

Faculdade de Engenharia da Universidade do Porto



**A local delivery system for extracellular vesicles
to promote tissue regeneration**

Alice Quintas Pinheiro

DISSERTATION

Mestrado em Engenharia Biomédica

Supervisor: Doutora Susana Santos

Co-supervisor: Mestre Andreia Silva

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Autor - Alice Quintas Pinheiro

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Resumo

As vesículas extracelulares (EV), particularmente os exossomas, são atualmente alvo de intensa investigação. As EV são mediadores fisiológicos de comunicação intercelular que refletem o fenótipo das células que as secretam, e que têm a capacidade de modular o fenótipo das células alvo. Estas características das EV têm vindo a ser exploradas para diferentes aplicações, nomeadamente para o desenvolvimento de biomarcadores de diagnóstico e prognóstico de diferentes doenças, bem como para o desenvolvimento de novas terapias, em particular na área do cancro, mas também da medicina regenerativa. Vários estudos *in vivo*, e também alguns ensaios clínicos, têm vindo a usar EV como veículos de entrega de biomoléculas funcionais. Nestes estudos, EV nativas ou modificadas têm sido administradas através de diferentes vias. Os primeiros estudos em modelos animais e pacientes humanos com administração sistémica de EV demonstraram várias limitações na biodistribuição e retenção das EV administradas. Assim, foi colocada a hipótese de que a incorporação das EV em biomateriais capazes de funcionar como um sistema de entrega poderia constituir uma potencial solução para ultrapassar estas limitações, potenciando o uso eficaz das EV. Deste modo, esta tese teve como objetivo o desenvolvimento de um sistema de entrega de EV que permita a libertação local e controlada das mesmas para efeitos de regeneração óssea.

No trabalho desta tese, começou-se por otimizar as condições de cultura de diferentes linhas celulares imunes humanas, THP-1, KG-1 e Jurkat, nomeadamente em relação à concentração de soro do meio de cultura, de forma a obter uma produção de EV com alto rendimento e com grande pureza. As EV produzidas foram caracterizadas por microscopia electrónica de transmissão, “*nanoparticle tracking analysis*”, e “*western blotting*” para marcadores exossomais, o que permitiu demonstrar que, para todas as linhas celulares testadas, a população de EV isolada era enriquecida em exossomas. No entanto, a linha celular KG-1 produziu os níveis mais elevados de EV, mantendo a viabilidade celular mesmo a concentrações de soro de 0.1 % (v/v), e por isso estas células foram selecionadas para a produção das EV utilizados para a otimização das condições de incorporação num sistema de entrega.

O sistema de entrega construído consistiu numa matriz 3D em quitosano funcionalizada com camadas de quitosano (Ch) e poli- γ -ácido glutâmico (γ -PGA) misturado com EV, depositadas alternadamente num sistema *layer-by-layer*. A incorporação das EV no sistema de entrega foi observada por microscopia confocal de fluorescência, usando EV previamente marcadas com um corante fluorescente, mas não pôde ser confirmada por espectroscopia de infra-vermelhos. De seguida, o perfil de libertação das EV do sistema de entrega foi testado

in vitro através da análise dos sobrenadantes do ensaio de libertação por microscopia electrónica de transmissão, após 0, 12 e 24 h de incubação, tendo sido possível identificar EV nestes sobrenadantes concentrados, em particular às 12 e 24 h de libertação. A nível funcional, o sistema de entrega produzido e funcionalizado com EV foi testado para a sua capacidade em promover o recrutamento, bem como a diferenciação osteogénica e condrogénica, de células mesenquimais multipotentes/estaminais do estroma (MSC), responsáveis pela formação do osso. Através de ensaios de microscopia de vídeo em tempo real, foi possível demonstrar que as EV incorporadas no sistema de entrega promoveram um aumento significativo da motilidade das MSC. Para além disso, resultados de expressão génica nas MSC mostraram que o sistema de entrega funcionalizado com Ch e γ -PGA foi pro-condrogénico, mas que a presença de EV não levou a aumentos significativos da diferenciação das MSC.

Em conclusão, foi possível incorporar EV no sistema 3D *layer-by-layer* de Ch e γ -PGA desenvolvido, que se mantiveram funcionais após libertação. As EV secretadas pelas células KG-1 promoveram o recrutamento das MSC através do aumento da sua motilidade, tal como já descrito para EV de células dendríticas primárias, enquanto que o sistema de entrega funcionalizado com o Ch e γ -PGA promoveu *per se* a diferenciação condrogénica das MSC. Em conjunto, os resultados obtidos suportam um efeito favorável da incorporação das EV num sistema de libertação controlada, sobre as MSC, sugerindo que as EV podem ser mediadores quimiotáticos deste tipo celular para locais de lesão óssea, onde o sistema de entrega produzido pode estimular a sua diferenciação, promovendo a reparação e regeneração do tecido ósseo.

Abstract

Extracellular vesicles (EV), in particular exosomes, are currently the subject of intense research. EV are natural mediators of intercellular communication, reflecting the phenotype of the secreting cell, and potentially modulating the phenotype of target cells. These characteristics of EV are being exploited for different applications, namely the discovery of biomarkers for disease diagnosis and prognosis, and the development of new therapies, particularly in the cancer field, but also in regenerative medicine. Several *in vivo* studies using EV as delivery carriers of active biomolecules have been reported, including a few human clinical trials. In these studies, different routes of EV administration and the use of natural or modified EV formulations have been reported. Pioneering studies have been uncovering several limitations in EV biodistribution and retention in animal models and human subjects, when these are administered systemically. Consequently, it was hypothesized that incorporation of EV in biomaterial delivery systems could contribute to overcome these limitations. Thus, this thesis aimed to develop an EV delivery system for local and sustained EV release, particularly for bone regeneration.

For this thesis, cell culture conditions for different human immune cell lines, THP-1, KG-1 and Jurkat cells, in terms of serum concentration in the media, were optimized, in order to obtain higher yields of EV production, and with higher purity. EV produced were characterized by transmission electron microscopy, nanoparticle tracking analysis and western blotting for exosomal protein markers, and found to be enriched in exosomes for all cell lines tested. However, it was the cell line KG-1 that produced EV at higher yields, while still maintaining high cell viability at serum concentrations of 0.1 % (v/v), and thus these cells were selected as EV producers for further use in optimizing the incorporation into a delivery system.

The EV delivery systems developed consisted of chitosan 3D scaffolds functionalized with layers of chitosan and EV-containing poly- γ -glutamic acid (γ -PGA), alternated in a layer-by-layer system. EV entrapment in the delivery system could be confirmed by laser scanning confocal microscopy, using fluorescently labelled EV, but not by Fourier-transform infrared spectroscopy. Then, the release profile of EV from the delivery system was evaluated *in vitro* by transmission electron microscopy of release supernatants, at 0, 12 and 24 h of incubation, and EV could be detected in concentrated supernatants, particularly at 12 h of release. At the functional level, EV-loaded delivery systems produced were tested for their capacity to promote recruitment, and osteogenic and chondrogenic differentiation of multipotent mesenchymal stromal/stem cells (MSC), responsible for bone formation. Using time-lapse

video microscopy we showed that EV entrapped in the delivery system were able to promote significant MSC motility. Also, gene expression analysis results indicate that while Ch and γ -PGA delivery systems were pro-chondrogenic, the presence of EV did not increase MSC differentiation further.

In conclusion, EV could be entrapped in the layer-by-layer Ch and γ -PGA 3D systems, and were functional upon release. The KG-1-secreted EV used, similarly to those of primary Dendritic Cells, induced an MSC recruitment-promoting behaviour, increasing their motility, while the Ch and γ -PGA delivery system *per se* promoted chondrogenic differentiation. Together, our results support a beneficial effect of EV entrapment and controlled release, upon MSC, suggesting EV as chemoattractants for this cell type into places of bone injury, where one can envisage the delivery system may enhance their differentiation, promoting tissue repair and regeneration.

Key words: Bone regeneration, delivery system, extracellular vesicles, mesenchymal stem cells

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Cofinanciado por:



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Abbreviations

AFM	Atomic force microscopy
ATR-FTIR	Attenuated total reflection-Fourier-transform infrared spectroscopy
BBB	Blood brain barrier
BC	Bottom compartment
bFGF	Basic Fibroblast Growth Factor
CCl_4	Carbon tetrachloride
cDNA	Complementary DNA
CFSE	5-carboxyfluorescein diacetate succinimidyl ester
Ch	Chitosan
Cx43	Connexin 43
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cells
DDS	Drug delivery system
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
DTH	Delayed-type hypersensitivity
ER	Endoplasmic reticulum
EV	Extracellular vesicle
FBS	Fetal bovine serum
FasL	Fas ligand
FDA	Food and Drug Administration
FTIR	Fourier-transform infrared spectroscopy
γ -PGA	Poly-gamma-glutamic acid
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IL	Interleukin
iPS	Induced pluripotent stem cell
i.v.	Intravenous injection
IFN- γ	Interferon- γ
LB	Luria Broth media
LbL	Layer-by-layer
LSCM	Laser scanning confocal microscopy
MCP-1	Monocyte chemoattractant protein 1
miRNA(s)	MicroRNA(s)
MSC	Multipotent mesenchymal stromal/stem cell

MV	Microvesicles
MVB	Multivesicular bodies
Mw	Molecular weight
MWCO	Molecular weight cutoff
NTA	Nanoparticle Tracking Analysis
PBS	Phosphate-buffered saline
PI	Propidium iodide
PGA	Poly-glutamic acid
PLGA	Poly(D,L-lactide-co-glycolide)
P/S	Penicillin/streptomycin
rhBMP-2	Recombinant human bone morphogenetic protein-2
RNA	Ribonucleic acid
Rluc	Renilla luciferase
RS	Raman spectroscopy
RT	Room temperature
RVG	Rabies virus glycoprotein
RT-qPCR	Quantitative real time polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
SFM	Serum-free media
SMSC	Synovium mesenchymal stem cells
SPR	Surface plasmon resonance
SN	Ultracentrifugation supernatant
TC	Top compartment
TEM	Transmission electron microscopy
TPP	Tripolyphosphate
tRPS	Tunable resistive pulse sensing
UC	Ultracentrifuged

Chapter 1

This chapter was adapted into a manuscript that was submitted for publication, and that is included as an annex (Annex 1) to this thesis.

Alice Pinheiro, Andreia M. Silva, José H. Teixeira, Raquel M. Gonçalves, Maria I. Almeida, Mário A. Barbosa, Susana G. Santos. **Extracellular Vesicles: Intelligent delivery strategies for therapeutic applications.** (submitted)

Introduction

The response to tissue injury requires well-orchestrated communication between the cells responsible for its different stages, from the initial inflammation to repair/regeneration. In the last decades, the roles of Extracellular Vesicles (EV), secreted by cells, in these processes have been the subject of intense research [1]. Three different types of EV secreted by cells have been reported and characterized, based on their origin, release mechanism and properties: apoptotic bodies, microvesicles and exosomes. Although the complete distinction of these different vesicle populations is still hindered by methodological limitations, the last two types of vesicles have deserved most attention from the scientific community, due to their biological properties [2]. Nonetheless, due to the persisting difficulties in fully distinguishing these vesicle populations, and in agreement with the recommendations of the International Society of Extracellular Vesicles, in this thesis the designation EV will be preferentially used.

The most intensely studied EV population is that of exosomes, nanometre-sized vesicles formed in the endosomal network and secreted by the majority of cells, including tumor cells [3] [4]. Exosomes are reported to play key roles in intercellular communication, without cell-to-cell contact. They have been reported to participate in different homeostatic biological processes, including antigen presentation, delivery of active biomolecules to target cells, and even cell waste management (initially thought to be their only function) [5]. These natural functions of exosomes have been explored for their application as therapeutic mediators in different fields, from cancer treatment to novel tissue regeneration strategies. However, they may also contribute for the spreading of infecting pathogens and pathologies, namely cancer metastasis [6].

EV have also been greatly explored as biomarkers of disease, because their composition is related to the status of the cell from which they derive. Recent studies have shown that the characterization of exosomes in fluids, particularly in the blood, urine, saliva, breast milk and amniotic fluid can strongly contribute for the diagnosis of various diseases, namely in cancer [7] [8]. This makes the use of EV as biomarkers to be applied in areas such as diagnosis, prognosis, as well as real-time monitoring of therapy of different pathologies by determining their levels and composition in body fluids [7] [9] [10].

On the other hand, EV can be obtained *in vitro* from different cells, and are reported to be stable when administered *in vivo*, which are important advantages for their application as a drug, or as drug delivery system. In addition, they can be obtained from the patient own cells, an important advantage regarding their immune compatibility [11]. Indeed, considering their clinical value, EV have been increasingly investigated in the delivery systems field. Many studies have been reporting their use to deliver different molecules such microRNAs (miRNAs), siRNAs, proteins or drugs to target cells [2]. However, despite the increasing research in EV field, there are several difficulties in their isolation and characterization process. Many isolation procedures have been described but in all of them, a very low EV yield is reached. In addition, there is a lack of characterization methods able to efficiently evaluate EV standardized production from different cell culture batches. Both issues constitute a big obstacle to make EV clinical applications succeed. On the other hand, when administered *in vivo*, exosomes are readily cleared from circulation. Furthermore, the mechanisms controlling the specific delivery of exosomes to a specific target are fairly unknown and need to be studied harder. These limitations are the main motivations underlying this master thesis. Thus, they will be further addressed in the following sections, along with possible strategies to overcome them.

1.1 - Cell-secreted extracellular vesicles

1.1.1 - Extracellular vesicles: characteristics and methods of isolation

Most cells are capable of releasing different types of EV, which are generally divided in three classes: exosomes, microvesicles, and apoptotic bodies. Each type of EV can be classified based on different criteria, such as origin in the cell, size, and enrichment of specific protein markers. Figure 1.1 establishes a close relationship between the sizes of the three different vesicles. However, this classification is still controversial with different authors reporting different sizes for the same EV population.

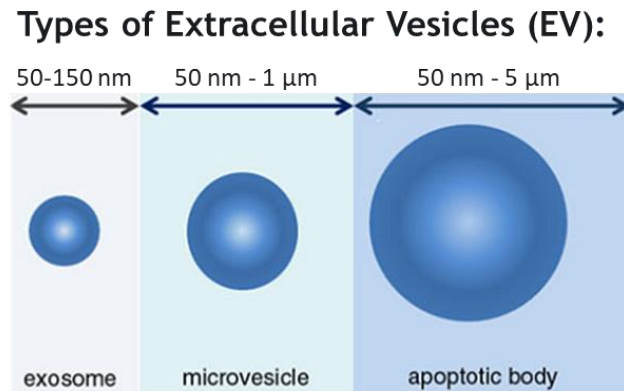


Figure 1.1 - Schematic representation of the sizes of the different types of extracellular vesicles that can be secreted by cells. Exosomes may range from 50 to 150 nm (are the smallest). Next, microvesicles can range from 50 nm to 1 μ m. The largest vesicles are the apoptotic bodies and can reach 5 μ m. Adapted from [12].

The microvesicles originate by direct budding from the plasma membrane and their size is quite variable (50 nm - 1 μ m) [13]. As for the apoptotic bodies, they are released when the cell is undergoing apoptosis [14], and are an even more heterogeneous population, ranging in size from 50 nm to 5 μ m [10]. On the other hand, exosomes are characterized by being the smallest EV, with sizes in the range of 50-150 nm, being derived from the intraluminal bud of multivesicular bodies (MVB), and that are released after fusion of these MVB with the plasma membrane (shown in Figure 1.2) [10]. As a common feature among them, all types of EV are lipidic vesicles that carry a protein content in their core, as well as on their surface. They also carry different types of RNAs, including messenger RNA and miRNAs, DNA, and even different metabolites. Importantly, these biomolecules are functional, even when delivered to target cells. There are three main mechanisms by which EV interact with target/recipient cells: endocytosis; direct fusion with the plasma membrane of the recipient cell; and receptor-mediated endocytosis following receptor-ligand interaction between EV and the recipient cell [15].

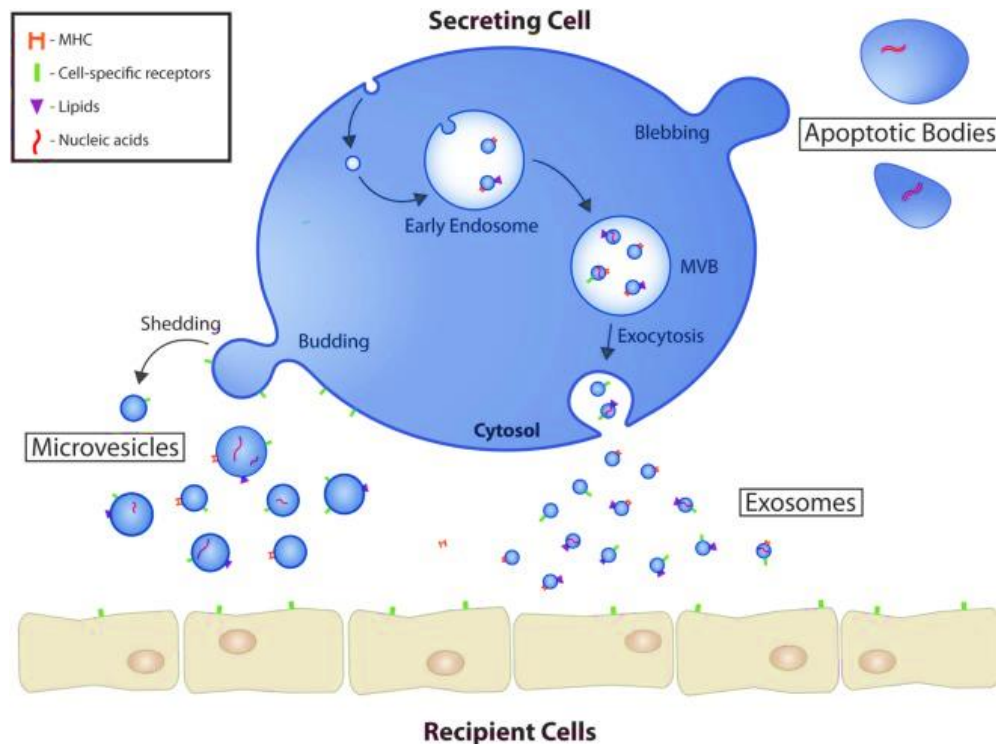


Figure 1.2 - Extracellular vesicles biogenesis, release, and mode of action. Cells secrete different types of extracellular vesicles, including microvesicles (MV), exosomes and apoptotic bodies. Apoptotic bodies are formed during programmed cell death. MV are released by cell membrane budding of, whereas exosomes have their origin in multivesicular bodies (MVB). Upon release, EV travel body fluids until they reach a target cell. Since EV carry biomolecules such as proteins, messenger RNA, microRNA (miRNA) and DNA, they are able to modulate the phenotype of target cells. Adapted from [16].

Upon secretion, these different EV are present together in body fluids or supernatants of *in vitro* cell cultures, and separation methodologies are required to allow the study of each EV population. Different methods have been developed and optimized in the last years to isolate these EV populations, particularly exosomes, with different degrees of efficiency. These methods include differential centrifugation with a final step of ultracentrifugation, or ultracentrifugation in density gradients, phase separation, size-exclusion chromatography, immunoprecipitation and filtration. Differential (ultra)centrifugation is still the method most commonly reported for the isolation of EV, although EV pellets obtained are generally a mix of exosomes, microvesicles and soluble proteins secreted by cells, since many proteins have sedimentation coefficients similar to EV [14]. In order to isolate pure EV populations, soluble protein aggregates, denser than EV, may be removed by ultracentrifugation using sucrose cushions or gradients [14] [17].

Size-exclusion chromatography also allows the separation of EV from contaminant soluble proteins. Importantly, it is perhaps one of the most complete methods for the separation of different EV populations, since apoptotic bodies, microvesicles and exosomes are eluted in different fractions of the eluate [18]. Due to their easy implementation, exosome isolation by phase separation is becoming increasingly popular, being available in commercial kits

nowadays. However, a major drawback of this method is the common precipitation of microvesicles and soluble proteins alongside exosomes [19].

Up to date, centrifugation in density gradients and size-exclusion chromatography are the preferred techniques for the isolation of pure exosome populations [20]. However, the yield of isolation is usually very low and the isolation protocols are time-consuming, impairing a wider use of these methods.

Considering the difficulties in isolating pure populations of the different types of EV, it is of utmost importance that the vesicles isolated are extensively characterized [17]. However, distinguishing and quantifying the different subtypes of EV remains a difficult task because of several similarities in size and physical properties of the different subtypes, and the lack of specific markers for each one of them [14].

1.1.2 - EV characterization: methods of analysis

In order to characterize the isolated EV several techniques are usually combined. So far, the main techniques used are transmission electron microscopy (TEM), atomic force microscopy (AFM), flow cytometry, western blotting, nanoparticle tracking analysis (NTA), dynamic light scattering (DLS), tunable resistive pulse sensing (tRPS) and surface plasmon resonance (SPR). More recently, Raman spectroscopy has also been applied to the characterization of EV.

A primordial technique used in the field of EV biology, and still the most used for determining EV morphology, and even to visualize their release from cells is transmission electron microscopy (TEM). This technique also allows to determine the size of individual EV with minimal sample processing. Biochemical information about the EV can also be obtained with this method, by using immunogold labelling. However, this technique has limitations related to preparation of the sample, since it must be dehydrated and analysed under vacuum [21]. The AFM is also used for the characterization of EV, however, few studies follow this approach, due to the need of immobilization of the vesicles under study. Moreover, the most suitable EV immobilization method, that could retain the physiological properties of the vesicles, is still being discussed. Besides retrieving information regarding vesicles size and morphology, AFM also allows characterizing their mechanical properties [22] and potentially their interactions, for instance with cells.

For a more detailed biochemical analysis, EV are usually characterized based on the proteins they carry on their surface [7]. Tetraspanins (CD37, CD53, CD63, CD81 and CD9) are highly enriched in EV, and thus are generally accepted as EV markers. CD63 is perhaps the most reported EV marker [23] [24]. However, several positive and negative markers of EV should be tested simultaneously. The specific detection of these markers by antibodies is usually achieved by flow cytometry or western blotting. Flow cytometry allows simultaneous multi-parameter analysis on EV. However, a disadvantage of this technique is that common flow cytometers cannot distinguish light scatter from one single EV, and thus, their analysis by flow cytometry usually requires an additional step of vesicle coupling to carriers of larger size [21]. Multiple EV are often count as a single EV, precluding an accurate analysis of EV concentration and size [25]. Thus, the use of larger beads with EV absorbed, followed by

fluorescent antibody labelling, is still the most common solution to characterize EV by flow cytometry [14]. Protein markers can also be assessed by western blot, the main disadvantage is that the analysis is of a protein pellet, and not individual vesicles. This technique can be associated to immunoprecipitation, using the classical EV markers (CD9, CD63 or CD81), and analysing the immunoprecipitated vesicles directly by western immunoblotting for the detection of additional markers [26].

To analyse changes in the amount of EV produced in different conditions and also to determine EV concentration and size distribution, for analysis of the isolated populations and for standardization of functional assays, the most used methodologies are nanoparticle-tracking analysis (NTA) and dynamic light scattering (DLS). NTA is the most widely used technique to assess EV size and concentration [21] [27]. In general, this technique gives information about EV size distribution and concentration in solution, and allows real-time virtual visualization of the vesicles [28]. In some equipment, fluorescence can be combined with this method, by labelling EV directly with fluorescent dyes or immunolabelling specific molecules with fluorescent-conjugated antibodies [21]. Concerning NTA limitations, it is known that instrument-specific variables affect the characterization of EV, limiting the analysis of very heterogeneous populations [29]. In addition, the use of fluorescently-labelled antibodies for the analysis of EV increases specificity, but may limit the analysis to specific subpopulations, as discussed by Rafal *et al.* [21]. Similarly to NTA, dynamic light scattering is a technique used for EV size characterization and EV distribution with the variance of scattered light intensities detected in a specific angle and then compared to the wavelength of light [7]. Importantly, this technique allows determining the surface charge of the vesicles, an important parameter for their internalization by target cells. In recent years, a few works were published reporting the use of tunable resistive pulse sensing (tRPS) to characterize EV suspensions. This technique calculates EV absolute size, their concentration in the sample and their surface charge [21]. These values are calculated in comparison with reference particles with known volume and concentration [27]. However, Maas *et al.* concluded that different operation variables could affect significantly EV quantification [27].

Other tools are being developed to improve accuracy and detail in EV characterization. For instance, surface plasmon resonance (SPR) allows real-time and label-free detection of ligand binding to target receptors located on a sensing film [30]. Im *et al.* performed EV populations characterization by using a nanohole-based SPR sensor, being 10 000 times more sensitive than western blotting analysis, and allowing a fast characterization of EV [31]. In addition, Raman Spectroscopy (RS) analyses chemical vibrational modes of the samples due to wavelength shifts [32]. RS generates a spectrum with qualitative and quantitative information to describe chemical composition of EV. With this, the use of specific protein biomarkers is not required. Unlike all the previously described techniques, RS provides label-free determination of chemical composition of even a single EV in a short time [33]. Due to these advantages, this technique is being increasingly investigated as a fundamental analytical tool in EV characterization field [34].

Despite all the technical advances achieved in the last years, basic EV characterization techniques are far from being used in diagnostics. In addition, they are still very limited for the characterization of EV to be administered in *in vivo* models, and to quantify their distribution in the body upon administration. The important role of EV, in particular as potential therapeutic tools, makes it necessary to further improve the techniques of

quantification and characterization of these vesicles. To reach clinical practice, a fast sample characterization, based on a fingerprint characterization of EV populations, must be achieved, and improved experimental settings and routines of analysis must be implemented [27] [35].

1.1.3 - Therapeutic potential of EV

It is nowadays recognized that EV play important roles in intercellular communication, which renders them a natural mechanism for transferring information between cells. In particular taking advantage of their nanosize, EV are being explored as nanodevices for therapeutic applications [36]. Our group recently reviewed the potential contributions of EV to the different stages of tissue repair and regeneration, as illustrated in Figure 1.3.

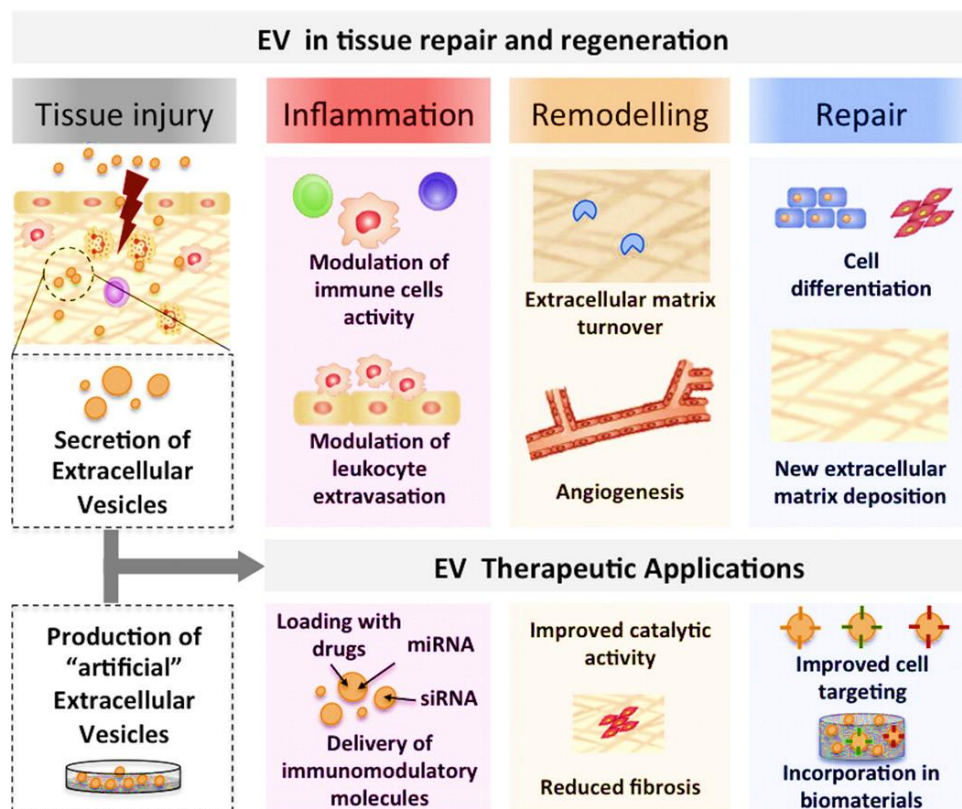


Figure 1.3 - Extracellular Vesicles are natural and tunable delivery vehicles with clinical potential in tissue repair and regeneration. EV are produced by virtually all cell types in the body and their content varies with physiological conditions, playing crucial roles during the inflammatory, remodelling and repair stages of tissue regeneration. Furthermore, natural EV can be modified to carry miRNAs, mRNAs, proteins and drugs of interest, functioning as improved therapeutic vehicles. Adapted from [37].

Naturally, EV carry active biomolecules that are loaded into their core or incorporated in their membrane through cell endogenous mechanisms [10] [38] [39]. The content of EV constitutively secreted by a cell reflects its physiological condition, and will impact on their

effect on target cells. Different roles have been described for EV, that may contribute to the different stages of tissue repair/regeneration. For instance, researchers previously showed that the RNA cargo of MSC-derived EV is different according to the MSC tissue source [40]. Also, EV secreted by immune cells in different stages of activation may elicit either immunostimulatory or immunosuppressive responses on target cells [41]. In addition, EV have been described for many years as having a role in bone matrix mineralization [42].

In order to improve the therapeutic potential and to standardize the effects of EV, the investigation into modified and artificial EV has been increasing. Much like with synthetic nanoparticles, different types of molecules such miRNAs, messenger RNAs, siRNAs, proteins, and even drugs, can be specifically incorporated into EV, which become the transporters of these molecules to target cells [43]. EV may be loaded with different molecules either exogenously or endogenously. In the first approach, the molecule of interest is included into EV after their isolation and purification, while the endogenous approach is based on active encapsulation during EV biogenesis, via transforming the EV-producing cells with the selected molecule [43].

EV have been most explored in the cancer field, where their potential roles and applications are more intensely researched. Their use in immunomodulatory therapies or directly anti-tumor therapies has been recently reviewed [17]. Recently, the anti-tumor therapies based on anti-cancer drug loading on EV have been the most studied. Saari *et al.* studied the cytotoxic effect of EV isolated from prostate cancer lines cultures, and loaded with the antimitotic cancer drug Paclitaxel, upon the same type of cancer cell [44]. They showed that loaded EV decreased cell viability, and generated better cytotoxic effects than the free drug, which was mediated by EV internalization by cancer cells. Also, using EV loaded with high dosage of Paclitaxel, Pascucci *et al.* showed great results *in vitro* in pancreatic cancer therapy [45]. This study introduced the ability of EV secreted by MSCs, which were previously loaded with Paclitaxel, to incorporate the drug during vesicle biogenesis. Interestingly, these EV retained anti-tumor activity *in vitro*. Many other studies reported the use of chemotherapeutics loaded in EV, supporting their use as drug vehicles as a good alternative to traditional cancer therapy. This is mainly due to a great reduction in toxic side effects, since EV biodistribution to target tissues may be increased by choosing appropriate parental cells for EV production [46] [47]. However, many different ways to further enhance effects and tailored targeting of these EV are being tested, so that EV therapies become a reality in clinical applications.

EV have also been studied for the development of novel therapies for neurodegenerative disorders, which affect increasing proportions of the world population. Haney *et al.* took advantage of the ability of EV to cross the blood-brain barrier (BBB) and incorporated catalase in the vesicles using different methods, in order to restore the reduced levels of redox enzymes, commonly observed in the brains of Parkinson's patients. A significant amount of catalase-loaded EV was detected in the brain of mice tested after intranasal administration, particularly in their neurons and microglial cells, which resulted in a neuroprotective effect [48]. Similarly, EV were suggested as therapeutic vehicles in Alzheimer's disease. Alvarez-Erviti *et al.* have shown the ability of engineered EV (from self-derived dendritic cells, DC), loaded with siRNA against BACE1 protein, to cross the BBB in mice, working as a treatment for Alzheimer's disease. Importantly, these EV were injected intravenously but non-specific effects in other tissues were not detected [49]. This work, along with other studies in the

field, is very promising for the EV-based treatment of different neurodegenerative diseases. However, some issues need to be overcome to achieve clinical trials. For example, the fact of the RVG peptide is exogenously expressed in EV surface in many studies targeting the brain, and the fact that the receptor for this peptide is often downregulated in Alzheimer's disease brains, makes this practice unable to proceed to the next investigation step before further *in vivo* studies [50].

Although cancer and neurodegenerative disorders are the fields where EV research is most intense, using EV as delivery systems in other areas, such as tissue repair and regeneration, has also gained increasing attention, particularly in models of hepatic failure, renal and myocardial injury. Interestingly, Wang and colleagues used EV derived from human umbilical cord-derived MSCs to study their capacity in preventive prophylaxis of acute graft-versus-host disease, in a mouse model of allogeneic hematopoietic stem cell transplantation. EV were intravenously administered, and results demonstrated a decrease in *in vivo* manifestations of acute graft-versus-host disease along with decrease of mortality [51].

In the field of tissue repair, EV secreted by MSC have been the most studied for their regenerative capacity. Many studies have been demonstrating their positive effects on the recovery of homeostasis, as well as on the regeneration and repair of damaged tissues. This suggests a therapeutic effect of MSC-derived EV that can be used as an aid to various therapies [52] [53].

Different injury models have been used to test the potential of MSC-derived EV as therapeutics. For instance, Li *et al.* studied the effect of EV derived from umbilical cord-MSCs in a carbon tetrachloride (CCl₄)-induced mouse liver fibrosis. This treatment inhibited hepatic lobule destruction, hepatocyte apoptosis and epithelial-to-mesenchymal transition, and ameliorated fibrosis [54]. Many other studies used EV of different origin as delivery systems for the treatment of the same model of CCl₄-induced liver fibrosis in mouse, reporting an amelioration of the condition [4] [55] [56]. Using a renal injury model, Gatti *et al.* demonstrated significant effects in reduction of epithelial tubular cell damage in an ischemia rat model, by using MSC-derived EV [57]. However, this knowledge has not yet been translated to human clinical trials [58]. EV derived from MSC were also shown to reduce infarct size in a mouse model of myocardial ischemia/reperfusion injury [59]. In a similar approach, Ibrahim *et al.* used EV from human cardiosphere-derived cells in a chronic myocardial infarction murine model. Exosome administration inhibited apoptosis of cardiomyocytes and interestingly, promoted their proliferation, helped by an increase of angiogenesis [60]. The regenerative effects of EV from MSCs were also reported in models with osteochondral defects [40]. In the study from Zhang *et al.*, MSC-derived EV were intra-articular administered in an osteochondral defect created on distal femurs of adult rats. After 12 weeks, a full reparative response of the femurs was observed in rats exosome-treated. In addition, only fibrous repair tissues were found in the control rats, PBS-treated. This was the first study reported the positive effects of EV derived from MSC in cartilage repair [61].

In our group, we have been focusing on the crosstalk between immune cells and MSC, particularly on the way immune cells can influence MSC behaviour. Our previous work showed that DC are able to recruit MSC [62], and that DC-secreted EV are main effectors of DC-mediated recruitment [63].

In the different studies published so far exploring the therapeutic potential of EV *in vivo* and in clinical trials, the strategy for vesicle administration is highly variable, using different routes and in different dosages. Furthermore, several EV-based therapies have been promoted for commercialization by several companies, for which EV dose, administration route, and timeplan of administration are still undisclosed and not well defined [64].

1.2 - Strategies for EV delivery

1.2.1 - Systemic delivery of EV

To reach the target tissue or organ, EV can be administered via different routes, such as intravenous, intraperitoneal, oral, intranasal, and subcutaneous (Figure 1.4), that may lead to their systemic distribution.

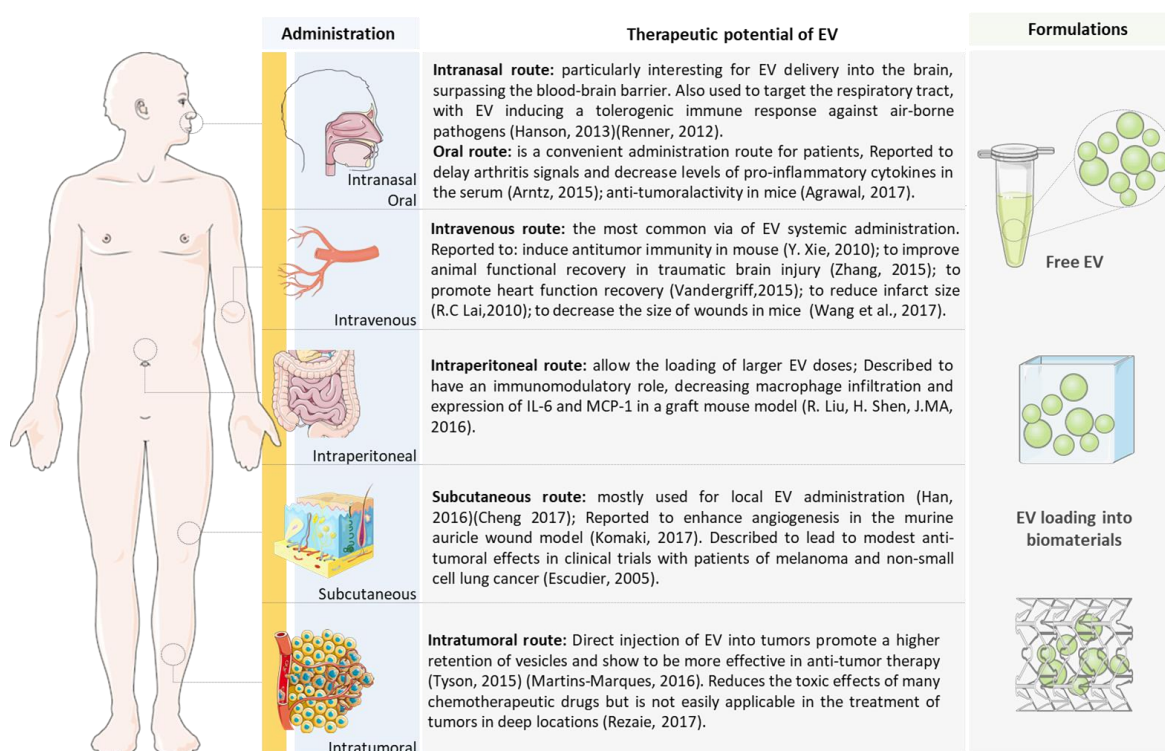


Figure 1.4 - Routes of administration of Extracellular Vesicles. EV may be used *in vivo* as a drug. They can be administered either as free vesicle suspensions or loaded in drug delivery systems that allow their controlled release along time. Depending on the clinical application intended, they can be administered in different body locations, which will affect their time of retention in circulation and in target organs, as well as their body biodistribution.

1.2.1.1 - EV intravenous administration

The most widely used strategy for EV *in vivo* delivery is the intravenous injection (i.v.), considered the fastest way to deliver drugs and also EV, due to their direct delivery to

systemic circulation. In one of the earliest studies dedicated to determine EV biodistribution, reporter EV derived from HEK 293T cells were injected subcutaneously in athymic mice and their biodistribution followed by *in vivo* imaging [65]. These EV were coupled to *Gaussia* luciferase and biotin, allowing conjugation with labeled streptavidin for non-invasive *in vivo* fluorescence imaging, and also analysis of EV distribution by bioluminescence, including in biofluids such as blood and urine, upon the addition of luciferase substrate. As early as 30 min after administration, EV could be detected in liver and spleen of mice, being detected in low amounts in the brain, heart and muscle at all timepoints analyzed. In accordance, EV were detected at the highest concentration in blood at 30 min after administration, peaking in the urine at 60 min post-injection [65]. In a later work, Manca *et al.* tested the biodistribution of fluorophore-labeled milk-derived EV upon i.v. and oral administration. After 24h, EV administered i.v. accumulated in liver and spleen, while those administered by oral route accumulated only in small intestine [66]. The i.v. route has been used in the majority of the *in vivo* studies investigating EV as new anti-cancer therapies. For instance, i.v.-injected EV were already demonstrated to work as potent vaccines for induction of antitumor immunity, in the mouse [67]. In one of the first studies performing EV administration i.v., EV ameliorated functional recovery in a mouse model of acute kidney injury [68]. Several other studies performing systemic i.v. injection have been successful in ameliorating different brain conditions, implicating that EV in circulation are capable of crossing the BBB [69] [70]. Indeed, in one of the first studies using siRNA-loaded EV, discussed above, EV injected intravenously, could reach the mouse brain, resulting in specific gene knockdown [49]. In further studies, MSC-derived EV injected in the tail vein were capable of improving animal functional recovery in traumatic brain injury [71] and cerebrovascular accident [72]. This capacity of EV to readily reach the brain is a great advantage comparing to most of the synthetic delivery systems under development, that usually need to be functionalized in order to improve their penetrance across the BBB. The i.v. administration of EV has also been explored in the cardiovascular field, using vesicles of different cellular origin. In the study by Vandergriff *et al.* the authors show that EV isolated from cardiosphere stem cells cultures, when injected in the tail vein of mice with induced dilated cardiomyopathy, promoted heart function recovery by reducing apoptosis and fibrosis [73]. Furthermore, i.v. administration of EV derived from MSC were shown to reduce infarct size in a mouse model of myocardial ischemia/reperfusion injury [59]. The systemic administration of EV was also previously shown to improve local cutaneous wound healing. The recent work from Wang and colleagues suggested that EV derived from human adipose MSC decreased the size of incisional dorsal wounds in mice, upon intravenous injection [74].

Intravenous administration allows, with a minimally invasive procedure, that EV reach their target anywhere in the body, even locations distant from the place of injection, like internal organs. However, the short half-life index of EV in circulation after injection is one of the major limitations of this route of administration [75]. Indeed, as demonstrated by several *in vivo* studies, EV are readily cleared from circulation, with some works reporting a calculated half-life close to only 2 minutes [75]. Most commonly, EV are cleared to liver and lungs [75]. In a detailed study by Morishita *et al.*, EV from B16BL6 melanoma cells were radioactively labeled and injected intravenously. By collecting blood and organs at different timepoints, and measuring radioactivity, the authors showed that, 30 min after injection, only 1 % of injected EV were still in blood. At the same timepoint, almost 40 % of the injected dose could be detected in the liver, and 10 % in the lungs, a distribution pattern that was

specific for those vesicles [76]. In a follow-up study, the same group indicated that the rapid clearance of EV from circulation after i.v. injection was mainly driven by macrophages, reason why the injected EV usually accumulate in the liver [77].

Although EV have a certain degree of specific tissue targeting, which is usually considered better than synthetic nanoparticles [78] [79], it is important to understand that EV clearance from circulation into different off-target organs might be an important drawback, for instance if vesicles are being used for the delivery of cytotoxic drugs. Furthermore, it is necessary to take into account that there are consequences of maladministration, which may be irreversible [10]. Indeed, serious consequences may occur when over-dosing EV is reached. For instance, Smith *et al.* reported a mouse died by asphyxiation with accumulation of EV in lungs, despite all the positive effects reported in regeneration of many organs [80].

1.2.1.2 - EV intraperitoneal administration

Another common method of EV administration is via the intraperitoneal route. Studies like the one by Liu *et al.* performed intraperitoneal administration of EV to modulate the immune response to a vein graft performed distantly, in the carotid of mice [81]. In this work, EV isolated from cultures of adipose tissue-derived MSC, and labeled with the PKH26 dye, were administered intraperitoneally and could be found after 30 min in the aortic endothelium, supporting the systemic distribution of the EV administered by this route. More interestingly, mice groups receiving the EV showed thinner neointima in the grafted carotid, which was correlated with a decreased macrophage infiltration and decreased expression of IL-6 and MCP-1 in the graft [82]. Of note, the intraperitoneal administration route allow the loading of larger EV doses, which may represent an advantage compared to other routes of systemic administration [3]. However, injected EV are also rapidly diluted to unwanted sites due to the big area of the peritoneal cavity [83].

1.2.1.3 - EV oral administration

Oral administration is the most convenient route for pharmaceutical products intake, due to ease of administration, facilitating patients' compliance to treatment. However, in this route EV face important obstacles. The intestinal barrier, changes in pH along the gastrointestinal tract and the characteristics of the intestinal microflora are further aspects that EV need to overcome to reach their target tissue or organ. Rezaie *et al.* suggested that this type of administration is mostly successful for delivery in luminal epithelial surface of the gastrointestinal tissues than in other target tissues [83]. Comparatively to other routes of administration, few studies were published so far reporting EV administration by oral route. Moreover, the works published mainly report the administration of milk-derived EV. In 2015, Arntz *et al.* reported the first oral delivery of bovine milk-derived EV with effects in arthritis [84]. Researchers used two different mouse models of arthritis and tested the capacity of these EV to delay the appearance of the disease and the evolution of the inflammatory phenotype. After EV administration, by oral gavage or in drinking water, histology analysis confirmed the delay of the arthritis as well as a decrease of cartilage and bone marrow inflammation. Serum levels of pro-inflammatory cytokines were also reduced [84]. Agrawal *et*

al. used EV from bovine milk for oral delivery of the chemotherapeutic drug Paclitaxel. To demonstrate the therapeutic effect of these vesicles, they used a female athymic nude mice model with lung tumor xenografts. Results showed a higher effect of drug-loaded EV on the inhibition of tumor growth, compared to control groups treated with PBS, the free drug or EV alone. Importantly, they observed a reduced systemic toxicity and inflammation when loaded EV were administered orally, comparing to their intravenous administration [85]. Thus, from this study, oral administration appears as a good alternative to decrease toxic side effects of EV systemic administration and to make EV-based therapies more efficient. The same group reported similar observations for milk EV loaded with the anti-cancer drug curcumin [86]. Upon oral administration of these loaded EV in mice bearing cervical tumor xenografts, tumors had lower volumes compared to control animals. However, curcumin could be found in lungs, liver and brain, in a dose dependent on EV dose administered [86].

1.2.1.4. EV intranasal administration

The intranasal route is also used for EV administration, with the first studies reporting this approach for the induction of a systemic immune response against air-borne pathogens using EV from infected macrophages [87], and to promote tolerogenic immune responses to air-borne allergens using bronchoalveolar lavage fluid-derived EV [88]. However, this route is also particularly interesting for the delivery of pharmaceutical products to the brain, either by entering the nearby circulation followed by crossing of the BBB, or directly through olfactory and trigeminal nerve cells [89]. Moreover, EV passage to circulation via the nasal route diminishes EV loss, because it avoids the intestinal and hepatic metabolism [90]. Indeed, this kind of administration showed great results in studies for Parkinson's disease therapy in mouse models of this condition, having neuroprotective effects [48]. Furthermore, the EV intranasal administration was reported to be more effective in retaining the vesicles in brain tissue than their systemic administration via intravenous injection in the tail vein [91].

Comparing different routes of administration, Yan *et al.* used EV from human umbilical cord MSCs in a murine model of hepatic oxidant injury, the CCl₄-induced acute liver failure. EV administration was tested orally and by injection in the tail vein, with different doses being tested. Both investigated routes showed similar effects, which gives advantage to the oral route due to the ease of administration. The results demonstrated significant antioxidant effects in CCl₄- and H₂O₂-injured cells. In addition, cell viability increased after EV administration. However, the mechanism underlying EV effect in hepatic oxidative stress remained unclear [92].

It is important to note that the classification of systemic and local routes of administration is not entirely linear. There are routes of administration, like the intraperitoneal, the intranasal or the subcutaneous, that are most commonly considered routes of local delivery. However, they can also be considered routes of systemic delivery, depending on the situation. In the case of subcutaneous injection, it can be used for local administration or be considered systemic administration (as detailed in the next section), when intended to act far from the site of administration, achieving systemic circulation. Intraperitoneal administration is considered systemic, but in fact it is mainly used to reach the gut, without necessarily going through the systemic circulation. Also, intranasal

administration can be used to reach the systemic circulation, but it is mainly exploited for its proximity to the brain.

Altogether, the different routes of systemic administration have some common limitations. The distance and barriers that EV have to travel until reaching their target cells, implies that most vesicles do not reach the target, and instead accumulate in other organs. Common constraints influencing EV biodistribution include passage through physical barriers such as capillary endothelium, gastrointestinal epithelium and the BBB, uptake by non-target tissues, including entrapment in their extracellular matrix, phagocytosis by immune cells, particularly macrophages, and clearance in urine. Ultimately, this implies that higher EV doses have to be administered in order to achieve the intended biological effect at the target tissue. One possible strategy to overcome these problems is to increase EV tropism to the tissue of interest. This may be achieved by EV functionalization with surface proteins binding ligands on target tissues. In fact, Tominaga and colleagues demonstrated that the transfection of surface proteins from donor cells in EV-producing cells functioned as strategy to harness EV with special tropism to the brain [93]. Thus, it remains a challenge in the EV delivery field to find the responsible proteins for different targets, in order to achieve natural organ tropism for EV. Another possible strategy to increase the chance of EV reaching their target tissue is their modification with “don’t eat me” signals, such as CD47, which allows escaping the engulfment by immune cells [94]. Alternatively, to minimize the adverse effects of EV systemic administration, they can also be delivery directly to the target tissue.

1.2.2 - Local delivery of EV

Local drug administration is generally associated with reduced side effects [10], which will likely be the case also for EV. Furthermore, local administration of EV may overcome the often-reported low retention rates of the vesicles at specific target sites, compared to non-specific organs.

One of the first studies reporting the local effect of EV dates back over a decade, to 2006, when Kim *et al.* demonstrated that EV derived from genetically modified murine bone marrow DC (overexpressing FasL), when administered locally, could resolve inflammation in a murine model of delayed-type hypersensitivity (DTH), and suppress the signals of arthritis in a collagen-induced arthritis mouse model. They administered EV via local injection in the footpad and via systemic intravenous injection, and concluded that both were effective in suppression of the DTH response and in the treatment of collagen-induced arthritis. However, EV injected locally were not detected in the animals liver or spleen, suggesting the effect of EV was not mediated systemically [95].

In the cancer field, intratumoral injection of EV as an anti-cancer treatment strategy has been greatly explored. Indeed, some biodistribution studies reported unmodified EV to be unsuccessful in tumor-specific delivery upon i.v. administration [96] [97]. Alternatively, direct injection of EV into tumors has been shown to be a much more effective anti-tumor therapy. Indeed, the work of Smyth *et al.* suggests that the intratumoral injection of DIR-labeled EV derived from 4T1 cells allows much higher retention of the vesicles inside the tumor, than their i.v injection in the tail [11]. Furthermore, intratumoral EV injection has been reported as efficacious in tumor growth inhibition. In the work of Martins-Marques *et al.*, EV loaded

with luciferin and the chemotherapeutic agent doxorubicin, were injected in mouse tumors. Tumor growth was then monitored by bioluminescence imaging and a cytotoxic effect specifically in tumor cells was demonstrated. Interestingly, they concluded the presence of the gap junction protein connexin43 (Cx43) embedded in EV membrane improved the release of EV content into target cells, contributing to the reduction of cardiotoxicity of free doxorubicin injected systemically [98]. Intratumoral injection may be generally associated with reduced toxicity in non-target tissues. However, similar results using local or intravenous administration have also been reported [46].

Although intratumoral EV injection is apparently more effective in tumor eradication, this type of administration is much more invasive than systemic administration, and thus unlikely to be as well accepted by patients [83]. In addition, it is not easily applicable for the treatment of tumors in deep body locations. However, injectable systems are also being developed that may facilitate delivery by minimally invasive surgery, and when surgery is required, for example for removal of the tumor, a delivery system could be implanted for the removal of cancer cells potentially remaining at the site. Indeed, a recent study by Palamà *et al.* leads us to speculate that an approach similar to the one they followed may be applicable for EV delivery. The authors reported the use of a poly- ϵ -caprolactone scaffold loaded with the anti-inflammatory drug dexamethasone in the treatment of osteosarcoma based on pre-and-post-operative surgery [99]. Results demonstrated a sustained drug delivery and thus, a good strategy of localized tumor therapy.

Another route of EV local administration often used is the subcutaneous injection. Investigations have shown that administration of EV by subcutaneous injection may be an effective anti-cancer treatment. An interesting study suggested the use of EV derived from M1-polarized macrophages as a cancer subcutaneous vaccine adjuvant [100]. A melanoma mouse model was used, and EV were injected subcutaneously in their right flank. To verify the EV distribution after administration, they were labeled with the lipophilic fluorescent dye Dil. Using an *in vivo* imaging system after animal's sacrifice at 7 days after injection, EV were localized in inguinal and axillary lymph nodes near the injection local. This observation suggests that EV did not achieve systemic circulation in the lymphatics but mainly accumulated in lymph nodes near the place of injection, where they promoted an increase in the secretion of proinflammatory cytokines [100].

Since EV efficiency is dependent on their administration route, Hao *et al.* compared effects of EV administered via subcutaneous injection and via intradermal injection in antitumor immunity. This was performed in a mouse model with induced tumors in the thighs. To monitor their distribution upon administration, EV were labeled and, despite the proximity of both injection routes to the tumor, by the intradermal route, labeled EV were detected in 3.8 % of lymph nodes cells, against only 0.9 % of cells when subcutaneous route was used. These results are in agreement with the mice survival rate upon the two different administration routes: intradermal route demonstrated to be more successful in antitumor immunity than subcutaneous administration [101]. Interestingly, the first clinical trials (phase I) using EV performed repeated injections of EV, derived from autologous dendritic cells (DC), into melanoma and non-small cell lung cancer patients using the intradermal (10 % of the dose) and subcutaneous routes (90 % of the dose). Nonetheless, only modest antitumoral effects were achieved [102]. More recently, a phase II clinical trial for the treatment of non-small cell lung cancer used conventional chemotherapy, combined with DC-derived EV for

cancer treatment. In this trial, EV were administered exclusively by intradermal injection leading to an increase of 32 % in the progression-free survival of patients [103]. Importantly, Dai and his team developed a Phase I clinical trial whereby EV derived from ascites of colorectal cancer, were subcutaneously administered to test their effectiveness in colorectal cancer treatment. Patients tested received EV only, or EV combined with GM-CSF. Both were shown to be safe, but only patients who received EV with GM-CSF developed an antitumor cytotoxic T lymphocyte response [104].

In the area of tissue repair and regeneration, subcutaneous injection of EV has also been reported. Komaki *et al.* injected subcutaneously placental tissue-derived EV into auricular wounds. They observed the capacity of this therapy to enhance angiogenesis in the murine auricle wound model [105]. In a mouse model of fat graft, animal flanks were subcutaneously injected with fat tissue, simultaneously with EV derived from normal or hypoxia pre-conditioned adipose tissue-MSCs, or with PBS. The subcutaneous administration of EV and fat tissue lead to an improvement in graft survival and integration, compared to control flanks injected with fat and PBS, suggesting the subcutaneous route of administration can be used for local EV administration and effects [106].

Zhang *et al.* demonstrated the ability of weakly intra-articular administrations of human MSC EV to repair critically-sized osteochondral defects in rat models. After 12 weeks, regeneration was observed with hyaline cartilage and subchondral bone very similar to the original one [61]. As demonstrated by this study, short-term repeated doses of EV should be administered during the regeneration process to achieve good results. This kind of EV delivery is not comfortable for a patient, and is pointed as one of the major drawbacks of this approach, for clinical application [107].

Although there are not many studies using this type of administration, intracavernous injection was performed by Chen and his team to demonstrate EV effects in erectile dysfunction. The EV used were derived from adipose stem cells, and tested in a type 2 diabetes mellitus rat model. Results showed an improvement on erectile dysfunction in rats, by decreasing apoptosis of penile tissue. Despite positive results, many issues were unclear in this study, namely how long EV remained in the organ, what was the EV dose administered and the specific time of their administration, and what was the mechanism of action [108].

Depending on the therapeutic action intended and the tissues to be targeted, different routes of administration of EV can be chosen. Overall, this choice will determine the pharmacokinetics and biodistribution of administered EV. Further modulation of these parameters might be achieved by EV incorporation into delivery systems as further discussed in the next section.

1.3 - Delivery Systems: from Drug to EV Delivery

A drug delivery system (DDS) is a system that is used as a media or carrier for administering a pharmaceutical product to a patient, to achieve a therapeutic effect [109]. Similarly to free pharmaceutical products, DDS may be administered in patients either by parenteral routes (e.g., intravenous, subcutaneous, intramuscular, intranasal, etc.) or gastrointestinal routes (e.g., oral, rectal, etc.), requiring different features to reach their target. Moreover, different profiles of drug release from DDS may be desired depending on the action intended. With this in mind, many different DDS have been developed, and include nanoparticles, liposomes, microspheres, gels, scaffolds, among many other formulations [110]. An important characteristic that must be achieved by the different DDS synthesized is the controlled release of the pharmaceutical products they carry. Different sustained drug release formulations have shown that a long-term exposure of the tissue to a certain drug concentration is better than an increase of drug concentration, to achieve the pretended drug effects. Most of them intend to avoid a burst release and a concentration decay in blood [111].

Local and systemic delivery of free EV do not allow to control the retention at the target site, consequently impairing the achievement of therapeutic concentrations. Also, the “pharmacokinetics” and biodistribution of delivered EV is neither controlled nor fully clarified. This issue may be overcome by the incorporation of vesicles into appropriate delivery systems that permit a timely and controlled release of EV *in vivo*. Controlled release will increase EV availability in the injury site for longer periods, maximizing their effect. In fact, EV have been used in combination with biomaterials of different types in a tissue engineering approach to treat several different lesions.

Hydrogels constitute the class of biomaterials most used for EV delivery *in vivo*. In fact, they are very attractive for EV incorporation, since their mechanical properties can be tuned into a large extent by different techniques, including by using biocompatible cross-linkers [112]. Thus, hydrogels with very small pores may be synthesized, providing a slow release rate of the vesicles. A gradual degradation of the material makes a prolonged release effective, and decreases side effects of bulk and fast EV administration, such as accumulation in non-target organs [113] [114]. Moreover, they can be easily modified with different moieties for improved nanoparticles attachment to the hydrogel, which may be translated to EV. Most importantly, hydrogels can be administered by different methods, including injection [112].

Zhang *et al.* reported the use of human MSCs-derived EV in the therapy of traumatic brain lesions in rats. In that work, the EV were incorporated into three-dimensional type I collagen scaffolds to mimic the extracellular matrix. When compared to the controls used (liposomes mimicking the primary lipid / phospholipid content of the EV), there was an increase in angiogenesis and neurogenesis, as well as a decrease in neuroinflammation [115].

Also, Liu *et al.* tested the incorporation of EV isolated from cultures of human iPS-derived MSC in a photoinduced imine crosslinking hydrogel glue, useful for cartilage repair due to its biocompatibility, and its capacity of cartilage integration. This approach has many advantages when compared to other EV administration modes, such as the ability of retaining EV in the cartilage defect site or the ability of achieve an accurate fit and even distribution in

irregularly shaped tissue defects. To monitor EV release from the glue, vesicles were labeled with the lipophilic fluorescent dye Dil prior to incorporation in the biomaterial, and release conditions were then simulated *in vitro*. Released EV could be detected by nanoparticle tracking analysis and red fluorescence of Dil-labeled EV were found in the cytoplasm of chondrocytes also entrapped in the glue, suggesting a successful endocytosis of the released EV. With this approach, the authors could demonstrate a released of about 100 million EV/mL every day, resulting in a major therapeutic dose to the surrounding cells [107].

EV delivery using biomaterial scaffolds has also been reported, particularly in recent studies. Huang *et al.* reported positive effects of EV derived from dental pulp cells on odontogenic differentiation *in vivo* and *in vitro*. In this study, EV were isolated and embedded in collagen sponges, and included on the root canal space [116]. Another study compared the effects in osteogenesis of EV/B-TCP combination scaffolds with only pure B-TCP scaffolds. EV-incorporating scaffolds were more effective in the bone repair on a rat critical-sized calvarial bone defect model, with large amounts of *de novo* bone formation [117]. In a recent work, Xie and colleagues functionalized decalcified bone matrix scaffolds with MSC-derived EV and investigated the angiogenic and osteogenic potential of these constructs *in vivo*. Regenerative potential of this scaffold was evaluated in a model of ectopic subcutaneous bone formation in nude mice. EV enhanced bone formation and increased vascularization in the grafts [118].

Chitosan-based materials have also been reported as delivery system for EV. A recent study demonstrated the use of chitosan hydrogel loaded with EV derived from miR-126-3p-overexpressing synovium mesenchymal stem cells (SMSCs) in wound repair. The material incorporating EV was implanted on the wound bed, providing a controlled release of the vesicles, and was monitored daily until day 14 after surgery. The results demonstrated that release of SMSC-miR-126 EV increased re-epithelization, deposition of collagen and generated new vessels and their maturation at the wound site [119]. Another study reported similar results with chitosan/silk hydrogel loaded with gingival mesenchymal stem cells derived EV in *in vivo* experiments. As the previous study, a diabetic rat was tested and positive effects on the diabetic rat skin wound repair were verified [120]. In an innovative approach, Huleihel *et al.* tested ECM bioscaffolds, produced by decellularization of connective tissue, as natural EV carriers. They found that EV naturally anchored in the native matrix could survive the decellularization protocols applied and, when implanted, could promote cell growth and proliferation, cell migration and cell differentiation. They also showed that EV have specific miRNA signatures associated to their source tissue, essential information to improve clinical applications [121].

Despite the good functional results reported so far, the use of carriers for EV delivery is still little explored, and advantages and disadvantages of these systems, compared with free EV administration, are not yet well characterized. For instance, studies addressing biodistribution and pharmacological kinetics parameters of EV entrapped in scaffolds are very limited. Another point that needs to be clarified is how the controlled release of the EV from these scaffolds is achieved *in vivo*. Many characteristics of scaffolds composition and structure may be tuned for optimal controlled release of EV from different origins, such as scaffold porous size, interconnectivity between porous, method of EV adhesion to the carrier, among others. Thus, in the near future, it is necessary to improve the design of carriers to use with EV.

In the scope of this thesis, delivery system synthesized with chitosan and poly-gamma-glutamic acid was developed for the entrapment of EV. The advantages of using such a system will be discussed in the next section.

1.3.1 - Chitosan and poly- γ -glutamic acid: Biomedical Applications

Ch is a linear polysaccharide that contains copolymers of β -(1 $\rightarrow$ 4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). It is a natural polycationic polymer obtained by the partial deacetylation of chitin, readily available from insects and crustaceans. It is biodegradable, biocompatible and nontoxic [122].

Research based on nanotechnology and polymer science have been showing great advances in the use of chitosan-based films in strategies such as coating, extrusion, layer-by-layer assembly, among others. For example, in food industry, chitosan-based films are being used for packaging [123]. In the environmental field, chitosan is a great alternative to synthetic pesticides, being used in the synthesis of environmentally friendly pesticides, contributing to a sustainable agriculture [124]. In the health sciences field, chitosan has been investigated as an antimicrobial agent, a blood anticoagulant and as a biomaterial in wound dressings, stent coatings, antibacterial coatings, biosensors, systems of controlled drug delivery and tissue engineering, including bone regeneration [122] [124] [125] [126] [127]. Usman *et al.* reviewed several chitosan-based materials and their potential application in the biomedical field [126]. Importantly, Food and Drug Administration (FDA) approved chitosan for tissue engineering and drug delivery. This biopolymer was also approved as safe by FDA for wound dressing applications and for dietary use [128]. Moreover, many cytotoxicity studies have been performed in order to clarify chitosan biodegradability and biodistribution *in vivo*, further supporting its safety [129].

Poly-glutamic acid (PGA) is a polymer of glutamic acid that can be distinguished in two isoforms, based on the carboxy group establishing the monomers bonding: α -PGA or γ -PGA, if the attachment is on carboxy group α or γ , respectively. Due to its characteristics, only γ -PGA will be described in more detail for the purpose of this work.

Poly-gamma-glutamic acid (γ -PGA) is naturally produced by several gram-positive bacteria, mainly from *Bacillus* species [130]. Contrarily to chitosan, it is an anionic polymer. It has good biodegradability, biocompatibility, water solubility and non-toxic effects [131]. Its use has been reported in food supplements and pharmaceutical. Its potential toxicity has been investigated [132]. Prodhomme *et al.* reported no toxic effects on the human B-cell line EHRB, and no toxicity after injection of 1 mg γ -PGA in a mouse model [133]. Hence, γ -PGA has been gaining importance in biomedical applications.

1.3.2 - Chitosan as a delivery system

Depending on their application, chitosan-based DDS can be used in many different formulations, mainly as nanoparticles or microparticles, hydrogel scaffolds and films. Importantly, for every chitosan-based DDS synthesized, many parameters can be modified in order to achieve specific characteristics in the final structure. They include the chitosan concentration used, the pH of the chitosan solution, the solvent used, the method chosen for

the synthesis of the structure, the use of chemically-modified chitosan derivatives, and the inclusion of crosslinking agents. Modification of any of these parameters affects the physicochemical properties of the chitosan structures, their mechanical properties and their porosity. Consequently, these modifications affect also the enzymatic degradability of chitosan and their biodistribution *in vivo* [134].

Several methods can be used to produce drug-loaded chitosan nanoparticles. Nevertheless, its low solubility in biological fluids and its insolubility in organic solvents are limitations for the synthesis of formulations. To overcome this problem, chitosan DDS may be synthesized in acidic conditions and, more interesting, derivatives with improved hydrophilicity have been developed [135], by modifying chitosan by N-substitution, O-substitution or free radical graft copolymerization [136]. Chitosan-based DDS have been reported for the delivery of labile molecules, such as peptides/proteins. For instance, incorporation of insulin in chitosan microparticles was tested, using tripolyphosphate (TPP) and glutaraldehyde as chitosan cross-linking agents. Incorporation of glutaraldehyde during microparticle synthesis was proven to be effective in preserving insulin activity [51].

Chitosan nanoparticles have also been investigated for the delivery of nucleic acids. Chitosan positive charge interacts with their negative charge, resulting in a polyelectrolyte complex, which protects the nucleic acids from degradation [135]. Chitosan particles have also been used for the delivery of different drugs. For instance, Zhang *et al.* tested the use of different carboxymethyl chitosan derivatives to function as nano DDS for docetaxel. Results suggested different physicochemical characteristics of the nanomaterials, such as different released profiles and cell cytotoxicity, with O-carboxymethyl chitosan exhibiting the strongest cytotoxicity against tumor cells [137]. In another study, Jain *et al.* realized that glutaraldehyde-crosslinked chitosan nanoparticles loaded with variable concentrations of docetaxel had a drug profile release *in vitro* that could be divided in 3 phases. Firstly, a burst release of the drug was observed, mainly from the surface of nanoparticles, secondly, there was a controlled drug release from the matrix, and finally, a steady release was reported, due to polymer degradation. Overall, about 68-83 % of loaded docetaxel was released, significantly decreasing viability of cancer cells *in vitro*, compared to blank control nanoparticles [138].

In addition, loaded chitosan micro/nanoparticles may also be integrated in more complex DDS. A recent study produced aspirin-loaded chitosan nanoparticles incorporated in an asymmetric collagen-chitosan membrane. This delivery system was used in a critical-sized cranial defect on rats, and results showed an effective controlled release of the drug along time [139]. Also in this context, Karri *et al.* incorporated curcumin in chitosan nanoparticles that were then loaded into a collagen scaffold. Results *in vitro* showed an effective controlled drug release. *In vivo* results show enhanced wound closure when compared to the placebo scaffold groups and the administration of free curcumin [140].

Besides being effective in the controlled release of many different compounds, chitosan particles have also a biodistribution pattern *in vivo* that favors their use as DDS in specific applications. As nanoparticles could be detected in bone marrow, chitosan nanoparticles may be usable for bone imaging and targeting strategies. However, it should be noted that nanoparticle biodistribution is greatly dependent on particle size. Nanoparticles with less than 5 nm diameter are usually removed by renal clearance due to their capacity to pass through the glomerular membrane. On the other side, nanoparticles with a diameter larger

than 200 nm are typically eliminated by the reticuloendothelial system. Thus, nanoparticles between this size range (5-200 nm) avoid both clearance mechanisms and can circulate in blood for a longer period [141]. Interestingly, exosomes size range is within these values.

Chitosan can also be processed into hydrogels and scaffolds, three-dimensional crosslinked polymer networks with the ability of retaining large quantities of water. Due to their capacity to transport molecules and drugs, chitosan hydrogels are extensively studied for biomedical applications [142]. Chitosan-based hydrogels have been used for different purposes, including immunomodulation, cancer treatment and tissue engineering. In this field, Nafee and colleagues created a chitosan-based thermogel loaded with alendronate, a drug used in the treatment of bone conditions that has low effects when free administered orally. It was possible to modulate thermogel properties, for example, by increasing alendronate concentration, which resulted in a hydrogel with higher density and lower porosity. Previous work from our group has used chitosan scaffolds alone and in combination with pro-inflammatory molecule, fibrinogen, or inflammation resolution mediator, resolvin, to promote bone regeneration [143]. Furthermore, a combination of chitosan with β -glycerophosphate resulted in a hydrogel with a time sustained drug release *in vitro*, up to 65 days [144]. Despite their hydrophilic nature, chitosan hydrogels have also been tested for the delivery of hydrophobic drugs. This was achieved by functionalization with hydrophobic moieties. Marin *et al.* used palmitoyl glycol chitosan to incorporate the model hydrophobic drug denbufylline [145]. Chitosan may also be combined with other materials to tune their drug delivery properties. A 2003 study reported for the first time the development of a bilayer chitosan-gelatin scaffold with different pore size by changing the pre-freezing plate temperature and concentration of chitosan-gelatin solution. Indeed, a lower concentration of the solution increased the pore size [146]. The use of gelatin, due its viscosity, helped preventing drug leakage and, the cationic density of gelatin increased at pH $\approx$ 4, contributing to a firm gel formation.

More recently the field of chitosan-based-self-healing hydrogels became of great interest. A self-healing hydrogel can self-reconstruct after its injection, allowing the simultaneous delivery of drugs of interest. Li and colleagues demonstrated that a chitosan-self-healing hydrogel was more effective in delivering an anti-tumor drug (Taxol), than a chitosan hydrogel [147].

More complex compounds may also be incorporated into chitosan hydrogels. Venkatesan *et al.* produced chitosan hydrogels with carbon nanotubes and tested their antimicrobial activity. The use of free carbon nanotubes is limited due its dispersion, but their incorporation in the chitosan hydrogel revealed to be a great strategy, greatly improving the antimicrobial activity of the nanotubes in a dose-dependent manner [148]. Nanoparticles and microparticles may also be loaded into a chitosan scaffold. A recent study demonstrated the effect of chitosan-hyaluronic acid nanocomposite scaffold loaded with antioxidant and anti-inflammatory Andrographolide lipid nanocarrier in the enhancement of wound healing. A controlled release of the drug was achieved up to 72 h. This approach proved to have potential in wound healing applications [149].

Chitosan can also be processed into films by solvent casting, usually followed by a crosslinking agent [150]. Films have many great characteristics, such as edibility, biocompatibility, non-toxicity and low cost. This chitosan formulation represents an

important form for drug delivery, due to the mucoadhesive properties of this biomaterial and their capacity to open epithelial tight junctions. These are great characteristics that promoted the investigation of chitosan as a DDS for the gastrointestinal system and skin, and as a coating material for other biomaterials [135]. Varshosaz and colleagues investigated the release of lidocaine from a crosslinked chitosan film to, in a future approach, deliver lidocaine into the oral cavity (for the treatment of oral mucositis) [151]. Importantly, this study confirmed that chitosan films crosslinking improves their physical and mechanical properties, thermal stability and water resistance, allowing their application onto other biomaterials [142].

1.3.3 - Poly- γ -glutamic acid as a delivery system

As for chitosan, γ -PGA has been explored for the synthesis of DDS. The most common formulations include nano/microparticles and hydrogels. It was previously demonstrated that PGA molecular mass influences its drug delivery properties [130]. In addition, γ -PGA DDS may be combined with other biomaterials, rendering them suitable for different applications. Aguirre *et al.* produced poly(D,L-lactide-co-glycolide) acid (PLGA) nanoparticles for doxorubicin delivery and modified its surface with γ -PGA and folic acid. Importantly, at pH 7.4, only 12 % of doxorubicin release was observed, which suggests this system as a good therapeutic vehicle for cancer [152]. In another study, the effect of caboxymethyl chitosan/PGA microparticles loaded with Levofloxacin as a hemostatic in wound dressings was verified. This system was demonstrated to be pH sensitive and a sustained release profile of levofloxacin was achieved in *in vitro* drug release experiments. Thus, this mechanism has potential to be a candidate carrier for drug delivery system [153].

PGA-based injectable hydrogels for drug delivery were also previously reported. Ma and colleagues formulated a hydrogel with hyaluronic acid and poly- γ -glutamic acid carrying bovine albumin as a drug model. Results showed a sustained albumin release behaviour in *in vitro* experiments, which was reduced by hydrogel crosslinking degree [154]. In a previous study, a DDS system was produced by mixing PGA with a small quantity of chitosan, resulting in a vehicle with a thermo-sensitive behaviour with sol-gel transition at body temperature. This platform was used for the loading of human bFGF, which had a controlled release both in *in vitro* and *in vivo*. This release profile could be explained by the positive charge of bFGF that allows electrostatic interaction with PGA. Thus, a slow PGA degradation allowed a slow bFGF release [155].

1.3.4 - Combining chitosan and poly- γ -glutamic acid in a layer-by-layer delivery system

The combination of chitosan cationic characteristics, with anionic polymers such as γ -PGA, has been studied in our group for several years. Pereira *et al.* investigated the capacity of chitosan and γ -PGA to assemble into nanoparticles by using a co-precipitation method [156]. Another investigation reported the incorporation of diclofenac in chitosan and γ -PGA nanoparticles and its release at physiological pH [157]. Moreover, considering the electrostatic interactions that are established between these polymers, we investigated their potential for the synthesis of layer-by-layer (LbL) self-assembly systems for drug release.

Antunes *et al.* demonstrated that chitosan could interact electrostatically with γ -PGA and developed a structure with a maximum of six alternated layers composed by chitosan or γ -PGA [158]. A similar approach was reported by Cardoso *et al.*, which synthesized chitosan and γ -PGA self-assembled polyelectrolyte multilayer films by using a LbL method. They further used this system for the delivery of IFN- γ *in vitro*, that was proven to be able to maintain protein structure and could retain high amounts of the cytokine. In addition, the assembly process could be made in the absence of organic solvents, improving the biocompatibility of this delivery system [159].

1.4 - Objectives

EV are important players in cell communication and, as discussed above, are promising as new therapeutic strategies for different diseases. Nonetheless, their clinical application is being impaired, among other factors, by their rapid clearance upon administration and broad body biodistribution. Hence, it was hypothesized that EV entrapment in a DDS could control local vesicle dose and bioavailability along time after administration. Thus, the main goal of this thesis was to develop an EV delivery system that could be used for the controlled release of the vesicles upon local administration *in vivo*. In order to achieve our aim, three main tasks were delineated:

1. Select a human immune cell line for the production of EV at high yield and optimize cell culture conditions for EV production and isolation.

In vitro cell culture conditions for EV production were optimized for the human immune cell lines KG-1, THP-1 and Jurkat. EV were isolated from cell culture supernatants and characterized.

2. Develop an EV delivery system.

A chitosan 3D scaffold was synthesized and functionalized with chitosan/ γ -PGA in a layer-by-layer system, in which EV could be entrapped. EV entrapment was confirmed by laser scanning confocal microscopy, and their release from the scaffold was studied in physiological conditions simulated *in vitro*.

3. Determine the effect of EV entrapped in the chitosan-based delivery system on the modulation of human MSC phenotype.

The 3D delivery system developed was used for EV entrapment and delivery *in vitro* to human MSC cultures. The effect of entrapped EV on modulating MSC migration, motility and differentiation was tested by transwell migration assay, time-lapse video microscopy, and gene expression analysis, respectively.

Chapter 2

Methods and Materials

2.1 - Cell culture

In this work, we tested 3 different human leukemia cell lines - KG-1, THP-1 and Jurkat - to select the most adequate for EV production with high yield.

THP-1 and Jurkat cells growth medium was RPMI-1640 (Corning), supplemented with heat inactivated Fetal Bovine Serum (FBS) 10 % (v/v) (Biowest) and penicillin/streptomycin (P/S) 1 % (v/v) (Invitrogen). KG-1 cells growth medium was IMDM (Life Technologies), with the same supplements described above. FBS heat inactivation was performed by incubation at 57 °C, for 30 min. All cells were grown in 75 cm² cell culture flasks (BD Falcon) until confluence, and maintained at 37 °C with 5 % CO₂. After unfreeze, cells were used until a maximum of 8 passages, to avoid major changes in their original [160] characteristics. Importantly, cells were subjected periodically to tests for mycoplasma contamination, and found to be negative.

For EV production cells were cultured in EV producing media: RPMI or IMDM media supplemented with FBS 10 % or 0.1 % and P/S 1 %, previously ultracentrifuged (at 100 000x g, for 2 h, at 4 °C).

Functional assays were performed using human primary multipotent mesenchymal stromal/stem cells (MSC), isolated from the tibia of patients undergoing knee surgery. All procedures were previously approved by the Ethics Committee of Centro Hospitalar São João. MSC growth media was DMEM (Corning) supplemented with MSC-qualified heat inactivated FBS 10 % (v/v) (Gibco) and penicillin/streptomycin 1 % (v/v) (Invitrogen). Cells were seeded in 175 cm² cell culture flasks (BD Falcon) until confluence, and maintained at 37 °C with 5 % CO₂. MSC from passage 4 to 7 were used in this work. MSC were previously characterized for the presence/absence of characteristic surface markers, and for their differentiation potential, and found to follow the criteria defined by ISCT for MSC definition [161].

2.2 - Cell viability Assays

KG-1, THP-1 and Jurkat cells were seeded in EV producing media, in duplicate in 6-well plates, at a density of 2×10^6 cell/well/2 mL of media. Cells were incubated at 37 °C for 48 h, and representative optical photographs were taken at 0 h, 24 h and 48 h. After the 48 h, cells were collected, washed with phosphate buffered saline (PBS), and stained using the Annexin V/PI cell apoptosis kit (BD Falcon), according to the manufacturers instructions. Briefly, cells were incubated for 15 min, on ice, in Binding Buffer 1x containing Annexin V-FITC (2 μ L in 250 μ L) and Propidium Iodide (PI; 5 μ L in 250 μ L). Then 250 μ L Binding Buffer 1x were added and cells analysed in a FACSCalibur flow cytometer, acquiring 10 000 events. Unstained cells and matched IgG isotype control were used to determine background controls. Data was analysed using FlowJo software and late apoptotic/dead cells defined as AnnexinV⁺PI⁺ double positive cells.

2.3 - EV production, isolation and characterization

2.3.1 - EV production and Isolation

For EV production, cells were incubated at a density between 2×10^5 and 3×10^5 cells/mL, for 48 h, in EV producing media. After 48 h, cell culture media was collected and centrifuged at 2000 g, for 20 min, at 4 °C; the supernatant was recovered and centrifuged at 10 000 g, for 20 min and 4° C and the supernatant was again recovered and ultracentrifuged at 100 000 g, for 2 h, at 4 °C, in a Beckman Optima L80-XP ultracentrifuge. The resulting supernatant was used as control (SN1), and the obtained pellets were resuspended in PBS pH 7.4 or in Tris NaCl-HCl pH 5, as indicated. For EV washing, resuspended EV pellets from different tubes were added together and ultracentrifuged again in the same conditions as before. EV pellet was resuspended in PBS pH 7.4 or Tris NaCl-HCl pH 5, and ultracentrifugation supernatant collected to use as control (SN2). All samples were stored at - 80 °C.

2.3.2 - EV characterization

EV produced were characterized by Transmission Electron Microscopy (TEM), Nanoparticle Tracking Analysis (NTA) and western blot.

For TEM, EV were adsorbed on 400-mesh formvar nickel grids and negatively stained with uranyl acetate 5 % for 2 min. Then, samples were analysed in a Jeol JEM 1400 electron microscope.

To evaluate EV concentration, NTA was performed. In short, EV suspensions were diluted 1:100 in 1 mL of filtered PBS 1x and then analysed in a NanoSight NS300 with NTA3.0 software.

Protein markers were tested by western blot, specifically CD63, an endosomal protein marker highly used for EV characterization, and calnexin, a protein marker of the endoplasmic reticulum, and which should be absent from EV. Ultracentrifugation supernatants and parent cell lysates were used as controls. Samples protein amount was

quantified using DC™ Protein Assay (Bio-Rad), following the manufacturer instructions. Samples were then prepared in non-reducing conditions, denatured at 65 °C during 15 min and loaded in a SDS-PAGE 10 % polyacrylamide gel (Bio-Rad). Resolved proteins were transferred to nitrocellulose membranes. Membranes were blocked with non-fat milk, and then incubated overnight, at 4 °C, with primary antibody anti-human CD63 (BD Pharmingen™) or anti-human calnexin (Abcam). On the next day, membranes were washed and incubated with HRP-conjugated secondary antibody (GE Healthcare). Membranes were then incubated 2 min with ECL substrate (from GE Healthcare) for the development of a chemiluminescent signal, that was detected with autoradiographic films.

2.4 - Production of EV delivery system

The EV delivery system developed consisted of a chitosan scaffold functionalized with a chitosan/poly-γ-glutamic acid/EV Layer-by-Layer, and was produced in three main steps. First, chitosan, chitosan scaffolds and poly-γ-glutamic acid were produced, and then scaffolds were functionalized with a Layer-by-Layer system along with EV incorporation. All steps are described in detail in sections 2.4.1, 2.4.2 and 2.4.3.

2.4.1 - Chitosan scaffolds production

Chitosan 3D scaffolds were prepared by freeze-drying, as previously described by our group [162]. Briefly, a 2 % (w/v) chitosan solution was prepared by hydration of chitosan with Milli-Q-water, overnight at 4 °C, followed by dissolution in acetic acid 0.2 M, overnight at 4 °C. The solution was centrifuged (5000 rpm, for 5 min) for air bubbles removal, and casted in 48-well plates. Plates were then frozen at -20 °C, overnight, in a flat surface, and freeze-dried at -80 °C for 48 h. The scaffolds obtained in each well, were removed from the plate and cut in the shape of cylinders with 2 mm diameter and 2 mm height with appropriate cutting material, as depicted in Figure S1.

The obtained scaffolds were disinfected and re-hydrated by impregnation under vacuum with a gradient of ethanol solutions, under sterile conditions. Firstly, scaffolds were incubated with absolute ethanol for 10 min, then ethanol 70 % for 30 min for disinfection, and finally with ethanol 50 % and 25 %, for 10 min each. Scaffolds were then washed two times with Tris NaCl-HCl pH 5 and kept in this solution at 4 °C until further use. All solutions used were filtered with 0.22 µm filter.

2.4.2 - Poly-γ-Glutamic Acid production

With the aim of producing chitosan sponges with chitosan-PGA layer-by-layer coating, γ-PGA production was required.

Poly-γ-Glutamic Acid was produced as previously described by our team [156]. The γ-PGA isolated in this work was produced by *Bacillus subtilis* IFO3335. *B.subtilis* were thawed and grown in Luria Broth (LB) solid medium with 1.5 % Agar. Colonies were picked, grown in liquid

Luria Broth media until reaching the exponential phase of growth, and then inoculated into γ -PGA producing media.

To isolate γ -PGA, culture medium was centrifuged at 12 000 g, for 1 h at 4 °C. The pellet with cells was washed with NaCl 0.14 M. The supernatant of first and second centrifugations was recovered, the pH of this solution adjusted to 3.0 with H₂SO₄ 6 M and then incubated at 4 °C for 12 h, with the aim of eliminating polysaccharides. The solution was then filtered with filter paper and mixed into three volumes of ethanol 99 % for protein precipitation, followed by centrifugation at 13 000 g for 10 min at room temperature (RT). The pellet was resuspended in Tris-HCl buffer 0.2 M pH 8.0 and the resulting solution dialyzed (MWCO: 10 KDa) against Tris-HCl buffer 10 mM pH 8.0, at RT, for ~18 h. The dialyzed solution was lyophilized at -80 °C for 3 days, and then dissolved in 8 mL of Tris-HCl buffer 10 mM pH 8.0. The solution was centrifuged at 12 000 g for 1 h at RT and then, the supernatant incubated with 20 μ L/mL of proteinase k for 12 h at 37 °C to promote α -peptides removal. After the 12 h, the solution containing γ -PGA was dialyzed against ultrapure water for ~18 h and then lyophilized. The dry matter was weighted for yield assessment, and then resuspended with ultrapure water. To confirm the molecular weight (Mw) of the γ -PGA isolated, an SDS-PAGE was performed. γ -PGA samples were prepared in reducing conditions and resolved in a NuPAGE Bis-Tris polyacrylamide gel. The gel was then stained with Alcian Blue basic dye, for visualization of blue bands containing the anionic γ -PGA.

2.4.3 - Scaffolds functionalization with a chitosan/ γ -PGA Layer-by-Layer system

Chitosan scaffolds were cut to the appropriate size and shape, before being combined with Ch and γ -PGA to make a multilayered system. These systems were prepared by the dipping method [163], consisting in the alternate immersion of chitosan 3D scaffolds in Ch 2 % and γ -PGA 0.2 mg/mL solutions at pH 5, according to the diagram in Figure 2.1. Between each layer, the scaffolds were washed with Tris NaCl-HCl pH 5 for 5 min. For EV incorporation into the system, 1×10^{10} EV from KG-1 cells (per scaffold) were mixed with the γ -PGA solution. Alternatively, the same volume of the washing ultracentrifugation supernatant (SN) was entrapped in the system and used as control.

The final LbL systems had in total 6 layers, 3 of Ch and 3 of γ -PGA. All the steps were performed under vacuum and in sterile conditions.

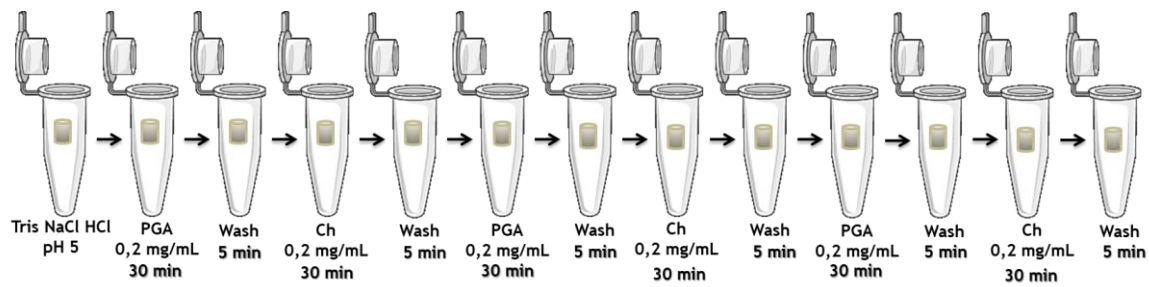


Figure 2.1 - Scheme of synthesis of the LbL system on Ch scaffolds. From the left to the right, are represented the solutions applied, under vacuum, for the construction of the system in each chitosan scaffold. EV (or ultracentrifugation supernatant) were diluted in the γ -PGA solution due their negative charge. Washes were performed with Tris NaCl-HCl, pH 5.

2.5 - Delivery system characterization

2.5.1 - Confocal microscopy

To confirm EV entrapment in the LbL system, EV were previously stained with the dye PKH26. Briefly, EV were labelled with PKH26 0.5 μ M dye (Sigma Aldrich), for 5 min at 37 °C. Excess free dye was then washed with PBS in VivaSpin® centrifugal columns (10 kDa cut-off). Red-labelled EV were entrapped in the LbL system as described in section 2.4.3. Functionalized scaffolds were then immersed in Vectashield mounting media and imaged by laser scanning confocal microscopy (LSCM; Leica TCS SP2 or SP5). Images were then processed for Z-projections in Fiji software. Importantly, the same acquisition settings were used for all conditions.

2.5.2 - FTIR

EV entrapment was also tested by Attenuated total reflection-Fourier-transform infrared spectroscopy (ATR-FTIR), in a FTIR Spectrometer Frontier (PerkinElmer). All measurements were done at room temperature. Functionalized and non-functionalized hydrated scaffolds were placed on the ATR accessory and compressed against it to achieve a flat contact between the sample and surface crossed by the analysis beam. Films of EV, SN, chitosan and γ -PGA solutions were also dispensed on the ATR accessory and spectra acquired. After data acquisition, a background correction was performed, and spectra of Tris NaCl-HCl films was subtracted from samples spectra.

2.5.3 - EV release assay

To evaluate EV release from the LbL system over time, release assays were performed. Briefly, after chitosan sponges functionalization with the LbL system, scaffolds were incubated in 250 μ l PBS 1x (pH 7.4), at 37 °C and 300 rpm. The scaffold was transferred to new tubes with PBS 1x, at different time points: 0 h, 12 h and 24 h. The supernatant of each timepoint was then concentrated on VivaSpin® centrifugal columns with 100 kDa cutoff, by

centrifugation at 2500 xg, for 15 min. Concentrate was resuspended and preserved at -80 °C until analysis by TEM, performed as described in section 2.3.2.

2.6 - MSC transwell recruitment assay

The capacity of EV to promote MSC recruitment was evaluated by transwell migration assay (Figure 2.2), as previously described by our team [164]. Briefly, transwell assays were set up in 24-well companion plates (bottom compartment; Figure 2.2A) with inserts (top compartment; Figure 2.2A) containing membranes of 8 µm-pore size (BD Falcon™). Due to the limited space between the bottom and top compartment for the placement of the chitosan scaffolds synthesized, inserts were elevated using *ependorf* tubes lids, as depicted in Figure 2.2B. Inserts were coated with 100 µL bovine gelatin (1% (w/v), 1 h, 37° C), washed with PBS 1x and then seeded with 0.2x10⁶ MSC. Chitosan scaffolds alone or chitosan scaffolds functionalized with LbL system, with or without KG-1 EV or ultracentrifugation supernatant from EV pellet wash (SN), were placed in the bottom wells, as depicted in Figure 2.2A. KG-1 EV and SN, and FBS 30 % (v/v) were used as controls. Serum-free DMEM was used in the assay. MSC were allowed to migrate for 12 h and then inserts were fixed with paraformaldehyde 4 % (w/v). The inside of insert membranes were cleaned from non-migrated cells using cotton swabs and washed with PBS 1x. Membranes were cut from the inserts, mounted on slides, and nuclei of migrated cells labelled using Fluoroshield with DAPI mounting media. Number of migrated cells was quantified by high throughput imaging using the IN Cell Analyzer 2000, followed by image processing using IN Cell Investigator software (both GE Healthcare).

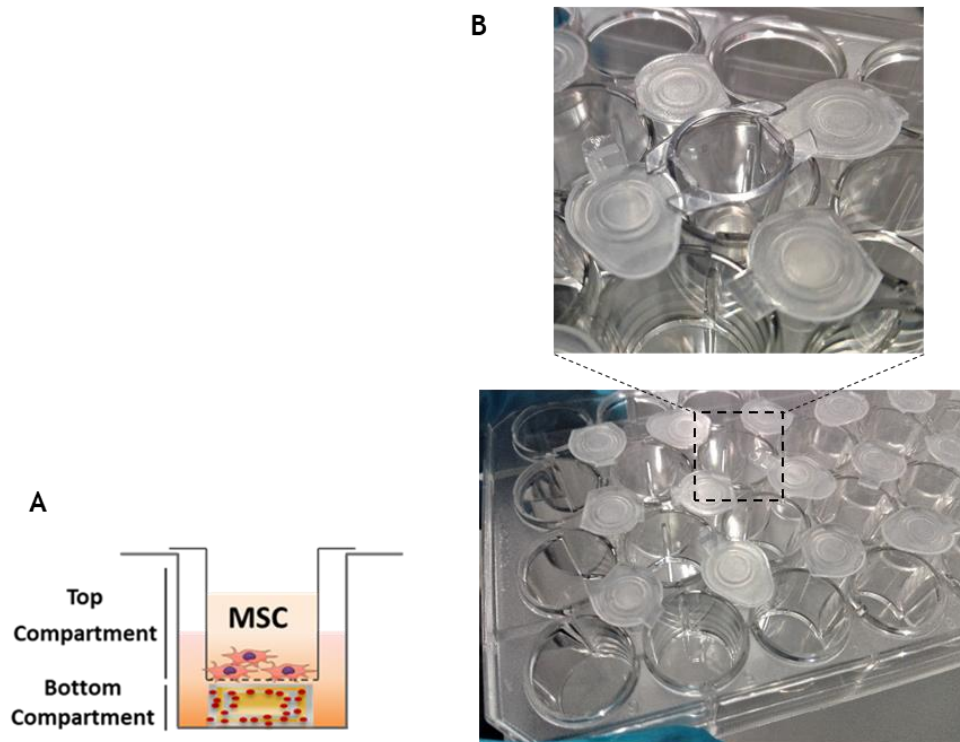


Figure 2.2 - Transwell migration assay setup to test MSC migration in the presence of different stimuli. **(A)** Schematic representation of the transwell system used to test MSC migration. The delivery system with entrapped KG-1 EV or ultracentrifugation supernatant (SN), chitosan scaffolds functionalized with LbL system only, chitosan scaffolds alone, or KG-1 EV or SN only were added to the Bottom Compartment (BC), and used as a chemoattractant stimuli gradient for MSC migration. MSC were seeded in the inserts containing membranes of 8 μm -pore size (Top Compartment, TC), and allowed to migrate for 12 h. **(B)** Representative photograph of the 24-well plate used for the migration assay. So that the insert did not touch the scaffolds placed in the BC, sterile microtube caps were placed between wells to create height and thereby allow a higher position of the inserts.

2.7 - Timelapse MSC motility assay

As an alternative method to test MSC motility under the influence of different stimuli, 6×10^3 cells/well were seeded in 24-well plates (bottom compartment), using MSC growth media (Figure 2.3). In the following day, media was replaced by serum-free DMEM (SFM) or MSC growth media previously ultracentrifuged and diluted to a FBS concentration of 1% (v/v). Inserts with 8 μm -pore size membranes (BD Falcon™) were placed on the wells (top compartment) and chitosan scaffolds alone or chitosan scaffolds functionalized with LbL system, with or without KG-1 EV or SN, were placed inside the inserts. Cells were then imaged by time-lapse video microscopy in an IN Cell Analyzer 2000 (GE Healthcare), for 12 h, in a controlled environment at 37 °C and 5% CO₂. Brightfield images from different fields of each well were taken every 10 minutes over 12 h. All images were converted to a time-lapse video using FIJI software and single cell movement analysed with MTrackJ plugin for FIJI software. The total distance travelled per cell for each condition was determined.

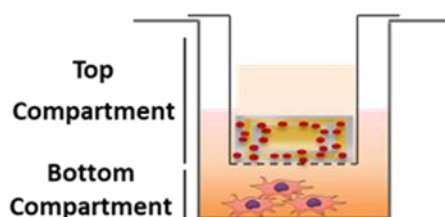


Figure 2.3 - Schematic representation of the system used for MSC motility assay in the presence of different stimuli. MSC were seeded in the wells of a tissue culture plate (bottom compartment). After 24 h, the delivery system with entrapped KG-1 EV or ultracentrifugation supernatant (SN), chitosan scaffolds functionalized with LbL system only or chitosan scaffolds alone were added to inserts containing membranes of 4 μm -pore size, that were placed in the wells (Top Compartment, TC). Cells were directly stimulated with KG-1 EV or SN for control. The described system was incubated in an IN Cell Analyzer 2000 for 12 h and images were obtained by time-lapse video microscopy, in order to evaluate MSC motility.

2.8 - MSC differentiation assay

The potential of KG-1 EV to enhance MSC differentiation was assessed by gene expression analysis, as previously described by our group [63] [165]. A total of 8×10^4 cell/well MSC were seeded in 6-well plates (bottom compartment; Figure 2.4) using MSC growth media. After 24 h, cell culture media was changed to DMEM supplemented with FBS 1 % (v/v) (Biowest) and P/S 1 % (v/v) previously ultracentrifuged. Inserts with 4 μm -pore size membranes (top compartment; Figure 2.4) were placed on the wells and chitosan scaffolds alone or chitosan scaffolds functionalized with LbL system, with or without KG-1 EV or SN, were placed inside the inserts. Cells were stimulated with KG-1 EV or SN alone as control. Osteogenesis inducing media, consisting of DMEM supplemented with FBS 1% (v/v) (Biowest), P/S 1% (v/v), dexamethasone 10^{-7} M, β -glycerophosphate 10^{-2} M and ascorbic acid 5×10^{-5} M (all supplements from Sigma-Aldrich) was used as positive control. Chondrogenic inducing media consisting of DMEM 4.5 g/L glucose supplemented with ascorbic acid 5×10^{-5} M (Sigma-Aldrich), proline 40 mg/mL (Sigma-Aldrich), sodium pyruvate 100 $\mu\text{g}/\text{mL}$ (Gibco), Insulin-Transferrin-Selenium 100 $\mu\text{g}/\text{mL}$ (Corning), dexamethasone 10^{-7} M (Sigma-Aldrich) and TGF- β 3 (R&D Systems) was also used as positive control.

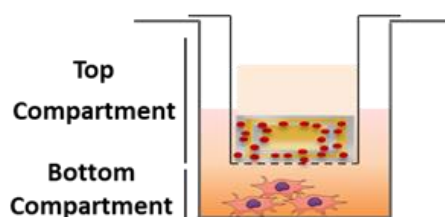


Figure 2.4. Schematic representation of the transwell system used to test MSC differentiation in the presence of different stimuli. The delivery system with entrapped KG-1 EV or ultracentrifugation supernatant (SN), chitosan scaffolds functionalized with LbL system only or chitosan scaffolds alone were added to the Top Compartment (TC). Cells were directly stimulated with KG-1 EV or SN for control. The influence of the stimuli on MSC differentiation seeded in the Bottom Compartment was evaluated by gene expression analysis after 3 and 7 days.

At day 3 and day 7 after stimulation, cells were lysed with TRIzol (Invitrogen). For gene expression analysis, RNA was extracted following the manufacturer protocol, quantified by NanoDrop ND-1000 (ThermoFisher Scientific) and its integrity analysed by agarose gel electrophoresis (Figure S2). DNA was digested using a TURBO DNA-free Kit (Life Technologies) and cDNA synthesized using random hexamers and SuperScript® III Reverse Transcriptase (Life Technologies), following manufacturer protocol. Quantitative real time PCR (RT-qPCR) was performed using SYBR green-based assays, using SYBR Green PCR Mastermix (Bio-Rad), diluted cDNA template, and gene specific forward and reverse primers. The sequence of the primers used is in Table 2.1. RT-qPCR reactions were performed in a iQ5 Real-Time PCR Detection System (Bio-Rad), according to the following conditions: denaturation for 3 min at 95 °C, followed by 40 cycles of cDNA denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s. A melting curve of 81 cycles with a temperature decrease step of 0.5 °C in 30 s, until 55 °C are reached, was generated at the end of the run to assess primer specificity. PCR products were also analysed by agarose gel electrophoresis (Figure S3). GAPDH was used as a reference gene and relative gene expression calculated by the $\Delta\Delta C_q$ method.

Table 2.1 - Sequence of the primers used in the RT-qPCR analysis performed on MSC, for the genes RUNX2, SOX9 and GAPDH.

Gene	Primer sequence
RUNX2	Forward: 5'- CCTGA ACTCTGCACCAAGTC -3'
	Reverse: 5'- GAGGTGGCAGTGT CATCATC -3'
SOX9	Forward: 5'- TTCCTCCTGCCTTTGCTTGT -3'
	Reverse: 5'- CGTGCTTGAAACATTCCCAGAAC -3'
GAPDH	Forward: 5'- CCTCAAGATCATCAGCAAT -3'
	Reverse: 5'- CCATCCACAGTCTTCTGGGT -3'

2.9 - Statistical analysis

Data were analyzed using GraphPad Prism 5.02 (GraphPad Software, Inc.). Normality of data distribution was first analyzed using the D'Agostino and Pearson omnibus normality test. For comparisons of multiple groups of non-normal data, the Kruskal-Wallis test, followed by Dunn's multiple comparisons test was used. For analysis of time-lapse video microscopy data, a Two-way ANOVA test, followed by Tukey's multiple comparisons test was used. Statistical significance was considered for $p\text{-value} < 0.05$.

Chapter 3

Results

3.1 - Optimization of cell culture conditions for EV production

The yield and characteristics of EV depend on the cell population producing them [166] and on the culture conditions used [167]. Here, we sought to find a human immune cell line that would produce high amounts of EV in low serum concentrations, to help reduce protein contamination of isolated EV. In this work, two immune cell lines of myeloid lineage - KG-1 and THP-1 -, and one immune cell line of lymphocytic lineage - Jurkat - were tested for their production of EV.

Before EV production, KG-1, THP-1 and Jurkat cells were cultured in media with different serum concentrations, namely standard conditions with media with FBS 10 % (v/v), ultracentrifuged media with FBS 10 % (v/v), and ultracentrifuged media with FBS 0.1 % (v/v). Cells were monitored for up to 48 h in these culture conditions. Results illustrated in Figure 3.1 show an increase in the number of cells in culture along time, for all cell lines and in all the culture conditions tested, indicating that even low FBS concentrations are adequate for cell proliferation. After the 48 h cells were harvested and stained for Annexin V and PI, to determine if there was any increase on the percentage of cells undergoing apoptosis (Annexin V+) or late apoptotic/dead cells (Annexin V+PI+). Results are illustrated in Figure 3.2A, and quantified across different experiments in Figure 3.2B. The viability of the KG-1 and THP-1 cell lines was found unaffected by the use of ultracentrifuged FBS, even when it was reduced to 0.1 %, with no detectable differences in the percentage of Annexin V+ apoptotic, or Annexin V+ PI+ dead cells in any of the conditions tested. On the other hand, viability of the Jurkat cell line was maintained in ultracentrifuged FBS, but was decreased when the percentage of serum was reduced to 0.1 %, when comparing to standard FBS conditions, although this difference was not statistically significant. Thus, KG-1 and THP-1 cells were cultured in media with 0.1 % ultracentrifuged FBS for EV production, while Jurkat cells were cultured in media with 10 % ultracentrifuged FBS.

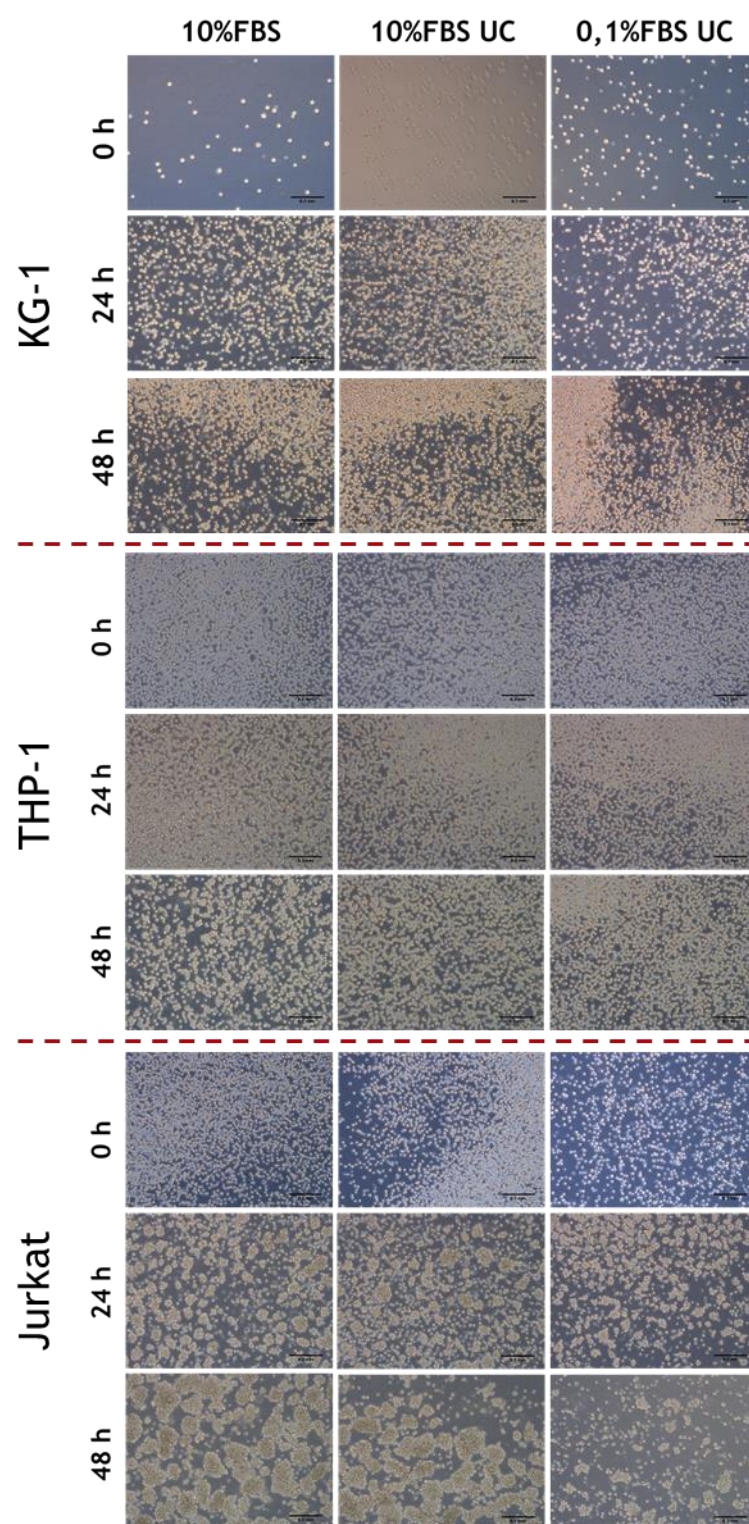


Figure 3.1 - Morphology in culture of all tested cell lines. KG-1, THP-1 and Jurkat cells were cultured at the density of 3×10^5 cell/mL in media with different conditions of serum concentration (10 %, 10 % ultracentrifuged (UC) and 0.1 % UC), and photos were taken in different time points (0 h, 24 h and 48 h). Scale bars: 0.2 mm. (n=3 independent experiments).

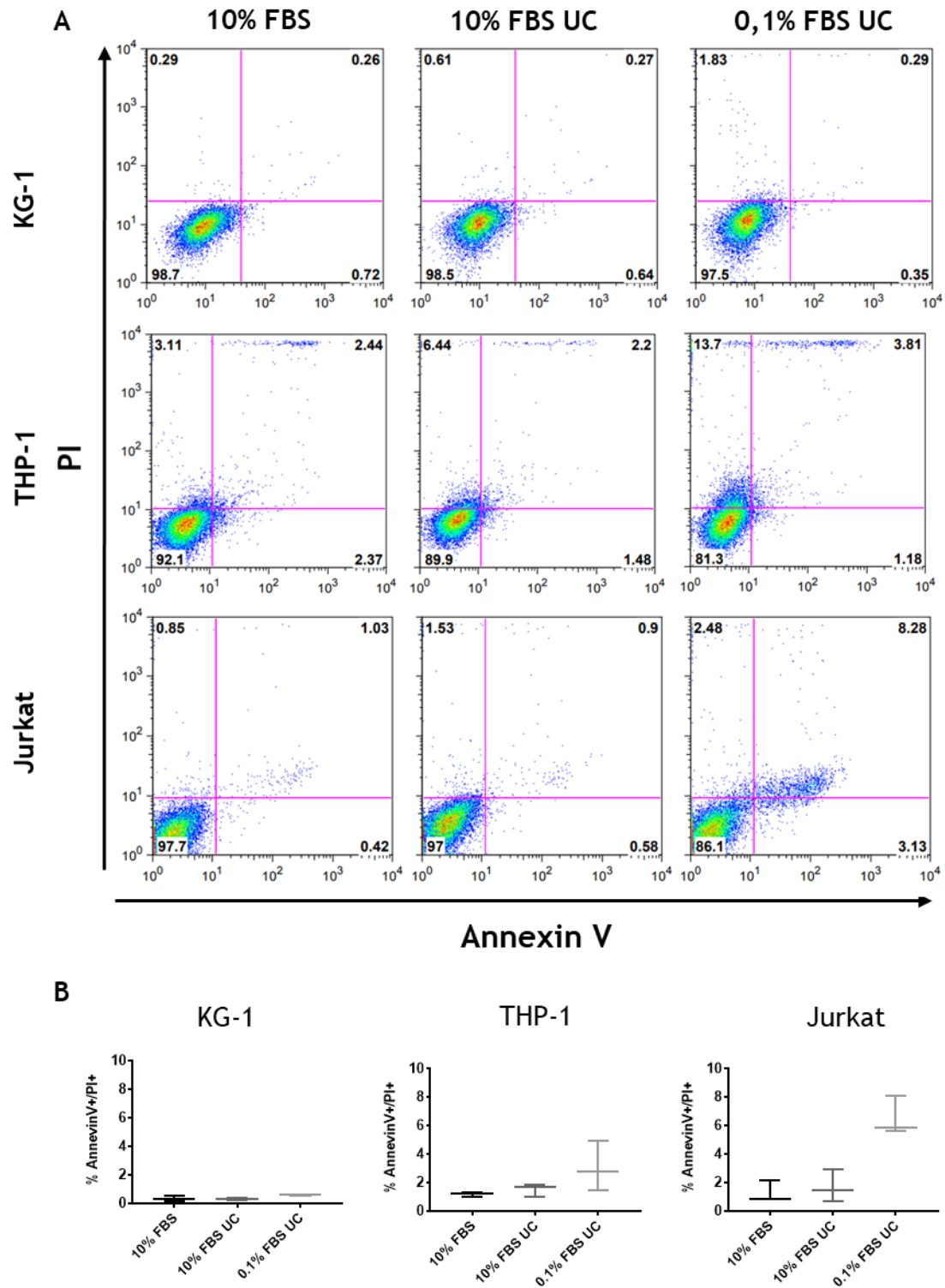


Figure 3.2 - Characterization of cell viability of the three cell lines tested, when cultured in different serum conditions. (A) Representative Annexin V vs Propidium Iodide flow cytometry dot plots for all cells and serum conditions tested, after 48 h of cell culture. (B) Percentage of Annexin V⁺/PI⁺ double positive cells in the different serum conditions, representing the percentage of cell death. No statistical differences were found by Kruskal-Wallis statistical test (n= 3 independent experiments).

3.2 - Immune cell EV secretion and characterization

After cell culture conditions optimization, EV from each cell line were isolated by ultracentrifugation as previously described and performed in our lab [63]. EV pellet washing with PBS 1x was also tested in order to remove non-EV contaminants. Then, washed and non-washed EV were characterized for their morphology, size and protein markers, using TEM, NTA and western blot. The results obtained are detailed in Figure 3.3. The TEM analysis shows successful isolation of EV for all cell lines tested, with the characteristic cup-like morphology and a size under 200 nm (Figure 3.3A). With this analysis, it was possible to perceive a more contaminated preparation, with protein aggregates (dark spots), in the images obtained for the EV isolated from Jurkat cell line. Also, we could verify that the supernatant from the first ultracentrifugation contained more protein and lipid clusters, than that of second ultracentrifugation, as expected. Data of size distribution and concentration were obtained by NTA and the results are shown in Figure 3.3B and Figure 3.3C, respectively. NTA data reported an average size of 200 nm for EV derived from KG-1 cells, and around 150 nm for EV derived from THP-1 and Jurkat cells (Figure 3.3B). EV concentration was higher for non-washed EV populations isolated from KG-1 cells (more than 2.0×10^{11} particles/mL), than isolated from the other cell types (around 1.0×10^{10} particles/mL for THP-1 and 1.5×10^{10} particles/mL for Jurkat), as showed in Figure 3.3C. Importantly, EV pellet washing seemed to lead to a great loss of EV, except for Jurkat cells (Figure 3.3C), but without increased particle number in the supernatants. Western blot was also performed to analyze protein markers, namely CD63 as an exosome marker and calnexin as an ER marker, that should be negative on EV. According to the results presented in Figure 3.3D, CD63 was enriched in EV of KG-1 and Jurkat, relative to cell lysates and was present but not enriched in EV from THP-1, indicating the isolated EV populations were enriched in exosomes. No CD63 was detectable in the ultracentrifugation supernatants. In addition, analysis of calnexin confirmed the purity of our EV suspensions, free from cellular organelles like the ER, as it was only detectable in cell lysates, and was absent in EV (Figure 3.3D).

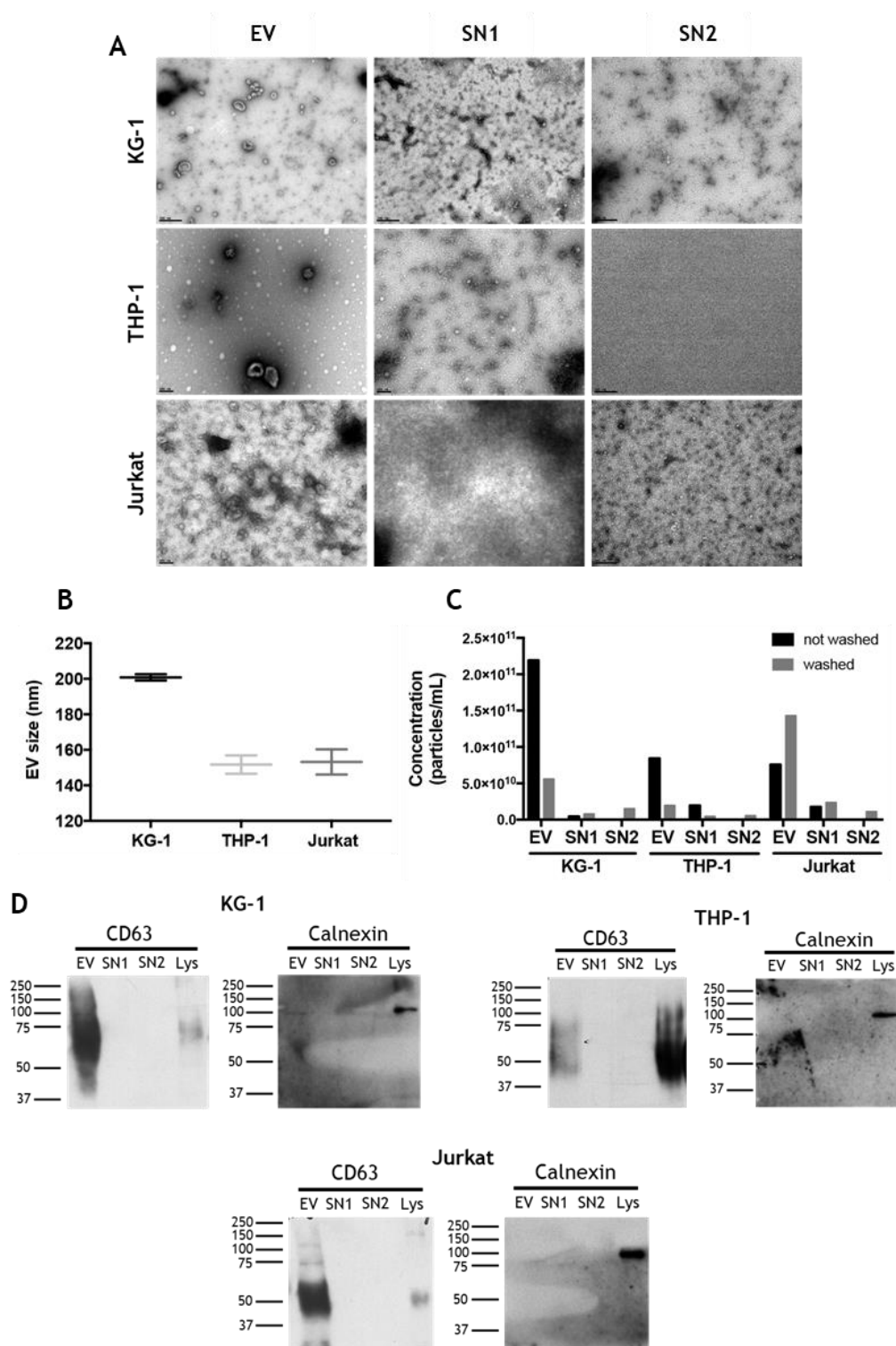


Figure 3.3 - Characterization of EV from KG-1, THP-1 and Jurkat cell lines. **(A)** Representative TEM images of EV and supernatants of the first ultracentrifugation (SN1) and the ultracentrifugation after the PBS wash step (SN2), for all cell lines tested. **(B)** NTA analysis of average EV size for each cell line tested. KG-1 EV size is around 200 nm, and for the remaining cell lines EV size is around 150 nm. **(C)** Concentration (in particle/mL) of EV isolated from all tested cell lines. KG-1 cell line, selected for further works, had the highest concentration of EV when compared to the other cell lines. **(D)** Representative western blot for detection of CD63 and calnexin in EV, ultracentrifugation supernatant before (SN1) and after EV pellet washing (SN2), and in cell lysates, for each cell line tested.

Considering the high yield of EV production by KG-1 cells, and preserved cell viability upon culture in low serum conditions comparing to the other cell lines, EV produced by KG-1 cells were selected to be further used in our work. KG-1 cells were cultured and EV were regularly isolated using the standardized protocol that included a washing step. Washing and pellet resuspension was performed in Tris NaCl-HCl pH 5 in order to obtain EV suspensions suitable to be entrapped in the delivery system developed. A summary of the characterization results for the different EV pools is shown in Figure 3.4. For each cell culture batch, KG-1 cell viability was assessed at the time of EV isolation by trypan blue exclusion assay, and found to be always above 80 % (Figure 3.4A). TEM analysis of the isolated EV revealed the presence of a high amount of EV in vesicle preparations and no detectable protein contaminants (Figure 3.4B). As expected, EV pellet washing with Tris NaCl-HCl helped to eliminate protein aggregates contaminants, as supported by the clear background (Figure 3.4B). Furthermore, EV could not be found in the washing ultracentrifugation supernatant SN2, revealing the efficiency of the EV isolation process used.

NTA analysis of the EV isolated showed they have an average size of 150 nm (Figure 3.4C). The average concentration of the EV suspensions was 2.3×10^{11} EV/mL (Figure 3.4D).

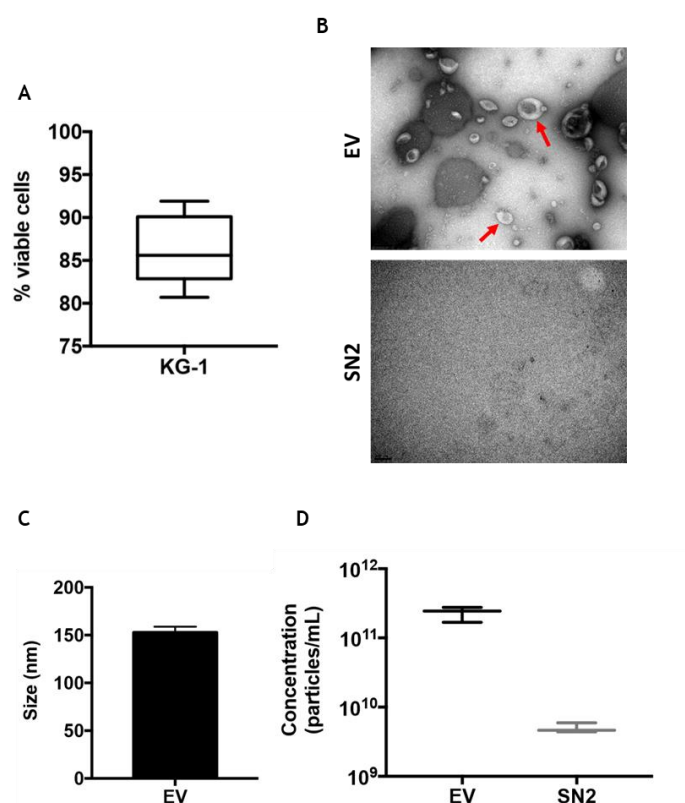


Figure 3.4. Characterization of EV isolated from KG-1 cells. (A) Percentage of cell viability for each EV isolation (n=9 independent experiments). (B) Representative TEM images of EV isolated and the supernatant (SN2) resulting from the washing ultracentrifugation. Red arrows are pointing to EV, evidencing their cup-shape morphology. Scale bar: 100 nm, and 100 000 x magnification. (C) Size average of isolated EV population. (D) Number of EV per mL in the different EV pools made (n=3 different EV pools).

3.3 - Delivery system component characterization

For γ -PGA biosynthesis, *B. subtilis* was cultured following the procedure previously detailed in Chapter 2. γ -PGA colonies obtained are represented in Figure 3.5A. *B. subtilis* was cultured in γ -PGA producing media and the γ -PGA synthesized and secreted by the bacteria was isolated and purified. To estimate the molecular weight (Mw) of γ -PGA isolated, a SDS-PAGE was performed and is depicted in Figure 3.5B. γ -PGA range its Mw from 20 to 50 kDa (Figure 3.5B). The final yield was ~200 mg γ -PGA/L of culture media.

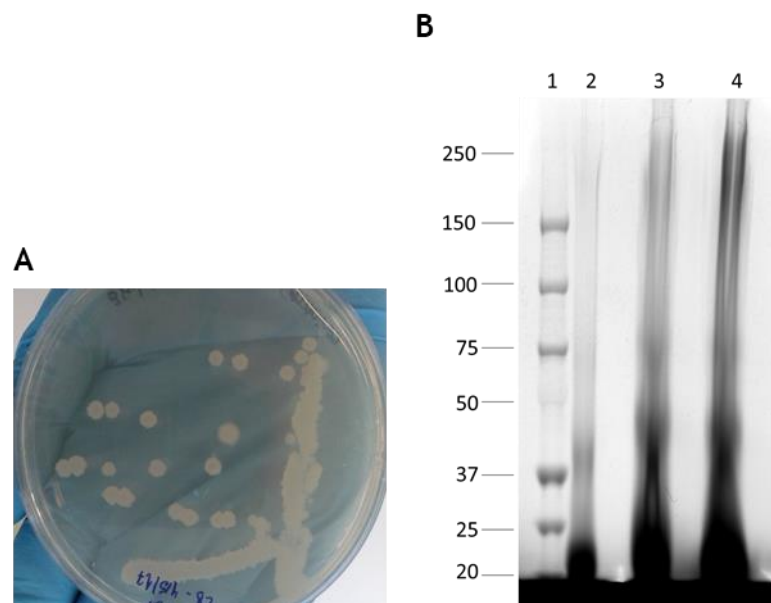


Figure 3.5. (A) *B. subtilis* culture in Agar. (B) SDS-PAGE to determine the Mw of γ -PGA produced. Lane 1 corresponds to Mw standards, in kDa, as indicated, and the next lanes (2,3 and 4) were loaded with different amounts of γ -PGA (80 μ g, 240 μ g and 400 μ g, respectively).

On the other hand, chitosan scaffolds were produced by freeze-drying as previously described [162] and cut into 2x2 mm cylinders (Figure S1) for subsequent use in building the LbL system synthesis. EV were incorporated into the γ -PGA solution, due to their negative charge, and LbL with 6 layers (3 of Ch and 3 of γ -PGA) were produced.

3.4 - Layer-by-Layer system is able to entrap EV

After synthesis of the delivery system, entrapment of labelled EV was evaluated by LSCM. Results are illustrated in Figure 3.6, and show abundant red labelled EV incorporated along the scaffolds structure. As controls LbL were produced using labelled ultracentrifugation supernatant (SN) control or empty (just Ch and γ -PGA). Empty LbL showed no red fluorescence at all, and only a few red spots were detected in the scaffolds functionalized with SN-containing LbL. The red fluorescence of labelled EV was detectable around the chitosan structure, as expected if layers are being formed around the scaffold (Figure 3.6, higher magnification image).

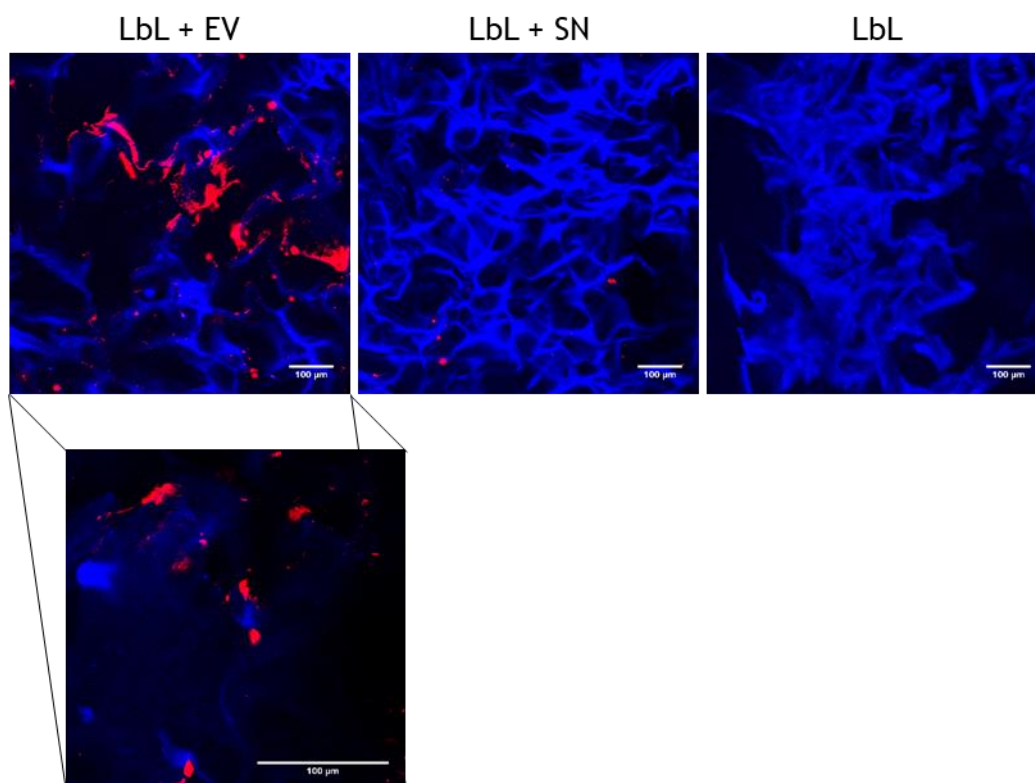


Figure 3.6 - Layer-by-layer system with entrapped EV. Representative laser scanning confocal microscopy images of chitosan scaffolds functionalized with the LbL system and with entrapped PKH26 (red) labelled EV (LbL+EV), or with PKH26-labelled ultracentrifugation supernatant (LbL+SN), or the system alone (LbL). A detail of entrapped EV on the surface of the Ch scaffold is shown in the higher magnification picture. Ch scaffold structure is visible in blue (autofluorescence). Scale bar: 100 μm . (n=2 replicates)

As confocal microscopy does not have enough resolution to image each EV, we sought to confirm EV presence on LbL by complementary approaches. So, EV alone, LbL empty, LbL with SN and LbL with EV were all analysed by FTIR. The comparative analysis is presented in Figure 3.7 and shows very similar spectra for all the LbL containing samples, with no detectable peak shift due to the presence of EV. Thus, it is possible to verify a similar peak profile for LbL, LbL+EV and LbL+SN conditions. All these spectra present characteristic peaks of Ch. The absorption peaks at 1340 and 1280 cm^{-1} are characteristic of the C-N bond of amines I and II, respectively [168]. Although, the peak verified at approximately 870 cm^{-1} and 1150 cm^{-1} , corresponding to the saccharide structure. In addition, peaks appearing at 1030 and 1080 cm^{-1} are due to the vibration of the C-O bond. The peak observed at 1380 correspond to the -CH group [168].

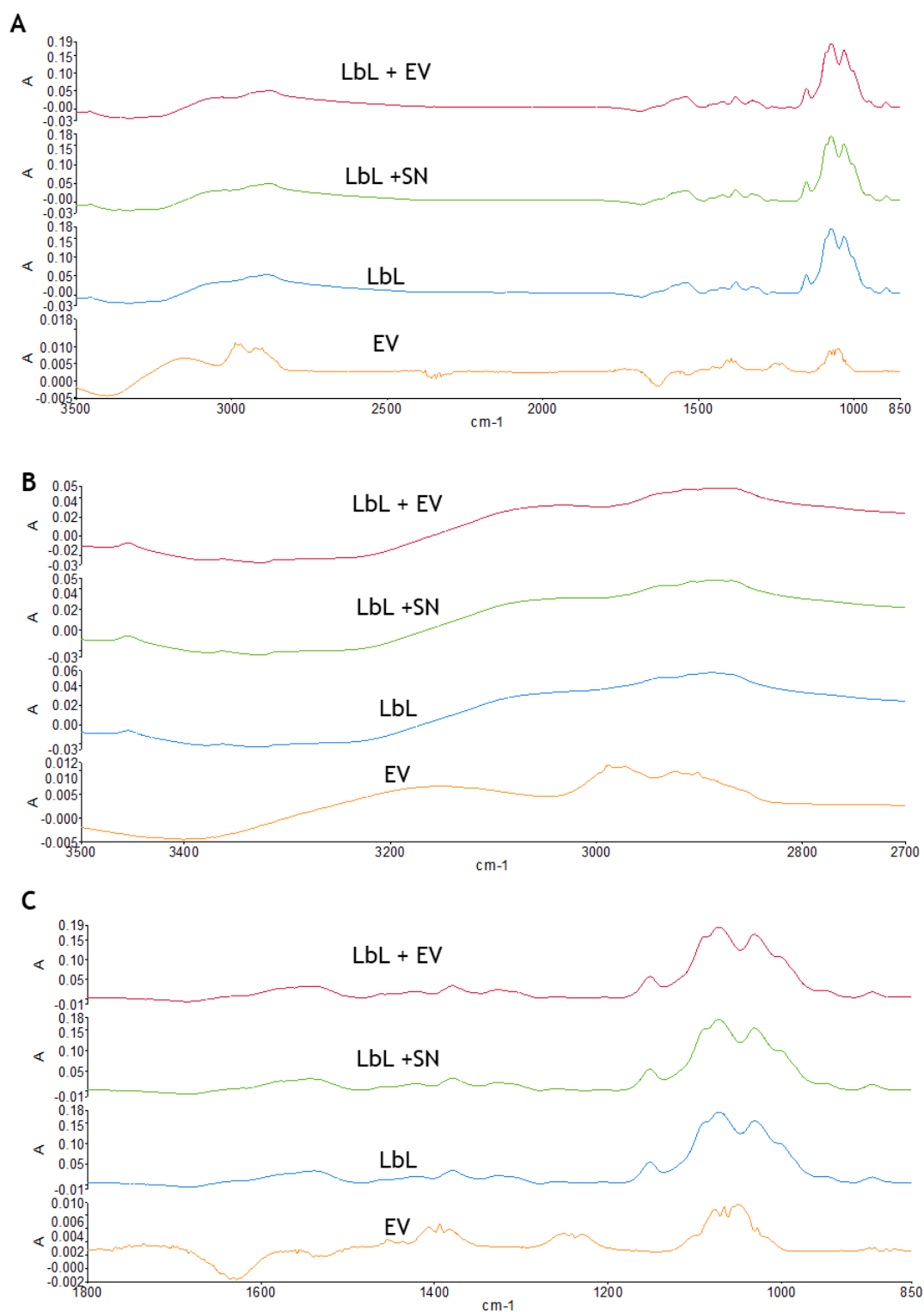


Figure 3.7 - FTIR spectra of EV-containing LbL systems. Representative images of the spectra obtained for the LbL + EV, LbL + SN, LbL alone and EV films. **(A)** Representation of all spectrum peaks (from 850 cm⁻¹ to 3500 cm⁻¹). **(B)** Detailed visualization of the FTIR spectra in the wavenumber region between 2700 and 3500 cm⁻¹. **(C)** Detailed visualization of the FTIR spectra in the wavenumber region between 850 and 1800 cm⁻¹. (n=2 replicates)

3.5 - Layer-by-Layer EV release profile

Next, we sought to analyse the release profile from the produced LbL. A release time course was set up and samples were collected at times 0, 12 h and 24 h of incubation at physiological pH, 37 °C and under agitation. First, we tried to quantify the released EV by measuring the amount of protein in the supernatants at the different time points. Protein concentration is a commonly used method for EV indirect quantification, but the protein levels released from the LbL systems were below detection (data not shown). Thus, supernatants were concentrated and analysed by TEM for the presence of EV. Results are illustrated in Figure 3.8, and show that it was possible to detect EV at 12 and 24 h timepoints, in the samples released from LbL+EV, but not from control LbLs. Moreover, EV seemed to be present in larger amount at 12 h (Figure 3.8; boxes in red are identifying some of them).

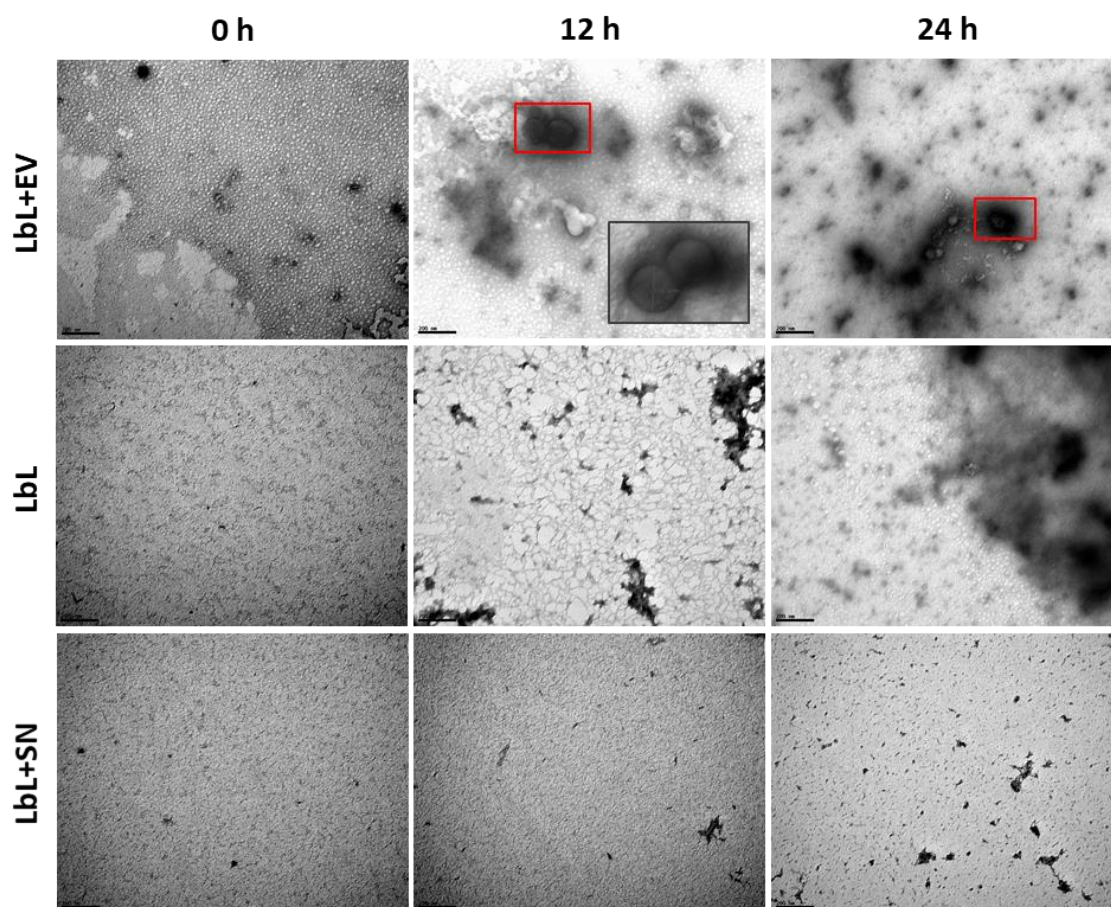


Figure 3.8 - EV release from LbL systems. Representative TEM images of the concentrated supernatants of the release assay at timepoints 0 h, 12 h and 24 h, for the LbL system with entrapped EV (LbL+EV), the control LbL system alone (LbL), and the LbL system with entrapped ultracentrifugation supernatant (LbL+SN). The presence of EV released from the LbL+EV at 12 h after incubation is highlighted by the red squares. Picture inset shows in detail the morphology of the EV released. Scale bar: 200 nm. Magnification: 80 000x. (n=1 experiment)

3.6 - Effect of EV from the delivery systems in MSC migration

Our previous results show a significant capacity of DC-derived EV to recruit MSC [63]. Thus, we next investigated the capacity of LbL-delivered EV to recruit MSC, using a transwell migration assay performed as previously described in our team [63]. Human MSC were seeded on TC and their migration capacity tested in the presence of different stimuli added to BC. However, the assay presented some technical difficulties, due to the size of the delivery system, that was too tall for the transwell set up. An adaptation was made, as described in the methods section. Results illustrated in Figure 3.9A show an example of an insert membrane with migrated MSC nuclei represented in blue (DAPI staining). The number of cells migrated per membrane was counted, and results are illustrated in Figure 3.9B, showing low numbers of migrated cells for all the tested conditions.

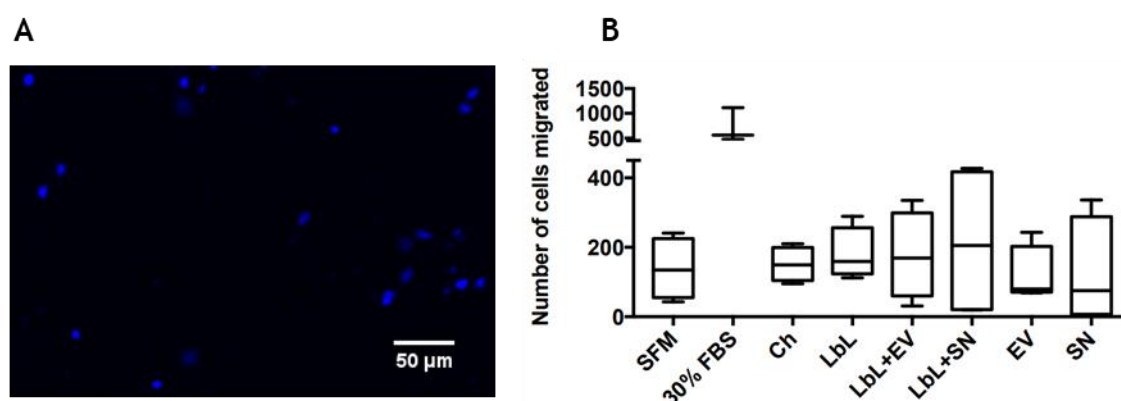


Figure 3.9 - MSC recruitment in a transwell migration assay, as influenced by different stimuli. **(A)** Representative image of the nuclei of MSC migrated through the transwell insert membrane (DAPI staining). Scale bar: 50 µm. **(B)** Quantification of MSC migration in the presence of different stimuli, namely Ch scaffolds alone, Ch scaffolds functionalized with LbL alone (LbL), or with EV (LbL+EV), or ultracentrifugation supernatant (LbL+SN). EV and SN alone were used as controls. Serum-free media (SFM) was used as negative control and FBS 30% was used as positive control. Statistical significance was tested by Kruskal-Wallis test, followed by Dunn's multiple comparison test, and no differences were found. (n=2 independent experiments)

As we could not exclude that the instability of the transwell system set up was conditioning the results obtained for MSC migration, we next evaluated the capacity of LbL-delivered EV to impact MSC motility by time-lapse video microscopy. Results are illustrated in Figure 3.10A and Figure 3.10B, and quantified in Figure 3.10C. MSC motility was quantified from the location of the nucleus in image each frame. Results indicate that when LbL included EV, MSC moved significantly more and faster, when compared to all controls, LbL+SN and LbL alone ($p < 0.0001$), and EV alone ($p < 0.001$). In addition, MSC appear to move more in response to Ch, to the empty LbL, and to EV alone than in the negative control condition (SFM), albeit not significantly. These results suggest a synergistic effect of EV and LbL delivery system on MSC motility. As expected, 30% FBS also increased MSC motility compared to SFM (negative control), but to a comparable level of Ch and empty LbL, and to a much lower extent than LbL+EV (Figure 3.10C).

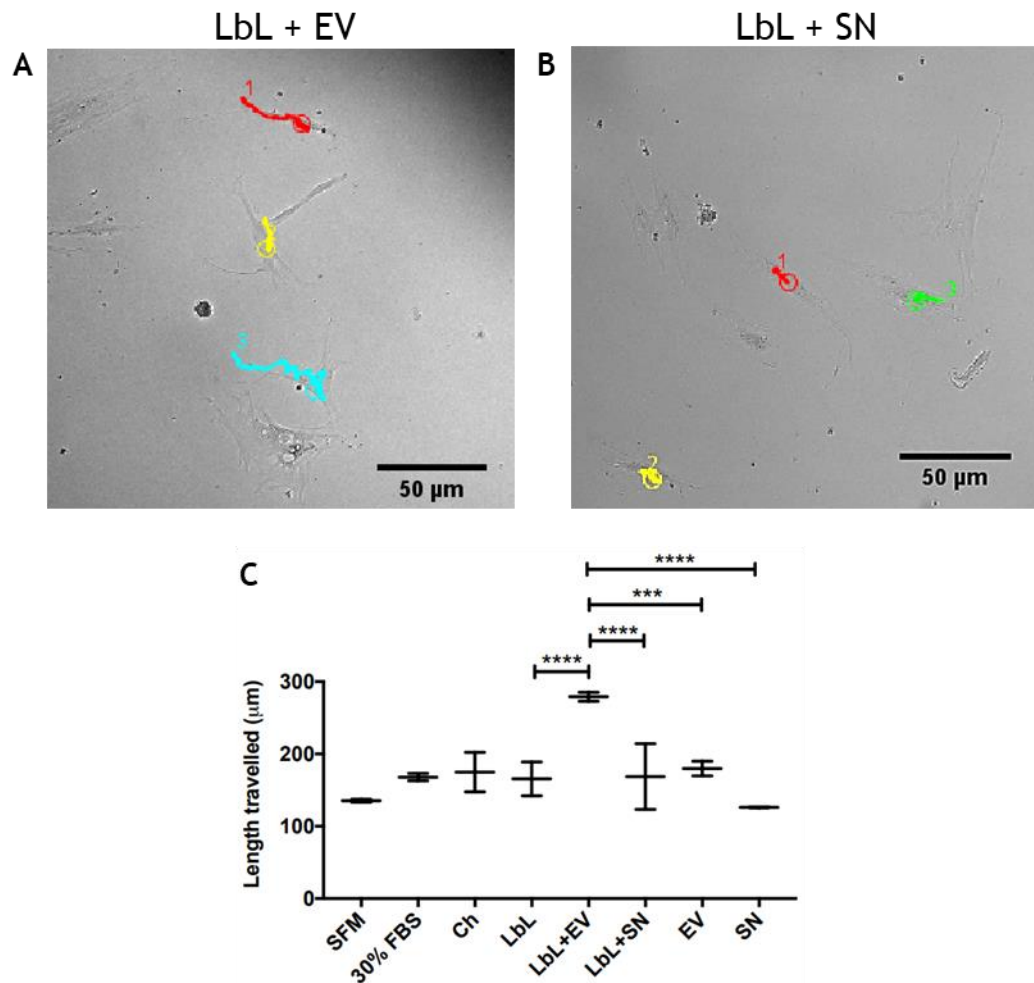


Figure 3.10 - MSC migration profile in time-lapse motility assays. (A) and (B) Representative image of some of the cell tracks determined. The videos obtained (by assembling the images obtained every 10 minutes for 12 h) were examined with MTrackJ plugin in FIJI in order to determine the pathways travelled by each cell analyzed. Scale bar: 50 µm (C) MSC travelled distance in the presence of the following stimuli: Ch scaffolds alone, Ch scaffolds functionalized with LbL alone (LbL), or with EV (LbL+EV), or ultracentrifugation supernatant (LbL+SN), EV and SN alone. Serum-free media (SFM) was used as negative control and FBS 30 % was used as positive control. ***p<0.001 and ****p<0.0001, as calculated by 2-way ANOVA followed by Tukey's multiple comparisons testing. (n=2 independent EV pools)

3.7 - Functional impact of LbL-mediated EV delivery on MSC differentiation

Finally, we investigated the influence of LbL delivered EV on MSC osteogenic and chondrogenic differentiation. A preliminary experiment was performed whereby MSC were exposed to LbL for 3 and 7 days, followed by RNA extraction for assessment of cell

differentiation by gene expression analysis of the levels of the osteogenic gene marker, RUNX2, and the chondrogenic gene marker, SOX9 (Figure 3.11).

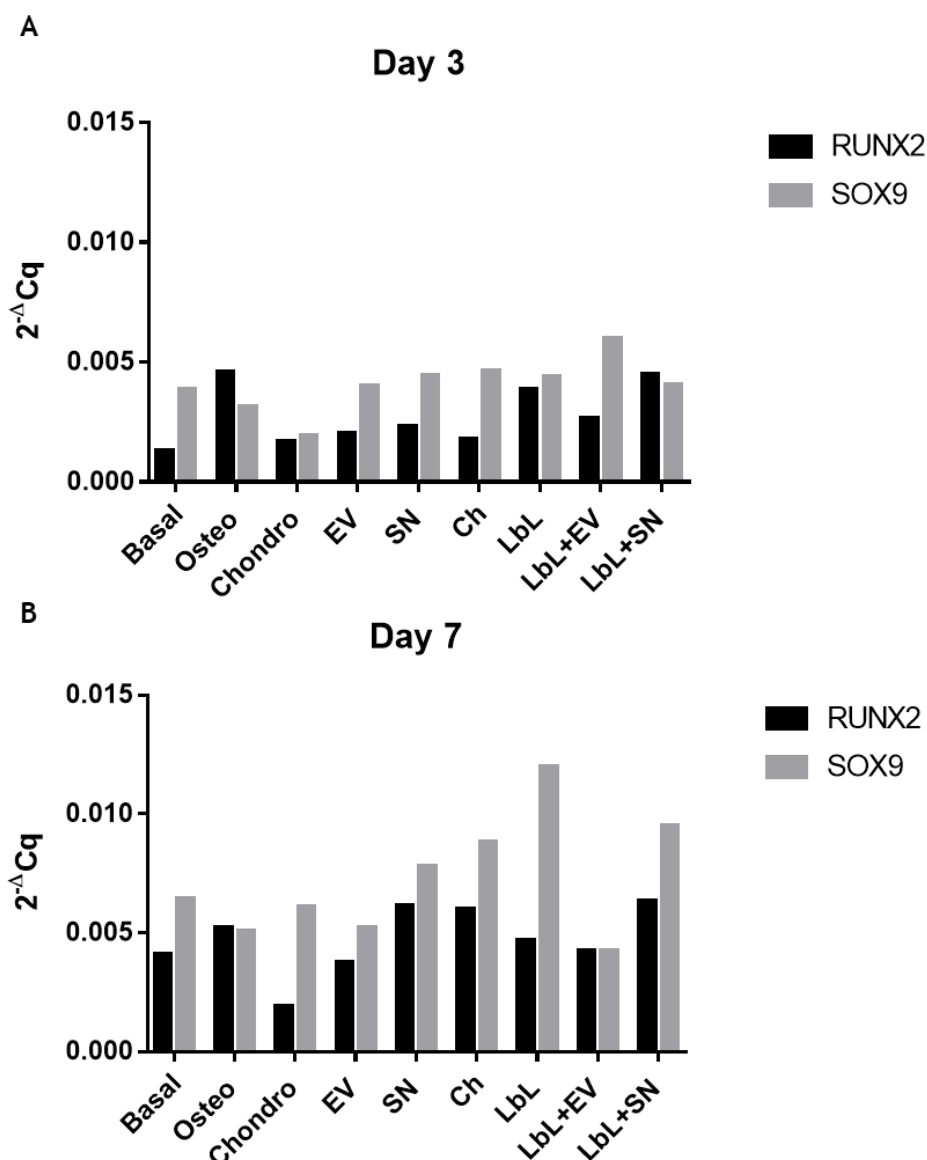


Figure 3.11 - Osteogenic and chondrogenic differentiation of MSC in the presence of LbL with and without EV. (A) MSC differentiation profile, after 3 days of culture. MSC showed more RUNX2 expression in the presence of the produced delivery system with different conditions (LbL alone; LbL+EV; LbL+SN). SOX9 was higher with LbL+EV. EV and SN alone were used as control. Osteogenesis (Osteo) and chondrogenesis-inducing media (Chondro) were used as positive controls. (B) MSC differentiation profile, after 7 days of culture. Gene expression was normalized to GAPDH. (n=1 experiment)

The results obtained show no significant impact of EV alone or entrapped in the delivery system on MSC differentiation (Figure 3.11). Nonetheless, at day 3, EV entrapment in the LbL system (LbL+EV) led to a slight decrease of RUNX2 expression by MSC, accompanied by an increase of SOX9 expression, compared to MSC in the presence of LbL alone or LbL+SN (Figure 3.11A). LbL-delivered EV also seemed to increase SOX9 expression compared to MSC stimulation with EV or SN alone. A slight increase in RUNX2 expression could also be observed for LbL+EV compared to EV alone, but to a lower extent than the osteogenic positive control

(Osteo). As expected, MSC stimulation with osteogenic media increased RUNX2 expression and decreased SOX9, relative to basal conditions. However, MSC stimulated with chondrogenic media, did not show a high SOX9 expression compared to basal conditions, at day 3.

Moreover, at day 7 (Figure 3.11B), LbL+EV showed reduced SOX9 expression, comparing with LbL alone or LbL+SN, while maintaining RUNX2 at similar levels. These results suggest LbL+EV could stimulate MSC osteogenic differentiation along time. At day 7, RUNX2 and SOX9 levels were similar for MSC stimulated with EV alone or entrapped, and the same was observable for SN. Contrarily to day 3, at day 7, Ch scaffold functionalization with LbL seemed to promote chondrogenesis as supported by a marked increase in SOX9 expression levels, compared to Ch alone and even to chondrogenic positive control (Chondro). From day 3 to day 7, MSC stimulation with osteogenic media maintained RUNX2 expression levels, while slightly increased SOX9 levels. In addition, chondrogenic media greatly increased the expression of SOX9 while maintaining RUNX2 levels.

Chapter 4

Discussion and future perspectives

The main goal of this thesis was to develop an EV delivery system that could be used for the controlled release of the vesicles upon local administration. In order to develop and test such a system it was important to have a reliable source of abundant EV. Thus, we started our work by investigating several immune cancer cell lines for EV production, due to their fast proliferation in *in vitro* cultures. We chose 3 different leukemia cell lines: KG-1, human monocytes derived from a patient with acute monocytic leukemia; THP-1, also human monocytes obtained from a patient with acute myelogenous leukemia; and Jurkat, human lymphocytes derived from an acute T Cell leukemia patient. Our focus on leukemia cells lines was motivated by the fact that these cells produce abundant EV, and also previous results from our group demonstrating that EV from human immune cells influence MSC biology [63]. In order to isolate EV with reduced protein contamination we optimized the cell culture conditions for these cell lines. This was motivated by the fact that FBS-derived protein components are major contaminants in EV preparations, often misleading the interpretation of results regarding EV biological functions [169] [170]. When reducing FBS concentration, it was crucial that cells would not undergo apoptosis, which would contaminate our EV preparations with apoptotic vesicles. From the results obtained for the different cell lines cultured in reduced serum conditions, we concluded that, when Jurkat cells were cultured in medium with 0.1% ultracentrifuged FBS, their viability was affected, with increased cell death (double positive cells for both Annexin V and PI), when compared with the remaining cell lines in the same percentage of serum. The viability of myeloid cell lines THP-1 and KG-1 cells was not affected by the reduced FBS concentrations. However, after isolation of EV for each of the cell lines, KG-1 cells were shown to secrete EV in a much higher concentration, when compared to THP-1 cells in the same culture conditions.

The EV populations isolated from KG-1, THP-1 and Jurkat cell cultures were characterized by complementary standard techniques, commonly applied in the EV field [169]. EV isolated from all cell lines had a size and morphology within the range described for exosomes [171], carrying also the exosomal protein marker CD63, indicating that the isolated vesicle

populations were enriched for exosomes. In particular, KG-1-secreted EV presented a cup-like morphology and a size in the range of 50 - 200 nm. Of note, EV average size distribution could be obtained by NTA and TEM, showing concordant values for both techniques. According to NTA analysis, the average EV concentration obtained was 2×10^{11} particles/mL, which is a good yield compared with EV derived from other cell lineages [172].

After optimization of cell culture conditions for KG-1 EV production, the isolated EV were incorporated into the delivery system under development. The concept of LbL self-assembly has been devised several decades ago [173], and is increasingly reported in different formulations for potential biomedical applications, from implant coatings to drug delivery systems [174]. Briefly, a LbL system consists in an alternating deposition of oppositely charged polymers. It is an innovative system, since it allows the incorporation of high concentrations of biological drugs, that may include chemical compounds, proteins, nucleic acids and even particles [163]. In addition, this system allows a local and controlled delivery of the cargo it carries, for instance when the pH of the environment changes, and can be adjusted by changing the number of layers that compose it, or the architecture of the scaffold to be used [175]. Several studies have been exploring the synthesis of such system with the most varied polymers, including with chitosan [176], and for applications also in bone tissue engineering [175]. The work of this thesis was focused on the use of a pH sensitive LbL system with Ch and γ -PGA as a coating for Ch scaffolds. The negative charge of the γ -PGA allows it to have electrostatic attraction with positively charged polymers such as Ch [177]. Also, if the drug to be incorporated has a net positive charge at pH 5.5, it is incorporated in the Ch layer, while if its charge is negative, as is the case of EV, then it is incorporated in the γ -PGA layer. The combination of these two biomaterials had been exploited in bone regeneration field [177] [178], which motivated their choice for application in our system. Hsieh *et al.* showed that it is possible to combine them in a matrix with interconnected pores with effects on the adsorption of cells, advantageous for several applications in tissue engineering [177]. In another approach, the same authors associated a similar system with entrapped rhBMP-2 (a protein known to promote cartilage and bone regeneration), and a potential therapy for bone defect was proposed [179]. Previously in our group, Ch and γ -PGA have been successfully combined into a LbL system based on films deposition on 2D surfaces [158], and shown to be efficient for the controlled release of IFN- γ [143]. However, to the best of our knowledge, the coating of 3D Ch scaffolds with a Ch and γ -PGA LbL was not previously reported. Thus, in the current work, we optimized this LbL system for the functionalization of 3D chitosan scaffolds, and also for the incorporation of more complex and bigger mediators than proteins, as is the case of EV.

In order to visualize EV entrapped in the delivery system, we previously labelled them with the lipophilic dye PKH26, which has been used by us and others to label EV lipid bilayer and follow their internalization into cells [63] [180]. To ensure the specificity of the observed staining, SN control was labelled in the same way as EV, before incorporation in the LbL delivery system. The results obtained showed a few bright labelled dots in the control samples LbL with entrapped SN, which are likely dye aggregates, formed by PKH26 [181]. Nonetheless, presence of labelled structures in EV-containing samples were much more abundant and surrounding the chitosan scaffold. Several attempts were made to further confirm EV presence in LbL systems. However, FTIR analysis could not confirm the EV presence in the delivery systems, as the spectra of LbL+EV was similar to that of the controls.

Similar results were obtained by Tao *et al.*, for the detection of EV incorporated into chitosan scaffolds [119]. This may be attributed to the small contribution of EV to the total composition of the delivery system, which includes a chitosan 3D scaffold further coated with Ch and γ -PGA. In fact, mainly peaks corresponding to chitosan are identifiable in the spectra obtained. Furthermore, chemical bonds present in EV components and in Ch are very similar, which hinders the success of this technique for the characterization of EV-containing LbL systems [182]. Also, SEM (Figure S4) and TEM microscopy approaches were previously attempted in the group, but without success, due to the irregularities of the scaffolds, which contribute to a high background with small spherical shapes that does not allow for a conclusive identification of EV. To overcome these difficulties, we will perform immunostaining of the EV entrapped in the LbL system using specific markers such as CD81, CD9, CD63, followed by fluorophore- or gold-conjugated antibodies and confocal or electron microscopy imaging, respectively. Other EV labelling methodologies can also be performed as alternatives, such as EV carrying Renilla luciferase (Rluc) or stained with hydrophilic permeable compounds such as carboxyfluorescein succinimidyl ester (CFSE) [183] [184].

An important property of delivery systems is the release profile of the compounds they carry. Thus, the LbL system was tested for the release of EV in conditions that mimic a physiological environment, namely in terms of temperature, pH and fluids dynamics (agitation). In our test system, the physiological pH is particularly important, since it allows the disruption of the electrostatic interactions that were established between chitosan and γ -PGA layers (at pH 5), and consequently, the release of EV entrapped. Our first approach to characterize the EV release was to quantify the amount of protein released along time. This was performed as quantifying protein amounts is an indirect measure of EV quantity, but the protein amounts released were below the detection limits of the tested protein quantification kits. Then we tried a qualitative approach, and focused on the detection of the vesicles in the supernatant of the release assay by TEM, at different timepoints after incubation in physiological-like conditions. Importantly, it was possible to confirm EV presence, particularly at 12 h of incubation. In order to better characterize the EV release profile, further experiments should be performed to analyse the release supernatants, both at earlier and later timepoints. In addition, the amount of EV loaded into the scaffolds could also be increased, in order to facilitate their detection by quantitative techniques, such as protein quantification and NTA, commonly employed to analyse EV concentration. Since we could detect less EV in release supernatants at 24 h after incubation, it is suggested that EV release from the system may be rather fast. On the other hand, since the bone regeneration process is a slow process, it could be important to increase the release time of EV, which could be achieved by increasing of the number of layers in the LbL system [163]. Another approach could be the use of blocking layers of less-diffusive polyelectrolytes (such as graphene oxide) that can enhance the mechanical durability of the layers [163]. Although it was possible to observe released EV at 12 h and 24 h of incubation, this assay provided only a qualitative analysis. To obtain quantitative results, our next approach would be to resort to more sensitive and specific methods for detecting EV, for example, by using the ExoELISA Kit, which would allow a CD63 antibody-based quantification of EV, and indirectly assess the quantity of EV released [185].

The ability of DC-derived EV to recruit MSC has been studied in our group [63] and is a very interesting feature for promoting bone regeneration, since these cells are able to

differentiate into osteoblasts, the bone-forming cell population. To confirm that EV delivered in the LbL system could maintain their functional properties, we investigated their capacity to promote MSC recruitment, using two different assays. The first consisted of a transwell recruitment assay, as previously used in our group to show EV recruitment potential [63]. However, in order to accommodate the size of the delivery systems, there was the need to perform a home-made adaptation to rise the inserts where MSC were seeded, and thus the experimental set up may have become unstable and potentially tilted (albeit this was not visible with the naked eye) influencing the MSC migration results. To circumvent those technical difficulties, we set up an alternative assay to test the capacity of KG-1 EV entrapped in the LbL delivery system to promote MSC motility, evaluating it by time-lapse video microscopy. This assay showed that EV-containing LbL were able to promote a significant increase in MSC motility, relative to all controls, including the LbL system alone or with entrapped SN, or the EV alone. The fact that the LbL system alone and the EV alone led to slight increases in MSC motility, albeit not significant, lead us to speculate that the effect observed with EV-loaded LbL systems results from the synergistic action of both. The effect observed for LbL alone is in accordance with previous observations, in which chitosan/ γ -PGA LbL films promoted a slightly higher MSC migration than TCPS alone [186]. Finally, it should be highlighted that the capacity of KG-1 EV to promote MSC migration is in line with similar results we obtained for primary human DC-EV [63], and reported by others for MEC-1 lymphocytic leukemia cell line [187]. Also, it is necessary to take into account that these results should be further validated by using more MSC donors. In addition, the delivery system should also be tested with EV isolated from human primary DC [63] or other human primary myeloid cell type, and not only from leukemia cells, since the effect of tumor immune cells upon MSC migration could be different from normal immune cells. Importantly, these results indirectly show that EV were in fact entrapped in the LbL delivery system, supporting our observations of labelled EV in the scaffold and our TEM results of EV release along time, also demonstrating that they remain functional upon release.

Finally, we also tested the influence that KG-1-derived EV could have in the capacity of MSC to differentiate into the osteogenic and chondrogenic lineage. These are important processes for bone regeneration, particularly by endochondral ossification, whereby a cartilageneous template is first formed and calcified along time to form new bone [188]. Thus, the gene expression analysis of RUNX2, a marker of early osteogenic differentiation [189], and SOX9, a marker of early chondrogenic differentiation, was performed. EV alone, do not seem to significantly affect MSC differentiation, since RUNX2 and SOX9 levels are similar to basal conditions at both timepoints tested. This is in agreement with previous results reported for the role of DC-derived EV in MSC differentiation [63]. On the other hand, EV entrapment seems to promote chondrogenesis compared to basal conditions at day 3, but the effect was not as noticeable at day 7. In addition, LbL alone seems to promote chondrogenesis, from day 3 to day 7, and thus an effect of LbL component materials on MSC differentiation, even in the presence of EV, cannot be excluded. This is in agreement with the literature, since the ability of these materials to promote new bone formation through endochondral ossification has already been reported [190]. Importantly, from day 3 to day 7, EV entrapment seems to promote MSC osteogenesis due to an increase of RUNX2 expression and a decrease of SOX9 expression. Of note, these results should be further validated for different MSC donors, in order to confirm our conclusions. Gene expression studies will be repeated and the effects at protein level will be evaluated by ALP activity and alizarin red

staining for osteogenesis, and Toluidine blue staining for chondrogenesis. Considering our results for the role of LbL+EV on MSC migration, this EV delivery system may be particularly adequate to promote bone regeneration. In fact, EV entrapped in LbL system greatly increase MSC motility, and the delivery system itself may promote MSC differentiation into the chondrogenic or osteogenic lineages, depending on presence or absence of EV respectively, and thus its implantation *in vivo* might promote MSC recruitment to places of bone injury followed by stimulation of their chondrogenic/osteogenic differentiation, enhancing bone regeneration.

Altogether, the results presented above indicate successful EV entrapment in a Ch and γ -PGA LbL system, assembled on 3D Ch scaffolds, and that those EV can be released and maintain their functional properties upon release. Our results support a beneficial effect of delivering entrapped EV, suggesting EV as chemoattractants for MSC, recruiting them to places of bone injury, where one can envisage the delivery system as enhancer of their differentiation, together promoting bone tissue repair and regeneration. In future studies, the contribution of LbL-delivered EV to bone regeneration should be validated, using *in vivo* models of bone injury, where this system could be implanted.

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Supplementary information

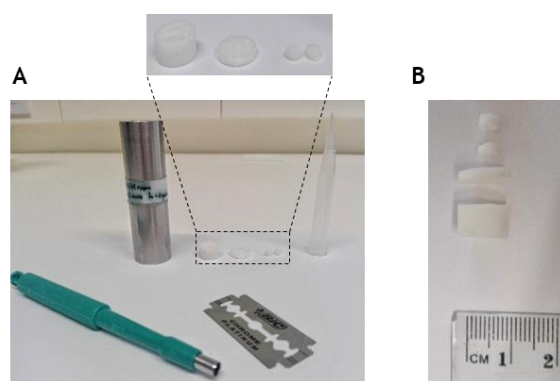


Figure S1 - Overview of all cutting steps of chitosan scaffolds until the desired size is achieved. **(A)** Chitosan scaffolds produced and equipment used to cut them. Chitosan scaffolds obtained by freeze-drying were trimmed with the blade to obtain a cylinder with flat tops. These scaffolds were first cut into 8x4 mm (diameter x height) cylinders, that were then punched with a 2 mm puncher (in green in the figure), to obtain 2x4 mm (diameter x height) cylinders. These scaffolds were then cut in half with the blade, into the final shape of 2x2 mm (diameter x height) cylinders used for the assays. From left to right: sponge originally obtained in the wells of the plate where the Ch solution is casted and subsequently lyophilized; after cutting to a height of 4mm with the materials previously described; cylinder obtained with a height of 2mm and diameter of 2mm. **(B)** Representative images of the scaffolds with different heights, in the different steps until obtaining the desired scaffold size (2x2 mm).

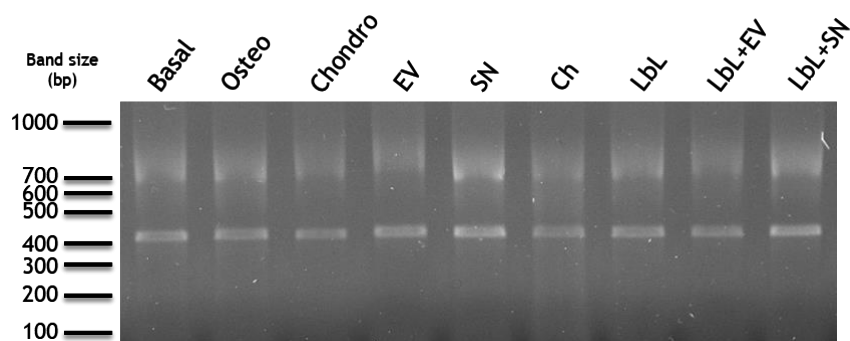


Figure S2 - Representative gel image of RNA extracted analyzed by agarose gel electrophoresis. As expected, two bands corresponding to ribosomal RNA 28S and 18S (from top to bottom) are visible, confirming RNA integrity.

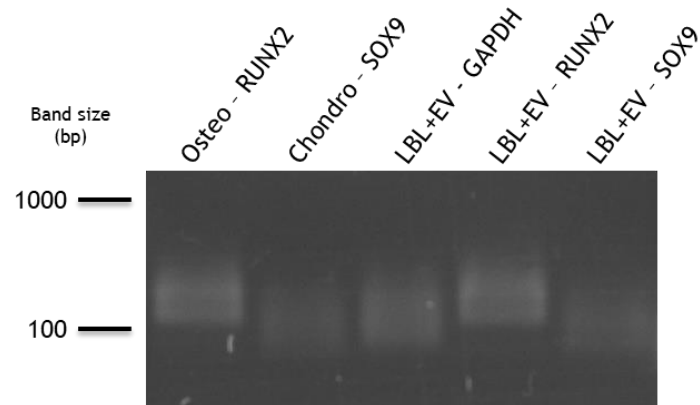


Figure S3 - Representative gel image of RT-qPCR products analyzed by agarose gel electrophoresis for RUNX2 in MSC stimulated with osteogenesis-inducing media (Osteo), SOX9 in MSC stimulated with chondrogenesis-inducing media (Chondro) and for GAPDH, RUNX2 and SOX9 in MSC stimulated with the LbL delivery system with entrapped EV (from left to right, respectively). Only one band was visible in each lane, and bands for the same gene but in different MSC-stimulating conditions had the same size, confirming the specificity of the primers used.

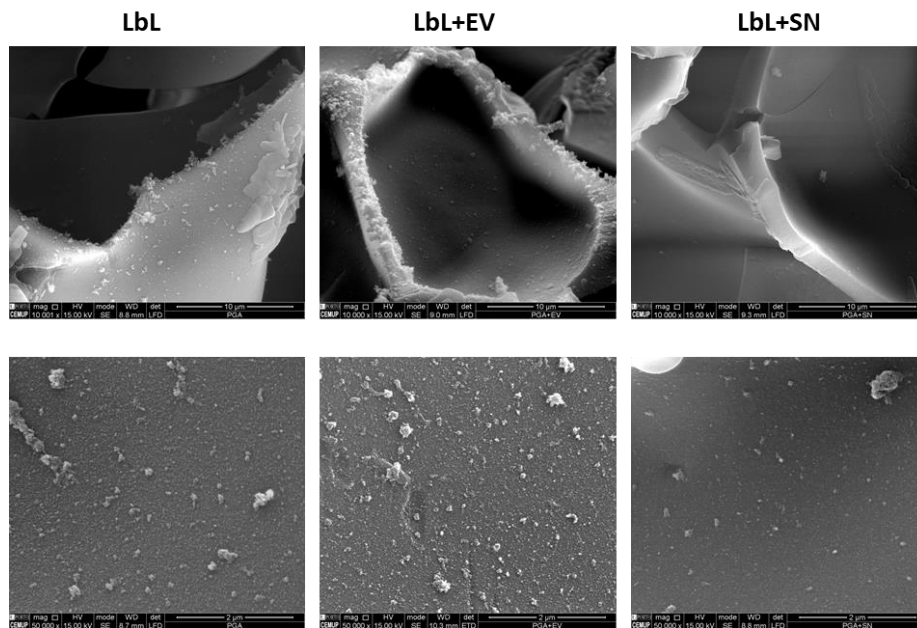


Figure S4 - Scanning electron microscopy analysis of scaffolds with entrapped EV, as previously performed in the lab. **(A)** Overview of chitosan 3D scaffolds functionalized with the LbL system alone (LbL), or with entrapped EV (LbL+EV), or ultracentrifugation supernatant (LbL+SN). Magnification: 10 000x. Scale bar: 10µm. **(B)** Detailed imaging of the surface of the chitosan scaffold with LbL, LbL+EV and LbL+SN. Magnification: 50 000x. Scale bar: 2µm. For the analysis, samples were coated by Au/Pd sputtering and images were acquired on a Quanta 400 FEG ESEM / EDAX Genesis X4M High Resolution Environmental Scanning Electron Microscope with X-Ray Microanalysis and Electron Backscattered Diffraction.

Annex 1

Alice Pinheiro, Andreia M. Silva, José H. Teixeira, Raquel M. Gonçalves, Maria I. Almeida, Mário A. Barbosa, Susana G. Santos. **Extracellular Vesicles: Intelligent delivery strategies for therapeutic applications. (submitted)**

Extracellular Vesicles: Intelligent delivery strategies for therapeutic applications

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Abstract

Extracellular vesicles (EV), in particular exosomes, have been the object of intense research, due to their potential to mediate intercellular communication, modulating the phenotype of target cells. The natural properties and functions of EV are being exploited as biomarkers for disease diagnosis and prognosis, and as nano-bio-carriers for the development of new therapeutic strategies. EV have been particularly examined in the field of cancer, but are also increasingly investigated in other areas, like immune-related diseases and regenerative medicine.

In this review, the therapeutic use of EV as drug delivery systems is described, balancing the advantages and drawbacks of different routes for their *in vivo* administration. Systemic and local delivery of EV are discussed, tackling the persisting difficulties in the assessment of their pharmacokinetics, pharmacodynamics and biodistribution *in vivo*. Finally, we discuss the future perspectives for incorporating EV into delivery systems and their use for an improved and controlled release of EV *in vivo*.

Keywords: exosomes, biodistribution, delivery system, nanoparticles.

1. Introduction

In the last decades, research on cell secreted Extracellular Vesicles (EV) has expanded exponentially. Their role in cancer has been by far the most described and explored, but their contribution to the processes of inflammation and tissue repair has also been under intense scrutiny. These have been recently reviewed by us [1].

Three main classes of EV secreted by cells have been reported and characterized, based on their origin, release mechanism and properties: apoptotic bodies, microvesicles and exosomes. The last two types of vesicles have deserved most attention from the scientific community due to their biological properties [2]. Microvesicles originate from the plasma membrane and their size can range from 50 nm to 1 μ m. Exosomes are smaller, with sizes in the range of 40-100 nm, are derived

from the intraluminal bud of multivesicular endosomes (MVE), and are released after fusion of these MVE with the plasma membrane [3]. These nanovesicles are secreted by most types of cells, including tumour cells [4] [5], playing a key role in intercellular communication, without cell-cell contact. In this review, exosomes will be particularly emphasized. However, the exact distinction of these different vesicle populations is still controversial, and partly hindered by methodological limitations, and thus wherever appropriate the designation EV will be preferred.

Upon secretion, the different types of EV are present in body fluids or supernatants of *in vitro* cell cultures, and thus to study each population, it is necessary to isolate them separately. Different methods have been developed and optimized, mainly intended to isolate exosomes, achieving different degrees of purity and yield. These methods include differential centrifugation with a final step of ultracentrifugation [6], ultracentrifugation in density gradients [6] [7], phase separation [8], size-exclusion chromatography [9] and immunoprecipitation, particularly based on the commonly used exosomal marker CD63 [6]. However, difficulties in isolating pure EV populations still persist, with most methods leading to the co-precipitation of non-EV contaminants. Consequently, it is of utmost importance that the vesicles isolated are extensively characterized, using standardized methods [10] [7] [11] [12]. So far, the main techniques used for EV characterization are transmission electron microscopy [6], [13], atomic force microscopy [14], nanoparticle tracking analysis [13] [15] [16], dynamic light scattering [17], flow cytometry (directly on EV or upon their capture on beads) and western blotting of common protein markers (e.g., CD63, CD81, CD9) [18] [19], and more recently, tunable resistive pulse sensing [13] [15], surface plasmon resonance [20], and Raman spectroscopy [21].

Independently of their origin, EV are lipidic vesicles that carry a protein content in their core, as well as on their surface, and may also contain different types of RNAs, including messenger RNA and microRNAs (miRNAs), DNA, and even metabolites [22].

Nonetheless, each type of EV has a detailed biomolecule signature. Importantly, these biomolecules are reported to be functional, when delivered to target cells [23] [24].

In this way, EV have been reported to participate in different homeostatic biological processes, including delivery of active biomolecules to target cells, antigen presentation to trigger an immune response, and cell waste management, initially thought to be their only function [25]. They may also contribute for the spreading of infecting pathogens and pathologies, namely cancer metastasis [26]. EV have also been greatly explored as biomarkers of disease, because their composition is related to the cell from which they derive. Characterization of EV levels and composition in biofluids, particularly in blood, urine and saliva, can contribute for the diagnosis, prognosis and monitoring of therapeutics in various diseases, cancer being the most explored [17] [27] [17] [3, 28].

EV have been increasingly investigated in the nanomedicine field, both as messengers in cell-cell communication and as intelligent systems for targeted delivery. Many studies have been reporting their use to deliver molecules such as miRNAs, siRNAs, proteins or drugs [2]. However, despite the increasing research in EV field, difficulties in their isolation, in terms of purity and yield, are obstacles to their successful translation into clinical applications. Furthermore, basic techniques for characterization of EV administered in *in vivo* models need substantial improvement, before going into clinical applications. The potential therapeutic role of EV makes it necessary to invest in quantification and characterization methodologies, before and after *in vivo* administration, in order to attain a fast and precise characterization of EV [15] [29]. On the other hand, it should be taken into account that EV are not retained for a long time in circulation when administered *in vivo*, reportedly due to the action of macrophages [30]. Furthermore, the mechanisms controlling the specific delivery of EV to a specific target are largely unknown and need to be further studied. Different approaches are being developed to overcome these barriers that limit the therapeutic use of EV, from vesicle functionalization to their incorporation into local delivery systems.

In this review, we discuss the different reported approaches for *in vivo* delivery of EV, including their systemic and local delivery, as vesicles suspensions or incorporated in more complex delivery systems. The limitations of each approach in terms of vesicles biodistribution and biological effects achieved are also appreciated. In parallel, different approaches to follow EV after administration in *in vivo* models are detailed. Finally, we compare the systems used for EV delivery with the ones commonly used for nanoparticles incorporation, suggesting some adaptations of the latter for the improved delivery of EV *in vivo*.

2. Therapeutic potential of EV: The importance of delivery methods

EV nanosize and natural ability to transfer information between cells, renders them promising candidates to be explored as nanodevices for therapeutic applications [31].

Naturally, EV carry active biomolecules that are loaded into their core or incorporated in their membrane through endogenous cell-regulated mechanisms [32] [33] [3]. The content of EV constitutively secreted by a cell reflects its physiological condition. For instance, researchers previously showed that the RNA cargo of MSC-derived EV is different according to tissue source [34]. Also, EV secreted by immune cells at different stages of activation may elicit either immunostimulatory or immunosuppressive responses in target cells [35].

In order to improve the therapeutic potential and to standardize the effects of EV, research has been increasing both in the modification of the content of naturally produced EV, and on the production of synthetic EV by functionalizing liposomes or small unilamellar vesicles functionalization [36]. Much like with synthetic nanoparticles, different types of molecules, such miRNAs, messenger RNAs, siRNAs, proteins, and even drugs, can be incorporated into EV, which become guided transporters to target cells. EV have been loaded with different molecules, exogenously or endogenously. In the first approach, the molecule of interest is included into EV after isolation, while the

endogenous approach is based on active encapsulation, particularly during exosome biogenesis, via transforming the producing cells with the selected molecule [37]. Many studies have focused on strategies for EV modification, and in comparing their properties with those of lipid-based nanoparticles, thus it will not be addressed in detail in herein [1, 31, 38] .

In cancer, EV have been explored for immunomodulatory therapies, or directly as anti-tumor therapies, as recently reviewed [7]. In fact, anti-tumor therapies based on anti-cancer drug loading on EV have been the most studied. For instance, EV loading with anti-mitotic drug Paclitaxel has been explored both by exogenous loading of the drug on isolated EV, that are then fed to the cancer cells [39], and by endogenous EV loading, using MSCs, which were previously loaded with Paclitaxel, to incorporate it during vesicle biogenesis [40]. Those studies reported that resulting EV decreased cell viability, and generated better cytotoxic effects than the free drug, which was mediated by EV internalization. Many other studies reported the use of chemotherapeutics loaded in EV, supporting their use as drug vehicles, and a good alternative/complement to traditional anti-cancer therapy. This is reportedly due to a great reduction in toxic side effects, since EV biodistribution to target tissues may be increased by choosing appropriate parental cells for EV production [41] [42].

EV have also been reported for the development of new therapies for several diseases, including neurodegenerative disorders. Their reported ability to cross the blood-brain barrier (BBB) has been exploited in different studies. Haney *et al.* used EV to increase the levels of catalase in the brain of mice, resulting in neuroprotective effects [43]. Similarly, EV (from self-derived dendritic cells) decorated with Lamp2b protein and neuron-tropic peptide from the rabies virus glycoprotein (RVG peptide), and loaded with siRNA against BACE1 protein, were able to cross the BBB in mice upon intravenous administration, without non-specific effects in other tissues [44]. These studies and others highlight the promise for EV-based treatment of different neurodegenerative diseases. However, some issues need to be overcome to achieve

clinical trials. For example, the RVG peptide is exogenously expressed in EV surface in many studies targeting the brain, but the receptor for this peptide is often downregulated in Alzheimer's disease [45].

Although cancer and neurodegenerative disorders are the fields where EV research is most intense, using EV as drug delivery systems in other areas, such as tissue repair and regeneration, particularly of the heart, has also gained increasing attention, and has recently been reviewed by us [1].

In the field of tissue repair, EV secreted by MSC have been the most studied for their regenerative capacity. Many studies have been demonstrating their positive effects on homeostasis recovery, as well as on the regeneration and repair of damaged tissues. This suggests a therapeutic effect of MSC-derived EV that can be used as an aid to various therapies [46] [47]. In particular, EV derived from umbilical cord MSC were explored in different contexts. For instance, to prevent acute graft-versus-host disease in a mouse model of allogeneic hematopoietic stem cell transplantation [38]. EV were intravenously administered, leading to a decrease in *in vivo* manifestations of acute graft-versus-host disease, along with decreased mortality [38]. Also, EV derived from umbilical cord-MSC were used to improve the fibrotic outcome of carbon tetrachloride (CCl₄)-induced mouse liver fibrosis. After 3 weeks of treatment, epithelial-to-mesenchymal transition of hepatocytes was inhibited and fibrosis ameliorated [48]. Many other studies used EV secreted by MSC from different tissues as delivery system for the treatment of the same model of CCl₄-induced liver fibrosis in mouse, reporting an amelioration of the condition [49].

Related to renal injury, Gatti *et al.* demonstrated significant effects in reduction of epithelial tubular cell damage in an ischemia rat model, by using MSC-derived EV [50]. However, clinical trials have not been performed yet, to demonstrate their effects in restoring the renal function in human chronic kidney disease [50]. EV derived from MSC were also shown to reduce infarct size in a mouse model of myocardial ischemia/reperfusion injury [51]. In a similar approach, Ibrahim *et al.* used EV from

human cardiosphere-derived cells in a chronic myocardial infarction murine model. Exosome administration inhibited apoptosis of cardiomyocytes and promoted their proliferation, helped by an increase of angiogenesis [52].

The regenerative effects of EV from MSC were also reported in models with osteochondral defects [34]. In the study from Zhang *et al.*, MSC-derived EV were intra-articular administered in an osteochondral defect created on distal femurs of adult rats, and PBS treatment was used as a control. After 12 weeks, a full reparative response of the femurs was observed in exosome-treated rats. In addition, only fibrous repair tissues were found in PBS-treated rats. This was the first study reporting the positive effects of EV derived from MSC in cartilage repair [53]. On the other hand, recent work also showed that EV from dendritic cells are able to recruit MSC [54].

In the studies published so far exploring the therapeutic potential of EV *in vivo* and in clinical trials, the strategy for vesicle administration is highly variable, occurring by different routes and in different dosages. Furthermore, several EV-based therapies have been promoted for commercialization by several companies, for which EV dose, administration route, and timeplan of administration are undisclosed, and not well defined [55].

Depending on the target tissue/organ and the effect intended, EV have been delivered systemically or locally, and either as vesicle suspensions or incorporated in more complex delivery systems. In the next sections, we discuss the advantages and disadvantages of the different delivery routes, from clinical and therapeutic standpoints.

2.1. Strategies for EV systemic delivery

To reach the target tissue or organ, EV can be administered via different routes, such as intravenous, intraperitoneal, oral, intranasal, and subcutaneous (Figure 1), that may lead to their systemic distribution.

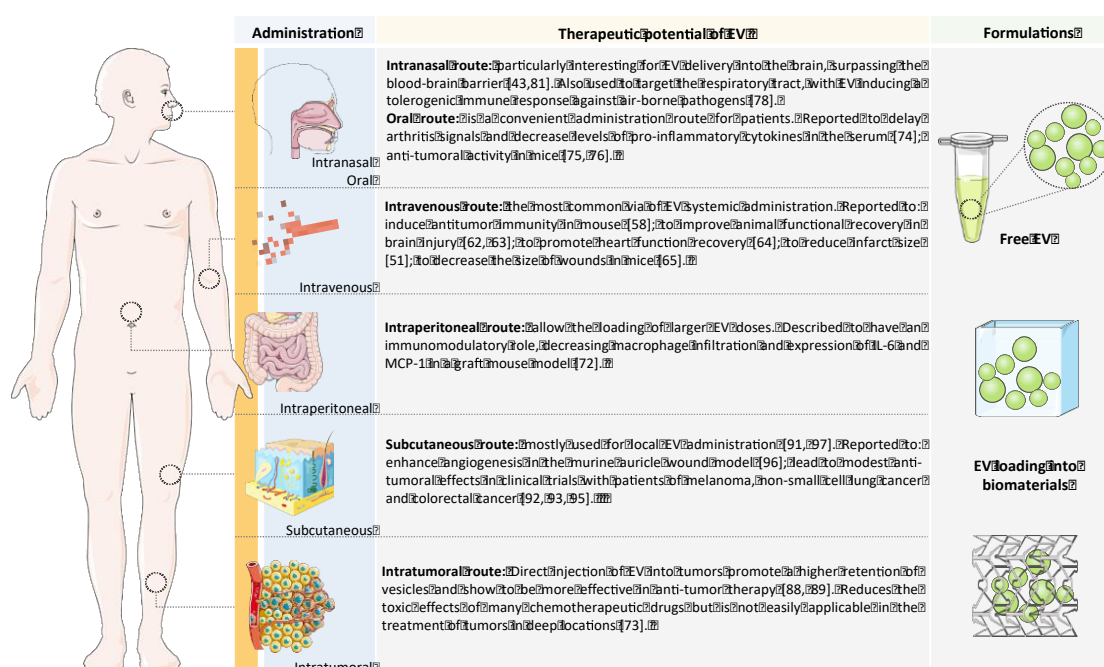


Figure 1. Strategies for EV administration *in vivo*. Studies with different animals models and also clinical studies with human subjects have been using different routes for EV administration, such as intranasal, oral, intravenous, intraperitoneal, subcutaneous, intratumoral, among others. The choice of the best route for EV administration route is mainly dependent on the target tissue and on the biological effect expected. Furthermore, it is related with the formulation used for EV preparation, which can be administered as free vesicles in suspension, or incorporated in a delivery system synthesized with different biomaterials that will allow their controlled release *in vivo*. These delivery systems include hydrogels, stiff scaffolds, decellularized matrices, among others. The use of the different routes for the administration of EV, and their preparation in different formulations, has been reported to lead to successful therapeutic effects.

2.1.1. EV intravenous administration

The most widely used strategy for EV *in vivo* delivery is the intravenous injection (i.v.), considered the fastest way to deliver drugs and also EV, due to their direct delivery to systemic circulation. In one of the earliest studies dedicated to determine EV biodistribution, reporter EV derived from HEK 293T cells were injected subcutaneously in athymic mice and their biodistribution followed by *in vivo* imaging [56]. These EV were coupled to *Gaussia* luciferase and biotin, allowing conjugation with labeled streptavidin for non-invasive *in vivo* fluorescence imaging, and also analysis of EV distribution by bioluminescence, including in biofluids such as blood and urine, upon the addition of luciferase substrate. As early as 30 min after administration, EV could be detected in liver and spleen of mice, being detected in low amounts in the brain, heart and muscle at all timepoints analyzed. In accordance, EV were detected at the highest concentration in blood at 30 min after administration, peaking in the urine at 60 min post-injection [56]. In a later work, Manca *et al.* tested the biodistribution of fluorophore-labeled milk-derived EV upon i.v. and oral administration. After 24h, EV administered i.v. accumulated in liver and spleen, while those administered by oral route accumulated only in small intestine [57]. The i.v. route has been used in the majority of the *in vivo* studies investigating EV as new anti-cancer therapies. For instance, i.v.-injected EV were already demonstrated to work as potent vaccines for induction of antitumor immunity in the mouse [58]. In one of the first studies performing EV administration i.v., EV ameliorated functional recovery in a mouse model of acute kidney injury [59]. Several other studies performing systemic i.v. injection have been successful in ameliorating different brain conditions, implicating that EV in circulation are capable of crossing the BBB [60] [61]. Indeed, in one of the first studies using siRNA-loaded EV, discussed above, EV injected intravenously, could reach the mouse brain, resulting in specific gene knockdown [44]. In further studies, MSC-derived EV injected in the tail vein were capable of improving animal functional recovery in traumatic brain injury [62] and cerebrovascular accident [63]. This capacity of EV to

readily reach the brain is a great advantage comparing to most synthetic delivery systems, that usually need to be functionalized in order to improve their penetrance across the BBB. The i.v. administration of EV has also been explored in the cardiovascular field, using vesicles of different cellular origin. In the study by Vandergriff *et al.* the authors show that EV isolated from cardiosphere stem cells cultures, when injected in the tail vein of mice with induced dilated cardiomyopathy, promoted heart function recovery by reducing apoptosis and fibrosis [64]. Furthermore, i.v. administration of EV derived from MSC were shown to reduce infarct size in a mouse model of myocardial ischemia/reperfusion injury [51]. The systemic administration of EV was also previously shown to improve local cutaneous wound healing. The recent work from Wang and colleagues suggested that EV derived from human adipose MSC decreased the size of incisional dorsal wounds in mice, upon intravenous injection [65].

Intravenous administration allows that EV reach their target anywhere in the body, even locations distant from the place of injection, like internal organs. However, the short half-life index of EV in circulation after injection is one of the major limitations of this route of administration [66]. Indeed, as demonstrated by several *in vivo* studies, EV are readily cleared from circulation, with some works reporting a calculated half-life close to only 2 minutes [66]. Most commonly, EV are cleared to liver and lungs [66]. In a detailed study by Morishita *et al.*, EV from B16BL6 melanoma cells were radioactively labeled and injected intravenously. By collecting blood and organs at different timepoints, and measuring radioactivity, the authors showed that, 30 min after injection, only 1% of injected EV were still in blood. At the same timepoint, almost 40% of the injected dose could be detected in the liver and 10% in the lungs, a distribution pattern that was specific for those vesicles [67]. In a follow-up study, the same group indicated that the rapid clearance of EV from circulation after i.v. injection was mainly driven by macrophages, resulting in the accumulation of injected EV in the liver [30].

Although EV have a certain degree of specific tissue targeting, which is usually considered better than synthetic nanoparticles [68] [69], it is important to understand that EV clearance from circulation into different off-target organs might be an important drawback, for instance if vesicles are being used for the delivery of cytotoxic drugs. Furthermore, it is necessary to take into account that there are consequences of maladministration, which may be irreversible [3]. Indeed, serious consequences may occur when over-dosing EV is reached. For instance, Smith *et al.* reported a mouse died by asphyxiation with accumulation of EV in lungs, despite all the positive effects reported in regeneration of many organs [70].

2.1.2. EV intraperitoneal administration

Another common method of EV administration is via the intraperitoneal route. Studies like the one by Liu *et al.* performed intraperitoneal administration of EV to modulate the immune response to a vein graft performed distantly, in the carotid of mice [71]. In this work, EV isolated from adipose tissue-derived MSC cultures, and labeled with the PKH26 dye, were administered intraperitoneally and could be found after 30 min in the aortic endothelium, supporting the systemic distribution of the EV administered by this route. More interestingly, mice groups receiving the EV showed thinner neointima in the grafted carotid, which was correlated with a decreased macrophage infiltration and decreased expression of IL-6 and MCP-1 in the graft [71]. Of note, the intraperitoneal administration route allows the loading of larger EV doses, which may represent an advantage compared to other routes of systemic administration [4]. However, injected EV are also rapidly diluted to unwanted sites due to the big area of the peritoneal cavity [72].

2.1.3. EV oral administration

Oral administration is the most convenient route for pharmaceutical products intake, due to ease of administration, facilitating patients' compliance to treatment. However, in this route EV face important obstacles. The intestinal barrier, changes in pH along the gastrointestinal tract and the characteristics of the intestinal microflora are further aspects that EV need to overcome to reach their target tissue or organ. Rezaie *et al.* suggested that this type of administration is mostly successful for delivery in luminal epithelial surface of the gastrointestinal tissues than in other target tissues [72]. Comparatively to other routes of administration, few studies were published so far reporting EV administration by oral route. Moreover, the works published mainly report the administration of milk-derived EV. In 2015, Arntz *et al.* reported the first oral delivery of bovine milk-derived EV with effects in arthritis [73]. Researchers used two different mouse models of arthritis and tested the capacity of these EV to delay the appearance of the disease and the evolution of the inflammatory phenotype. After EV administration, by oral gavage or in drinking water, histology analysis confirmed the delay of the arthritis as well as a decrease of cartilage and bone marrow inflammation. Serum levels of pro-inflammatory cytokines were also reduced [73]. Agrawal *et al.* used EV from bovine milk for oral delivery of the chemotherapeutic drug Paclitaxel. To demonstrate the therapeutic effect of these vesicles, they used a female athymic nude mice model with lung tumor xenografts. Results showed a higher effect of drug-loaded EV on the inhibition of tumor growth, compared to control groups treated with PBS, the free drug or EV alone. Importantly, they observed a reduced systemic toxicity and inflammation when loaded EV were administered orally, comparing to their intravenous administration [74]. Thus, from this study, oral administration appears an adequate choice to decrease toxic side effects of EV systemic administration and to make EV-based therapies more efficient. The same group reported similar observations for milk EV loaded with the anti-cancer drug curcumin [75]. Upon oral administration of these loaded EV in mice bearing cervical tumor xenografts, tumors had lower volumes

compared to control animals. However, curcumin could be found in lungs, liver and brain, in a dose dependent on EV dose administered [75].

2.1.4. EV intranasal administration

The intranasal route is also used for EV administration, with the first studies reporting this approach for the induction of a systemic immune response against airborne pathogens, using EV from infected macrophages [76]. Another study reported this approach to promote tolerogenic immune responses to air-borne allergens using bronchoalveolar lavage fluid-derived EV [77]. However, this route is also particularly interesting for the delivery of pharmaceutical products to the brain, either by entering the nearby circulation followed by crossing of the BBB, or directly through olfactory and trigeminal nerve cells [78]. Moreover, EV passage to circulation via the nasal route diminishes EV loss, because it avoids the intestinal and hepatic metabolism [79]. Indeed, this kind of administration showed great results in studies for Parkinson's disease therapy in mouse models of this condition, having neuroprotective effects [43]. Furthermore, the EV intranasal administration was reported to be more effective in retaining the vesicles in brain tissue than their systemic administration via intravenous injection in the tail vein [80].

Comparing different routes of administration, Yan *et al.* used EV from human umbilical cord MSCs in a murine model of hepatic oxidant injury, the CCl₄-induced acute liver failure. EV administration was tested orally and by injection in the tail vein, with different doses being tested. Both investigated routes showed similar effects, which gives advantage to the oral route due to the ease of administration. The results demonstrated significant antioxidant effects in CCl₄- and H₂O₂-injured cells. In addition, cell viability increased after EV administration. However, the mechanism underlying EV effect in hepatic oxidative stress remained unclear [81].

It is important to note that the classification of systemic and local routes of administration is not entirely linear. There are routes of administration, like the intraperitoneal, the intranasal or the subcutaneous, that are most commonly considered for local delivery. However, they can also be employed in systemic delivery, depending on the specific situation. In the case of subcutaneous injection, it can be used for local or systemic delivery (as detailed in the next section), when intended to act far from the site of administration, achieving systemic circulation. Intraperitoneal administration is considered systemic, but it is mainly used to reach the gut, without necessarily going through the systemic circulation. Also, intranasal administration can be used to reach the systemic circulation, but it is mainly exploited due to its proximity to the brain.

Altogether, the different routes of systemic administration have some common limitations. The distance and barriers that EV have to travel until reaching their target cells, implies that most vesicles do not reach the target, and instead accumulate in other organs. Common constraints influencing EV biodistribution include passage through physical barriers such as capillary endothelium, gastrointestinal epithelium and the blood-brain barrier, uptake by non-target tissues, including entrapment in their extracellular matrix, phagocytosis by immune cells, particularly macrophages, and clearance in urine. Ultimately, this implies that higher EV doses have to be administered in order to achieve the intended biological effect at the target tissue. One possible strategy to overcome these problems is to increase EV tropism to the tissue of interest. This may be achieved by EV functionalization with surface proteins binding ligands on target tissues. In fact, Tominaga and colleagues demonstrated that the transfection of surface proteins from donor cells in EV-producing cells functioned as strategy to harness EV with special tropism to the brain [82]. Thus, it remains a challenge in EV delivery to find the responsible proteins for different targets, in order to achieve natural organ tropism for EV. Another possible strategy to increase the chance of EV reaching their target tissue is their modification with “don’t eat me”

signals, such as CD47, which allows escaping the engulfment by immune cells [83]. Alternatively, to minimize the adverse effects of EV systemic administration, they can also be delivery directly to the target tissue.

2.2. Strategies for EV local delivery

Local drug administration is generally associated with reduced side effects [3], which will likely be the case also for EV. Furthermore, local administration of EV may overcome the often-reported low retention rates of the vesicles at specific target sites, compared to non-specific organs.

One of the first studies reporting the local effect of EV dates back over a decade, to 2006, when Kim *et al.* demonstrated that EV derived from genetically modified murine bone marrow DC (overexpressing FasL), when administered locally, could resolve inflammation in a murine model of delayed-type hypersensitivity (DTH), and suppress the signals of arthritis in a collagen-induced arthritis mouse model. They administered EV via local injection in the footpad and via systemic intravenous injection, and concluded that both were effective in suppression of the DTH response and in the treatment of collagen-induced arthritis. However, EV injected locally were not detected in the animals liver or spleen, suggesting the effect of EV was not mediated systemically [84].

In the cancer field, intratumoral injection of EV as an anti-cancer treatment strategy has been greatly explored. Some biodistribution studies reported unmodified EV to be unsuccessful in tumor-specific delivery upon i.v. administration [85] [86]. Alternatively, direct injection of EV into tumors has been shown to be a much more effective anti-tumor therapy. Indeed, the work of Smyth *et al.* suggests that the intratumoral injection of DIR-labeled EV derived from 4T1 cells allows much higher retention of the vesicles inside the tumor, than their i.v injection in the tail [87]. Furthermore, intratumoral EV injection has been reported as efficacious in tumor growth inhibition. In the work of Martins-Marques *et al.* [88], EV loaded with luciferin

and the chemotherapeutic agent doxorubicin, were injected in mouse tumors. Tumor growth was then monitored by bioluminescence imaging and a cytotoxic effect specifically in tumor cells was demonstrated. Interestingly, they concluded the presence of the gap junction protein connexin43 (Cx43) embedded in EV membrane improved the release of EV content into target cells, contributing to the reduction of cardiotoxicity of free doxorubicin injected systemically [88]. Intratumoral injection may be generally associated with reduced toxicity in non-target tissues. However, similar results using local or intravenous administration have also been reported [41].

Although intratumoral EV injection is apparently more effective in tumor eradication, this type of administration is much more invasive than systemic administration, and thus unlikely to be as well accepted by patients [72]. In addition, it is not easily applicable for the treatment of tumors in deep body locations. However, injectable systems are also being developed that may facilitate delivery by minimally invasive surgery, and when surgery is required, for example for removal of the tumor, a delivery system could be implanted for the removal of cancer cells potentially remaining at the site. Indeed, a recent study by Palamà *et al.* leads us to speculate that an approach similar to the one they followed may be applicable for EV delivery. The authors reported the use of a poly- ϵ -caprolactone scaffold loaded with the anti-inflammatory drug dexamethasone in the treatment of osteosarcoma, based on pre- and post-operative surgery [89]. Results demonstrated a sustained drug delivery and thus, a good strategy of localized tumor therapy.

Another route of EV local administration often used is the subcutaneous injection. Investigations have shown that administration of EV by subcutaneous injection may be an effective anti-cancer treatment. An interesting study suggested the use of EV derived from M1-polarized macrophages as a cancer subcutaneous vaccine adjuvant [90]. A melanoma mouse model was used, and EV were injected subcutaneously in their right flank. To verify the EV distribution after administration, they were labeled with the lipophilic fluorescent dye Dil. Using an *in vivo* imaging

system after animal's sacrifice at 7 days after injection, EV were localized in inguinal and axillary lymph nodes near the injection local. This observation suggests that EV did not achieve systemic circulation in the lymphatics but mainly accumulated in lymph nodes near the place of injection, where they promoted an increase in the secretion of proinflammatory cytokines [90].

Since EV efficiency is dependent on their administration route, Hao *et al.* compared effects of EV administered via subcutaneous injection and via intradermal injection in antitumor immunity. This was performed in a mouse model with induced tumors in the thighs. To monitor their distribution upon administration, EV were labeled and, despite the proximity of both injection routes to the tumor, by the intradermal route, labeled EV were detected in 3.8% of lymph nodes cells, against only 0.9% of cells when subcutaneous route was used. These results are in agreement with the mice survival rate upon the two different administration routes: intradermal route demonstrated to be more successful in antitumor immunity than subcutaneous administration [91]. Interestingly, the first clinical trials (phase I) using EV performed repeated injections of EV, derived from autologous dendritic cells (DC), into melanoma and non-small cell lung cancer patients using the intradermal (10% of the dose) and subcutaneous routes (90% of the dose). Nonetheless, only modest anti-tumoral effects were achieved [92]. More recently, a phase II clinical trial for the treatment of non-small cell lung cancer used conventional chemotherapy, combined with DC-derived EV for cancer treatment. In this trial, EV were administered exclusively by intradermal injection leading to an increase of 32% in the progression-free survival of patients [93]. Importantly, Dai and his team developed a Phase I clinical trial whereby EV derived from ascites of colorectal cancer, were subcutaneously administered to test their effectiveness in colorectal cancer treatment. Patients tested received EV only, or EV combined with the granulocyte–macrophage colony-stimulating factor (GM-CSF). Both were shown to be safe, but only patients who received EV with GM-CSF developed an antitumor cytotoxic T lymphocyte response [94].

In the area of tissue repair and regeneration, subcutaneous injection of EV has also been reported. Komaki *et al.* injected subcutaneously placental tissue-derived EV into auricular wounds. They observed the capacity of this therapy to enhance angiogenesis in the murine auricle wound model [95]. In a mouse model of fat graft, animal flanks were subcutaneously injected with fat tissue, simultaneously with EV derived from normal or hypoxia pre-conditioned adipose tissue-MSCs, or with PBS. The subcutaneously administration of EV and fat tissue lead to an improvement in graft survival and integration, compared to control flanks injected with fat and PBS, suggesting the subcutaneous route of administration can be used for local EV administration and effects [96].

Zhang *et al.* demonstrated the ability of weakly intra-articular administrations of human MSC EV to repair critically-sized osteochondral defects in rat models. After 12 weeks, regeneration was observed with hyaline cartilage and subchondral bone very similar to the original one [53]. As demonstrated by this study, short-term repeated doses of exosome should be administered during the regeneration process to achieve good results. This kind of EV delivery is not comfortable for a patient, and is pointed as one of the major drawbacks of this approach, for clinical application [97].

Although there are not many studies using this type of administration, intracavernous injection was performed by Chen and his team to demonstrate EV effects in erectile dysfunction (ED). The EV used were derived from adipose stem cells, and tested in a type 2 diabetes mellitus rat model. Results showed an improvement on ED in rats, by decreasing apoptosis of penile tissue. Despite positive results, many issues were unclear in this study, namely how long EV remained in the organ, what was the EV dose administered and the specific time of their administration, and what was the mechanism of action [98].

Depending on the therapeutic action intended and the tissues to be targeted, different routes of administration of EV can be chosen. Overall, this choice will determine the pharmacokinetics and biodistribution of administered EV.

2.2.1. EV loading into biomaterials

Local and systemic delivery of free EV do not allow to control the retention at the target site, and consequently to achieve the concentration required for the therapeutic effects. Also, the release kinetics and the biodistribution of delivered EV is neither controlled nor fully clarified.

This issue may be overcome by the incorporation of vesicles into appropriate biomaterial delivery systems that permit a timely and controlled release of EV *in vivo*. Controlled release will increase EV availability in the injury site for longer periods, maximizing their effect. In fact, EV have been used in combination with biomaterials of different types in a tissue engineering approach to treat several different lesions.

Hydrogels constitute the class of biomaterials most used for EV delivery *in vivo*. They are very attractive for EV incorporation, since their mechanical properties can be tuned to a large extent by different techniques, including by using biocompatible cross-linkers [99]. Thus, hydrogels with very small pores may be synthesized, providing a slow release rate of the vesicles. A gradual degradation of the material makes a prolonged release effective, and decreases side effects of bulk and fast EV administration, such as accumulation in non-target organs [100] [101]. Moreover, they can be easily modified with different moieties for improved nanoparticles attachment to the hydrogel, which may be translated to EV. Most importantly, hydrogels can be administered by different methods, including injection [99].

In a recent study, Liu *et al.* tested the incorporation of EV isolated from cultures of human iPS-derived MSC in a photoinduced imine crosslinking hydrogel glue, useful for cartilage repair due to its biocompatibility, and its capacity of cartilage integration. This approach has many advantages when compared to other EV administration modes, such as the ability of retaining EV in the cartilage defect site or the ability of achieve an accurate fit and even distribution in irregularly shaped tissue defects. To monitor EV release from the glue, vesicles were labeled with the lipophilic fluorescent dye Dil prior to incorporation in the biomaterial, and release conditions were then

simulated *in vitro*. Released EV could be detected by nanoparticle tracking analysis and red fluorescence of Dil-labeled EV were found in the cytoplasm of chondrocytes also entrapped in the glue, suggesting a successful endocytosis of the released EV. With this approach, the authors could demonstrate a released of about 100 million EV/mL every day, resulting in a major therapeutic dose to the surrounding cells [97].

EV delivery using biomaterial scaffolds has also been reported, particularly in recent studies. Huang *et al.* reported positive effects of EV derived from dental pulp cells on odontogenic differentiation *in vivo* and *in vitro*. In this study, EV were isolated and embedded in collagen sponges and included on the root canal space [102]. Another study compared the effects of exosome/ β -TCP combination scaffolds with only pure β -TCP scaffolds in osteogenesis. Exosome-incorporating scaffolds were more effective in the bone repair on a rat critical-sized calvarial bone defect model, with large amounts of *de novo* bone formation [103]. In a recent work, Xie and colleagues functionalized decalcified bone matrix scaffolds with MSC-derived EV and investigated the angiogenic and osteogenic potential of these constructs *in vivo*. Regenerative potential of this scaffold was evaluated in a model of ectopic subcutaneous bone formation in nude mice. EV enhanced bone formation and increased vascularization in the grafts [104].

Chitosan-based materials have also been reported as delivery system for EV. Tao *et al.* reported controlled-release of EV derived from miR-126-3p-overexpressing synovium mesenchymal stem cells combined with a chitosan hydrogel. Chitosan without EV showed lack of angiogenesis. *In vitro* experiments showed an enhancement in human dermal fibroblasts proliferation. *In vivo* experiments with a diabetic rat model, confirmed increased reepithelialization, angiogenesis and maturation of collagen matrix. Thus, chitosan functions as a biomaterial with controlled-release capacity that leads to a potential approach when combined with EV from a modified cell line [105]. Another study reported similar results with chitosan/silk hydrogel loaded with gingival mesenchymal stem cells derived EV in *in vivo* experiments. As the previous study, a

diabetic rat was tested and positive effects on the diabetic rat skin wound repair were verified [106]. In an innovative approach, Huleihel et al. tested ECM bioscaffolds, produced by decellularization of connective tissue, as natural EV carriers. They found that EV naturally anchored in the native matrix could survive the decellularization protocols applied and, when implanted, could promote cell growth and proliferation, cell migration and cell differentiation. They also showed that EV have specific miRNA signatures associated to their source tissue, essential information to improve clinical applications [107].

Despite the good functional results reported so far, the use of carriers for EV delivery is still little explored, and advantages and disadvantages of these systems, compared with free EV administration, are not yet well characterized. For instance, studies addressing biodistribution and pharmacological kinetics parameters of EV entrapped in scaffolds are very limited. Another point that needs to be clarified is how the controlled release of the EV from these scaffolds is achieved *in vivo*. Many characteristics of scaffolds composition and structure may be tuned for optimal controlled release of EV from different origins, such as scaffold porous size, interconnectivity between porous, method of EV adhesion to the carrier, among others. Thus, in the near future, it is necessary to improve the design of carriers to use with EV.

2.3. Future perspectives for EV controlled release: translation from the nanoparticles field

The use of hydrogels or biocomposite scaffolds for the controlled delivery of EV, still requires more studies, namely to explore methods of EV incorporation in different matrices, to tune the rates of vesicle release *in vivo* to fit different applications. These issues have been extensively researched in the field of nanoparticles for drug delivery. Many studies have been published reporting the use of hydrogels or solid

scaffolds combined with nanoparticles for the local controlled release of active compounds. Those studies may provide useful information on the development of similar systems to incorporate EV.

For applications in the Tissue Engineering field, hydrogels and scaffolds doped with bioactive proteins, nano/microparticles of bioactive materials, or nanoparticles encapsulating active compounds have long been described. In many cases, these nanoparticles are liposomes or have a major lipidic composition, resembling EV in many of their physical properties.

The capacity of self-assembled hydrogels to incorporate growth factors and liposomes, controlling their release, for instance by noncovalent interactions, makes them attractive for EV-delivery systems. Hydrogels showed no significant changes in mechanical properties upon vesicles incorporation and allowed bimodal drug release. When compared with controls, consisting of the individual factors solely loaded in the hydrogel, this system proved to be more efficient in promoting tissue regeneration, an effect mainly attributed to the controlled release of the incorporated factors [108]. Another study reported a hydrogel system with acidic liposomes for intratumoral injection in a tumor-bearing mouse model. A controlled release and prolonged action of these liposomes were reported, translating into an efficient antitumor action with decreased toxicity [109]. The controlled release of Fluconazole (FZL) loaded liposomes integrated in a hyalugel, as a novel ocular delivery system on male albino rabbits was used to overcome the limited penetration of FLZ in ocular fungal infection therapy, and results demonstrated an enhancing on corneal permeability and in sustained FLZ effect, as intended [110]. In addition, hydrogels decorated with nanoparticles and active compounds have also been used as coatings of materials used for biomedical applications. For instance, chitosan hydrogel films containing mesoporous silica nanoparticles loaded with ibuprofen were previously used to coat titanium surfaces, a material commonly used in orthopedic implants [111]. In our group chitosan and PGA have been combined as nanoparticles for drug loading [112], and

as a layer-by-layer systems, based on electrostatic interactions [113] for the delivery of molecules, like IFN- γ , when physiological pH disrupts the interaction between the layers [114]. Although EV have not been incorporated thus far in these systems, their negative charge should allow their incorporation based on electrostatic interactions, and their release when locally administered.

In recent years, printing techniques applied to the biomedical field have greatly evolved, and bioprinting is nowadays one of the methods most explored in the development of novel drug delivery vehicles. Using this technique, liquid materials with active pharmaceutical drugs may be printed onto surfaces or be directly incorporated in the milieu of 3D scaffolds, in an oriented and controlled manner [115]. Many rigid scaffolds and hydrogels loaded with different compounds by bioprinting have been reported in the literature. As an example, Akagi *et al.* described the fabrication of a layer-by-layer composite of poly(L-lactide) and poly(D-lactide), loaded with ovalbumin or ovalbumin encapsulated in poly- γ -glutamic acid (PGA)/L-phenylalanine ethyl ester nanoparticles [116]. The printing of scaffolds with nanoparticles loaded with active drugs allow the construction of complex structures that ultimately control at several levels the release of the biologically active compounds at target sites. The incorporation of EV in bioprinted scaffolds has not reported so far. However, a recent study performed for the first time a polyethylene glycol (PEG) hydrogel incorporating red blood cell (RBC) membrane-coated nanoparticles (RBC-NPs) produced by a light-based, rapid 3D bioprinting process. This is possible because this 3D bioprinting method is superior in resolution and speed when compared with traditional 3D printing [117].

Interestingly, 3D-like meshes synthesized by electrospinning were also reported for the incorporation of drugs and nanoparticles, including liposomes [118]. More recently, Masek *et al.* used a porcine model to test an improvement of transmucosal delivery of macromolecular therapeutics. This approach involved electrospun nanofibrous reservoir layers and mucoadhesive layers as a system for

effective administration of PLGA-PEG nanoparticles. A high porosity and great pore interconnectivity made possible the slow clearance of the loaded nanoparticles [119].

An alternative approach to increase EV bioavailability for therapeutic purposes could be achieved via their incorporation in osmotic pumps. Indeed, Hill and colleagues reported that incorporation of nanosuspensions of an undisclosed compound in the commercially available micro-osmotic pump Alzet® improved the availability of the compound in plasma upon pump implantation subcutaneously in mice [120]. In comparison with bolous subcutaneous injection of the nanosuspensions, osmotic pumps lead to a lower peak concentration of the compound of interest in the plasma, but which was maintained for a longer period of time, up to 7 days. Later on, Tian *et al.* showed it was possible to deliver nanostructured lipid carriers (NLC) incorporating fenofibrate, using osmotic pump tablets [121]. In this study, the release of integral NLC from the tablets could be observed *in vitro*, although great changes in their size occurred upon incorporation in the pumps. Importantly, in this approach NLCs were lyophilized to be incorporated into the osmotic pump, a process that seems to only mildly affect EV content, but not structure, being also suggested that their function is not affected provided lyophilization conditions are optimal [122].

The use of foams has also been explored for the delivery of nanoparticle therapeutics, particularly for topical applications of high drug doses in large areas or cavities of the body [123]. In 2015, Tang *et al.* reported two foam-based platforms incorporating nanoparticles for their controlled released, that were responsive to temperature or moisture [124]. When translating this approach into the EV field, foam formulations compatible with EV lipidic composition will have to be sought, to avoid their lysis or loss of biological activity. Furthermore, the method of foam formation will also have to be carefully considered, as higher pressures may lead to vesicle mechanical disruption.

3. Conclusion

EV, in particularly microvesicles and exosomes, have been increasingly tested for their therapeutical potential in different diseases. EV constitute an interesting option for drug delivery, because they are biocompatible, they can deliver many different cargo and target specific cells. Many *in vivo* studies, and even clinical trials, using EV for therapeutic purposes have been reported. However, the doses administered, the formulation of EV, and the route chosen for their administration greatly varies across studies. Administration techniques range from systemic to local, leading to different biodistributions and outcomes, including off-target effects, important parameters that have to be carefully taken into account to translate EV research into the clinics. A solid preclinical and clinical body of data behind EV-based therapies is still lacking, and many important questions remain unanswered. However, there is an obvious and strong movement towards the development of EV-based therapies. In order to minimize toxic action of EV in non-target organs, and to maximize the intended therapeutic effects, EV have been incorporated in biomaterials that can control their release. The future, will likely comprise more complex and tunable EV delivery systems, particularly using methodologies well established in the nanoparticles field, that are easily translatable to EV delivery.

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