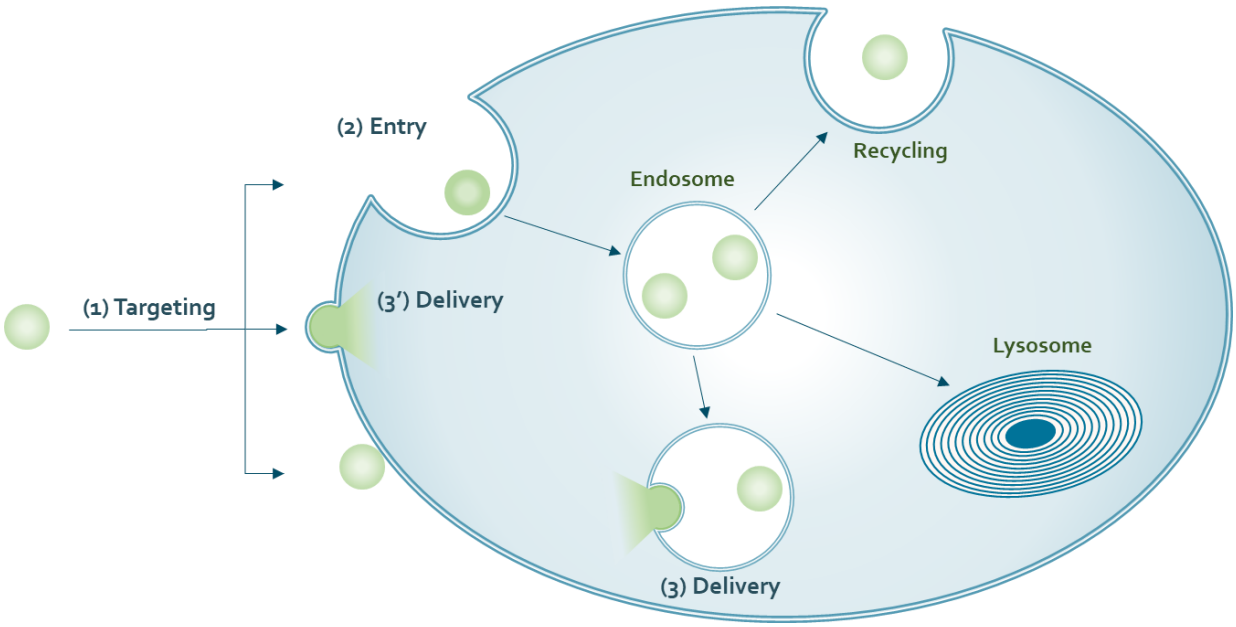


Figure 4 – The three steps of exosome uptake by acceptor cells. (1) exosomes are targeted to the acceptor cell. Docking could occur through specific molecular interactions or through unspecific macropinocytosis or micropinocytosis. (2) When entering the acceptor cell exosomes may be targeted to endosomes. (3) The Internalized vesicles release their content by fusion with the endosomal membrane. Alternatively, they can be re-released to the extracellular space or degraded. (3′) A different, direct route of content delivery to the acceptor cell could involve fusion of exosomes with the plasma membrane.

The first step relies on specific receptor-ligand interactions, several of which have been reported to have an impact on the specific type of internalization of the vesicles, as are the examples of the integrin CD11a and its ligand ICAM-1 and the tetraspanins CD81 and CD9 [37]. Nevertheless, the targeting specificity of these interactions is not yet entirely determined, with some studies reporting selectivity in the binding of these vesicles, depending on both the parent and the recipient cells[38], while others demonstrate that a wide variety of cells are able to incorporate exosomes secreted by a similar array of different donor cells. With this said, it should be further noted that the various forms of exosome uptake have also been shown to differ depending on the recipient cells rather than being solely reliant on the expression of certain exosome marker proteins [39], thus the importance of fully understanding and identifying the underlying mechanisms and targets responsible for the vesicle-cell interactions of interest.

Regarding the internalization of exosomes by acceptor cells, endocytosis is known as the main process responsible and five major routes have been identified and described: phagocytosis, macropinocytosis, clathrin-mediated, caveolin-dependent and lipid raft-dependent endocytosis[40].

Phagocytosis is a receptor-mediated function, mostly performed by specialized cells (e.g. macrophages and monocytes), which involves the engulfment of large particles (>0.5 μm) through invagination of the plasma membrane. This process is highly important in the regulation of immune response as it is used for antigen presentation, however it has also been shown to be performed by other non-phagocytic cells, such as epithelial and cancer cells, and several studies have identified it as a mechanism for the uptake of exosomes [41]. In fact, Feng *et al.* [42] reported a preferential internalization of leukaemia cell-derived exosomes by macrophages, when compared with other cell types, and that these vesicles are targeted to phagosomes and phagolysosomal compartments when within these cells. Additionally, using specific PI3-kinase inhibitors (enzyme involved in the phagocytic process) these authors were able to inhibit the uptake of exosomes in a dose dependent manner, further demonstrating that their entry occurs through phagocytosis. Similarly to this process, **macropinocytosis** involves the formation and projection of cell membrane ruffles that encapsulate a sample of the extracellular fluid and its components. Yet, this mechanism does not require specific receptor-ligand interactions and, therefore, there is no need for direct contact with the internalized material [41]. This endocytic pathway is also characteristic of antigen-presenting cells but can be stimulated in other cells types, additionally it has been shown to have a role in the uptake of exosomes, as is the example of the transfer of oligodendroglia-derived exosomes to microglial cells which was found to occur by macropinocytosis[43].

Another major endocytic pathway, identified for most cell types, is **clathrin-mediated endocytosis** (CME), which involves the formation of vesicles coated in clathrin (an intracellular protein) and formed through a sequential process either triggered by receptor-ligand interactions or performed in a constitutive matter. Furthermore, clathrin-independent pathways have also been described, an example of which is **caveolin-dependent endocytosis**, a mechanism involving small invaginations in the plasma membrane (caveolae) which can be internalized into the cell similarly to the clathrin-coated vesicles. Unlike the previously described, this mechanism doesn't allow for the uptake of large particles, however its range in size falls within that of exosomes (~120nm) and it has, accordingly, been widely associated with the uptake of these vesicles in a variety of works [40,41]. In their study, Horibe *et al.*[39] assessed the exosome uptake efficiency of different cell lines and found that the effect of the inhibition of caveolin and clathrin-dependent endocytosis varied depending on the tested cell, with COLO205 cells demonstrating complete inhibition of exosome uptake in both situations, while HCT116 cells were only affected by the latter. In contrast, the same was not observed for the remaining cell line tested. This study helps support that these endocytic routes are, in fact, relevant for the internalization of these vesicles, however, there isn't a universal mechanism for this process.

Indeed, lipid rafts have also been revealed to have a role not only in caveolin-dependent but also in clathrin/caveolin-independent endocytosis, otherwise known as **lipid raft dependent endocytosis**. These are sections of the PM rich in sphingolipids and cholesterol, as well as protein receptors, and are known to affect membrane fluidity and for their role in exosome biogenesis and trafficking [40]. Moreover, this lipid raft dependent pathway has also been shown to be involved in exosome uptake in studies such as those by Svensson *et al.* [44] who observed co-localization of these vesicles with cholera toxin subunit B, a known lipid raft marker, and a dose dependent inhibition of their internalization to membrane

cholesterol depletion. Also supporting the lack of involvement of clathrin or caveolin in this experiment, no co-localization was found with conventional ligands of CME and the inhibition of this pathway did not cause any disruption in the uptake of EVs. In contrast, the absence of caveolin-1 (a protein required for caveolae formation) was shown to increase the uptake of exosomes, thus demonstrating that this occurs merely through lipid rafts.

All the described endocytic pathways are summarized and represented schematically in **Figure 5.**

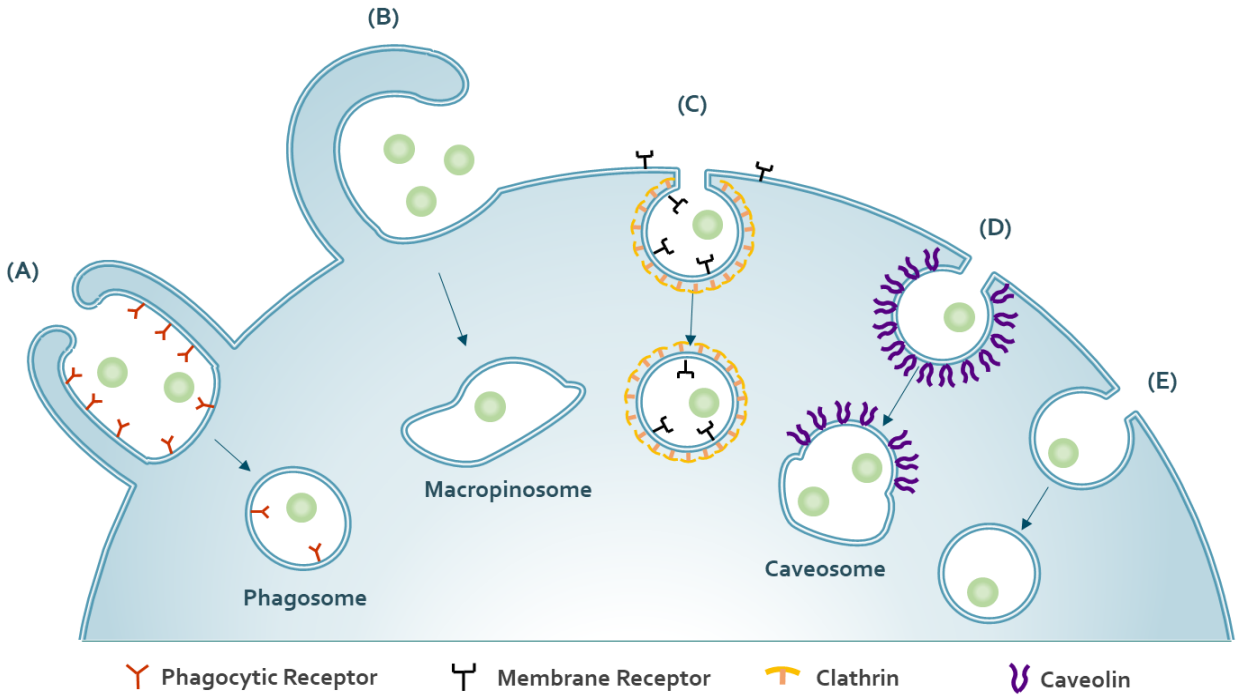


Figure 5 - Schematic representation of the endocytic pathways. Endocytosis is the main process responsible of the internalization of exosomes and other extracellular components into recipient cells. It can be divided in five different routes: **a)** Phagocytosis, **b)** Macropinocytosis, **c)** Clathrin-mediated endocytosis, **d)** Caveolin-dependent endocytosis and **e)** Lipid raft-dependent endocytosis. All of these are characterized by a specific set of molecules and mechanisms to them associated.

Once fully internalized, the final step of exosome uptake is the delivery of their cargo. Nevertheless, most studies focus only on the entry of the vesicles within the cell and their fate hasn't been widely explored. Three possible explanations for the final destination of exosomes are: 1) their re-secretion onto the extracellular space; 2) their degradation inside the endosomes/lysosomes; or 3) their fusion with the endosomal membrane, which would occur through still unknown molecular mechanisms (**Figure 4**). Another possible, alternative, approach for exosomal cargo delivery would be the direct fusion of these EVs with the PM. However, results mostly demonstrate that exosomes are fully internalized by the recipient cells and thus this mechanism is not very likely [36].

In conclusion, currently, there still isn't a consensus regarding exosome uptake and cargo delivery into recipient cells as the molecular and cellular mechanisms underlying this process are not yet fully identified or characterized. Therefore, it would be extremely beneficial to proceed to further investigation in order to fully understand and possibly control this biological process.

1.2. Mesenchymal Stem/Stromal Cells

Mesenchymal stem/stromal cells are non-hematopoietic, fibroblast-like cells, that have the ability to differentiate *in vitro* into multiple mesenchymal lineages such as the osteogenic, chondrogenic and adipogenic cell lines[45].

MSCs were first isolated and described from bone marrow (BM); however, since then it has become evident that these cells are not exclusive to the BM as they have been reported in most tissues, including adipose tissue, peripheral blood and several birth-associated tissues, like amniotic fluid, placenta and fetal membrane, umbilical cord and Wharton's jelly [46]. Nevertheless, and regardless of their abundance in several different tissues, BM is still considered as the prime source for MSCs and BM-MSCs are taken as a standard for the comparison of the quality of MSCs from other sources[46].

The designation "Mesenchymal Stem Cells" is commonly used to refer to all MSCs; however, this has given rise to some controversy as not all of these cells seem to present some of the characteristics of regular stem cells. Given this, the International Society for Cellular Therapy (ISCT) suggested that these cells start being termed "Mesenchymal Stromal Cells", which allows for the maintenance of the well-known acronym MSC. In this case, the extracted cells should only be accurately referred to as "stem" when it has been proved that they in fact have the ability to differentiate into multiple cell types *in vivo* and self-renew in the long-term while maintaining their multipotency[47].

Despite their characteristics, the identification of MSCs within a heterogenous population of cells is still quite challenging, mostly due to the inconsistency in characterization that exists between investigators. In a way to attenuate this issue, the ISCT proposed minimum criteria to define these cells: **1)** Adherence to plastic in standard culture conditions; **2)** Expression of a specific set of surface markers, CD105 (endoglin), CD73 (ecto 5' nucleotidase) and CD73 (Thy-1), and lack of expression of hematopoietic (CD45, CD34, CD14 or CD11b) and immune cell markers (CD79α or CD19 and HLA-DR); **3)** Ability to differentiate to osteoblasts, adipocytes and chondroblasts *in vitro*[48].

1.2.1. Therapeutic Potential

Regarding their therapeutic application, MSCs are quite promising, since they hold distinct advantages when compared with other cell types, namely their wide availability, ease of harvest and expansion, and, when compared to most stem cells, lack of ethical issues, which is a major problem in stem cell-based therapies, particularly those involving the destruction of human embryos[49]. All these features have increased the interest in these cells and promoted a widescale analysis of their intrinsic therapeutic properties.

Some of the better-known therapeutic characteristics of MSCs include their homing ability, multilineage potential, secretion of paracrine factors (e.g. anti-inflammatory molecules) and immunoregulatory effects. As a result, these cells are great candidates for the treatment of autoimmune, inflammatory and degenerative diseases[46].

The **homing** of MSCs, also known as tropism, is defined as their capacity to migrate to and reach a specific site of injury. The exact mechanisms used by MSCs for this migration and homing are still

unknown and it is therefore assumed that they are similar to those described for leucocytes with certain chemokines and receptors being involved. This characteristic of MSCs is very advantageous and highly sought for when it comes to cell-based therapies, as the direct interaction with the host tissues is very beneficial. However many factors influence the homing ability of MSCs like the culturing conditions, cell source and their delivery method, and therefore it is advantageous to track the location of the cells post administration, in order to understand their therapeutic efficiency [46]. In fact, MSCs have been shown to lodge in the lungs after infusion which could be an inconvenience when other tissues are the target of treatment, nevertheless it has also been seen that this preferential homing can be altered by pre-treatment with certain factors, as is the case of a vasodilator.[50]

The **multilineage potential** of MSCs is not only limited to mesenchymal lineages, as in reality these cells have been shown to also differentiate into cells of endodermal and ectodermal lineages like lung epithelial cells, neuronal cells, hepatocytes and insulin producing cells (IPCs).This was shown both *in vitro* and *in vivo* by Choi *et al.*[51] and Katuchova *et al.*[52], respectively, whose works focused on the application of these cells for the treatment of diseases such as type 1 diabetes. Combined, their results allow for the conclusion that MSCs could successfully be used for the treatment of this disease making use of this specific characteristic, as they were successfully differentiated into functional IPCs *in vitro* [51] and an initiation of pancreatic regeneration was registered along with an improve of hyperglycemia in experimental diabetic rats [52].

However, certain aspects of MSCs' clinical application are still unclear, more specifically those related with their properties *in vivo* after transplantation, as there are no certainties on whether they maintain their therapeutic properties in the long run or on the underlying mechanisms responsible for their effects.

In this context, results from several studies have provided interesting and promising results, demonstrating that MCSs do in fact exhibit elevated efficacy in a variety of disease models and have the ability to engraft and differentiate into functional cells of tissues from non-mesodermal lineages, as was previously mentioned. However, the engraftment ability of the cells has been shown to be very poor and short lasting, not providing a definite explanation for such high efficacies[53] and disproving the idea that their therapeutic effect is caused by direct replacement of injured cells.

New hypotheses suggest that the healing properties of MCSs' may be the result of alternative modes of action that modulate the surrounding cellular microenvironment, such as the **secretion of regulatory and trophic factors** (i.e. growth factors, cytokines and chemokines) or of **extracellular vesicles**, whose interaction with recipient cells can influence their phenotype by facilitating the transfer of the latter molecules[53]. Collectively these secreted factors and vesicles can be referred to as the secretome of MSCs and have also been a target of extensive research over the years. Data suggest that the MSC secretome does in fact have therapeutic properties, and it has been applied towards the treatment of a number of medical conditions. As an example, recently Khatab *et al*.[54] assessed the anti-osteoarthritic (OA) effects of the MSC secretome in a mouse collagenase-induced OA model and compared them to those of whole MSCs. In this study, the administration of the secretome of MSCs was shown to lessen structural changes and pain associated with OA, and these effects were seen to be at

least as effective as those seen after the injection of MSCs themselves, demonstrating the potential that the secretome of MCSs can have as a therapeutic approach. Furthermore, the effects of the MSC secretome have also been demonstrated to be enhanced by *in vitro* modification approaches[55], which provides novel possibilities for the application of MSCs in clinical settings.

The secreted extracellular vesicles of MSCs have also been of large interest over the past few years and several studies have already shown the great potential that they may have. This will be further discussed in the next chapter, where the main focus is the application of exosomes from MSCs as therapeutic agents in several different pathological conditions.

1.3. MSC-derived exosomes as therapeutic agents

Considering, as was previously mentioned, that exosomes are known to participate in a plethora of biological activities by maintaining some of their parent cell's intrinsic properties, these vesicles have been seen in the past few years as potential alternatives for whole cells in the treatment of certain diseases and conditions. Also, their low immunogenicity, easier preservation and transfer, as well as high stability *in vivo*, are major advantages when compared with cell-based therapies[56].

As MSCs represent one of the most common cell types applied in experimental therapies, yielding high therapeutic efficacies in several disease models, mainly through secretion of paracrine factors and consequent stimulation of host cells, these cells appear to be an attractive source for the production of exosomes. In fact, MSC-derived exosomes have been an object of study, mostly when it comes to their application in animal models of disease where MSCs have already been shown to exert some action, having achieved promising results[57].

In 2010, Lai *et al.*[7] revealed that previously reported action of MSC-conditioned media (CM) on cardioprotection during myocardial ischemia/reperfusion injury, was related to existing vesicles with exosome-like characteristics. Once purified, the exosome preparation was applied and seen to reduce infarct size in a mouse model of the same condition, even in concentrations much lower than that of the CM. Furthermore, it was also possible to rule out any intervention of circulating cells (like immune cells or platelets), thanks to the chosen mouse model, hereby demonstrating that exosomes are in fact cardioprotective components in MSC paracrine secretion.

Other studies have also shown the applicability of MSC-exosomes in conditions like therapy-refractory graft-versus-host disease (GvHD)[6]. In their work, Kordelas *et al.* treated a therapy resistant GvHD patient with an exosome-enriched fraction of MSC supernatants. Results showed that the exosomes produced immunosuppressive effects on the patient's immune cells and, upon a long duration of treatments, that GvHD symptoms were clearly improved with patient stability being observed for several months. This was one of the first clinical cases from which it was possible to conclude that MSC-derived exosomes may provide a safe and effective tool to treat inflammation associated diseases.

More recently, Rager *et al.*[58] assessed the effects of exosomes isolated from BM-MSCs in the prevention of necrotizing enterocolitis (NEC). The results obtained were similar to those registered for the administration of BM-MSCs, with the intraperitoneal injection of exosomes resulting in improved

bowel wall function, as well as in the reduction of NEC incidence and severity, in a murine model. This again suggests that exosomes may be the main paracrine factors of these cells responsible for the protection of the intestines from NEC, therefore representing a novel, cell-free, preventative therapy for this condition.

The therapeutic potential of BM-MSC-derived exosomes has also been tested for the treatment of several eye diseases. In 2017, Mead *et al.*[59] determined the effect of MSC-exosomes in the survival of retinal ganglion cells (RGC), the loss of which is one of the current leading causes of blindness. The results of *in vitro* assays showed significant neuroprotective and neuritogenic effects after treatment with exosomes, which was concordant to the *in vivo* assays where these vesicles promoted the survival and regeneration of RGC axons, while partially preventing axonal loss and RGC dysfunction. The authors were also able to assess that these therapeutic effects were exerted through miRNA dependent mechanisms. This way, this study comes as an indication that BM-MSC-derived exosomes can be applied for the treatment of traumatic and degenerative ocular diseases.

Additionally, as previously mentioned, research has also focused on the use of exosomes, as well as other MSC-derived EVs, as delivery agents that can be used to carry certain drugs directly to the site of interest. In this context, Pascucci *et al.*[35] demonstrated that MSCs are able to release exosomes loaded with, in this case, an anticancer drug (Paclitaxel). The drug was seen to significantly maintain its anti-tumour effect, indicating that its pharmacological activity was not affected during the packing into the vesicles. These results were promising in this setting, opening the possibility for the use of MCS and their excreted vesicles as drug delivery systems.

Similarly, Tian *et al.*[60] also demonstrated the promising application of MSC-exosomes for drug delivery for cerebral ischemia therapy. In this work, a peptide with high affinity to integrin $\alpha_v\beta_3$ in reactive cerebral vascular endothelial cells after ischemia [c(RGDyK)] was conjugated to the surface of the exosomes, in order to improve their targeting ability. Subsequently, these exosomes were intravenously administered to a mouse model of stroke and it was seen that they were able to target the lesion region of the ischemic brain. Also, the same exosomes were then loaded with curcumin and the results showed the successful suppression of the inflammatory response and cellular apoptosis in the lesioned region, which was more effective than when curcumin or exosomes were applied alone. Given the results, this study also sheds light on the application of exosomes as highly specific drug delivery agents.

It is easy then to understand the scope of possibilities for the application of MSC-derived exosomes as therapeutic agents. In fact, numerous studies have been developed in a wide range of conditions, from heart disease to immunological disorders, all yielding proof that these vesicles present the broad therapeutic effects that had already been attributed to their parent cells. However, the major challenge in the use of MSC-derived extracellular vesicles is the fact that they may be highly heterogenous, depending on cellular source, state and environmental conditions. To reduce the impact of this problem, it is essential to select the most effective MSC source for the production of these vesicles, by comprehension of the correlation between their therapeutic effect and that of their parent cells, as well as the establishment of the specific molecules inside exosomes which mediate their healing potential. This way, it will be possible to optimize their production towards enhanced therapeutic efficiency[56,61].

2. Study Setting and Objectives

The present study fits within the scope of the work developed by PhD student Miguel Fuzeta, in which the main objective is the development of a scalable production of human mesenchymal stem/stromal cell (MSC)-derived exosomes, in vertical-wheel bioreactors (PBS Biotech), with the future intent of applying these vesicles to cancer therapy. Therefore, the main goal of this work is to perform an initial characterization of the functional activity of these exosomes, in a way to understand what effects they may have on cells from different disease and healthy cell lines and determine the mechanisms underlying those effects, thus possibly establishing a baseline for future assays. This will be attempted for exosomes obtained from MSCs of different tissue sources and healthy donors, thus conceivably also allowing for a comparison between these parameters and the determination of their contribution towards the effects of their secreted vesicles.

Firstly, however, it is important to note that in order for their denomination to be accurate these isolated vesicles should not be called exosomes but small vesicles with exosome-like properties, since, as of this moment, there are no methods for isolation procedures that can 100% guarantee their sole isolation, due to overlapping characteristics with other vesicles. Nonetheless, for the sake of brevity the term “exosome” will be used to mention these vesicles, for the length of this work.

With this in mind, and as the purpose of this thesis can be divided into two main objectives, in a first approach these vesicles’ samples will be used for treatment of two human cancer cell lines and a human brain microvascular endothelial cell line, a major component of the blood-brain barrier, with the aim of monitoring their proliferation and, for one of the tumour cell lines, the capacity of *in vitro* invasion through Matrigel in response to the treatment with MSC-derived exosomes. Furthermore, exosomes will also be isolated from other sources, following the same isolation protocol, and characterized in order to be used as controls in parallel experiments. Finally, the determination of the molecular mechanisms by which these vesicles are taken into the cells is also of interest, and so a confocal microscopy approach is intended. Consequently, in this work, the labelling of these vesicles will be attempted with an immune-fluorescence approach, in opposition to a lipid membrane staining method, and further characterization of stained exosomes will also be performed.

3. Materials and Methods

3.1. Cell Lines and Cell Culture

Human breast cancer (MCF-7), human non-small cell lung cancer (A549), human embryonic kidney 293 (HEK293) and human brain microvascular endothelial (hBMEC) cell lines were obtained from ECACC (European Collection of Authenticated Cell Cultures).

MCF-7, A549 and HEK293 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO™), supplemented with 10% heat-inactivated (HI) fetal bovine serum (FBS, GIBCO™), 100 lU/mL penicillin and 100 mg/mL streptomycin (1% PenStrep, GIBCO™).

hBMEC cells were cultured in Roswell Park Memorial Institute Medium (RPMI-1640) supplemented with 10% HI FBS, 10% Nu-serum, 1% MEM-vitamins, 1% PenStrep, 1% MEM non-essential aminoacids and 1% Sodium pyruvate.

The cells were maintained in culture in T-Flasks, at 37ºC in a humidified atmosphere with 5% CO_2 and passaged by chemical detaching with Tryple™, when ~90% confluence was reached.

3.2. Serum Inactivation

Exosome-depleted FBS (GIBCO™), was inactivated by heat (Heat-inactivation, HI). Briefly, the serum was thawed at room temperature and swirled every 10-15 min. After completely thawed, it was submerged in a temperature-controlled water bath at 56ºC for 30 min and gently swirled every 5-10 min. The temperature of the water bath was monitored using a control bottle with water and a suspended calibrated thermometer. After the 30 min, the serum was removed from the bath, gently swirled again and allowed to cool at RT. The heat inactivated serum was then stored at -20ºC, until use.

3.3. Exosome Samples

Exosomes derived from MCF-7, A549 and HEK293 cell lines were isolated from cells' conditioned media using total exosome isolation reagent (Invitrogen). Briefly, the cells were cultured in DMEM supplemented with 10% FBS and 1% PenStrep until 80-90% confluence was reached. Afterwards, cells were washed two times with PBS and medium was exchanged to DMEM supplemented with 10% HI Exosome-Depleted FBS, for 48 h. The conditioned media was then collected and centrifuged at 2,000x*g* at room temperature (RT) for 30 min. The supernatant was transferred to a centrifuge tube and 1/2 of the volume of total exosome isolation reagent was added and well homogenized. This solution was incubated at 4ºC overnight (12h - 18h) and subsequently centrifuged at 10,000x*g* at 4ºC for 1 h. The resulting supernatant was discarded, and the exosome-pellet was resuspended in PBS (high-grade) in order to achieve a concentration factor of 40. The total protein concentration of the samples was determined using a Pierce™ BCA Protein Assay Kit (Thermo Scientific™), following manufacturer's instructions, and the exosomes were stored at -80ºC until further use.

As a control, DMEM medium supplemented with 10% HI Exosome-Depleted FBS, not conditioned by cells was also processed by the same method.

MSC-derived exosome samples isolated from umbilical cord matrix (donors UCM#40 and UCM#45, UCM-MSCs(1) and UCM-MSCs(2), respectively), bone marrow (donors M79A15 and F99A18, BM-MSCs(1) and BM-MSCs(2), respectively) and adipose tissue (donor L140326, AT-MSCs) were provided by PhD student Miguel Fuzeta, as they were obtained in the scope of his work. Briefly, the cells were initially expanded in T-flasks and cultured in DMEM supplemented with 5% human platelet lysate (hPL). Subsequently, they were inoculated in vertical wheel bioreactors and grown attached to microcarriers, in the same medium. For conditioning, the medium was exchanged to basal DMEM for 48h. Exosomes were then isolated from the conditioned medium using total exosome isolation reagent, as described.

3.4. Protein extraction and Western blotting analysis

Cells conditioned for exosome isolation were washed twice with PBS and resuspended and lysed in catenin lysis buffer (1% Triton X-100, 1% Nonidet-P40 in deionized PBS) supplemented with 1:7 proteases inhibitor (Roche Diagnostics GmbH, Germany), for 10 min at 4ºC. The lysates were collected, vortexed three times, for 10 seconds each time, and centrifuged at 14,000 rpm at 4ºC for 10 min. The supernatant was kept and the total protein concentration was determined by Bradford method.

30 µg and 20 µg of total protein of exosome samples and of whole cell lysates, respectively, were prepared with Laemmli sample buffers (1x and 4x), denaturated at 100ºC for 5 min and separated by electrophoresis in 12% polyacrylamide gels.

Gels were transferred onto nitrocellulose membranes (RTA Transfer Kit, BioRad), using Trans-Blot Turbo Transfer System (BioRad), following the manufacturer's instructions. Transfer was confirmed with Ponceau S staining solution and non-specific binding sites were blocked for 1 h with 5% (w/v) non-fat milk in PBS-Tween-20 (0.5% v/v). Subsequently, the membranes were incubated in an agitator overnight at 4ºC with primary antibody [anti CD63, 556019, BD Pharmingen™, diluted 1:100 in 5% (w/v) non-fat milk in PBS-Tween-20 (0.5% v/v)].

The membranes were then washed three times with PBS-Tween-20 (0.5% v/v), for 5 min, and incubated for 1 h, at room temperature, with the proper secondary antibody, conjugated with horseradish peroxidase [m-IgGκ BP-HRP: sc-516102, Santa Cruz Biotechnology, diluted 1:2000 in 0.5% PBS-Tween-20 (0.5% v/v)]. Afterwards, they were washed five times with PBS-Tween-20 (0.5% v/v), for 5 min and developed by adding ECL substrates (Pierce) and chemiluminescence was captured by Fusion Solo equipment (Vilber Lourmat).

3.5. Nanoparticle Tracking Analysis

Particle concentration and size distribution of the isolated exosome samples were determined using a NanoSight LM10 instrument (Malvern, Worcestershire, UK) and NTA 3.1 software. Samples were diluted in PBS (high-grade), to a final volume of 1.5 mL, to achieve a particle concentration ranging from 5×10^8 to 2×10^9 particles/mL. Ten runs of 30 seconds were recorded for each sample, at 20ºC, with a

camera level of 13, and analysed with a detection threshold of 13. All parameters were optimized by pre-testing with 100 nm silica microspheres (Polysciences).

3.6. Cell Viability assays

For the establishment of the appropriate cell density for the viability experiments, MCF-7 and A549 cells were seeded in 96-well plates at different cell densities (1×10^5, 5×10^4, 2.5×10^4, 1.25×10^4, 3125 and 780 cells/well), and as a control, wells containing fresh medium were used. After 24 h, a PrestoBlue™ (PB™, Life Technologies) viability assay was performed by washing the cells twice with PBS and adding 100 µL of DMEM with 10% PB™ reagent to each well. Fluorescence intensity was measured using a microplate reader (FilterMax F5, Molecular Devices) with 535 nm excitation and 595 nm emission, for 6 h, every 1 h, meanwhile the cells were incubated at 37ºC, 5% CO_2.

To determine the influence of media exchange on the viability of MCF-7 and A549 cells,1×10^4 cells were seeded in 96-well plates and cultured for 24 h. After this time, cells were treated by exchanging the medium to DMEM+10% HI exosome-depleted FBS, or, as control, the medium was exchanged for fresh culture medium. Viability was assessed at 24, 48 and 72 h time points through PB™ Viability and MTT [3-(4,5 dimethylthiazol-2-yl-2,5 tetrazolium bromide)] assays. PB™ viability assay was performed as described and the fluorescence intensity measured for 4 h. For the MTT assays, after incubation in the different media, cells were washed with PBS and 100 µL of DMEM and 20 µL of MMT (5 mg/mL) were added to each well, followed by incubation at 37ºC for 3.5 h. The reaction was stopped with the addition of 150 µL of 40 mM HCL in isopropanol and MTT formazan formed was spectrophotometrically read at 590 nm in a microplate reader. The viability values were calculated from the fluorescence intensity (at 3.5 h time point)/absorbance values as a percentage of the control cells.

For the assessment of the effect of media changes for hBMEC cells, a PB™ viability assay was conducted. 5×10^4 and 1×10^4 cells were seeded in a 96-well plate and cultured in fully supplemented RPMI-1640 (control) or DMEM+10% HI exosome-depleted FBS for 24 h. The media were then exchanged for fresh corresponding media and the cells were left to incubate for 48 h. Viability was then determined as previously described. In addition, hBMEC cells were also seeded in rat-tail collagen type I coated 96-well plates at different cell densities (5×10^4, 2.5×10^4, 1.25×10^4, 3125 and 780 cells/well), as a control wells containing fresh medium were used. After 48 h, a PB™ viability assay was performed and fluorescence intensity was measured as described, for 4 h, every 30 min.

For the assessment of the effect of exosome and conditioned media samples in MCF-7, A549 and hBMEC cells, 1×10^4 cells were seeded in a 96-well plate and cultured in exosome-depleted medium for 24h. The cells were washed twice with PBS and treated with 100 µL of exosome solutions at different total protein concentrations (200, 100, 50, 25, 10 or 5 µg/mL) or 100 µL of conditioned medium solutions. Dilutions were performed using PBS and medium supplemented with HI exosome-depleted serum, for the conditioned medium solutions the volumes used for the dilutions were the same as those used for the 100 µg/mL solution of the correspondent exosome samples. As a control, cells were treated with a solution of medium and PBS, using the same volumes as the prepared exosome solutions (0 µg/mL).

After 48 h of incubation, PB™ viability assays were performed as described and the viability values were calculated.

3.7. Transwell Invasion Assays

The invasion capacity of A549 cells through Matrigel was assessed using a 24-well-transwell migration assay (Corning® BioCoat™ Matrigel® Invasion Chambers) with cell culture inserts containing an 8 µm pore size PET membrane treated with a thin layer of Matrigel (Falcon®). The inserts were rehydrated with basal DMEM for 3h, at 37ºC, according to manufacturer's instructions.

After rehydration, the medium was removed and 750 µL of exosome-depleted medium were added to the bottom chamber. Cell suspensions (250 µL) were prepared in DMEM+10% HI exosome-depleted FBS, containing 5×10^4 cells as well as different dilutions of exosome samples (5, 10, 25 and 50 µg/mL), in PBS, and added to the upper chamber. As a control, cell suspensions were prepared with either a solution of medium and PBS, using the same volumes as the prepared exosome solutions (0 µg/mL), or with processed basal DMEM diluted in medium, in the same volumes as the prepared exosome solutions. An initial experiment where cells were resuspended in either normal culture medium or exosome-depleted medium, was also conducted to assess the influence of the different media in the invasion capacity of the cells.

Following a 48h incubation period, at 37ºC, the inserts were washed with PBS and non-migrated cells were removed from the upper side of the chamber with cotton swabs dipped in PBS. Migrated cells were fixed in cold methanol (4ºC) for 10 minutes. After complete drying, the membranes were removed using a scalpel, placed in a microscope glass slide and stained with VECTASHIELD® mounting medium with DAPI (Vector Laboratories). The slides were stored at 4ºC overnight or -20ºC for longer periods. Cells of ten independent fields were counted under a fluorescence microscope (Zeiss), using the ImageJ software setting "Analyse Particles" [Size ($pixel^2$): 800-1000; Circularity: 0.1-1.0] and the mean number of migrated cells per condition was determined by the average of all fields. Results are presented as the fold change in migration of the cells when compared to the control (0 µg/mL).

3.8. Fluorescent Labelling of Exosomes

Exosomes from HEK293 cells were labelled with an anti-CD63 antibody coupled with FITC (0.04 mg/mL mouse anti-Human CD63 FITC, EXBIO) in three different experiments. Initially, a volume equivalent to 6.5×10^8 particles of exosome specimens was diluted in 500 µL of PBS+0.2% BSA and then mixed with 20 µL of the labelled antibody and incubated for 2h, at RT, in the dark. For the following experiments, volumes equivalent to 9.6×10^9 and 1.1×10^{10} particles of exosome specimens were mixed with 50 µL of the labelled antibody, and incubated at different conditions (2h, at RT and overnight, at 4ºC, respectively) in the dark. Any necessary dilutions were made in PBS+0.2% BSA. For a blank control, the antibody was replaced by PBS+0.2% BSA.

To wash out unbound antibodies, the samples were transferred to an ultrafiltration unit (Amicon®, Merk) with a 100 kDa cut-off and three centrifugations at 5000 rpm for 1 min were performed, with the

addition of PBS+0.2% BSA for each to wash the sample. The membrane was thoroughly washed with PBS+0.2% BSA and the labelled exosomes solutions were stored at 4ºC, in the dark.

To determine the effectiveness of the washout steps, particle concentration and size distribution of the samples were determined using NTA, as previously described, and later compared to that of the original exosome samples.

To confirm the effectiveness of the labelling strategy, fluorescence emission spectra were obtained from 480 nm to 600 nm, in a spectrofluorometer (Fluorolog®-3-21, HORIBA Scientific, USA) for an excitation wavelength of 460 nm. Quartz cuvettes (0.5 × 0.5 cm) from Hellma Analytics were used.

4. Results

4.1. Establishment of conditions for MCF-7, A549 and hBMEC cells viability assays

To determine the effect of the MSC-derived exosome samples in the viability of the cell lines to be tested, a fluorescence-based PrestoBlue™ (PB™) viability assay was chosen. In this assay, resazurin is reduced to resorufin upon entering the cell. Since resorufin is highly fluorescent and this reduction only takes place in metabolically active cells, the increase in fluorescence can be used to quantify cell numbers [62].

Initially, it was then necessary to establish the conditions in which the assays would be performed, considering that all cell lines are different and there was no prior knowledge on the behaviour of these cells in the conditions intended. For this, increasing cell densities of MCF-7 and A549 cells were seeded in 96-well plates and assayed after 24 hours, with a PB™ viability assay for 6 hours, in an attempt to determine the density of cells and the appropriate time point at which the assessment of the relative viabilities should be performed.

As can be seen in the plots in **Figure 6A**, for both cell lines, the fluorescence intensity increased along with the number of seeded cells and, in a linear way, with the passage of time, as would be expected. Nevertheless, this increase overtime is linear only up until the 4-hour time point, when it starts to be less significant, an occurrence which was more noticeable for the higher cell densities. Given this, it was determined that using a cell density of $1.0x10^4$ cells/well (represented as ▲) and performing fluorescence intensity measurements for up to 4 hours after treatment was sufficient for the intended assays, as in these conditions it is possible to accurately follow the reduction of the reagent by the cells and, consequently, determine their viability.

Subsequently, it was necessary to determine whether exchanging the medium of cells during the assay had some influence on their viability, seeing as, for the assays using exosome samples, medium supplemented with HI exosome-depleted serum must be used, in order to ensure that there is no interference of other vesicles (present in FBS) in the obtained results[63]. For this, both cell lines were seeded in regular culture medium and after 24 hours the media were exchanged for DMEM supplemented with 10% HI exosome-depleted FBS. PB™ and MTT viability assays were performed after 24, 48 and 72 hours of treatment, and relative cell viability (%) was determined by comparison with the cells cultured in regular medium for the whole duration of the assay (control). The results are represented in **Figures 6B** and **6C.** As is made evident by the graphs, there is some impact on cell viability after the sudden exchange of the medium, for both cell lines. Thus, it was decided that the cells should be seeded directly in medium containing exosome-depleted serum, in order to allow for a better adaptation.

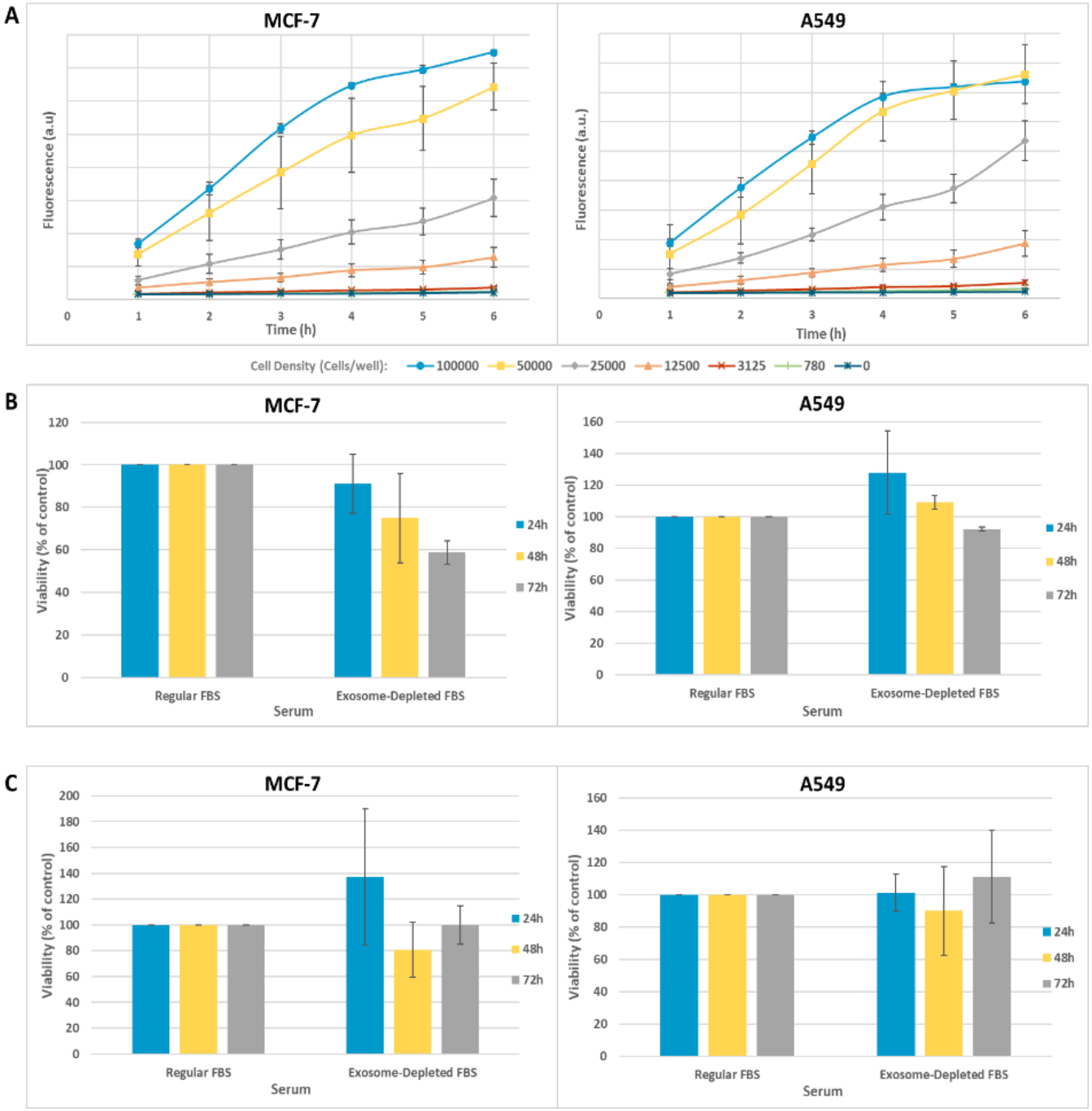


Figure 6 - Establishment of the viability assays for MCF-7 and A549 cells. 1×10^4 cells should be seeded per well and cultured in DMEM supplemented with 10% Exosome-depleted FBS. A) Determination of the cell density required for the proper tracking of resazurin reduction and consequent cell viability assessment. Fluorescence intensity was measured for 6 hours after a 24-hour incubation period. For MFC-7 and A549. **B**) Cell viability assessed with PrestoBlue™ viability assay for MCF-7 and A549 cells after 24, 48 and 72 hours of exposure to medium exchange from regular culture medium to DMEM supplemented with 10% HI exosome-depleted FBS. The media of control cells were exchanged for regular fresh culture media and their viability was admitted as 100%. **C**) Cell viability assessed with MTT viability assay for MCF-7 and A549 cells after 24, 48 and 72 hours of exposure to medium exchange from regular culture medium to DMEM supplemented with 10% HI exosome-depleted FBS. The media of control cells were exchanged for regular fresh culture media and their viability was admitted as 100%. Results are represented by the mean of 3 replicates ± SD.

Similarly, this same optimization of experiment conditions was performed for hBMEC cells, although in separate assays. Firstly, the exchange in medium was assessed, this time more in an attempt to understand if the cells would remain viable when cultured, and subsequently assayed, in DMEM medium, as they are usually maintained in RPMI-1640. The assay was similar to the previously described, being that, in this case, the relative viability of the cells was determined, using a PB™ viability assay after 48 hours, with the cells having been seeded directly in DMEM+10% HI Exosome-depleted FBS, and by comparison with cells cultured in regular medium for the whole duration of the assay (control). Results (**Figure 7A**) demonstrate that the viability of the cells is not significantly reduced by the alteration of the culture media. In fact, when a higher density of cells was assessed, a significant increase in cell viability for the cells cultured in exosome-depleted DMEM culture medium was seen, when compared to the control. However, it should be noted that a considerable amount of cell detachment was observed in the control wells where the highest amounts of cells were plated. Given this, it was concluded that hBMEC cells can in fact be assayed in DMEM culture medium, however they should be seeded in coated plates, in order to avoid cell detachment and, consequently, obtain more reliable results. Accordingly, in a subsequent assay, similarly to what was done for the previous cell lines, increasing cell densities of these cells were seeded in rat-tail collagen type I coated 96-well plates, in DMEM supplemented with exosome-depleted serum, and assayed after 48 hours, with a PB™ viability assay for 4 hours. Results from this experiment (**Figure 7B**) are in agreement with what had been previously seen for MCF-7 and A549 cells and therefore it was concluded that a cell density of $1.0x10^4$ cells/well (represented as ♦) and an assay duration of up to 4 hours would be the appropriate conditions for the future experiments.

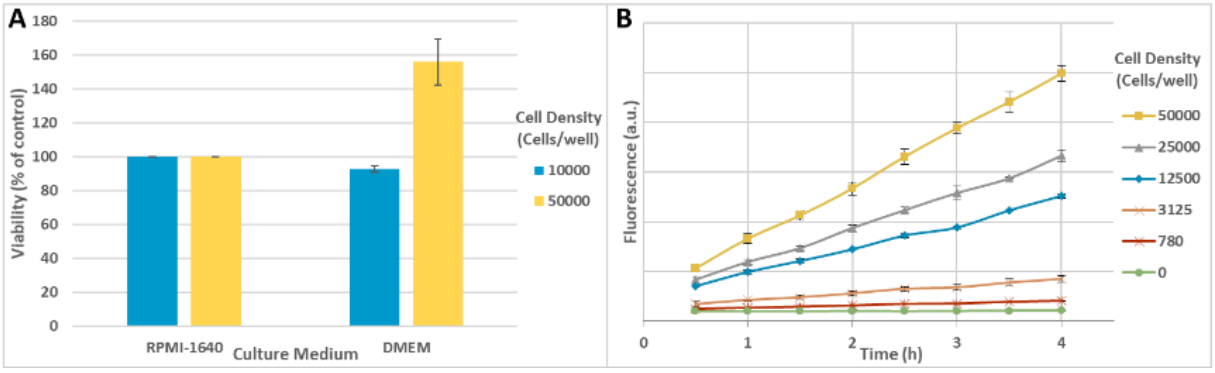


Figure 7 - Establishment of the viability assays for hBMEC cells. $1×10^4$ cells should be seeded per well (coated with rat-tail collagen type I) and cultured in DMEM supplemented with 10% exosome-depleted FBS. A) Cell viability assessed with PrestoBlue™ viability assay for hBMEC cells cultured in either regular culture medium (RPMI-1640) or DMEM supplemented with 10% HI exosome-depleted FBS. Cells cultured in RPMI medium were used as control and their viability was admitted as 100% **B)** Determination of the cell density required for the proper tracking of resazurin reduction and consequent cell viability assessment. Fluorescence intensity was measured for 4 hours after a 48-hour incubation period. Results are represented by the mean of 3 replicates ± SD.

4.2. Influence of MSC-derived exosomes on MCF-7, A549 and hBMEC cell viability

As aforementioned, one of the main goals of this study was the determination of the effects that MSC-derived exosomes have in the viability of cells from different cell lines. With this objective in mind, four different exosome samples, obtained from Umbilical Cord Matrix (UCM), Adipose Tissue (AT) and

Bone-Marrow (BM) mesenchymal stem/stromal cells cultured in vertical-wheel bioreactors, were diluted, in order to achieve six solutions with concentrations up to 200 µg/mL of total protein, for the UCM-MSC derived samples, and 100 µg/mL, for the remaining. MCF-7, A549 and hBMEC cells were then seeded in the conditions previously determined and exposed to these solutions, after 48 hours of incubation a PB™ viability assay was performed. The graphs in **Figure 8** were created using the values of viability calculated for the cells exposed to exosome samples, relatively to the cells to which no exosomes were added (control – 0 µg/mL).

By analysing the results, it was possible to see that the three cell lines had different responses to the treatment with these exosomes. In the case of MCF-7 cells (**Figure 8A**), a similar pattern was noted in the viability of the cells after treatment with all four samples. As it seems, exposure to smaller concentrations of MSC-exosomes (5-50 µg/mL) had a stimulating effect, increasing cell viability, however, in contrast, the cells subjected to higher concentrations of exosomes, mainly the 100 µg/mL solutions, were shown to have lower viability values in comparison, in one case even demonstrating a 10% loss in viability when compared to the control (UCM-MSCs(2) represented as ▌).

Similarly, A549 cells demonstrated a comparable pattern (**Figure 8B**) to that presented for the MCF-7 cells, with the least concentrated (25-50 µg/mL) samples causing a slight increase in cell viability, when compared with the control, and with the cells treated with the highest dose (100 µg/mL) presenting a lower relative viability. This pattern was slightly different for the UCM-MSC-derived exosome sample (represented as ▌), for which it was seen that the most stimulating is the 10 µg/mL solution and that the solutions with higher concentrations (25-100 µg/mL) caused a slight loss in cell viability. Nevertheless, the variation in cell viability, observed for these cells is not very significant when compared with what was seen for the MCF-7 cells, being that the maximum variation observed was an increase of ~20%, as a response to treatment with the 50 µg/mL solution of BM-MSC-derived exosomes (represented as ▌).

Regarding the hBMEC cells, there was no cohesive response pattern that could be observed between the different sources of exosome samples tested (**Figure 8C**). Firstly, treatment with lower concentrations of UCM-MSC-derived exosomes (represented as ▌), was shown to not have a significant impact on these cells, as their viability values upon treatment remained relatively close to that of the control (100%), however, once cells were exposed to the most concentrated solutions (50-100 µg/mL), a stimulating effect could be observed, with an increase in cell viability. In contrast, for the AT-MSC-derived sample (represented as ▌) there is an inverse response, being that treatment with higher doses (25-100 µg/mL) led to a slight decline in cell viability, while lower concentrations had a stimulating effect, increasing cell viability, comparable to what had been seen for A549 and MCF-7 cells. Finally, the sample that demonstrated a more significant effect on the viability of these cells was the BM-MSC-derived one (represented as ▌), to which the response was a loss of cell viability, when compared to the control, which was more pronounced for the cells treated with the 50 and 100 µg/mL concentrations.

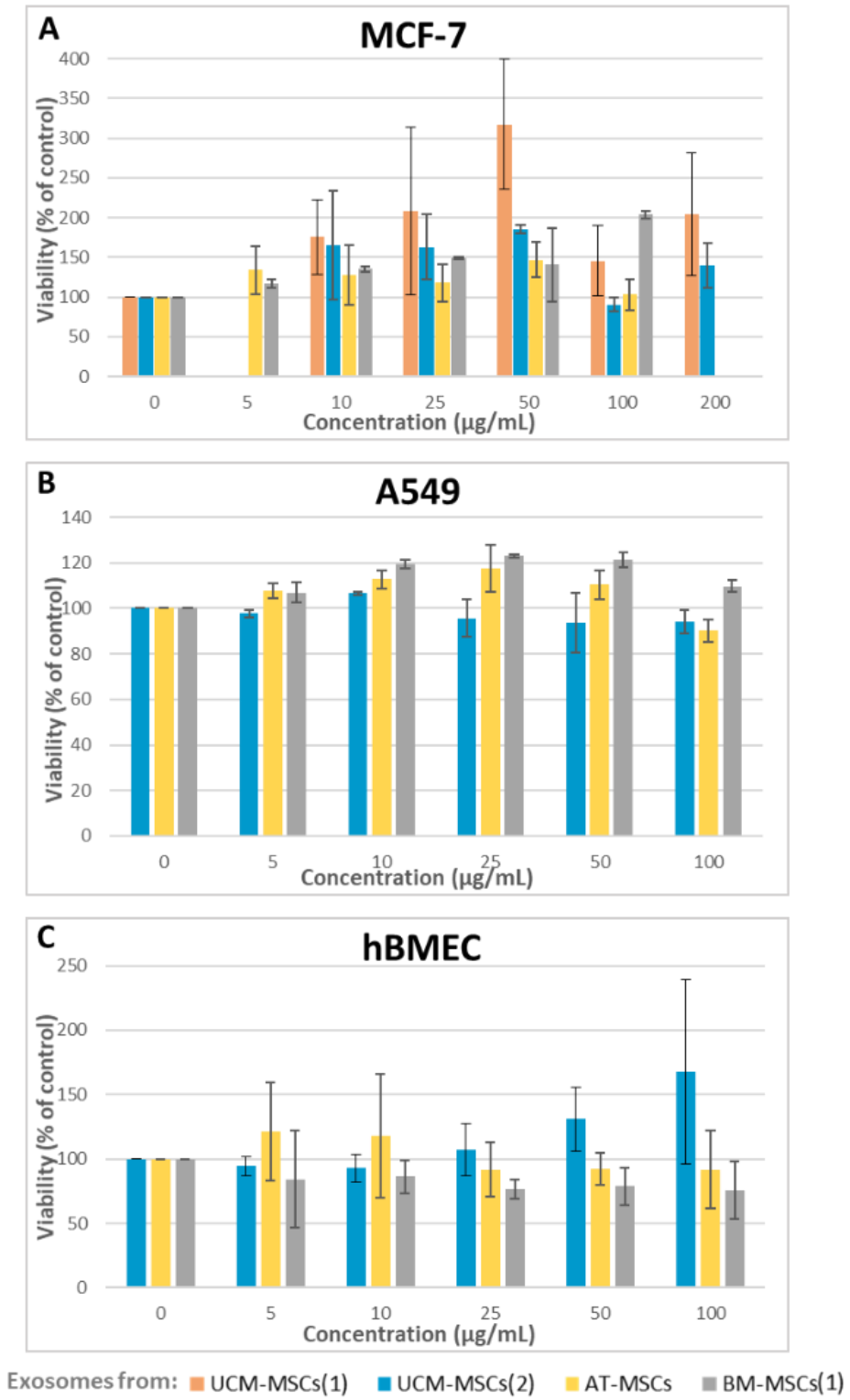


Figure 8 - Cell viability assessed with PrestoBlue™ viability assay for MCF-7 **(A)**, A549 **(B)** and hBMEC **(C)** cells after 48 hours of treatment with increasing concentrations of exosome samples from three different MSC sources (Umbilical Cord Matrix – UCM-MSCs(1) and UCM-MSCs(2) – Adipose Tissue – AT-MSC – and Bone Marrow – BM-MSC(1)) cultured in bioreactors. Untreated cells received medium with PBS and no exosome samples and their viability was admitted as 100%. Results are represented by the mean of 3 replicates ± SD.

Overall, these results demonstrate that some effects are exerted over the cells by the experimented exosomes, which can be different depending on the assayed cell line and, in some cases, on the source of the tested exosome samples. Therefore, this should be further assessed in a way to understand the mechanisms underlying these effects.

4.3. Biophysical and biochemical characterization of isolated control exosomes

As suggested in MISEV2018 [64], when the functional activity of specific exosome samples is being studied, certain controls should be employed and produce minimal effects over the tested cells. Considering this, several negative or background control situations were established and for some cases an isolation of exosomes was performed using a total exosome isolation reagent, similar to what had been done for the MSC-derived samples.

On the one hand, “mock” exosomes from DMEM culture medium were isolated, that is medium that had not been conditioned by cells was processed following the same protocol as that for exosome isolation, and on the other hand, vesicles were extracted from HEK293 cell conditioned medium, as these cells have not been described to have intrinsic therapeutic properties and, thus, can be used as an exogenous negative control in this situation. Furthermore, specifically for the cancer cell lines, exosomes were extracted from medium conditioned by the cells to be assayed, in this case MCF-7 and A549 cells.

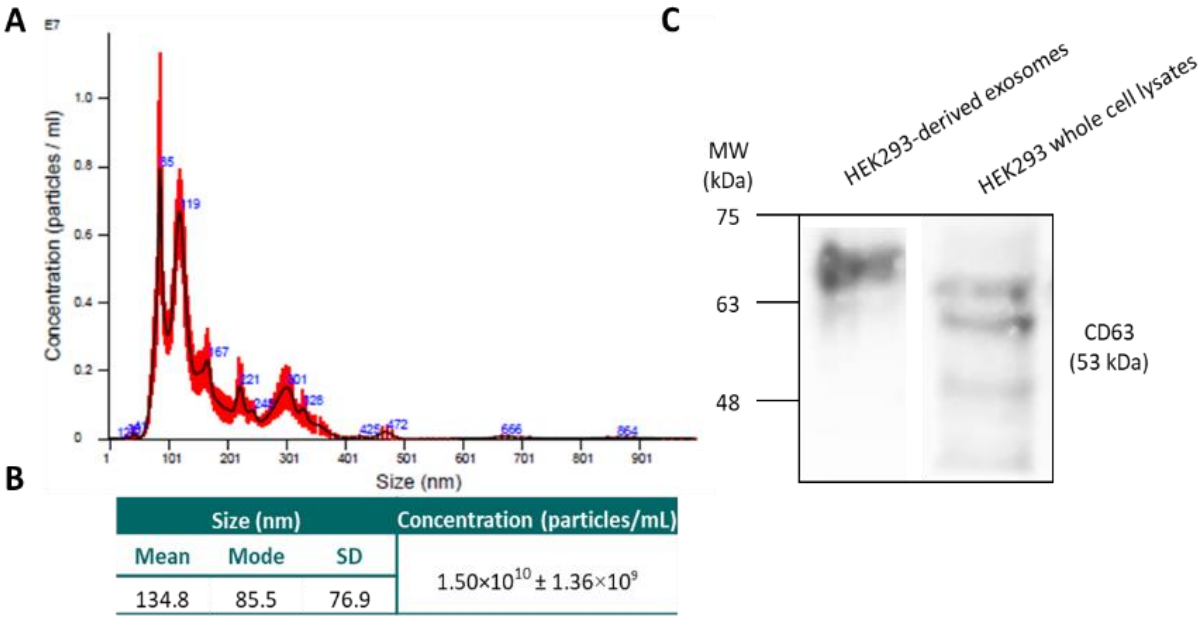
After isolation, for all samples, the total protein concentration was determined using the bicinchoninic acid (BCA) assay (**Table 2**). This quantification was performed in a way to standardize the following experiments relatively to those performed with the MSC-derived exosome samples, as it represented a comparable straightforward parameter to quantify the dose of the samples for the treatment of the cells.

Table 2 - Protein concentration, determined using BCA assay, and corresponding standard deviation (SD), for the exosome samples isolated from MCF-7, A549 and HEK293 cells and non-conditioned culture medium (DMEM+10% HI Exosome-depleted FBS). For the HEK293 and non-conditioned medium samples, (1) represents the sample later used for treatment of MCF-7 and A549 cells, and (2) of hBMEC cells.

Sample	MCF-7	A549	HEK293		DMEM + 10% HI Exosome-depleted FBS	
			1	2	1	2
Protein concentration (µg/mL)	337.3	950.5	695.2	601.8	735.2	513.5
SD of Protein concentration	11.0	24.7	25.5	12.4	30.9	61.9

Moreover, in a way to demonstrate that the isolated samples were in fact composed of extracellular vesicles, more specifically exosomes, and not just undefined extracellular particles, two different assays were performed. The first was a Nanoparticle Tracking Analysis (NTA) and the second a Western Blot assay, which allowed for the determination of the size distribution of the particles in question as well as the particle concentration of the samples and for the assessment of the presence of specific exosome

markers, respectively. An example of the results from these experiments is depicted in **Figure 9** and was obtained for a HEK293-derived exosome sample.



B

Size (nm)			Concentration (particles/mL)
Mean	Mode	SD	$1.50\times10^{10} \pm 1.36\times10^{9}$
134.8	85.5	76.9	

Figure 9 – **Characterization of an isolated HEK293-derived exosome sample. A)** Particle concentration and size distribution assessed by Nanoparticle Tracking Analysis (NTA). **(B)** Mean and mode size (nm), and standard deviation (SD) of results, of the vesicles present in the HEK293-exosome sample, assessed by NTA. **C)** Confirmation of the presence of a specific exosomal marker in the tested sample through Western Blot. The tetraspanin CD63 (53 kDa) was detected both in the cell lysates (positive control) and the tested exosome sample, in different forms.

NTA quantification (**Figure 9A**) demonstrated that, overall, the size distribution of the particles in this sample falls inside the range expected for the desired vesicles, as the diameter of the majority of the vesicle population was found to be between 60 and 200 nm. In fact, as can be seen in **Figure 9B** the mean size calculated for this specific population of particles was of 134.8 nm, yet the mode, i.e. the particle size most commonly found in the distribution, was of 85.5 nm meaning that, regardless of the fact that there is some heterogeneity in the sample, possibly caused by co-precipitation of medium components or even aggregation of the vesicles, the greater part of these particles seem to present diameters representative of the target vesicles.

Furthermore, immunoblot analysis (**Figure 9C**) allowed for the detection of the tetraspanin CD63, a protein marker widely associated with exosomes, in the tested sample as well as in the protein lysates of the producing cells. However, this protein seems to be more enriched in the exosome sample when compared to the HEK293 whole cell lysates, for which different forms of this protein were identified, as a single, more intense, band was obtained.

Combined, these results corroborate the existence of exosomes in the isolated samples, and therefore these samples represent a suitable control for the performed experiments.

4.4. Influence of vesicles isolated from same cells and other background controls on MCF-7, A549 and hBMEC cell viability

Once isolated and characterized these controls were then diluted in order to achieve six solutions, as was described for the MSC-derived samples, to which all three cell lines were exposed and after 48 hours a PB™ viability assay was performed. In addition, the influence of culture medium conditioned by UCM-MSCs and HEK293 cells, in the viability of these cells was also assayed, in a way to better understand whether the effects previously seen were in fact related to the presence of exosomes or solely to other soluble factors secreted by the cells that may have been present in the samples. The graphs depicted in **Figure 10** were then created using the values of viability calculated for the cells exposed to these controls, relatively to the cells that received no treatment (control – 0 µg/mL).

For the tumour cells (MCF-7 and A549, **Figures 10A** and **10B**, respectively), it was seen that both, treatment with exosomes isolated from self-conditioned culture medium and with non-conditioned culture medium processed with the same isolation protocol, did not alter the viability of the cells. On the contrary, for MCF-7 cells, the treatment with exosomes derived from a different cancer cell line (A549) had a stimulating impact, inducing cell proliferation, which could be explained by the fact that extracellular vesicles derived from cancer cells have been demonstrated to have a role in tumorigenicity and tumour-related pathologies, and thus could be stimulating the growth of these cells[19]. When it came to the samples obtained from HEK293 cells, treatment with conditioned medium (CM) was shown to not affect the viability of both cell lines and, for A549 cells, the same was true for the exosome samples. On the other hand, MCF-7 cells demonstrated to be slightly stimulated by these samples, as their viability was seen to increase after treatment, in a dose-independent manner.

Regarding the hBMEC cells (**Figures 10C**), results were consistent with what was observed for the A549 cells, when it comes to the HEK293-derived exosome and conditioned medium samples. However, treatments with increasing concentrations of processed DMEM culture medium supplemented with 10% HI exosome-depleted FBS led to a great decline in cell viability which was enhanced for the higher doses. Finally, medium conditioned by umbilical cord matrix (UCM)-MSCs was seen to slightly induce cell proliferation in these cells, which was not observed for A549 cells as their viability was maintained relatively close to the control after treatment with this sample.

All in all, these results were significantly different from those obtained for the MSC-derived samples and thus help support the specificity of their effect over the assayed cells.

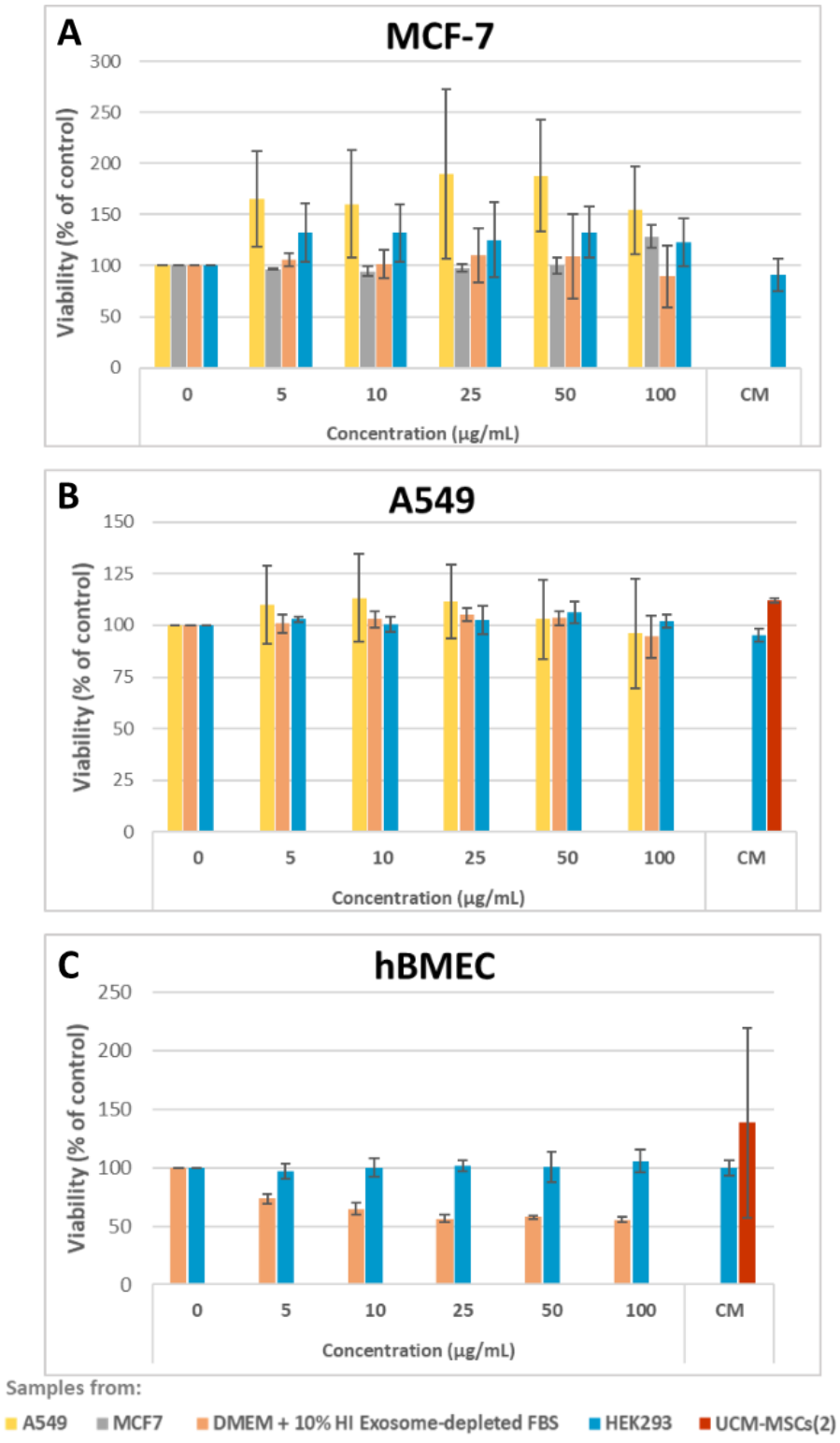


Figure 10 - Cell viability assessed with PrestoBlue™ viability assay for MCF-7 **(A)**, A549 **(B)** and hBMEC **(C)** cells after 48 hours of treatment with increasing concentrations of exosome or conditioned medium (CM) samples from four different sources (HEK293, A549 and MCF-7 cells, and Umbilical Cord Matrix MSCs – UCM-MSCs(2)) and increasing concentrations of processed DMEM culture medium supplemented with HI exosome-depleted serum. Untreated cells received only medium with PBS and their viability was admitted as 100%. Results are represented by the mean of 3 replicates ± SD.

4.5. Influence of MSC-derived exosomes on A549 cells invasion capacity *in vitro*

To complement the previous experiments, the invasive capacity of A549 cells after exposure to MSC-derived exosomes was tested using a transwell migration assay with cell culture inserts containing an 8 µm pore size PET membrane. The chambers were coated with a thin layer of Matrigel™ matrix, a solubilized basement membrane preparation extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma and rich in extracellular matrix proteins such as laminin, collagen IV, heparan sulphate proteoglycans, entactin and nidogen. This coating of Matrigel™ mimics the extracellular matrix and consequently acts as a barrier to tumour cell invasion and migration through the membrane pores, and thus allows for a better approximation to the *in vivo* environment [65].

Firstly, and similarly to what was done for the viability assays, it was necessary to determine whether medium exchange to DMEM supplemented with exosome-depleted serum affected the invasion capacity of these cells. For this, A549 cells were resuspended in either normal or exosome-depleted media and then added to the Matrigel™ coated chambers. After 48 hours of incubation, non-migrated cells were removed from the upper side of the chamber and invading cells were fixed, stained with DAPI and counted under a microscope. Results are presented in **Figure 11** as the fold change in migration of the cells when compared to the control (cells resuspended in DMEM+10% HI FBS).

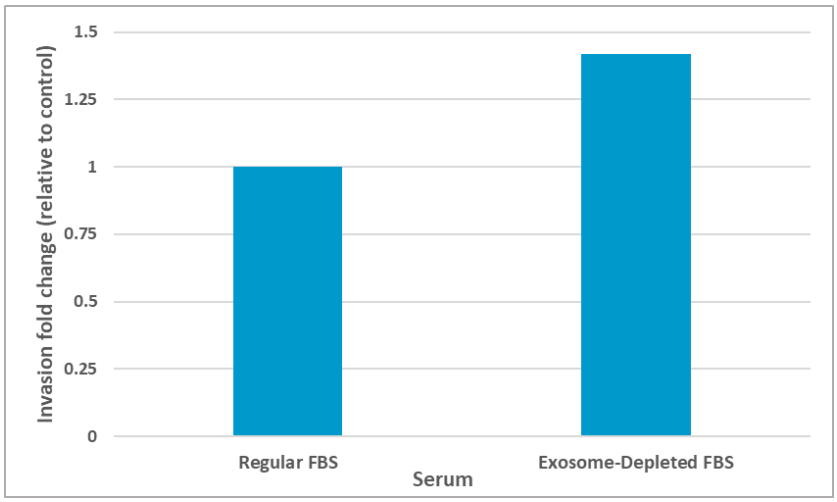


Figure 11 - Invasion potential of A549 cells when resuspended in DMEM culture medium supplemented with 10% HI exosome-depleted FBS and left to incubate for 48 hours. Results are represented as the fold change in invasion relatively to the control cells, which were resuspended in regular culture medium (DMEM+10% HI FBS).

As can be seen in the graph, culturing in this medium does not have a negative impact on the invasive capacity of these cells, and in fact this was seen to be increased under these conditions. Therefore, it was considered that it would be feasible for future assays to be performed with A549 cells using medium supplemented with exosome-depleted serum.

With this established, it was then possible to assess the effect of MSC-derived exosomes on the invasion potential of this cell line. For this, the cells were resuspended in culture medium containing increasing concentrations of three MSC-derived exosome samples and after 48 hours of incubation the results were obtained as described. The different doses of exosome samples used were chosen taking into consideration the results obtained in the viability assays, that is, for the BM and AT-derived samples concentrations from 10 to 50 µg/mL were used, as it was for these samples that a shift in cell proliferation response was observed, while for the UCM-derived sample a 5 µg/mL was also tested. Furthermore, besides the MSC-derived samples, an exosome sample obtained from HEK293 cells and a sample of non-conditioned culture medium (Basal DMEM) processed following the exosome isolation protocol were likewise used for treatment of these cells, once again with the intent of serving as negative controls. The results from these experiments are depicted in **Figure 12** as the fold change in migration of the cells relatively to the control (cells to which no exosomes were added).

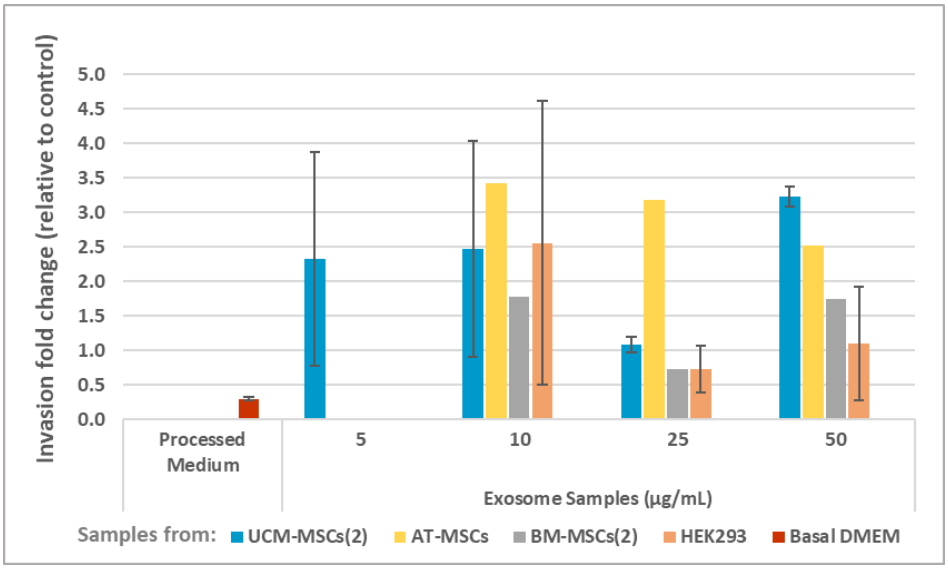


Figure 12 - Invasion potential of A549 cells after a 48-hour incubation period with increasing concentrations of exosome samples from three different MSC sources (Umbilical Cord Matrix –UCM-MSCs(2) – Adipose Tissue – AT-MSC – and Bone Marrow – BM-MSC(2)), cultured in bioreactors, and HEK293 cells, and with processed basal DMEM culture medium. Results are represented as the fold change in invasion relatively to control cells, to which no exosomes were added (cells were resuspended in DMEM medium supplemented with exosome-depleted FBS and PBS). UCM-MSCs(2) results are represented by the mean of 2 independent experiments ± SD. HEK293 and Basal DMEM results are represented by the mean of 3 independent experiments ± SD.

Overall, the results shown in **Figure 12** demonstrate that co-incubation with MSC-derived exosomes has a significant effect on the invasive capacity of A549 cells as, despite some exceptions, an increase of at least 2-fold was seen in the number of invading cells, when compared to the control. More specifically, the response pattern was similar when it came to treatment with both UCM and BM-MSC-derived exosomes, that is, for the 25 µg/mL dose there was a decline in the number of invading cells which was not observed after treatment with the other concentrations of exosome samples. Furthermore, the AT-MSC-derived sample led to the most significant rise in migration of these cells, which seemed to be slightly affected as the concentration of the samples increased.

Regarding the control samples, cells subjected to exosomes obtained from HEK293 cells demonstrated a behaviour similar to those who received the BM-MSC-derived samples with the slight

difference that when a higher concentration of exosomes was tested there seemed to be no change in the number of invasive cells when compared to the control. Nevertheless, it should be noted that a great variability between the results from different experiments was observed, which is made evident by the standard deviations represented in the graph. Finally, treatment with non-conditioned basal DMEM medium, processed with the same isolation protocol, was seen to affect the invasive capacity of these cells significantly, which was not expected. However, this sample seemed to be degraded and thus these results could be a consequence of its state.

In general, these results support what was seen with the viability assays, corroborating that these MSC-derived exosomes do in fact have an effect over these cells, which may vary depending on their source.

4.6. Establishment of a protocol for fluorescent labelling of exosomes

As mentioned in the objectives section, one of the main goals of this work was to assess the mechanisms involved in the uptake of MSC-derived exosomes into the different cell lines tested. For this, it was first necessary to determine a method to fluorescently label these vesicles. The most common labelling technique for extracellular vesicles is through lipophilic dyes like PKH or Carboxyfluorescein succinimidyl ester (CFSE), nevertheless these dyes will often lead to false positive signals as they'll non-specifically label the vesicles along with other cellular components and unbound dyes may even stain the recipient cells [66]. Given this, it was thought that staining of exosome membrane markers using an immune-fluorescence strategy would be ideal for the indented assays, as it would allow for the specific targeting of these vesicles (avoiding the described false positives) and possibly for a better insight on the mechanisms involved in their internalization.

With this in mind, exosomes were once again isolated from HEK293 cells, considering that these are easier to cultivate in larger quantities, and subsequently characterized with an Immunoblot analysis as previously described, in a way to confirm the presence of the tetraspanin CD63 as an antibody for this exosome marker conjugated with fluorescein isothiocyanate (FITC) was chosen for the labelling of the vesicles. Subsequently, three staining experiments were performed in different conditions. Firstly, 20 µL of the labelled antibody were mixed with 6.5×10^{8} particles of exosome specimens and incubated for 2 hours, at room temperature. For the second and third experiments around 1.0×10^{10} particles of exosome specimens were mixed with 50 µL of labelled anti-CD63 antibody and incubated for 2 hours, at room temperature, and overnight, at 4ºC, respectively. As a blank control, exosomes were processed in the same conditions used in the first experiment and the antibody was replaced by PBS+0.2% BSA. To wash out unbound antibodies, for all experiments, an ultrafiltration was performed.

After labelling, a fluorescence emission spectrum was obtained from 480 nm to 600 nm using an excitation wavelength of 460 nm, for each of the labelled samples as well as the control. This allows for a comparison of the labelling efficiency in each experiment. Results are represented in **Figure 13**.

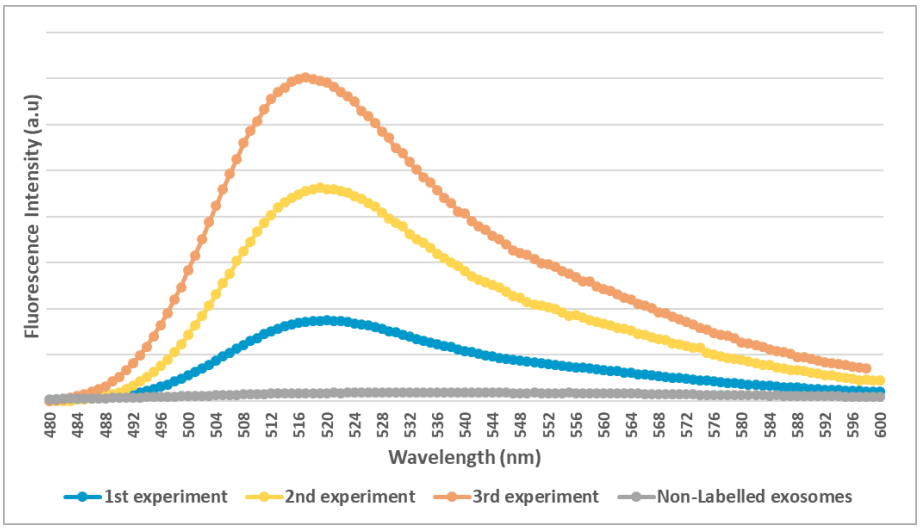


Figure 13 – Fluorescence spectroscopic analysis of HEK293-derived exosomes. Emission spectra of exosomes labelled with an anti-CD63 antibody coupled with FITC in three different experiments and non-labelled exosomes processed in the same conditions as the first experiment (control). Excitation wavelength was 460 nm.

By analysis of the represented spectra, it was possible to conclude that the conditions in which the exosomes are labelled do in fact have an impact on the effectiveness of the staining and their detection through fluorescence spectroscopy. As it seems, the second and third experiments, in which greater quantities of exosomes were stained with an increased volume of labelled antibody, relatively to the first experiment (●), resulted in higher fluorescence intensity values, consequently indicating the existence of more labelled vesicles. Furthermore, incubation overnight at lower temperatures (third experiment, ●) appeared to also result in a more efficient labelling when compared to the conditions applied in the second experiment (●, 2 hours, at room temperature).

To further confirm these results, a calibration curve for the quantification of the FITC conjugated anti-CD63 antibody was traced. Given the very low concentration of particles and antibody, the calibration cannot be performed spectrophotometrically and the fluorescence signal of FITC was used due to the higher sensitivity of fluorescence detection. From this the concentration of the labelled samples was determined, as well as the ratio of antibody or FITC molecules per particle for each of the samples. The calibration curve, along with its equation, is represented in **Figure 14**, and the calculated values are shown in **Table 3.**

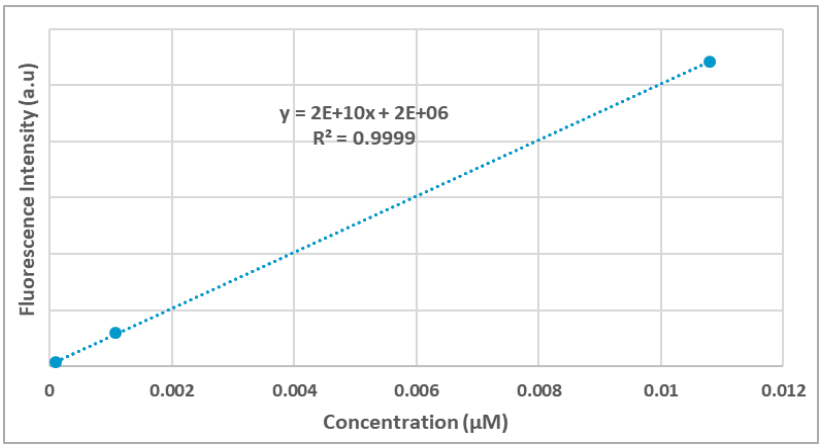


Figure 14 – Calibration curve for anti-CD63 FITC quantification. Fluorescence intensity values were obtained by the integration of the emission spectra obtained for three diluted solutions of the stock antibody with concentrations ranging from 1.08×10^{-2} to 1.08×10^{-4} µM, from 480 nm to 600 nm, for an excitation wavelength of 460 nm.

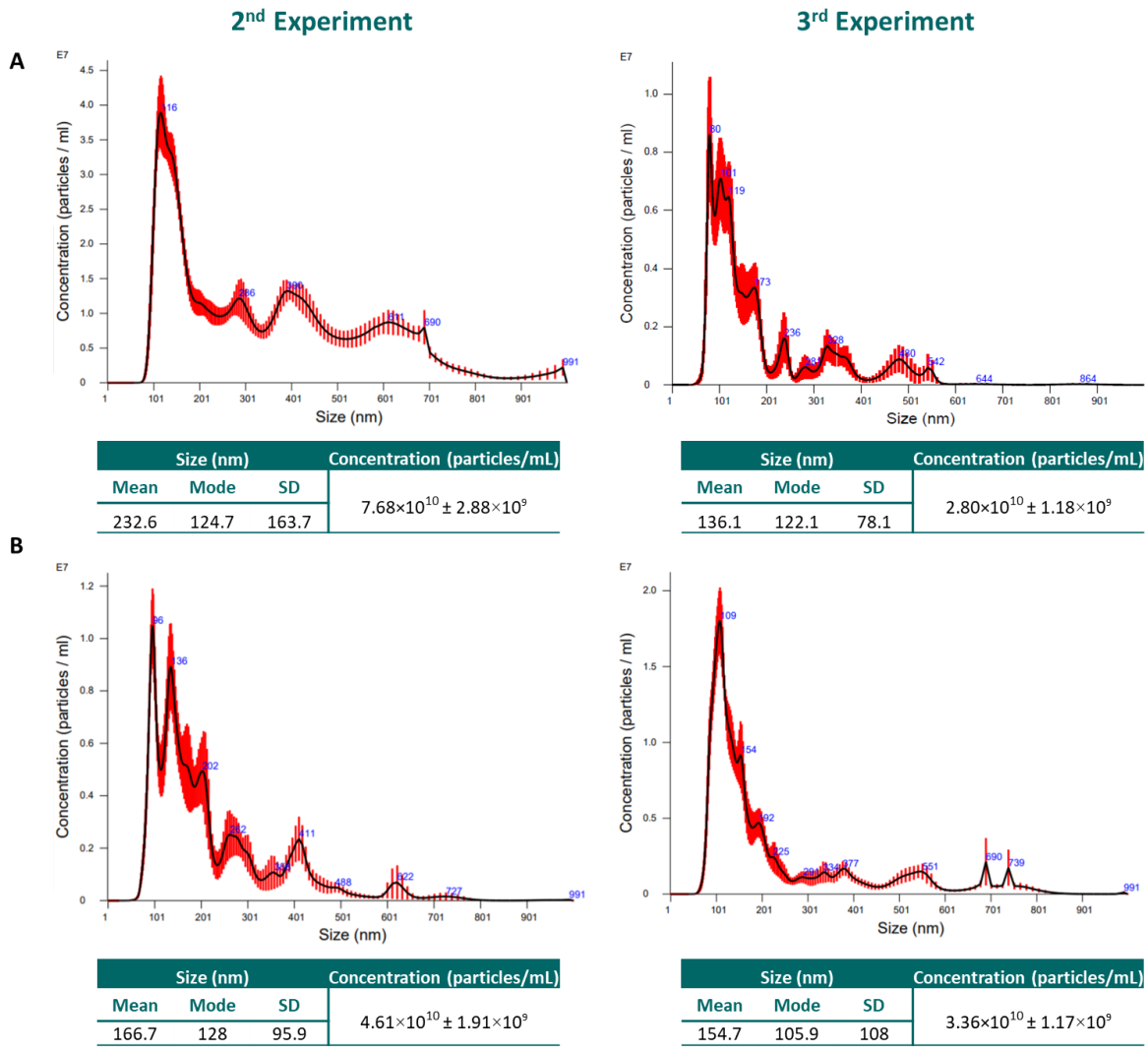
Table 3 – Anti-CD63 FITC concentration, determined using the fluorescence intensity values obtained by the integration of the emission spectra obtained from 480 nm to 600 nm, for an excitation wavelength of 460 nm (Figure 13), for exosomes labelled in three different experiments. The ratio of antibody molecules per particle was calculated using the total number of particles in the sample, obtained through NTA, and the FITC molecules per particle ratio was calculated admitting a FITC to antibody stoichiometry of 4-7:1 (provided by the manufacturer).

	Concentration (µM)	Anti-CD63 molecules/Particle	FITC molecules/Particle	
			Min	Max
1st Experiment	2.1×10^{-3}	98	392	686
2nd Experiment	4.9×10^{-3}	162	650	1137
3rd Experiment	7.9×10^{-3}	142	569	996

As expected, the values calculated for the antibody concentration for each of the different samples were in agreement with what was previously seen for the fluorescence intensity values, however, when it came to the number of antibodies and, consequently, FITC molecules bound to each vesicle it was seen that in fact a higher ratio was achieved with the second experiment rather than the last. Nevertheless, despite this, the difference between these two experiments is not very significant, which may indicate that the condition that mostly contributed to a higher efficiency of labelling in the last two experiments, when compared with the first, was the larger amount of anti-CD63 FITC co-incubated with the exosomes.

Finally, to assess whether the purification of the samples using an Amicon® ultrafiltration unit was successful in the retrieval of the stained vesicles and whether there was any aggregation of the exosomes caused by either the labelling or the purification steps, an NTA assay was performed for the samples stained in the second and third experiments. The results from this analysis, as well as those obtained for the exosome samples in question before labelling, are co-represented in **Figure 15**, in order to facilitate a direct comparison.

In general, it can be concluded that for both experiments the protocols to which the samples were subjected did not affect the distribution in size of the vesicles or the mode diameter of the population, indicating that there was little to no aggregation of the particles caused by the labelling. Furthermore, the particle concentration of the stained solutions was close to what had been seen for the initial samples, meaning that no significant amounts of exosomes were lost and thus validating this protocol for the intended purposes.



	Size (nm)			Concentration (particles/mL)
	Mean	Mode	SD	
A – 2nd Experiment	232.6	124.7	163.7	$7.68\times10^{10} \pm 2.88\times10^{9}$
A – 3rd Experiment	136.1	122.1	78.1	$2.80\times10^{10} \pm 1.18\times10^{9}$
B – 2nd Experiment	166.7	128	95.9	$4.61\times10^{10} \pm 1.91\times10^{9}$
B – 3rd Experiment	154.7	105.9	108	$3.36\times10^{10} \pm 1.17\times10^{9}$

Figure 15 – Particle concentration, size distribution, mean and mode size (nm), and standard deviation (SD) of results, obtained by Nanoparticle Tracking Analysis (NTA) for the HEK293 exosome samples used for the second and third experiments, before **(A)** and after **(B)** labelling.

In conclusion, the results obtained from this experiment indicated to the effectiveness of these labelling strategies. Nevertheless, further assays should be performed in order to assure the specificity of the chosen technique.

5. Discussion

Over the years, the development of stem cell-based therapies has attracted much attention towards mesenchymal stem/stromal cells (MSCs), which are multipotent cells present in adult tissues of different sources and are characterized by their vast therapeutic properties. In fact, these cells have proven to be effective as cell-based therapies in several disease models without raising any safety concerns *in vivo* as numerous clinical trials progress. Nevertheless, the mechanisms underlying their therapeutic properties are still not certain, although recent evidence indicate that most of them might be related to their secretome. Amongst the secretome of MCSs are exosomes, small membrane-vesicles that aid in the transfer of proteins, lipids and RNA molecules to target cells, thus playing an important role in intercellular communication in numerous biological contexts. These vesicles have been the target of many studies occurring over the past years and are currently seen as promising components of MSCs' secretome, providing a possible alternative to cell-based therapies that already involve MSCs.

With this in mind, this study was developed with the intent of elucidating the biological functionality of the exosomes isolated from human mesenchymal stem/stromal cells (MSCs), obtained in the scope of the framework of the PhD studies of Miguel Fuzeta who is aiming at the scalable production of these vesicles expanding the cells in bioreactors and, in future works, their application for drug delivery in cancer therapy.

For this, the first approach was to determine the effects of these MSC-derived exosomes on the proliferation of two adenocarcinoma cell lines (MCF-7 and A549) and one representative of the blood-brain barrier (hBMEC): Proliferation was assessed using a fluorescence-based PrestoBlue™ (PB™) viability assay and, for lung adenocarcinoma (A549) invasion through Matrigel was also evaluated in transwell platforms.

Results obtained from the viability experiments (**Figure 8**) were comparable for both tumour cell lines and demonstrated a similar pattern for all of the MSC-derived exosomes assayed. In general, a slight stimulating effect was noticed when these vesicles were applied in lower doses, which was seen to be attenuated when the cells were exposed to the more concentrated exosome preparations. In contrast, when it came to the response of hBMEC cells to this treatment, no cohesive pattern was observed for the different samples. However, these cells seemed to be overall more affected by the MSC-exosomes than the previous ones, with the exception of the UCM-MSC derived sample, which led to a slight increase in cell viability. Furthermore, for the A549 cells, as mentioned, these viability assay results were complemented with the assessment of their invasive capacity *in vitro* when exposed to these vesicles, achieved using a transwell migration assay with Matrigel coated cell culture inserts which allowed for an approximation to the *in vivo* environment. In general, the results obtained from this experiment (**Figure 12**) demonstrated that the presence of these exosomes stimulated the invasive capacity of this cell line, with results varying between samples from different sources.

Overall, the results from these experiments indicate that MSC-derived exosomes do in fact have a functional effect over the cells tested, which was not observed for exosomes from other sources

(HEK293). Nevertheless, these effects were seen to change with the cell line in test and with the source of the MSC-exosomes. Despite being more obvious for hBMEC cells, the results obtained for MCF-7 and A549 cells also demonstrated notable differences in the variation of cell viability from source to source and, for the UCM-derived samples, even between both donors, regardless of the fact that there was a similar response pattern after treatment with all samples. This was not unexpected as it has been previously reported that the properties of these cells vary with their source and donor [67], and thus, the same would be true for their secreted vesicles.

Nonetheless, it is worth emphasizing that these are only preliminary results and despite the fact that these vesicles stimulate and promote the proliferation of cancer cells it might not be indicative of their effect *in vivo,* as the conditions in which they were assayed do not truly resemble the tumour microenvironment and thus it might be possible that in *in vivo* conditions there would be more favourable results. Therefore, at this point these results shouldn't be seen as discouraging when it comes to their application for cancer therapy. In fact, in their work, Mendt *et al.*[68] reported comparable observations when assaying exosomes isolated from BM-MSCs cultured in conditions similar to those used to obtain these vesicles. When applied for *in vitro* treatment of pancreatic cancer cells, these MSC-derived exosomes were overall not seen to cause an increase in apoptosis, in some cases even demonstrating a slight decrease in the percentage of apoptotic cells, relatively to the control. Nonetheless, when tumour-bearing mice were treated with these vesicles their survival rate was seen to be slightly improved comparatively to that of the mice treated solely with their vector (control). These results could then be promising when it comes to the application of MSC-exosomes for cancer therapy, as, all in all, they show the possibility of a large-scale production of GMP compliant exosomes with positive *in vivo* outcomes, despite of what was observed *in vitro*. In addition, as mentioned, the main goal for these vesicles is their use for drug delivery, an application which was also shown to be possible by these authors, who were able to effectively load siRNA into the vesicles and consequently demonstrated significantly increased treatment efficacies in both testing conditions.

In a similar way, the same should also be noted for the results obtained for the hBMEC cells, as the observation that these cells seem to be affected by treatment with the tested vesicles might not be truly indicative of their possible effects *in vivo*. As a matter of fact, these cells were assayed since there is interest in their application as an *in vitro* model of the blood-brain barrier (BBB), in a way to possibly elucidate the capacity of these vesicles to cross this barrier and, hopefully, allow for their future application for treatment of neurodegenerative and other brain related disorders. Therefore, it is relevant to understand that the conditions to which these cells would be subjected when mimicking the BBB [69,70] are substantially different to those used in these assays, and consequently the impact caused by these vesicles might also be changed. Also, several studies have shown that systemic administration of MSC-derived exosomes allows for the delivery of drugs into target regions of the brain [60] and also for the recovery of neurological function and neurovascular plasticity [71,72] in mouse models of stroke, indicating that these vesicles are indeed capable of crossing the BBB without causing adverse effects on the tested subjects, and consequently providing a promising outlook on the application of the produced MSC-derived exosomes.

The establishment and isolation of controls for the mentioned experiments was performed taking into consideration MISEV2018 [64], where the authors suggest the importance of using certain negative controls which should result in minimal functional effects, consequently allowing for the substantiation that the impact caused by the MSC-derived samples was in fact related to the presence of purified exosomes and not associated with other factors.

Following their isolation, certain characterizations were proceeded, beginning with the quantification of their total protein concentration, using a BCA assay. This parameter was used mostly for the preparation of the exosome solutions later utilized in the viability and invasion assays, for both the controls and the MSC-derived samples. It might be suggested that other parameters would be more appropriate for the quantification of these samples, e.g. the total number of particles which can be obtained by Nanoparticle Tracking Analysis (NTA), as medium components might have co-precipitated with the vesicles, consequently resulting in an overestimation of the concentration of the samples. Nevertheless, it was not possible to perform a NTA quantification for all of the isolated samples, due to low resulting yields after isolation and lack of time. Furthermore, for the MSC-derived samples, a comparison between calculated ratios of total protein per vesicle was performed (**Table 4**) and it was possible to conclude that there is a consistent correlation between these two parameters for all of the samples, which was expected as all exosomes were isolated using the same methodology, consequently resulting in similar yields. This comparison then supports the use of the total protein concentration for the quantification of the dose of the samples for the treatment of the cells.

Table 4 – Ratio of total protein per particle for the exosome samples, from three different MSC sources (Umbilical Cord Matrix – UCM-MSCs(1) and UCM-MSCs(2) – Adipose Tissue – AT-MSC – and Bone Marrow – BM-MSC(1) and BM-MSC(2)), used for treatment of cells in viability and transwell invasion assays. Total protein concentration was determined using BCA assay and total number of particles was determined by NTA. Results were provided by PhD student Miguel Fuzeta.

Exosome Sample	UCM-MSCs(1)	UCM-MSCs(2)	AT-MSCs	BM-MSCs(1)	BM-MSCs(2)
Protein/particle (fg/particle)	2.94	3.20	4.03	6.12	4.49

Besides this, NTA and western blot analysis (**Figure 9**) were performed for some of the isolated controls, and in general the obtained results were representative of the presence of vesicles with exosome-like characteristics and were in agreement with what has been obtained for MSC-derived vesicles isolated in static conditions, i.e. grown in flasks rather than the vertical-wheel bioreactors, similar to how these controls were obtained. Although it was not possible to perform these analyses for all of the samples, these results allow for a general idea of the properties of the isolated controls, and therefore support the suitability of these samples for the experiments.

Finally, as there was the intention of using a confocal microscopy approach for the determination of the mechanisms involved in the uptake of MSC-derived exosomes, the labelling of HEK293-derived exosomes was attempted using an immune-fluorescence strategy. This was thought to allow for a more specific targeting of these vesicles when compared to the more commonly used lipophilic dyes, possibly circumventing issues regarding their use. Results from three separate experiments indicated that the

amount of labelled antibody co-incubated with the exosomes as well as the number of vesicles to be labelled were the parameters that mostly influenced the effectiveness of the labelling strategy. Also it was seen that the protocol applied for the removal of unbound antibodies, and consequent purification of the labelled vesicles, was adequate for the intended purposes, as it did not affect the distribution in size of the vesicles or the particle concentration of the samples, indicating that it doesn't cause aggregation of the particles and there isn't a significant loss of vesicles during this process. Once again, these results are only preliminary since, due to time constraints, it was not possible to proceed to further testing. In fact, fluorescence fluctuation spectroscopy (FFS) analysis is intended and expected to allow for a more in-depth characterization of these vesicles and of the overall labelling strategy [73].

6. Conclusion and Future Perspectives

The use of exosomes as an alternative to MSCs has been seen as a way to overcome the issues involved with the use of whole cells as, regardless of not having proliferative abilities, these vesicles have a much higher safety profile, lower immunogenicity and *in vivo* stability. However, their properties, when compared to their parent cells are still not certain, and, therefore, the determination of their function when applied in a biological setting is of interest.

All in all, this work provides an outlook on the biological properties of exosomes derived from three different sources of MSCs (AT, UCM and BM), demonstrating that the vesicles isolated from the conditioned medium obtained from the expansion of cells in bioreactors are not inert when co-incubated with different cell lines and, consequently, highlighting the possibility for their future application for therapeutic purposes. The main objective of this experiment was to determine an overall behaviour pattern of cells in response to the treatment with these vesicles which was achieved for the two tumour cell lines (MCF-7 and A549) assayed. However, further experiments are still needed in order to confirm these results and to achieve a better understanding of how these effects are produced.

Given this, for future works there is the intent of performing more specific assays, in order to better elucidate on the actual mechanisms underlying MSC-exosomes' effects, such as the assessment of exosome uptake and intracellular routes, determined using fluorescence microplate readers and confocal fluorescence microscopy, respectively. As mentioned, permeability studies using an *in vitro* blood-brain barrier model (achieved with hBMEC cells) are also intended, in order to clarify the potential of their application for treatment of neurodegenerative disorders. Moreover, and considering that the main goal for the production of these vesicles is their use for drug delivery in cancer therapy, their loading with therapeutic molecules will be attempted and thus different encapsulation techniques will be tested.

Finally, it is worth mentioning that the production of these vesicles from medium conditioned by MSCs cultured in vertical-wheel bioreactors seems to be yielding vesicle concentrations in the same order of magnitude as those obtained by other authors who reported a large-scale production of GMP-compliant exosomes from bone marrow–derived MSCs [68]. The method used for the isolation of these vesicles was a precipitation based one, using a total exosome isolation reagent, which allows for a fast recovery of the intended vesicles with good yields, nevertheless, this technique has been reported as having increased chances of contamination with medium components or even polymeric materials. With this in mind, it would be of interest to experiment with other isolation techniques, such as a sucrose cushion ultracentrifugation which has been shown to allow for better yields with high purity, by Chopra *et al.*[74] when compared with ultracentrifugation and with the same total isolation reagent used.

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