UNIVERSITAT POLITÈCNICA DE VALÈNCIA

ESCUELA TÉCNICA SUPERIOR INGENIERÍA INDUSTRIAL VALENCIA

BIOMEDICAL ENGINEERING MASTER THESIS

EXOSOME-BASED THERAPY WITH BIOMATERIALS APPLICATION IN THE REGENERATION OF THE CENTRAL NERVOUS SYSTEM

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Resume

Regenerative medicine is a field based on helping tissues to heal by offering growth factors or improving environment and conditions to enhance regeneration. One of the biggest aims of regenerative medicine is Central Nervous System injuries due to lack of regeneration of this system unlike Peripheral Nervous System. Many kinds of researches are carried on to find solutions to this problem.

Lately, exosomes therapy has grown in this field with a solution. Exosomes are small vesicles secreted by many kinds of cells and work as transporters between cells, carrying RNA and proteins which activate different metabolic pathways. These carriers can be used as drug transporters to deliver certain substances in harmed areas. This is the basis of exosomes therapy, which has been applied in several pathologies and cases.

Exosomes therapy appeared as a replacement of stem cell therapy because exosomes derived from stem cells have been demonstrated to carry the desired factors to achieve the same effect and offer a huge advantage being less immunogenic than stem cells, which means that the answer of the host body is going to have less aggressive respond to the addition of these foreign bodies.

Exosomes are not only based on pure exosomes therapy but there are ways of improving its performance in therapy. It is needed especially to extend delivery time to maintain the effect during a large period of time. In order to do this exosome therapy has also been studied companied by biomaterials such as hydrogels and bioscaffolds which has some effects reducing inflammation and making more comfortable the environment.

The main disadvantages of exosome therapy are the low efficiency in isolate and mass produce these vesicles to be used them in clinic. Looking for solutions some research teams have been testing artificial exosomes based in two different methodologies of creating exosomes instead of naturally collect them from cells.

Exosomes have demonstrated to be a very useful tool in regenerative medicine but not only in applications in Central Nervous System but in all kinds of applications that require drug delivery, and also it is appearing its information providing role as natural biomarker associating some proteins and RNA to expression of concrete pathologies. The research on exosomes is growing with lot of potential applications and opportunities.

Key words: Exosomes, regenerative therapy, isolation, biomaterials, CNS, extracellular vesicles, hydrogels, bioscaffolds, cancer, biomarkers, Drug delivery system.

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Exosome-based therapy with biomaterials application in the regeneration of the central nervous system

Daniel Roldán Herrero

1. Introduction

1.1 Central Nervous System and its regeneration

Regeneration en human body or other living beings is an essential ability and it is very common to finds these repair mechanisms in wide number of species and tissues. This regeneration can be achieved in 3 different levels of complexity, cellular level, tissue level and organ level.

Some cell regeneration are simple and can be achieved by normal mitosis of cells of the same type, such as it occurs in passive cell replacement, because cells constantly die by apoptosis mechanism and they are replaced by other new cells. However, these are small issues compared to the main goals of regenerative research and some higher levels of regeneration need some other cells with higher potential of differentiation. Stem cells are the most important part of any regenerative research or regenerative therapy due to the ability of generate several differentiate paths in its division. The capacity of this differentiation depends of the type of stem cell. It is known stem cells can be classified in 4 orders: unipotent, multipotent, pluripotent or totipotent. Stem cells are the origin cells in any organism and they can be found in an embryonic state. Due its capacity of generate other types of cells, embryo grows up and becomes in a full organism with many different kinds of tissues with unique properties that altogether allow the organism to do complex actions and functions (Ramírez 2009).

Stem cells are also found in adults in some tissues such as bone marrow or fat, but instead of being all totipotent stem cells, adult stem cells have a limited capacity of generate other kinds of human cell. These stem cells can be unipotent if they only can generate one kind of cell, multipotent if they can differentiate into cell types of a particular lineage (endoderm, mesoderm and ectoderm) and pluripotent, which can generate any kind of cell of any lineage but cannot create a full organism as totipotent (Ramírez 2009).

In case of injury, human body can regenerate lost cells using stem cells and regenerative therapies are focused in this way. One main target of regenerative research is the central nervous system or CNS, due to its lack of regenerative ability.

The nervous system is one of the most important systems in human body due to its functions of communication with other tissues in the body and with the external environment and processing of all information received. It also has more complex functionalities such as learning, thinking, memory or emotional responses. All these functionalities are achieved by neurons, which are the main cellular type in nervous system. Neurons have just one function, receive different stimulations of other neurons or peripheral afferent organs and convey the signal by a depolarization wave through its axon and communicate to other neuron by the synapsis process. The high specialization of these cells allows nervous system to achieve all complex functions mentioned before but this specialization means lack of a lot of basic functionalities in other cells that without them a cell could not live alone. Neurons are the main kind of cell but not the only one. Apart from neurons, in the nervous system it is found the neuroglia, that is made up of different cells such as astrocytes, oligodendroglia (CNS), Schwann cells (SNP) and others. Neuroglia does not participate into communication between

neurons but offer support for this function by capturing some neurotransmitters or increasing communication speed with myelin sheaths. Neuroglia also offers metabolic and physic support, isolation of neurons and maintenance of the media. Without neuroglia, neurons could not achieve its function, even they could not survive (Cruz 2013).

The nervous system is divided in two parts: central nervous system (CNS) and peripheral nervous system (PNS). CNS is composed of brain and spinal cord and is the main part of nervous system, the one that process information and make up answers to the stimulation that reaches SNC through all the neurons net. On the other hand, PNS has no relation with the processing of information and its purpose is just sends the information in both ways. The way directed to CNS is called afferent path and the way directed to the rest of the body, specifically to effector muscles is known as efferent path.

Back to the regeneration, there are similarities and differences between these two parts of nervous system (Stoll, Jander, and Myers 2002) in the regeneration matter and as it has been said, nervous system is one of the most important systems in the human body and could be said the most important one, and its repair has a huge importance

In general, growth and remodeling mechanisms are activated by an injury, and in case of the nervous system, a nervous system injury. This nervous system injury could be traumatic injury, interruption of blood supply or degenerative diseases and all these causes can damage axons in peripheral nerves. The observation of these peripheral nerves has lead investigations to conclude that a vigorously regeneration and re-grow is presented. Also it seems to reestablish synaptic connections both with other neurons and targets in the periphery under favorable circumstances (Chen-Wishart 2014).

However these observations does not appear in case of CNS damaged neurons and lead to permanent harm such as blindness in case of damage in the optic nerve or paralysis if the harm is done into the spinal cord

As it is said in Neuroscience book, successful in PNS depends on two characteristics. The first one is related to gene expression and the response mechanism of the neuron which can support axon regeneration. Interruption of the axon elongation reactivates these genes but not in the case of CNS. The second characteristic involved in a correct regeneration of neurons in PNS is a beneficial environment when the gene expression is activated. The environment has to support and guide regrowing axons and these environment should be activated also by the detection of any damage or degeneration (Chen-Wishart 2014) and the responsibility of this task belongs to neuroglia and macrophages, which has to remove fragments of the lost part of the axon and myelin, which could inhibit axonal growth

In contrast, in CNS there are some circumstances that make impossible to reactivate growth in neural axons. In adults, the activation signal must be higher than in a developing CNS. Also damaged myelin sheaths cannot be removed in its whole and this problem inhibits the axonal regeneration (Figure 1). The explanation of this inhibition is based on some hostile molecular and cellular media which are regular components of myelin sheaths (Stoll, Jander, and Myers 2002). It also appears additional inhibitors when astrocytes react to CNS injury. However, CNS

axons can sometimes regenerate successfully if they are into more supportive environment. In conclusion regeneration of adult CNS nerves is held in gene suppression.

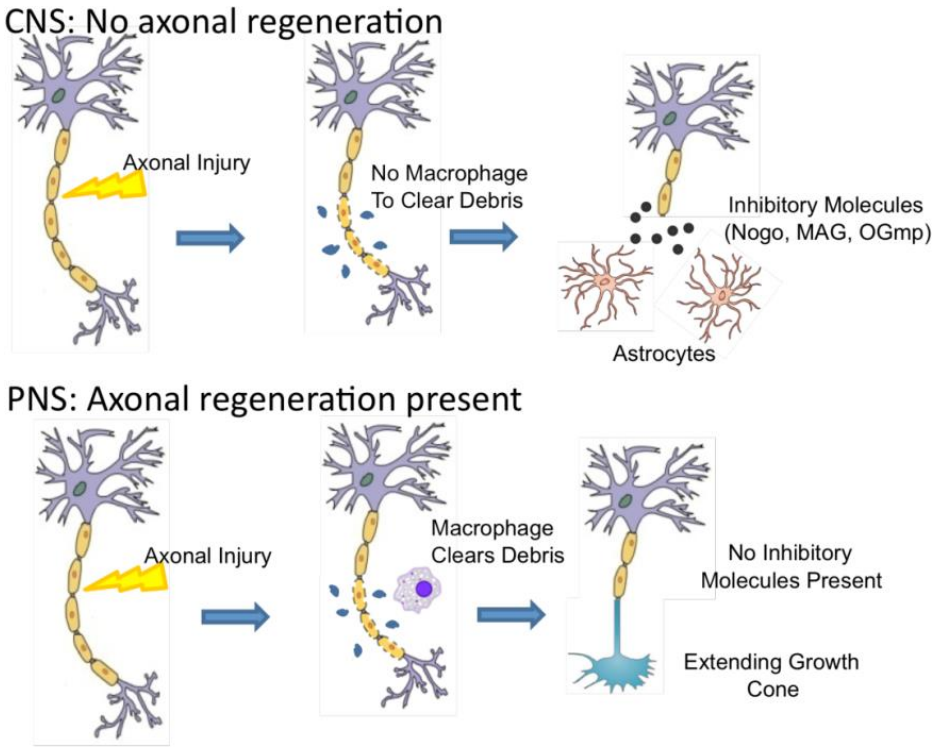

Figure 1. Attempts of regeneration in CNS and PNS (Strittmatter 2010)

1.2 Blood Brain Barrier (BBB)

In terms of trying some different approaches on the CNS regeneration, BBB matter has an essential role. The blood-brain barrier is an isolation barrier that works as a protector of the CNS (Figure 2); preventing the access of many substances to the brain and spinal cord with the purpose of maintain CNS homeostasis. This goal is achieved by a very tight physical construction of the barrier, with tight junctions, adherent junctions and gap junctions, which allows only small molecules and proteins to enter by diffusion. The rest of the blood components have to enter through specific transporters. So in the end, BBB is a full, close barrier that protects CNS allowing the entrance only to recognizable substances (Dong 2018).

BBB should not be disrupted because of a lot of pathologies associated with that condition, loss of the homeostasis, formation of cerebral edema and potentially stimulates a second injury (Yuan et al. 2019). However, there are certain pathologies that can disrupt BBB such as TBI, multiple sclerosis, Parkinson´s disease and Alzheimer´s disease and BBB state is a major issue on all these diseases. Other liable of this disruption is tumor. The most common brain tumors are gliomas. These gliomas grow and can damage BBB but due to the angiogenesis in the process of oncogenesis. New blood vessels are formed and it is called BBTB or blood-brain tumor barrier. Its permeability is higher in tumor areas and slight or null in peripheral areas and some therapy approaches are crossing BBTB for targeting glioma cells (Wanjale and Kumar 2017).

For this reason BBB has a huge relevance in CNS regeneration research and therapy. Any kind of drug, molecule or particle used in those researches to target glioma or any structure inside BBB has to pass through BBB and cannot be avoided. This has been a problem in CNS regeneration with drugs due to the impossibility of crossing that barrier. Recently, there have been some approaches for brain drug delivery systems such as viral vectors (Check 2005), nanoparticles (Saraiva et al. 2016), exosomes (Prathipati, Zhu, and Dong 2016), delivery through active transporters (J. Zhu and Dong 2017), brain permeability enhancer (Haqqani et al. 2013), non-invasive techniques to enhance brain drug uptake (Jinlong Zhang et al. 2017), alteration of administration routes and others (Sarin 2009).

In order to search strategies to deliver drugs is necessary to understand the concept of BBB and know consequences of its disruption. But there is need for more studies on delivery strategies searching efficient delivery systems that could be applied into therapy (Dong 2018).

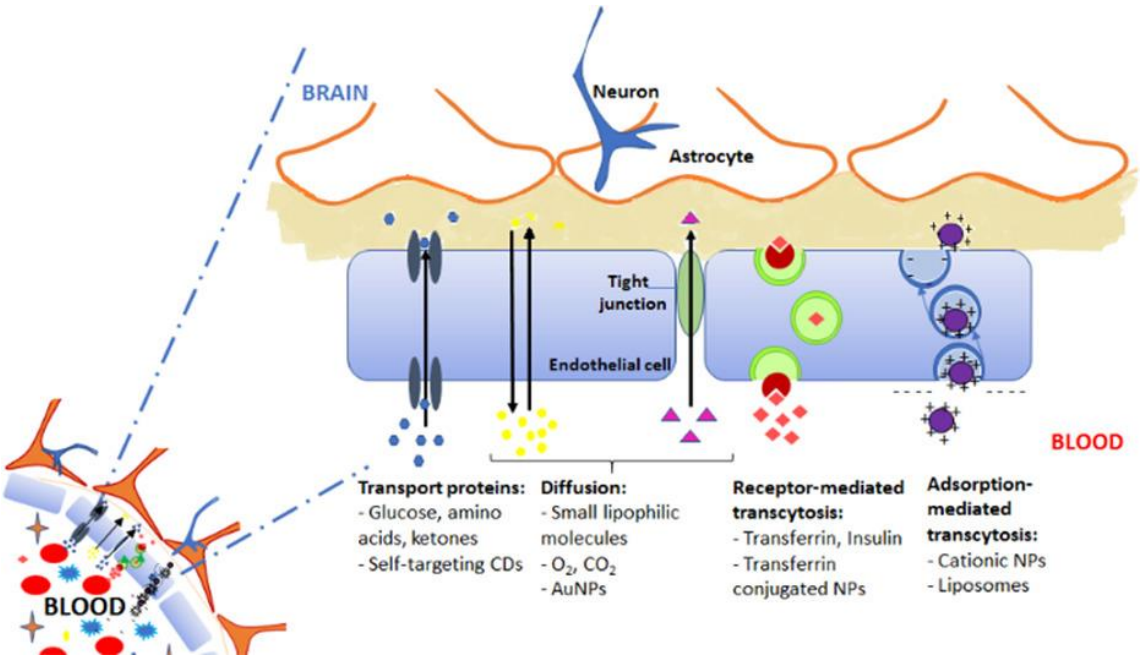

Figure 2. Functions of Blood Brain Barrier (Y. Zhou et al. 2018)

1.3 Types of nervous system injury

Nervous system is complex, and disorders in this system had been studied for a long time, especially CNS disorders, due to the importance of this part of the system, which has the highest value to the organism. Some of these disorders are: trauma, infections, degeneration, structural defects, tumors, blood flow disruption, autoimmune disorders…

A common injury studied in regeneration research due to its relevance and frequency is traumatic brain injury or TBI. This is an acute condition that can lead patient to disability or even death, and it is not a rare disorder because in 2019 there were registered 13 million cases of TBI related patients with complications combined United States and Europe (Rubiano et al. 2015). Most of the affected people are male young adults, and the cause of the TBI is usually traffic accidents or violence (Quinsey et al. 2018), being ex-soldier a common case type. TBI

has two categories depending of severity of the injury. It can be mild TBI (mTBI) or severe TBI (Y. Xiong, Mahmood, and Chopp 2018). mTBI is not well understood but there are evidences of chronic traumatic encephalopathy in case of repetitive mTBI (Goetzl et al. 2019). Studies are more focused on severe TBI. The aim in TBI therapy is to prevent harm from what is called secondary injuries because the primary injuries have irreversible condition harm.

TBI is a condition where brain shows itself harmed by many types of causes or situations. In this same direction of understanding pathologies in CNS, can also be found Spinal Cord Injuries (SCI) which, as it can be thought, refers to damage done into the spinal cord instead of the brain.

SCI is a severe neurogical trauma with also high morbidity and mortality that can be divided in two main processes: primary and secondary injuries. Though the pathophysiological mechanism of SCI is still unclear it has been probed to lead to some serious damage such as vascular damage and disruption of BBB. Since the spinal cord is the connecting pathway for most neural control mechanisms, any damage in spinal cord can cause dysfunction in almost all biologica system (Ozturk et al. 2018). As it was in TBI, spinal cord injuries are followed by inflammation, oxidative stress, motor neuron apoptosis, necrosis and autophagy and because of being part of CNS and not PNS, neurons have myelin sheaths for high speed signaling and regeneration is almost impossible in this circunstances.

Other major problem in the brain stability is infection, caused by foreign bodies such as virus, bacteria, fungal agents or even prions. Infections in the CNS represent a significant source of morbidity and mortality throughout the world. Some of these severe infections are meningitis, caused by Streptococcus, N. meningitides or S. pneumoniae among others bacteria or fungus. Similar symptoms are presented in tuberculosis, which is other possible infection of CNS. Other infections produce encephalitis or brain abscesses. The problem with theses pathologies is that they do not only cause physical damage in CNS but can involve other organ systems (Rushing and Burns 2001). For this reason, and the relevance of the functioning of the CNS, infections have to be considered, specifically when therapy planning includes surgical intervention.

However, damage in CNS can appear without any foreign body, just due to the protective mechanisms of the body, which in case of alteration can damage other tissues. This is the case of autoimmune pathologies such as diabetes or arthritis, but the pathology with high relevance on CNS is multiple sclerosis. Multiple sclerosis is a neurodegenerative, chronic, not contagious pathology of unknown origin, which affects more in young women (29-33 years old). It is characterized by demyelization, edema, remyelintaion and axonal damage (Felipe and Morcuende 2012) produced by an aggressive immune response that harms BBB (Ruíz García and Solar Salaverri 2006). It is an unpredictable disease which the only exiting therapy of delaying its advance (Felipe and Morcuende 2012). Repetitive inflammatory episodes can lead the patient to severe physical disabilities (Zijian Li, Liu, He, et al. 2019). This is a very important disease with a lot or research on it because the impossibility of healing.

Finally, among all pathologies, there is one that appears in several body systems and this is tumors. Tumors appear due to an uncontrollable formation of cells that grows and in the end damage many structures and could move to other locations by its metastasis. CNS tumors are

relatively common, with more than 40000 cases annually in the United States. Most of them are benign but malignant ones are a problem being third leading cause of death in adolescents and young adults. Some of these tumors are gliomas, lymphomas or oligodendrogliomas (Buckner et al. 2007). Tumors have bad prognosis and this needs new strategies of research that can achieve stable and reliable therapies.

2. Exosomes

2.1 Regenerative medicine

As its name says, this field focuses on regenerate and repairs all kind of tissues or organs that might have been compromised and resulted injured or its full loss. Inside regenerative medicine it can be distinguish two main lines: regenerative research and regenerative therapy. First of all there is regenerative research, which move forward experimental techniques mainly related with in vitro induction of cellular activity that can achieve support to regenerative therapy. The two main objectives of regenerative therapy are studying cellular activity related with tissue regeneration and how cells response to different drugs and evaluate its toxicity. The other main objective is lead those studies to systems where in vivo cellular activity can be inducted in order to treat diseases and achieve that support previously mentioned to actual regeneration therapies and getting some clinical relevance (J. ichiro Jo, Gao, and Tabata 2019).

Cellular activity can be modulated by searching bio-functional molecules and creating a safe and stable environment. In this task, technologies based on biomaterials play an important role. Biomaterial researches propose biomaterials-based drug delivery system technology (DDS) which consists in a controlled release of a drug that target certain cells. The interaction between low-molecular weight substances used as drug and cells depends on the type of bio-functional molecule. Some of them interact with receptors and produce response inside the cell and others need to be integrated into cells to target intracellular substances. This interaction is also affected by ECM, that can induce several cellular behaviors and any modification of cellular activity must include local microenvironment similar to this matrix. Some of these solutions are scaffolds with different characteristics as the charge, wettability, biological affinity of their surface or the stiffness, roughness, pore size of the bulk property and porosity (Jafari et al. 2017; Ghasemi-Mobarakeh 2015; Carletti, Motta, and Migliaresi 2011).

The best way to modify cellular activity is through nucleic acids, which are the main molecules that could induce some changes in cellular activity. Through mRNA cells can increase gene expression, and mRNA is decoded into sequences of proteins thanks to RNAi units, which assembly amino acids into ribosomes, where the protein sequence is created (J. ichiro Jo, Gao, and Tabata 2019). However naked form of RNA cannot effectively exhibit their biological activities in the living systems and some strategies on designs of DDS are reviewed (Yin et al. 2014; W. Wang et al. 2013; R. Ni et al. 2016; Bakhtiar et al. 2014).

Regenerative research aims to modify cellular activity by regulating nucleic acids because of RNA regulates cellular gene expression. For this reason, regenerative research is focused in DDS that deliver substances such as nucleic acids into target cells. For example, a disease cell by cell can be created from a disease patient using nucleic acids due to differentiation and reprogramming techniques (Papp and Plath 2011). Other biomaterial approaches are combination of cell with 3D scaffolds due to its similarity to original cells environment. This similarity means a good approach in regenerative research and that is why 3D constructs are commonly used in this field (J. ichiro Jo, Gao, and Tabata 2019).

In order to create DDS of nucleic acids some materials have been proposed, such as cationized polymers (Singha, Namgung, and Kim 2011), cationized liposomes (F. Xiong, Mi, and Gu 2011) and ceramics (Thomas et al. 2015). However, biological expression of nucleic acids based on carriers is transient and is required to continue for long time period, and for that reason other alternatives have been presented such as cationized gelatin hydrogel, aiming to control release of nucleic acids inside cells (Doi, Jo, and Tabata 2012). The release occurs in a gradient form with disintegration of the hydrogel. A reverse transfection procedure has also applied following Sabatini et al. procedure (Ishikawa et al. 2012). This procedure has three advantages. The first one is that maintain a high level of biological expression even in the presence of serum. The second one is a high biological expression of nucleic acids and the last one cellular viability increased with reverse transfection (J. ichiro Jo, Gao, and Tabata 2019).

Regenerative therapy is the application in vivo of all regenerative studies searching a viable clinical solution. There are two approaches of therapy with nucleic acids. Nucleic acids can be delivered direct to cells in vivo or transplant cells that have been extracted and modified with nucleic acids or injection of exosomes from cells extracted.

Direct reprogramming has been paid much attention, and consists on converting differentiated cells to ones of different lineage by using transcription factors as nucleic acids. By using this method the result is allowing fibroblasts to contribute to cell-based tissue regeneration (D. Srivastava and DeWitt 2016). As well as promoting regeneration by grow factor is important, it is also important to regulate microenvironment such as angiogenesis (Madeddu 2005) and some regulation of inflammatory environment researches had been made (Eming, Wynn, and Martin 2017).

Through many years cell transplantation has been the most viable option in order to reprogram cells in vivo presenting good therapeutic potential due to its excellent ability of targeting and its biological properties. However, cell transplantation has become obsolete due to the low survival ratio in vivo, that differs a lot from in vitro studies (J. ichiro Jo, Gao, and Tabata 2019). One of the solutions proposed is a new technique in the last years that consist in using exosomes to deliver substances in vivo. Its advantage is its biological stability and being able to communicate to cells and interact with them. For this reason exosomes are expected to be used in many therapeutic approaches (Fatima et al. 2017). The use of exosomes is starting to show good performance in regenerative therapy (de Jong et al. 2014).

In regenerative therapy using DDS of nucleic acids it can be performed in two ways: through local injection or systemic administration. Systemic administration has few studies due to the need of establish previous results in local injection in order to attempt to test a systemic administration. The problem of local administration is to achieve a way to avoid diffusion away from the injection. Success of solutions depends on complexes size, which is better to be high, and also depends on steric hindrance of biomaterial chains present on the surface. Not only avoid diffusion away from the injection but also its degradation. One proposal is to create a nanomicelle carrier for mRNA, which goes inside and surrounded by a layer of polyethylene glycol (PEG). This material makes mRNA more stable in vitro and in vivo (Uchida et al. 2013). These nanomicelles can be used in regenerative therapy on cartilage (Aini et al. 2016) but also

in the brain (C. Y. Lin et al. 2016). The use of polymeric nanoparticles has showed to be effective in tissue regeneration(Gálvez-Gastélum et al. 2011).

If diffusion away from the injection is not controlled, performance of therapy has poor results. A low concentration of nucleic acids or the substance that allows a tissue to regenerate is shown as a decreased biological activity. For this reason is highly required to maintain those substances in the target cells, not only avoiding diffusion to other parts of the body but through time, maintaining enough concentration during calculated time to develop a good regeneration and this can be several weeks or months, depending on the tissue to be repair. This is the origin of DDS, a way to make nucleic acids remain around the injection site and continuously act on the target cells. DDS control release and the action of the substance delivered. Controlled release also reduces adverse effects that can be related to drug administration. So the researched point is which carrier is the best in DDS. There are three types of carriers for controlled release: biodegradable polymers, non-biodegradable polymers and ceramics. Different researches on DDS carriers have been made (J. ichiro Jo, Gao, and Tabata 2019). Most of them prefer to search biodegradable polymers due to the ability of these materials to be reabsorbed by the body. Depending on the target tissue and the problem to be solved, expression duration of DDS with nucleic acids can be consider long term duration in some hours or days. For this reason expression duration cannot be always a comparative measure to describe differences between carriers used. A compilation table has been created (Table 1) in order to show different carriers used and some comments on how carriers had been created or results on the experiments.

Different from others controlled release systems; a cationized gelatin hydrogel was developed (J. ichiro Jo, Gao, and Tabata 2019) and released nucleic acids in the state of complex with the cationized gelatin fragments. This prevents enzymatic degradation and nucleic acids complexed with cationized gelatin fragments can interact by its electrostatic nature to be easily integrated into cells (J. ichiro Jo, Gao, and Tabata 2019). This method of using cationized gelatin hydrogels was successful in regenerative therapy for fibrosis (Aoyama et al. 2003), aortic aneurysms (Zhong et al. 2009), and autoimmune alopecia (Nakamura et al. 2008).

Another promising approach in regenerative therapy is genetic engineering of somatic stem cells; in particular the use of mesenchymal stem cells (MSC) has been investigated with very good results on various tissues (Rohban and Pieber 2017).

Material	Type of carrier	In vitro/in vivo	Expression duration	Comments	Ref
IFN-silicone + water soluble substance	Non-biodegradable	Both	30 days	Suppressed tumor growth in nude mice for about 100 days after a single administration.	(Kajihara et al. 2001)
polymethacrylate-based copolymers	Non-biodegradable	Both	No mention	Amorphous polymers having glass transition temperatures between 9 to > 150(o)C	(Thakral, Thakral, and Majumdar 2013)
poly(lactic acid)	Biodegradable	Both	20 days	Induce tumor regression, suppression of metastasis	(Egilmez et al. 2000)
polyanhydride	Biodegradable	Both	24 hours	Strong adhesive interactions with gastrointestinal mucus and cellular linings	(Mathiowitz et al. 1997)
poly(orthoester)	Biodegradable	In vitro	90 hours	Characterization of the enhanced the efficacy of POE microspheres by blending PEI, a well-characterized cationic transfection agent, into the POE matrix.	(Nguyen et al. 2008)
PLGA	Biodegradable	Both	28 days	Increased resistance to nuclease degradation, increased amounts of plasmid DNA (pDNA) uptake, and the possibility of control in dosing and sustained duration of pDNA administration	(Cohen et al. 2000)
poly(D,Llactide-co-4-hydroxy-L-proline)	Biodegradable	In vitro	30 days	The copolymer was synthesized by ring-opening polymerization of D,L-lactide (DLLA) with N-cbz-4-hydroxy-L-proline (HP) in the presence of stannous octoate (Sn(Oct)(2)).	(Zhenhua Li and Huang 2004)
poly(1,8-octanediol-co-citrate)	Biodegradable	Both	2 weeks in vivo	POC scaffolds can potentially support long-term biological cues to mediate tissue formation through non-viral gene delivery.	(X. Q. Zhang et al. 2009)
oligo(poly(ethylene glycol) fumarate)	Biodegradable	In vitro	42 days	Two formulations of OPF were synthesized from poly(ethylene glycol) of a nominal molecular weight of either 3.35K (termed OPF 3K) or 10K (termed OPF 10K)	(Kasper et al. 2005)
poly(2-aminoethyl propylene phosphate)	Biodegradable	In vitro	12 days	Sustained release of plasmid was achieved from PPEEA/DNA complexes as a result of PPE-EA degradation.	(J. Wang et al. 2002)
polypseudorotaxane	Biodegradable	In vitro	6 days	A cyclodextrin-based supramolecular hydrogel system with supramolecularly anchored active cationic copolymer/plasmid DNA (pDNA) polyplexes was studied as a sustained gene delivery carrier(Zibiao Li et al. 2012)	(Zibiao Li et al. 2012)
polysaccharide	Biodegradable	In vitro	20 days	Degradable hydrogels were used for the local and sustained delivery of a pDNA encoding for vascular endothelial growth factor (VEGF) in the ischemic hindlimbs of mice, and local pDNA release significantly improved the recovery of blood perfusion as compared with a bolus injection of VEGFencoding pDNA	(Kong et al. 2008)
silk elastin-like polymer	Biodegradable	In vitro	3 weeks	Hydrogels with higher silk content and concentration degraded at a slower rate compared to other analogs.	(Gustafson et al. 2009)
atelocollagen	Biodegradable	Both	40 days	A biocompatible natural polymer such as collagen or its derivatives acts as the carrier for the delivery of DNA vectors	(Ochiya et al. 2006)
Calcium phosphate	Ceramic	Both	40-60 days	Calcium phosphate nanoparticles can be carriers of therapeutic agents that would enable a con-trolled drug release to treat a given bone infection and at the same be resorbed in the body so as to regenerate hard tissue lost	(Uskoković and Uskoković 2011)

Table 1. Compilation of carriers used in regenerative therapy

2.2 What are exosomes?

Exosomes are part of what is called extracellular vesicles, which are bodies that are originated into some kinds of cells (Samal, Demir, and Pandit 2018). These extracellular vesicles or EVs have important roles as messengers, where the most common functionality is to be natural nanotransporters which transfer RNA and proteins into target cells. There are different kinds of EVs and can be categorized by its size, where can be found EVs with a diameter greater than 200nm and some others that are categorized as small EVs if its diameter do not reach to 200nm (Yao et al. 2019). There are more ways to classify these EVs such as its composition (CD63+/CD81+-EVs, Annexin A5, etc) or its parental cell (Mesenchymal cell, glia cells…).

As an example of EVs that are secreted apoptotic bodies are one of the largest EVs in terms of size, with a diameter of 500-4000nm. These vesicles are produces in the apoptotic mechanism, in which a cell by its interaction with immune system starts a controlled destruction mechanism. In this process some parts of the cell are secreted as vesicles known as apoptotic bodies which finally will be phagocytized by immune system cells (Akers et al. 2013).

Exosomes are one type of EVs with a very small size that depending of the study its diameter can oscillate into 40 and 150 nm (Jieyuan Zhang et al. 2016), but as it has been said; limits of this range can be slightly different. As most of EVs, exosomes are naturally created by invagination, as it can be seen in Figure 3, and they are specialized en communication cell to cell. This property has been used in several investigations where they work as therapeutic agents.

It is not clear when small EVs have to be called exosomes and when they are just microvesicles, in fact, important articles sometimes refers to both of them by the name of exosomes. This is due to similar mechanism of creation. Both are derived from intraluminal budding of multivesicular bodies (MVBs) (Lu and Huang 2020). Its main difference is the size because while exosomes have a smaller diameter, microvesicles can reach to 500 nm depending on the source (Batrakova and Kim 2015).

Production of exosomes is achieve by ESCRT or Endosomal Sorting Complex required for Transport, which consists in a four multi-protein complexes (ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III). These complexes are the ones that perform exosomes formation mechanisms apart from other mechanisms (Bobrie et al. 2011). At first, an early endosome is invaginated to the intracellular media by endocytosis mechanism. Then, Golgi network transform that early endosome into MVB full of vesicles in its lumen. MVB can have two different endings. The first one is a fusion with a lysosome for degradation. This is the case of MVB made of waste or substances that need to decompose in order to anabolize some other substances. The second option would be secretion of the content of the MVB. In this case, MVB membrane is fused with cell membrane and release into the extracellular space all vesicles contained, where it can be identified exosomes (Urbanelli et al. 2013).

They are produced by several kinds of cells but some cells have more interesting properties in its application in regenerative therapy. The most interesting exosomes are the ones that are produced by mesenchymal stem cells (MSC). These cells are multipotent and they have its origin in the mesoderm layer, what it means MSC can differentiate into osteocytes,

chondrocytes, hematopoietic stem cells, mast cells and fibroblasts. This collection of possibilities in terms to differentiate stem cells makes MSC a great source of application options in regenerative therapy (Socarrás-Ferrer et al. 2013). Some other cells that can secrete exosomes are mast cells(Liang et al. 2018), nerve cells (Ranjit et al. 2018) and activated platelets (Jiannan Li et al. 2017).

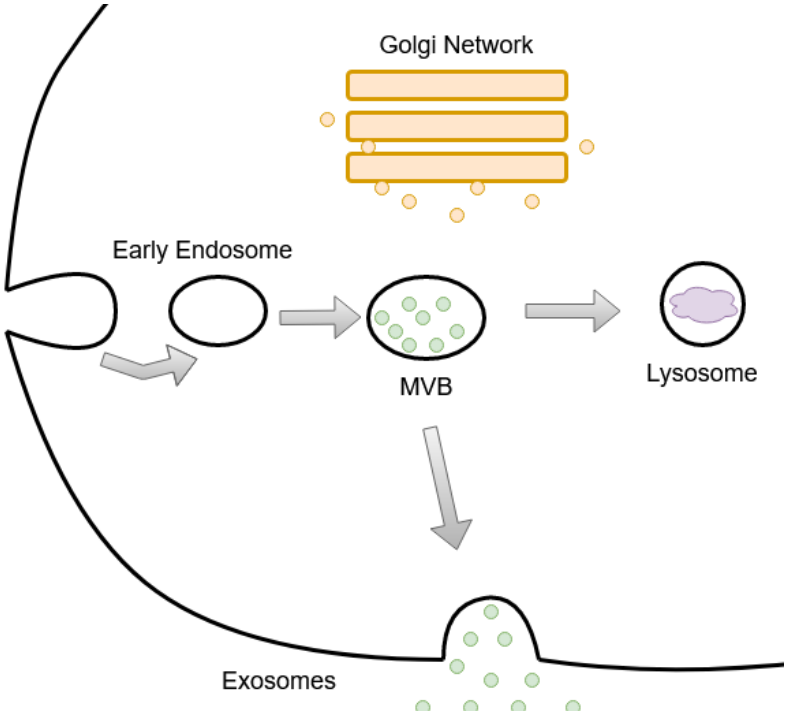

Figure 3. Exosome formation. MVB can release its content to extracellular space or fuse with lysosome.

Despite of having different ancestries or different progenitor cells; all exosomes share common surface proteins which help in researches to identify the existence of exosomes in the media. Some proteins are specific of a concrete cell source but exosomes show high levels of some proteins in common: tetrapanins (CD63, CD9, CD81), liposomal proteins such as LAMP2B, fusion proteins (Annexin, GTPase), HSC70 (which is a heat shock protein) and also TSG101, protein involved in the biogenesis of MVBs (Conde-Vancells et al. 2008).

The strength of exosomes as therapeutic factor is in its content, because the structure of the exosomes is a sphere made of a lipid bilayer which imitates cell membrane typology that created that exosome (Lopez-Leal and Court 2016). Inside the vesicle there is stored some content, which gives functionality to this transporter. The content is mainly an agglomeration of several proteins and some types of RNA such as mRNA, miRNA or siRNA (Yao et al. 2019) but also enzymes that catalyze some mechanisms initiated by this content. Lipid and protein composition in its membrane not only contribute to their physicochemical stability but also enable them to directly fuse with the plasma membrane of the target cell (Lu and Huang 2020). When the content enters the target cell and reaches intracellular media after fusion of both membranes, some mechanisms are activated or accelerated. Some mechanisms that regenerative research aims are the ones involved with growth and tissue regeneration, not

also the ones that are activated by these mechanisms but all mechanisms that could improve regeneration and some with autoimmune regulation and protective effects.

Exosomes secreted from B lymphocytes were demonstrated to be capable of presenting antigens and triggering effector T cell (Raposo et al. 1996) responses. These data suggest a role for exosomes in antigen presentation in vivo. The immune surveillance function of exosomes got attention on several research teams; and made progress in the use of exosomes in immunotherapeutic studies (Bell et al. 2016). Those investigations reported that exosomes could express peptide-MHC class II at the surface and promote the binding to MHC class II, which induces the previously mentioned strong stimulation to T cells.

Interaction between exosomes and target cells could be in 3 different mechanisms. The first one consists in an interaction of the exosome with receptors in the target cell. By this method, exosome does not enter inside the cell. The second method consists on the action of extracellular proteases that releases some proteins from the exosome surface that target receptor on the cell surface, which has even less contact the content of the exosome with the target cell. In these two first mechanisms the RNA and proteins inside the vesicle are not relevant and exosome just works as a membrane with proteins in the surface. The third option and the most interesting one in regenerative therapy is fusion of both recipients, the only mechanism that allows exosome to transfer genetic material. However, fusion with the plasma membrane could be limited by pH conditions. Mechanisms of endocytosis could be used to internalize exosomes (Urbanelli et al. 2013).

Soon, some of the processes, which exosomes could improve, start or accelerate, mentioned before were studied and establish some evidence of the benefit of exosomes in regenerative mechanisms of cells. Signaling properties, homeostasis maintenance, angiogenesis regulation, plaque and coagulation balance, cell proliferation and tissue regeneration were studied in the performance of exosomes with promising results (El Andaloussi et al. 2013; Iraci et al. 2016).

Then, studies of the messenger role of exosomes were started; due to its potential of carry substance from one cell to another and naturally incorporates its cargo on the target cell. The most commonly studied pathological function of exosomes is the transportation of tumorgenic factors produced by tumors, where exosomes have a crucial role. These tumorgenic factors are secreted by tumor cells and it has been reported to show angiogenesis activity which can induce formation os pre-metastasic areas in other systems (Costa-Silva et al. 2015). Other applications of exosomes have been investigated, in particular in neurodegenerative disorders by transferring the molecules involved in the disease and cause the malfunctioning (Quek and Hill 2017). In Alzheimer´s disease has been demonstrated that β-amyloid peptides were carried by exosomes to neighboring zones of the brain, producing the advance of the disease (Rajendran et al. 2006). Similar to Alzheimer´s disease, in Parkinson disease, α-synuclein, which is toxic was released by exosomes and this action contributed to the spread of aggregates that accelerate the pathology (X. Wu, Zheng, and Zhang 2017).

Exosomes are involved in other mechanisms and pathology progression of inflammation (Buzas et al. 2014), cardiovascular disease (Azevedo, Pedro, and Laurindo 2007), diabetes mellitus (Müller 2012), and virus infections (Mfunyi et al. 2015).

There are investigations on the exosomes performance in CNS such as their role in diseases of Parkinson and Alzheimer, but those are not the only exosomes pathways which have been studied. Pigment epitelium-derived factor, known as PEDF, has an important role in neuronal survival. It was proved that PEDF modified adipose-derived mesechymal stem cell-extracted exosomes made some improvements in cerebral ischemic reperfusion injury by the mechanisms or regulation of apoptotic factors and instigation of autophagy (X. Huang et al. 2018).

Other research on CNS suggests neuroprotectiveness property of oligodendrocytes-derived exosomes, due to its natural role of messenger between neurons and myelinated oligodendrocytes. This exosomes property was clearly identified in neuronal metabolism during stress conditions. Besides, the study came up with the fact that glutamate instigation has de ability to release oligodendroglia exosomes. This release is mediated by Ca^{2+} and NMDA (N-methyl-D-aspartate) receptors (Frühbeis et al. 2013).

All this studies confirmed the fact that exosomes can cross BBB and emerge at the target lesion site. Some studies research about how is the interaction between the barrier and exosomes and other EVs. These studies confirm, based in their experiments, that exosomes and EVs could be used for drug delivery into the brain(András and Toborek 2016), even in stress conditions (C. C. Chen et al. 2016).

All these roles of exosomes in several mechanisms suggest that they are part of natural relationship between cells, and this fact makes them good candidates for biological therapeutics and drug delivery system.

2.3 Isolation/Treatment procedures

Exsosomes are natural particles that have been used by the cells to achieve certain purposes. However, regenerative research focus not only in the study of these particles but in the use of them to establish some communications between cells that are not happening in the natural state of the body, guiding some regenerative factors from other cells such as MSC to tissues which needs repair and regeneration. This would allow accelerating natural regeneration of the tissue, even reaching to some regeneration pathways which would not be possible without these new techniques.

Previously to treatment procedures, there are some guides to obtain enough quantity of exosomes. Exosomes can be obtained of cell culture or if it is a better option obtain from other laboratories exosomes in media. In the case of cell culture is used fetal bovine serum, which reduces exosomes in the media. During 2 weeks and after some passages, cells produce more exosomes due to the lack of them in the media. When confluency reaches 80-90% the culture can be replaced and in case of getting enough passages the dissolution would be ready for the first step of its treatment (Jieyuan Zhang et al. 2016).

The first step in order to investigate exosomes is isolation and purification. By this, a separation of exosomes and the media is achieved and ensure that the isolated substances are only exosomes. For obtaining functionally data it is important to work with samples of high purity.

First step of isolation must be separation of exosomes from cell debris. The gold standard method for exosomes isolation is differential ultracentrifugation. The separation of exosome and cell debris is achieved by a low centrifugation of 2000g followed by a high centrifugation of 100,000g. This second centrifugation aims to sediment exosomes, however an ultracentrifugation is needed in order to reach 100,000g. In some studies, an intermediate centrifugation of 10,000g is used to pellet down and to separate cell organelles (TAYLOR, HOMESLEY, and DOELLGAST 1983). Depending on the study and the research team there are differences in parameters, specific in g force. In other studies first low speed centrifugation is set at 400g and se high speed centrifugation can be set in a range of 10,000-20,000g (Théry et al. 2006).Low speed centrifugation can be done in different ways, for example in 2 phases, the first one at 300g for 10 minutes and the second one at 2000g for other 10 minutes. By this dead cells and cellular debris are separated in two different moments. In fact, low centrifugates can be performed reaching 4000g to remove every big size body that could be present, even a third centrifugation can be performed after the high speed if this third can achieve better purity (Jieyuan Zhang et al. 2016). Alternatively to the low centrifugation other studies suggest filtration to clarify the media of cells and cell debris (Lamparski et al. 2002). The supernanat is often filtered with 0.22µm filter sterilizer but pores size can be different. Depending of the application, all these procedures might change, even some reported cases avoided high speed centrifugation in the process of a combination of a chitosan/silk hydrogel sponge with GMSC-derived exosomes. They did only low speed centrifugations and used a MWCO hollow fiber membrane to mix exosomes with hydrogel sponge (Shi et al. 2017). Different ultracentrigufation parameters of studies are listed in Table 2.

Application	Low speed		Medium-speed	High-speed	Other	ref
Exosomes/tricalcium phosphate combination scaffolds for bone regeneration	300g-10min	2000g-10min	4000g + wash + 4000g	100000g-2h	4000g	(Jieyuan Zhang et al. 2016)
Exosomes naturally equipped for drug delivery	400g	-	10000-20000g	100000-150000g	-	(Batrakova and Kim 2015)
Cellular capsules for drug delivery in Parkinson´s	-	2000g	10000g	100000g	-	(Samal, Demir, and Pandit 2018)
Exosomes for mediation of promoting axonal regeneration	1000g-5min	Filter 0.45µm + 2000g x2	10000g-30min	100000g-70min	100000g-70min	(Tassew et al. 2017)
MSC exosomes for attenuation of inflammation and demyelination of CNS	300g-10min	2000g-10min	10000g-30min	100000g-1h	100000g-1h	(Zijian Li, Liu, He, et al. 2019)
GMSC exosomes combined with hydrogel to accelerate wound healing	Low centrifuge (not g force mentioned)	Filter 0.22µm	5000g 30 min	MWCO hollow fiber membrane		(Shi et al. 2017)
Nanodrug for osteosarcoma consisting of doxorubicin and MSC exosomes	Isolation kit	3000g-10min	10000g-1h	Resuspend and 3000g-10min		(Wei et al. 2019)
Schwann cell-derived exosomes for axonal regeneration in the PNS	300g-10min	2000g-10min	10000g-30min	100000g-70min	PBS and 100000g-70min	(Lopez-Verrilli, Picou, and Court 2013)
Exosomes derived from high-glucose–stimulated Schwann cells for development of diabetic peripheral neuropathy	Low centrifuge (not g force mentioned)	Filter 0.22µm	10000g-30min	100000g-1h	-	(Jia et al. 2018)

Table 2. Comparative between centrifugations of different researches

However differential centrifugation is a limited technique due to the limitations in g force, the rotor type, the angle of rotor sedimentation, radius of the centrifugal force, pelleting efficiency and more factors (Cvjetkovic, Lötvall, and Lässer 2014).

Another rising isolation technique is the one based on polymer precipitation. With all new applications being on research, some polymers and macromolecules containing PEG (polyethylene glycol) have been created and commercialized aiming an easy way to isolate exosomes. The most commonly used commercial polymer precipitation-based product for exosome isolation is ExoQuick-TC™ from System Biosciences. The mix of exosome and these products do not require high speed centrifugation, just low speed (Batrakova and Kim 2015). This could be an option for those laboratories without an ultracentrifuge due to its ease to prepare and to use. A disadvantage from this technique is the high presence of contaminants in the pellet which should be just exosomes. Those contaminants are lipoproteins and polymer material (D. D. Taylor, Zacharias, and Gercel-Taylor 2011). Some studies have demonstrated that contamination can be reduced to a minimum using pre- and post- isolation steps. Before creating the mixture can be executed a removal or subcellular particles with centrifugation and after the isolation polymer can be remove with Sephadex G-25 column (D. D. Taylor and Shah 2015). Another commercial polymer used in exosomes isolation is Optiprep™ density gradient medium that is used in combination with ultracentrifugation (Gardiner et al. 2016).

Immunoaffinity technique takes advantage of exosome specific markers such as CD9, CD63 or TSG101 to specifically purify the vesicles of interest and could be used as another alternative (Samal, Demir, and Pandit 2018). Immunoaffinity chromatography is a process in which antibodies, covalently attached to beads, filters, or other matrices, bind to specific surface proteins or antigens on the target particle and non-target particles remain unbound (Batrakova and Kim 2015). Immunoaffinity uses magnetic beads conjugated with specific antibodies binding to proteins on exosomal surface (Mathivanan et al. 2010).

Aiming to fast diagnostics, new microfluidics techniques has some promising results. Microfluidics is in an early stage of development but shows a high pure exosome preparations capacity with minimal processing time (Liga et al. 2015).

After completing an isolation of exosomes there is a second step of purification. For this task, density gradient centrifugation is usually used to meticulously separate exosomes from other vesicular structures and protein aggregates. Using this technique it is aimed to separate exosomes in different fractions from those structures using sucrose density gradient (Batrakova and Kim 2015). Another technique used to purify exosomes is size exclusion chromatography or SEC, which rather than the molecular weight it uses hydrodynamic radius to achieve separation between molecules and the solution using columns of various matrices such as sepharacyl S-400 or sepharose, and takes this as advantage (Samal, Demir, and Pandit 2018). This alternative method is used in some researches (Baranyai et al. 2015; Böing et al. 2014; D. D. Taylor, Zacharias, and Gercel-Taylor 2011). Components with a small hydrodynamic radius are able to penetrate through the many small pores, like a maze, resulting in a longer time to elute. However, larger hydrodynamic radius substances such as exosomes are unable

to pass through that maze and leave early the column. SEC has some advantages as purification method due to, without affecting structural integrity and biological functions, being able to separate the vesicles by size, separating them from the smaller protein or lipid aggregates (Samal, Demir, and Pandit 2018).

Physical Characterization of exosomes

After isolation and purification is always recommended to make characterization test, in order to evaluate the final result of the isolation. As exosomes have extremely small size, their characterization becomes uncomfortable. The most used technique is TEM (transmission electron microscopy). It uses the stained samples to register some information about exosomes but the staining limits the capacity to obtain further biochemical information. The most relevant information is size distribution (Théry et al. 2006).

SEM (Scanning electron microscopy) is also used for morphological characterization of exosomes, but the problem with this technique is preparation of samples. Preparation of samples needs following steps of alcohol dehydration and air drying (Sokolova et al. 2011) or freeze drying(Kadiu et al. 2012). These methods require long time and can generate artifacts that make SEM a worse choice in clinical use. It also would need some improvement in throughput analysis due to the high importance in clinic (Mehdiani et al. 2015).

Nanoparticle tracking analysis, known as NTA, uses the principle of laser scattering and Brownian motion to visualize and analyze particles size and it could be another characterization technique for exosomes (Dragovic et al. 2011). This system offers advantages in analysis speed in an easy way of handling. On the other hand, this system is limited by its dynamic range (Samal, Demir, and Pandit 2018).

One rare system used to morphologic analysis of exosomes is atomic force microscopy (AFM). This was used to analysis the ultrastructure of a single exosome isolated. These analysis and the ones performed with field emission scanning electron microscopy (FESEM) showed the presence of extra vesicular channels connecting exosomes (Sharma et al. 2010).

Flow cytometry is also a common technique used in qualitative and quantitative physical analysis but is limited by exosomes small size (Lacroix et al. 2010) and in majority of analysis exosomes end useless for functional analysis (van der Vlist et al. 2012)

The last one of physical characterization techniques for exosomes exposed is TPRS (tunable resistive pulse sensing), that is based on the resistence generated by particles crossing through a hole and measures conductivity (Freyssinet and Toti 2010).

As a resume, in Figure 4 are listed all mentioned techniques in a figure extracted from the work of Samal and coworkers (Samal, Demir, and Pandit 2018)

Technique	Minimum Detectable Size (nm)	Preparation Time	Size Detection	Biochemical Detection
Dynamic light scattering	0.3	M	+	N/A
Scanning electron microscopy	100	H	+	N/A
Transmission electron microscopy	10–20	D	+	Immunogold labeling
Flow cytometry	200–500	H	–	Fluorescent antibody-tagging
Dedicated flow cytometry	70–100	H	–	Fluorescent antibody-tagging
Nanoparticle tracking analysis	25–40	M	+	Fluorescent antibody-tagging
Resistive-pulse sensing	70–100	M	+	N/A

Figure 4. Techniques used for the morphological characterization of exosomes(Samal, Demir, and Pandit 2018)

Chemical characterization of exosomes and other procedures

As it was said before, in regenerative therapy with exosomes the most important part is the content of exosomes. For this reason research teams also are interested in biochemical analyzing to determine protein and RNA profile content. Different proteins, miRNA, lipids and RNA can give a hint of the cell source that produced those exosomes and can help to elucidate the biogenesis of those isolated vesicles. The content of exosomes can provide a large volume of information about their biological pathophysiological functions (Samal, Demir, and Pandit 2018).

Different techniques are used in order to accomplish biochemical characterization. Mass spectrometry (MS) provides a detailed outline of proteins isolated and this information can be used to compare between different isolation methods (Tauro et al. 2012). However, the technique most commonly used for evaluation of proteins in EV preparations is western blot. Briefly explained, this method allows detecting specific proteins by denaturation and performing gel electrophoresis and binding of specific antibodies (S. C. Taylor and Posch 2014). SDS-PAGE can also be used as an alternative electrophoresis technique (Yao et al. 2019). Another use of antibodies that can be used es immunoelectron microscopy (IEM) which results of combination of microscopy and labeling with specific antibodies. Other suggestions to obtain qualitative and quantitative analysis of cargo biomolecules can be performed with FTIR, ELISA or RT-PCR

In order to provide a global outlook of the proteins related with vesicles, a study determined the content of vesicles as: CD81, CD9, CD63 and others from the tetraspanins family, annexins, integrins, TSG101, HSPs, ALIX, Rab proteins, syntenin-1, proteins from cytoskeleton such as actins, or tubulins, some metabolic enzymes and ribosomal proteins (Choi et al. 2013).

Apart from isolation and characterization procedures, there are others techniques used in exosome applications development. As investigation on exosomes goes further, clinical

applications start to appear and for this step capacity of developing exosomes for therapy use must be higher. This is what is called scalability of the process, in special scalability of exosomes isolation which allows processing large amounts of cell culture media and producing consistently high exosomes yield. For this purpose, tangential flow filtration (TFF) has been validated as a scalability method for EVs (Corso et al. 2017). This characteristic is very important due to the lack of experience in exosome development, which means significant production is a problem to address before exosomes can be taken into clinic use. Also microfluidics could be an option for a scalable method to isolate and prepare exosomes for clinic (Yao et al. 2019).

Aiming to orientate in which characterization techniques are commonly used there has been designed a list (Table 3) of techniques used in the same studies which were exposed in Table 2 where it was shown ultracentrifugation parameters

Application	Physical characterization		Chemical characterization	ref
Exosomes/tricalcium phosphate combination scaffolds for bone regeneration	TEM/SEM TPRS		Western blot	(Jieyuan Zhang et al. 2016)
Exosomes naturally equipped for drug delivery	TEM/SEM TPRS Flow cytometry	NTA DLS	Western blot MS	(Batrakova and Kim 2015)
Cellular capsules for drug delivery in Parkinson´s	TEM/SEM TPRS	NTA Flow cytometry	Western blot MS IEM	(Samal, Demir, and Pandit 2018)
Exosomes for mediation of promoting axonal regeneration	TEM/SEM		Western blot IEM	(Tassew et al. 2017)
MSC exosomes for attenuation of inflammation and demyelination of CNS	TEM/SEM Flow cytometry		Western blot ELISA RT-PCR	(Zijian Li, Liu, He, et al. 2019)
GMSC exosomes combined with hydrogel to accelerate wound healing	TEM/SEM TPRS Flow cytometry		Western blot FTIR	(Shi et al. 2017)
Nanodrug for osteosarcoma consisting of doxorubicin and MSC exosomes	TEM/SEM NTA		none	(Wei et al. 2019)
Schwann cell-derived exosomes for axonal regeneration in the PNS	TEM/SEM		Western blot IEM	(Lopez-Verrilli, Picou, and Court 2013)
Exosomes derived from high-glucose–stimulated Schwann cells for development of diabetic peripheral neuropathy	IEM		Western blot RT-PCR	(Jia et al. 2018)

Table 3. Use of exosomes characterization techniques in researches. In case of review, it is marked the techniques mentioned which can be used

Proposition of isolation pathways of exosomes

With all different properties and choices made in the previous studies mentioned it seems clear that characterization techniques can be used as each group prefer due to their possibilities and research goals. However isolation techniques are not that clear and it appears to be one main problem, the lack of verified isolation protocols, specific differential ultracentrifugation, which is performed in some many ways. Aiming to clarify this isolation procedure it has been generated the next diagram (Figure 5) where all mentioned procedures in researches have been compared and it is exposed a common general route to follow in the isolation of exosomes.

It was divided in two main paths. The principal difference of isolation procedures nowadays is the use of polymer solution which isolates exosomes without complex centrifugations. The other path is the one that lacks polymer solution and it is based all in centrifugation of different g forces and time. This path requires more time and higher speed centrifugations but there is no need of acquiring exosome solution. However, the use of isolation polymers makes easier exosome isolation and that is why more isolation polymers for exosomes are appearing in the market

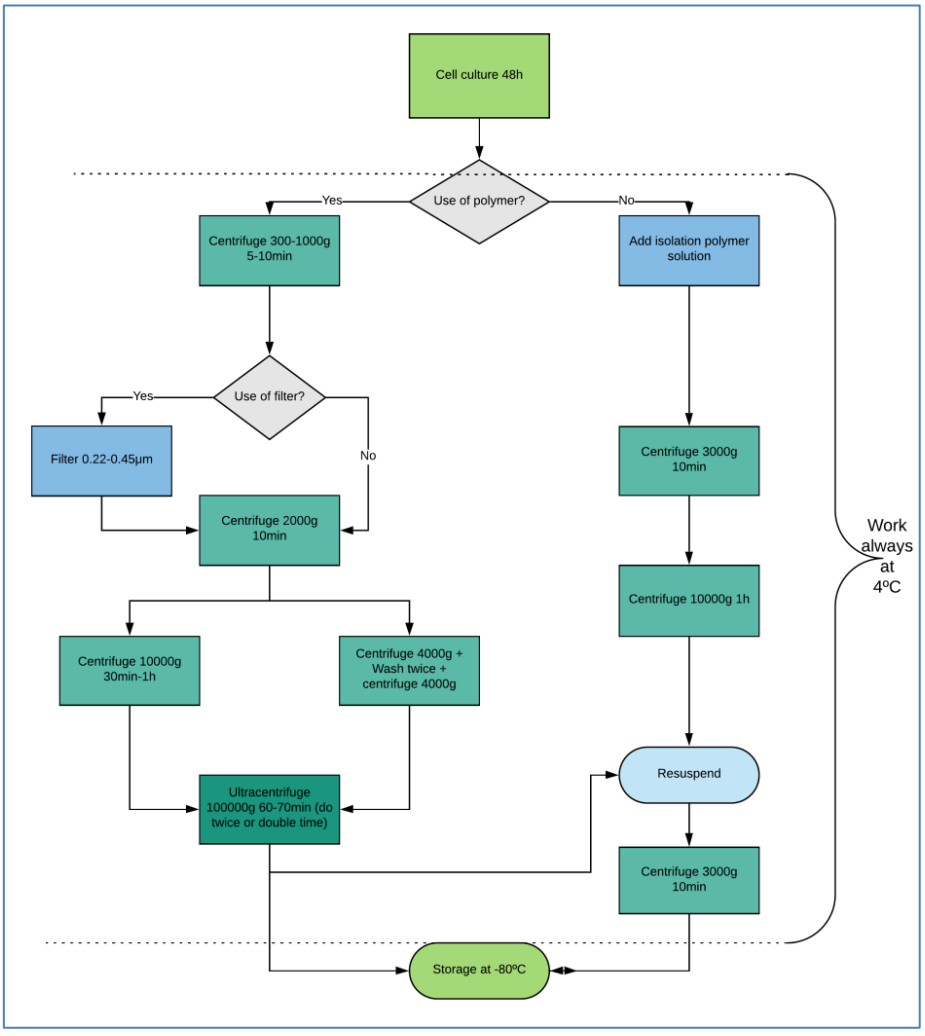

Figure 5. Proposed general procedure of exosomes isolation

3. Strategies of regenerative research with exosomes

3.1 Exosomes in Drug Delivery System (DDS)

In regenerative research is common the investigation and use of drug delivery systems (DDS). The objective of these systems is to target cells and tissues with specific biochemical drugs and success in making those drugs do its effect when it is required (Figure 6). One characteristic of good DDS is control release for two reasons. The first one is maintain all the substance in the messenger and do not waste it until the messenger has reached the target cell. The other reason is the need of life-time extension of drug supply to maintain cell response. Many intracellular processes need a long activation signal in order to produce enough effect, for example en regeneration and formation of new cells during some time. Other characteristics are accelerated permeation and absorption and targeting of drug (J. ichiro Jo, Gao, and Tabata 2019).

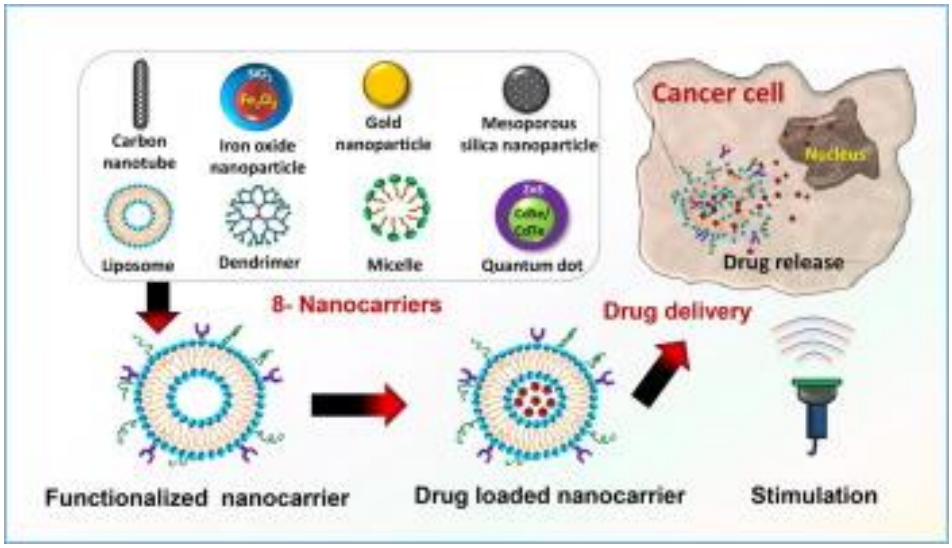

Figure 6. Operation of a DDS (Hossen et al. 2019)

This field of regenerative therapy is also known as gene therapy, and it focus on RNA drugs that can activate some metabolic routes. It covers a lot of applications, form gene replacement to vaccination. The key of these systems are finding or designing good messengers or vectors which can fit in the application required. Viral vectors have been used since long ago and have been used in many applications, but in more recent years there have been problems with some limitations and risks of this form of vector (Seow and Wood 2009).

Gene delivery vehicles (GDVs) are still being improved but also investigating some others due to those limitations. For example, adeno-associated viral vectors, which gained popularity in gene therapy due to its lack of pathogenicity has shown limitations as small packaging capacity, allows only DNA-based cargo, low probability of integration or reduced efficacy of repeat administration (Coura and Nardi 2007). Retroviral or lentiviral vectors are also used as GDVs due to the stable gene transfer of retrovirus or the efficiently expression of the lentiviral

vectors (Ellis 2005), however there have been discovered some insertional mutagenesis in patients and transcriptional silencing, especially in retrovirus, leads to reduced expression over time (Ellis 2005). Adenoviral vectors also triggers strong inmmune response against vehicle and transgene (Hartman, Appledorn, and Amalfitano 2008). There have been used liposomes vectors as GDVs but also presented limitations in immune recognition, transduction due to lower efficiency than viruses (Sakurai et al. 2008) and risk of inflammatory toxicity (Sellins et al. 2005).

Recently, new researches have demonstrated exosomes can act as natural GDVs, containing a large number of functional mRNAs and miRNAs (Valadi et al. 2007). Exosomes show their potential delivering oligonucleotides that can be exploited due to the research of delivery of mRNAs to human mast cell to produce murine proteins using murine mast-cell derived exosomes, acting like synthetic liposomes (Seow and Wood 2009). Depending of the progenitor cell, exosomes acquire different properties, for example, exosomes can be produced to have immunosuppressive effect if the progenitor cell has immunosuppressive ligands such as FasL (S. H. Kim et al. 2006). The same effect could be achieved with immunosuppressive cytokines treatment, such as interleukin-10 (S.-H. Kim et al. 2005). This characteristic makes them appropiate for long-time and repeated administration. But the main reason to use exosomes as GDVs is the favorable immunological properties they have. They are well tolerated in the blood due to their natural function of transporter and communication (Seow and Wood 2009).

Exosomes have been studied for use in several clinical therapeutic applications, especially for cancer (Vader et al. 2016). They try to use tumor-derived exosomes for drug delivery. For example using exosomes to deliver antigens to dendritic cells can cause an induction of a T-cell mediated immune response against tumor cells (Wolfers et al. 2001).

Investigation of exosomes as DDS has gone so far and established different methods of encapsulation the therapeutic agents, because incubation and using RNA contained in exosomes is not the only method used. Not all drugs needed in clinic are naturally produced by cells and it is needed to add to exosomes in an active or passive way. The main passive cargo-loading methods are incubation with exosomes and incubation with donor cells. In the first one exosomes isolated from donor cells are incubated with drug and drugs diffuse into exosomes along the concentration gradient (Luan et al. 2017). Examples of this method are mouse-lymphoma-derived exosomes incubated with curcumin (Sun et al. 2010) or loading catalase into exosomes extracted from RAW264.7 cells (Haney et al. 2015). Incubation with donor cells is the one mentioned above (ver capitulo 2.3). Drug is mixed with progenitor cells and as a result they secrete exosomes with that drug inside. As an example Paclitaxel was incorporated in exosomes by incubation stromal cells with the drug (Pascucci et al. 2014).

Those were examples of passive methods of loading drugs into exosomes and they are based on letting the own mechanisms of exosomes be the one that load the cargo. These methods are simple but the main problem it their low loading capacity, having a low efficiency. This would be a problem in clinic because, even though passive methods are enough for research and lab trials, their efficiency and capacity would collapse as a clinical treatment due to a high

demand of isolated and loaded exosomes. However, there are some active methods which offer higher production ratio and higher efficiency in loading exosomes.

Diffusion of drug into exosomes can be performed using a homegenenizer probe to do a sonication of donor cells mixed with drugs. The mechanical shear force destabilizes exosomes membrane allowing drug to diffuse into them (Luan et al. 2017), decreasing exosomes microviscosity after sonication. Membrane integrity is restored within an hour. This method has the property of not only load drug inside the exosomes but also in the outer layer of membranes. This allows a release in two phases. First exosomes of the outer layer are released and them it follows a release of the drug encapsulated (M. S. Kim et al. 2016). Another active method is loading by extrusion, which consist in loading the mixture of donor cells and drug into a syringe-based lipid extruder with porous membrane. The exosome membrane ends being disrupted and the drug mixed with them. However, membrane disruption by extrusion seems to change membrane properties such as zeta potential, which can cause toxicity as it was reported in breast cancer cell study using porphyrin-loaded exosomes from those cells, and with other methods toxicity does not appear (Fuhrmann et al. 2015).

Other methods are freeze and thaw in cycles, where the problem is a lower loading capacity than the previous active methods mentioned and possible aggregation of exosomes (Sato et al. 2016), or electroporation, a technique that creates small pores in the exosome membrane using electrical fields. Temporary pores allow drug to diffuse inside exosomes and the advantage is that membrane integrity is recovered. It has been used to load siRNA and miRNA into exosomes, however electroporation can cause RNA aggregations and instability (Wahlgren et al. 2012).

The use of surfactants as Saponin reduces membrane destabilization. The use of Saponin enhances loading capacity of exosomes compared with other methods and it is a good option to diffuse hydrophilic molecules into exosomes through their lipid membrane. The problem with Saponin is its hemolytic activity that should be considered and its use should be limited (Podolak, Galanty, and Sobolewska 2010).

The last approach is avoiding disruption and diffusion of drugs into exosomes, and focus on bind drugs to exosome membrane. In this particular way of delivery drugs using exosomes there are two methods: using chemistry methods to establish covalent bonds or using antibodies to bind antigen to surfaces. In chemistry it is known as click chemistry the use of copper-catalyzed alkyne cycloaddition and can be used to bioconjugate small molecules to exosomes surface in a rapid and efficient way. The best advantage of this method is lack of affection to exosome structure or function (M. Wang et al. 2015). In the other hand, antibodies are mainly used to give exosomes fluorescence property, and this method facilitates exosomes analysis (Higginbotham et al. 2016).

All loading methods mentioned are represented in the next table (Table 4) with its advantages and disadvantages and the studies where these methods were used.

Due to several forms of loading and research behind exosomes as drug carriers it is enough evidence of the role of exosomes in DDS which is increasing and will be continue increasing next years.

Examples of engineering exosomes for cargo delivery				
		Advantages	Disadvantages	Examples of Model drug
I) Passive loading	a) Incubation of exosomes and free drugs	Simple, do not compromise membrane integrity	Low drug loading efficiency	Doxorubicin(Yano et al. 2004), Paclitaxel(Pascucci et al. 2014) , Catalase(Haney et al. 2015)
	b) Incubation of the donor cells with free drugs	Simple, do not compromise membrane integrity	Low drug loading efficiency. Drugs may cause cytotoxicity to the donor cells	
II) Active loading	a)Sonication	High drug loading efficiency	Compromise membrane integrity	Catalase(Haney et al. 2015)
	b)Extrusion	High drug loading efficiency	Compromise membrane integrity	Porphyrin(Fuhrmann et al. 2015)
	c)Freeze/thaw	Medium drug loading efficiency. Liposome-exosome fusion	Aggregations	Porphyrin(Fuhrmann et al. 2015)
	d)Electroporation	Loading with large molecules such as siRNA, miRNA	Aggregations	let-7a miRNA(Ohno et al. 2013), MAPK1 siRNA(Wahlgren et al. 2012)
	e)Incubation with saponin	Enhanced drug loading	Toxicity	Catalase(Haney et al. 2015), Porphyrin(Fuhrmann et al. 2015)
	f)Click chemistry	Quick and efficient Better control over the conjugation site	Drug is not inside exosome	Azide-fluor 545 for *in vitro* tracking(Smyth et al. 2014)
	g)Antibody binding	Specific and easy to operate	Drug is not inside exosome	CD9 antibody with Alexa-647(Higginbotham et al. 2016)

Table 4. Comparison of advantage and disadvantages of different loading methods(Luan et al. 2017)

3.2 Exosomes as biomarkers

Another potential use of these small vesicles is their role as biomarkers, which refers to biological markers. The objective of biomarkers is to indicate the medical state of a patient by observing some signs from outside. Biomarkers show some information of physiology and can be estimated the state of some metabolic routes and consequently, physiological systems by the observation of concrete molecules, not only its presence or its non-presence but, sometimes, the quantity of them (Strimbu and Tavel 2010). They are defined as “almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction.” They are used in

clinic due to the simplicity of evaluate a patient by analyzing blood, plasma or other fluids or tissues. To consider a molecule as biomarker in some physiological or pathological process requires the determination of relevance and validity, referring to testing if biomarker´s ability to provide information has relevance and clinic interest; and if characterization of that biomarker is effective (Strimbu and Tavel 2010).

Biomarkers play a critical role in improving the drug development process as well as in the larger biomedical research enterprise (Strimbu and Tavel 2010). It has been a necessity of using biomarkers as surrogate outcomes in large trials of major diseases, such as cancer (Ellenberg and Hamilton 1989) and heart disease (Wittes, Lakatos, and Probstfield 1989), though discussed some times. They only could replace for clinical relevant endpoints if by using them all physiological or pathological process can be understood, but undoubtedly, biomarkers have an important role in diagnosis and treatment (Strimbu and Tavel 2010).

The use of exosomes as biomarkers has major relevance in cancer. During cancer development, cancer cells secrete more exosomes, with significant changes protein composition (Palazzolo et al. 2012). Exosomes show communication properties and be responsible for drug resistance (D. dan Yu et al. 2015) or metastasis to distant organs (Keyu Li, Chen, Li, et al. 2019). Other non-invasive biomarkers have been developed but studies have confirmed that these biomarkers are located in the exosomes (Gallo et al. 2012), what means that exosomes can be novel target in cancer diagnosis and prognosis (Yan Li et al. 2015).

A research study analyzed other 42 studies were cancer patients used some biomarkers to determine diagnosis and prognosis of the pathology. Studies had different profiles, exosomes isolation method was in some cases just ultracentrifugation and in other they used ExoQuick™ product or other exosomes isolation kit. Studies had patients of colon cancer; others had liver cancer, pancreatic tract cancer and gastric cancer. They evaluate a total of 50 biomarkers used in studies and exosomes show their association with overall survival, disease-free survival and Recurrence free survival in various types of cancer (Wong and Chen 2019).

That meta-analysis of biomarkers in cancer indicated that exosomes can be potential biomarker in cancer diagnosis and prognosis. Exosomes are found in blood, saliva and urine and that helps in their detection. The major problem is still isolation and treatment methods, which are not still clear and they are not efficient enough for clinic. Exosomal markers seem to reflect the situation in cancer cells in various types of cancer (Wong and Chen 2019).

Exosomes contain cell surface cancer antigens, which confers them the potential for therapeutic approaches in cancer vaccination. Some researches indicate that in cancer, there is a cell surface glycosylation, which can be detected in exosomes and it has been largely used as biomarker (Escrevente et al. 2011).

Exosomes role of biomarker in cancer is due to exosomal microRNA, which has the information of the cell of origin of exosomes. However, though it is known that circulating microRNA can be used as biomarker in clinic due to results of different studies, there is currently no collective view if which circulating exosomal microRNA are suitable candidates (Nedaeinia et al. 2017).

Though there is not a global agreement in these new questions, there are some evidences about some proteins of exosomes, especially tetraspanins. These proteins are a family of scaffolding membrane proteins, which are highly enriched in exosomes, as it was previously said. In 2009, CD63 was reported as potential marker in melanoma disease due to the high levels of this protein in melanoma patients (Logozzi et al. 2009). After that, in 2013, it was evidenced the correlation between CD63 and malignant cancer, due to the high levels of this protein in cancer patients compared to noncancer patients. This information could show CD63 potential in the role of cancer biomarker (Yoshioka et al. 2013). Another tetraspanin which was reported to have marker functions is CD81, this time its role is important in hepatitis C. This tetrapanin shows some association with inflammation and severity of fiborsis. For these observations, CD81 could be recognized as a potential marker for hepatitis C diagnosis and treatment response (Welker et al. 2012).

As it can be noticed, biomarker function of exosomes is not only related with cancer disease, for example they work also as biomarkers in other diseases, for example in TBI, which is later evaluated how exosomes can help in therapy.

Also exosomal proteins have shown potential as central nervous system diseases biomarker. In 2008, exosomal EGFRvIII may provide diagnostic information for glioblastoma (Skog et al. 2008) but also was reported in Alzheimer´s Disease patients accumulations ofexosomal amyloid peptides plaques in the brain (Rajendran et al. 2006). As it was supposed in other cases, exosomes could also have potential as biomarkers that help in early diagnosis of Alzheimer´s disease. But Alzheimer´s is not the only disease were exosomes were involved. Previosly was explained their role in Parkinson´s disease and some studies confirmed their potential as biomarkers in this disease due to aggregation of α-synuclein, which is released by exosomes in an in vitro model (Alvarez-Erviti et al. 2011). Other application as biomarkers in CNS is spongiform encephalopathies biomarker (Vella et al. 2007).

Another approach in exosomes function as biomarker is the analysis of urinary exosomes, which is easily obtained and less invasive. Proteins of these exosomes have shown also biomarker potential. In 2006, exosomal fetuin-A showed an increase in patients with acute kidney injury (AKI) (H. Zhou et al. 2006). Two years later, the same group could distinguish between AKI and chronic kidney disease and controls due to the finding of activating transcription factor 3 in patients with AKI (Hua Zhou et al. 2008). In 2008, a study about bladder cancer proposed exosomes as potential biomarkers for this kind of cancer. Some proteins from exosomes showed high diagnosis value. Some of them were associated with EGFR pathway, while other proteins involved were resistin or the alpha subunit of Gs protein, (Smalley et al. 2008). One year later, it was demonstrated that PCA-3 and TMPRSS2:ERG also could work as prostate cancer biomarkers (Nilsson et al. 2009)

Biofluid	Disease	Associated proteins	Reference
Plasma	Chronic hepatitis C	CD81	(Welker et al. 2012)
	Melanoma	CD63, caveolin-1, TYRP2, VLA-4,HSP70,HSP90	(Logozzi et al. 2009), (Peinado et al. 2012)
	Glioblastoma	Epidermal growth factor receptor VIII	(Skog et al. 2008)
	Prostate cancer	Survivin	(Khan et al. 2012)
	Plasma cell dyscrasias	c-src	(Di Noto et al. 2013)
Urine	Acute kidney injury	Fetuin-A, ATF 3	(H. Zhou et al. 2006), (Hua Zhou et al. 2008)
	Liver injury	CD26,CD81,S1c3A1, CD10	(Conde-Vancells et al. 2010)
	Barterr syndrome type 1	NKCC2	(Gonzales et al. 2009)
	Bladder cancer	EGF, α subunit of Gs, resistin, retinoic acid-induced protein 3, and so forth.	(Smalley et al. 2008)
	Prostate cancer	PSA, PCA3	(Nilsson et al. 2009)
Plasma, cell culture medium, and ascites	Human ovarian cancer	LICAM, CD24, ADAM10,EMMPRIN, claudin	(Sascha Keller et al. 2009), (Jianghong Li et al. 2009)

Table 5. Summary of exosomal proteins for clinical diagnostic applications (J. Lin et al. 2015)

Some of the examples of uses of exosomes as biomarkers in diseases mentioned and others are exposed in Table 5. All these studies have been previously compared and the information here presented is based on the work done by Jin Lin et al (J. Lin et al. 2015).

Returning to microRNA or exosomal nucleic acids, they have always seen has potential biomarkers, especially miRNA. As it was said previously, exosomes based therapy and its application study is a novel field where there are no global rules or protocols yet. This is observed as there is no exact and common knowledge for every research group about which RNA can be used as biomarker, but there are some evidences that could help the scientific community to establish some ideas to work on.

Exosomal miRNA is protected by RNase-dependent degradation and this makes its detection in plasma and serum stable (Urbanelli et al. 2013). In 2008 it was discovered that eight miRNas previously determined as biomarkers for ovarian cancer were present also in ovarian cancer and serum exosomes isolated from ovarian cancer patients (D. D. Taylor and Gercel-Taylor 2008). MiRNA was also found in an adenocarcinoma study in 2009, where circulating levels of tumor-derived exosomes were compared between adenocarcinoma patients and control subjects. They found similar patterns of miRNA between biopsies and exosomes derived from lung adenocarcinoma patients and it was, as it can be thought, different profile of the control group exosomes analysis (Rabinowits et al. 2009).

Prostate cancer diagnosis, which it has been said to use exosomal proteins as biomarker was demonstrated to also be helped by the analysis or miRNA. In 2008, circulating level miR-141 was reported as a potential diagnostic marker for prostate cancer (Mitchell et al. 2008). This means that exosomes, not only the proteins but its miRNA content can be used as biomarker in some diseases such as prostate cancer. This study was completed by others, where determined that miR-141 can be used as diagnostic biomarker but a tumor progression one. Also determined miR-375 was another biomarker for this type of cancer (Brase et al. 2011).

Exosomes have been evaluated as biomarker in other types of cancer, for example, in esophageal squamous cell cancer (ESCC), were high levels of exosomal miR-21 in serum was reported in 2013 had correlation with tumor progression and aggressiveness. This study also determined that the only source of that miRNA (miR-21) was exosomal miRNA (Tanaka et al. 2013). For this pathology more miRNAs were determined as potential biomarkers, such as miRNA-1246, which show a strong correlation with tumor diagnosis and progression but only if they are found in exosomes, due to the low correlation founded in case of miRNA-1246 from biopsy (Takeshita et al. 2013).

Apart from cancer, exosomal miRNAs have also demonstrated to potentially be biomarkers for cardiovascular diseases (Kuwabara et al. 2011) and renal fibrosis (Lv et al. 2013). As it was done with exosomal proteins, the next table (Table 6) includes all studies related to exosomal RNA (maily miRNA) which show fucntions of biomarker in several diseases.

Biofluid	Disease	Associated proteins	Reference
Plasma	Ovarian cancer	miR-21, miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-205, miR-214	(D. D. Taylor and Gercel-Taylor 2008)
	Lung cancer	miR-17, miR-3p, miR-21, miR-20b, miR-223, miR-301, let-7f	(Rabinowits et al. 2009), (Silva et al. 2011)
	Prostate cancer	miR-141, miR-375	(Mitchell et al. 2008), (Brase et al. 2011)
	Esophageal squamous cell cancer (ESCC)	miR-21, miR-1246	(Tanaka et al. 2013), (Takeshita et al. 2013)
	Breast cancer	miR-21	(Corcoran et al. 2011)
	Cardiovascular disease	miR-1, miR-133a	(J. F. Chen et al. 2006), (Kuwabara et al. 2011)
Cell culture medium	Gastric cancer	Let-7 family miRNAs	(Ohshima et al. 2010)
	Colorectal cancer	mRNAs	(Hong et al. 2009)
Urine	Renal fibrosis	miR-29c, CD2APmRNA	(Lv et al. 2013)(Lv et al. 2014)

Table 6. Summary of exosomal RNAs for clinical diagnostic application(J. Lin et al. 2015)

To conclude this section, where function as biomarkers of exosomas is evaluated it must be said that serum and urine are not the only fluids were exosomes can be found. Exosomes can be found in others biofluids and collected to be analyzed. This is an advantage to biomarker function due to the existence of easy ways of collecting exosomes for their isolation, characterization and analysis. The first biofluid that was observed to contain exosomes is saliva, and exosomes founded could be used as biomarkers (Palanisamy et al. 2010). Other biofluids that contains exosomes are amniotic fluid (S. Keller et al. 2007), which contains fetal exosomes and placenta (Gilad et al. 2008) were can be detected miR-526a, miR-527, miR-515-5p and miR-521.

3.3 Examples of regenerative therapy with exosomes

In this section there are explained two examples of applications of exosomes in concrete diseases such are TBI or lung cancer, but first, there are presented in Table 7 a list of several applications of exosomes in different phatologies

Applications	Sample source	Conclusions	References
Lung cancer	Human	Exosomes may serve as minimally invasive diagnostic applications.	(Cazzoli et al. 2013)
Cancer-directed immune response	Rat	Exosomes may distinctly affect the immune system	(Zech et al. 2012)
Preeclampsia	Human	Microvesicles can modulate immune cell responsiveness at different times of pregnancy and in preeclampsia.	(Holder et al. 2012)
Graft-versus-host Disease (GvHD)	Human	Mesenchymal stem cells-exosome therapy improved clinical GvHD symptoms significantly.	(Kordelas et al. 2014)
Type-1 diabetes mellitus (T1DM)	Mice	Exosomes exert ameliorative effects on autoimmune T1DM	(Nojehdehi et al. 2018)
Colorectal cancer	Human	Exosomes derived from hypoxic colorectal cancer enhance prometastatic behaviors and may provide new	(Z. Huang et al. 2018)
Cardiac ischemia-reperfusion injury	Mice	Exercise-derived extracellular vesicles might serve as a potent therapy for myocardial injury in the future.	(Bei et al. 2017)
Hepatocellular carcinoma	Human	Exosomal transfer of siGRP78 can suppress Sorafenib resistance in hepatocellular carcinoma.	(H. Li et al. 2018)
Steroid-induced femoral head necrosis (SFHN)	Rat	Exosomes affect SFHN osteogenesis and may develop a novel therapeutic agent for SFHN.	(Fang, Li, and Chen 2019)
Parkinson's disease	Mouse	Exosomes loaded with catalase produce a neuroprotective effect.	(Haney et al. 2015)
Autoimmune encephalomyelitis	Rat	Exosomes may be a promising cell-free therapy for multiple sclerosis	(Zijian Li, Liu, He, et al. 2019)
Central nervous system (CNS) trauma	Human	Exosomes can deliver siRNA into the CNS to decrease inflammasome activation.	(De Rivero Vaccari et al. 2016)
Traumatic brain injury (TBI)	Rat	Exosomes effectively improve functional recovery in rats after TBI.	(Y. Zhang et al. 2015)
Stroke	Rat	Exosomes can be employed for stroke treatment.	(Xin et al. 2013)

Table 7. Applications of exosomes in regenerative therapy

Exosomes can improve TBI

As it was previously described, TBI is an acute condition in the brain that can lead to disability or death. Taking advantage of communication cell to cell property of exosomes, there are applications in TBI were exosomes can help to improve results. Investigations suggest exosomes improve cerebral ischemic reperfusion injury by promoting autophagy and regulating apoptotic factors (Yuan et al. 2019) and also oligodendrocyte-derived exosomes have a neuroprotectiveness involvement during cell stress conditions. Some studies about applications of exosomes in TBI indicate the possibility of their use as a diagnosis and treatment tool. These studies are summarized in the next table (Table 8).

Source of exosome	Species	Results	References
Bone mesenchymal stem cell	Mouse	BMSCs-exosomes reduced the lesion size and improved the neurobehavioral performance and inhibited the expression of BAX, TNF-α, IL-1β.	(H. Ni et al. 2019)
Neuron	Mouse	Exosomal miR-21-5p produced a protective effect by suppressing autophagy in a TBI model.	(D. Li, Huang, Zhu, et al. 2019)
Microglia	Rat	Exo-miR-124 treatment promoted M2 polarization of microglia and improved hippocampal neurogenesis and functional recovery after brain injury.	(Y. Yang et al. 2019)
Microglia	Mouse	Exosomal miR-124-3p exerted a protective effect by inhibiting neuronal autophagy in scratch-injured neurons.	(D. Li, Huang, Yin, et al. 2019)
Adipose-derived stem cells	Rat	Significant recovery of function onmotor behavior as well as a reduction in cortical brain injury was observed in TBI after treatment with exosomes.	(Patel et al. 2018)
Brain tissue	Mouse	TBI derived exosomes manifested toxicity in primary neuronal cultures.	(B. Wang and Han 2018)
Microglia	Mouse	Microglial exosomal miR-124-3p inhibited neuronal inflammation in scratch-injured neurons.	(S. Huang et al. 2018)
Odontogenic stem cell	Rat	Human exfoliated deciduous teeth-originated exosomes can reduce neuroinflammation by altering microglia polarization.	(Ye Li et al. 2017)
Traumatic brain tissues	Mouse	155 circRNA are upregulated and 76 are downregulated.	(Zhao et al. 2018)
Plasma	Human	TBI patients exhibit differential protein expression in their exosomes.	(Moyron et al. 2017)
Multipotent mesenchymal stromal cells	Rat	Exosome treatment significantly increased the number of newborn endothelial cells in the lesion boundary zone and dentate gyrus, and significantly increased the number of newborn immature and mature neurons in the dentate gyrus as well as reduced neuroinflammation.	(Y. Zhang et al. 2015)

Table 8. Summarized studies appertain to the beneficial effects of exosomes in TBI (Yuan et al. 2019)

The work of Jiaying Yuan et al. (Yuan et al. 2019) compares and analyses knowledge about exosomes in TBI and studies which has been developed in this area. They expose that quantity of microvesicles and exosomes in TBI is higher than in normal circumstances. CSF-derived

exosomes in TBI were characterized and presented a diameter of 74-98 nm (Manek et al. 2018). Some exosomal proteins and RNAs showed high levels, so this indicates they can be potentially used as biomarkers in TBI (Agoston, Shutes-David, and Peskind 2017).

Exosomes modified with specific molecules and proteins were demonstrated to be helpful in the assistance of transportation stem cells to target injured areas crossing BBB and thank to their ability of low immunogenicity, therapeutic applications can reduce some side effects associated to current TBI treatments (Yuan et al. 2019).

Exosomes as liquid biopsy for lung cancer

There is a new concept which is called “liquid biopsies” which consist on collection body fluids such as blood, urine or saliva and analyzing it searching clinical information with high meaning as there is found in conventional biopsies. There are four major approaches in liquid biopsies: evaluating circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), tumor-educated platelet (TEP) and extracellular vesicles (EVs) (Cui et al. 2018).

There are some studies were mechanism of exosome upregulation in lung cancer was evaluated. The evidences founded were that the p53 pathway was involved in exosome secretion into the medium (X. Yu, Harris, and Levine 2006), SNARE family protein was a fundamental molecule involved in regulating exosomes release in lung cancer cells (Ruiz-Martinez et al. 2016) and Rab27A was reported to have some kind of relation with malign exosome secretion from lung adenocarcinoma cells (W. Li et al. 2014).

Exosomal cargo has potential as biomarker also for lung cancer, especially miRNA, for example miR-21 has relevance on angiogenesis and malignant information of the tumor (Y. Liu et al. 2016). Others as miR-197-5p or miR-4443 offers information about cisplatin (DDP) resistance (X. Qin et al. 2017) and other exosomal RNA and proteins show its relevance in radiation dose-related (Dinh et al. 2016), proliferation (Clark et al. 2016) or migration (Y. Wang et al. 2016).

Other application in development in lung cancer therapy is using exosomes as DDS. In vitro studies demonstrate anti-tumor effect of exosomes loaded with celastrol. This effect is higher than previously uses of free celastrol (Aqil et al. 2016). There are three advantages of using exosomes as DDS (Cui et al. 2018), as previously was mentioned: 1) exosomes can target specific cells and serve as cell to cell communication vehicle, 2) allows cargo reach intact and without degradation to delivery point thank to lipid bilayer and 3) exosomes are minimally immunogenic and toxic.

There have been some approaches in lung cancer with this use of exosomes and particularly exosome-based DDS showed anti-cancer activity against H1299 and A549 lung cancer cells (A. Srivastava et al. 2016).

As it was previously reported in those studies, exosomes have a great potential in lung cancer therapy not only as biomarker but as DDS too. All studies encourage developing new studies of lung cancer researching the use of exosomes and their potential uses in this disease.

3.4 Clinical trials

With the advance of researching in exosomes and their uses, there are appearing first clinical trials based in exosomes therapy and here some of them are synthetized. Clinical trials are very important in medical investigation due to their acknowledgment in the medical community as they are considered gold standard of application of new therapy based in new instrument, new protocols or new drugs. For this importance, clinical trials are a great step in exosomal investigation because success of clinical trials will determine the introduction of these therapies into clinic.

Most clinical trials do not report any documentation until some phase is finished or some important goal is achieved. For this reason, only few clinical trials are available to expose here.

The first clinical trial (Escudier et al. 2005) is a study of feasibility and safety of using dendritic cell derived-exosome-based in search of vaccination in melanoma patients. Exosomes from 15 patients were purified from day 7 autologous monocytes derived-DC cultures. Patients were enrolled from 2000 to 2002 and received four vaccinations. Evaluations were performed 2 weeks after de immunization, apart from de previous evaluation. 4 cases showed non progression and they continued with treatment. Large scale exosome production was achieved and also feasibility and safety. There were registered some minor responses, other stable and mixed responses. The goals of Phase I were achieve, due to not having grade II of toxicity but there is a need of improve for next phases.

Another 2 phase I clinical trials were available to watch its results. In the same line of developing cancer immunotherapy strategies, a clinical trial (Morse et al. 2005) is focused on using dendritic cell-derived exosomes (DEX) loaded with the MAGE tumor antigens in patients with non-small lung cancer (NSCLC). This study is also in phase I, which means primary objective is achieving feasibility and low toxicity using DEX vaccine. A total of 13 patients were enrolled but only 9 completed therapies in which 3 formulations of DEX were evaluated and they were well tolerated with adverse events of grade 1-2. Some immune responses were detected and some patients experienced long term stability of disease and activation of immune effectors.

The last clinical trial here exposed is a study (Dai et al. 2008) of using ascites-derived exosomes (AEX) in combination with the granulocyte-macrophage colony-stimulating factor (GM-CSF) in the immunotherapy of colorectal cancer. In this study a higher N of patients was achieved, enrolling 40 patients in two groups, one AEX and another with AEX plus GM-CSF. Both therapies resulted safe and tolerated but only AEX plus GM-CSF can induce beneficial tumor-specific antitumor cytotoxic T lymphocyte (CTL) response. As phase I study was successful in achieving feasibility and safety and demonstrated that use of exosomes in immunotherapy and an alternative choice in advance CRC.

All 3 clinical trials were in phase I and there are more but documentation was not available. However, they can be listed to observe that clinical trials with exosomes are developing and exosomes-based therapy is getting more importance and impact. In the Table 9 some clinical trials with exosomes therapy are listed.

Exosome resource	Application	Disease	Phase	Region	N	Ref /NCT NO.
DCs pulsed with antigenic peptides	Antitumor vaccine	Metastatic melanoma	I	France	15	(Escudier et al. 2005)
DCs pulsed with antigen peptides	Antitumor vaccine	Non-small lung cancer	I	United States	13	(Morse et al. 2005)
Acscites fluid	Antitumor vaccine	Colon cancer	I	China	40	(Dai et al. 2008)
IFN-γ-DCs pulsed with peptides	Antitumor vaccine	Non-small lung cancer	II	France	22	NCT01159288
Allogenic MSCs	Therapeutic to reduce inflammation	Diabetes mellitus	II-III	Egypt	20	NCT02138331
MSCs	Therapeutics to promote healing	Macular holes	I	China	44	NCT03437759
Autologous plasma	Therapeutics to promote healing	Ulcer	I	Japan	5	NCT02565264
Plant	Therapeutics to reduce inflammation	Polycystic Ovary	Not available	United States	176	NCT03493984
Plant	Therapeutics to reduce inflammation	Oral mucositis	I	United States	60	NCT01668849
Plant	For delivery of curcumin	Colon cancer	I	United States	7	NCT01294072
Tumor cells	For delivery of chemotherapeutics	Malignant pleural effusion	II	China	30	NCT01854866
Allogenic MSCs	For delivery of miR-124	Acute ischemic stroke	I-II	Iran	5	NCT03384433
MSCs	For delivery of KRAS (G12D) siRNA	Metastasic pancreatic cancer	I	United States	28	NCT03608631

Table 9. Clinical trials of exosome-based therapeutics and delivery nanoplatforms (Lu and Huang 2020).

4. Regenerative research based on exosomes used with Biomaterials for CNS repair

4.1 Bioscaffold with exosomes

Scaffolds are biocompatible polymers with microarquitecture designed to allow cellular adhesion, growth and proliferation. They are used in regenerative medicine in combination with cells to promote tissue regeneration. When scaffold, or bioscaffold, are made of absorbable materials, during healing process appears a degradation of the scaffold avoiding extracting it. Their structure is porous and they must have enough stiffness to mimic tissue that it is going to be repaired. By this, a scaffold should induce a positive interaction with the surrounding tissues.

Bioscaffold is a promising therapy in tissue and organ regeneration which prevent scar formation in severe injuries, as it was reported in nerve regeneration, where scaffold inhibited glia scar formation (Yuan et al. 2019). Different materials had been used and tested in many diseases. In TBI there have been scaffolds made of Polydimethylsiloxane (PDMS) (C. Chen et al. 2019), collagen-chitosan (Yan et al. 2019), silk fibroin (Moisenovich et al. 2019), extracellular matrix (Y. Wu et al. 2017), sodium hyaluronate collagen (Duan et al. 2016), and polycaprolactone (PCL) and PCL tricalcium phosphate (TCP) (Choy et al. 2013); for spinal cord injury scaffolds are made of poly (propylene furanate) polymer with collagen (B. K. Chen et al. 2018) and PDGF-MS-containing tubular scaffold (X. Chen et al. 2018) among others and some of them demonstrate that the use of human components such as marrow-derived mesenchymal stem cells (BMSCs) (Arulmoli et al. 2016).

As it can be seen, there are so many ways of forming polymer scaffolds and there is no a main option for most of the cases. The material used will depend of the tissue where the scaffold is planned to be implanted, its application and other properties involved related with the scaffold stability and tissue mimic. So there are some important data from biomaterials used in scaffold fabrication such as mechanical properties of biomaterials compared to human tissues, for example bone strength; and for this reason in Table 10 it is shown some of these properties of biomaterials.

While there is a consensus on the limited ability of CNS to regenerate, combination of sodium hyaluronate collagen scaffold and stem cells are able to deliver growth factors while also improving environment for the survival of stem cells and facilitate its regenerative function (Duan et al. 2016). BMSCs are gaining in research, especially in its use in transplantation in CNS injury situations and teams are investigating on promotion of axonal growth, improving angiogenesis and neuroprotective functions under stress situations (Assinck et al. 2017; Tetzlaff et al. 2011).

As it was shown, many biomaterials have been tested in order to promote regeneration with bioscaffolds, with or without stem cells, side effects appear and the most important in

scaffold-induced treatment is inflammation. Some chemicals and synthetic materials could potentially stimulate this reaction mediated by lysosomal damage (C. Chen et al. 2019).

Substance	Elastic modulus (GPa)	Strength (MPa)	Elongation (%)	References
Cortical bone (human femur, tested dry in compression)	14.7-19.7	167-215		(Reilly, Burstein, and Frankel 1974)
Cancellous bone (human distal femur, tested dry in compression)	0.298±0.224	5.6±3.8		(Kuhn et al. 1989)
Bioglass®	35	42		(Hench 1998)
HA	95	50		(Hench 1998)
PDLLA	1.4-2.8	27.6-41.4	3-10	(S. Yang et al. 2001)
PLGA	1.4-2.08	41.4-55.2	3-10	(S. Yang et al. 2001)
PLLA	2.4-4.2	55.2-82.7	5-10	(S. Yang et al. 2001)
PGA	>6.9	>68.9	15-20	(S. Yang et al. 2001)
PCL	0.21-0.34	20.7-34.5	300-500	(S. Yang et al. 2001)

Table 10. Properties of biomaterials used for scaffods design in tissue engineering.

In one study, one of the diseases mentioned before, TBI was treated in rats with multipotent mesenchymal stromal cell-derived exosomes. In this study were registrated an increase in newborn neurons and endothelial cells in the lesion site of the rats. Exosomes are thought to have abilities in modulation of immune answers to prevent TBI damage (Y. Zhang et al. 2015). Administration of exosomes to TBI rats enhace motor functions and inhibited inflammatory-related pathways (Patel et al. 2018). This gain more plausibility when appears a study (Ye Li et al. 2017) where exfoliated deciduous teeth stem cells-derives exosomes altered microglia polarization and reduce neuroinflammation after TBI. According to J.Yuan et al. (Yuan et al. 2019) “Mi-RNA in exosomes was involved in neuroinflammation after TBI. MiR-21 and miR-124 enriched in exosomes could reduce M1 (proinflammatory phenotype) polarization and promote M2 (anti-inflammatory phenotype) polarization”. In the case of BMSCs also have neuroinflammation inhibition properties thank to the ability of reducing TNF-α and IL-1β and they also modulate microglia polarization (H. Ni et al. 2019). Exosomal miR-124-3p and miR-21-5p from microglia and neuron suppress autophagy and that produce neuroprotective effects (D. Li, Huang, Yin, et al. 2019).

This contribution suggests exosomes can also modulate inflammatory in combination with scaffolds and solve the inflammation problem of bioscaffold treatment and they can be used in treatment not only for this reason but also their good ability of delivery drugs on target cells and lower immunogenicity than other external biomaterials (Tian et al. 2014).

The use of exosomes in combination with bioscaffolds and stem cells seem to improve diseases such as TBI. In this case is observed how exosomes can cross BBB and target lesion site and promote regeneration due to be MSCs-derived exosomes (Yuan et al. 2019). Other studies showed increased solubility, stability and bioavailability of curcumin (Sun et al. 2010).

One of the promising experimental uses of scaffolds is bone regeneration, where scaffold interact with bone tissues by acting as a support for mineralizing cells, ensuring an adequate environment for growth factors and nutrients, and being able to stimulate a proper communication among the resident mineralizing cells (Langer and Tirrell 2004).

In order to repair critical-sized calvarial bone defects another research was carried out with a combination of exosomes obtained from human-induced pluripotent stem cell-derived mesenchyme stem cells (hiPS-MSC-Exos) with tricalcium phosphate (β-TCP). This scaffold was tested in vitro and it was evaluated its effect on migration, proliferation and osteogenic differentiation of human bone marrow-derived mesenchimal stem cells. Exosome/ β-TCP combination scaffolds were implanted into defect areas of rats. New formed bone appeared at 8 weeks post-implantation. This new formation was not observed in pure β-TCP scaffolds and that means addition of exosomes into β-TCP scaffods could enhance osteogenesis. Besides, gene expression prolifing and bioinformatics analysis demonstrated that the new combination scaffold significantly altered the expression of a PI3K/Akt signaling pathway involved network of genes. This signaling pathway was the key mediator during the exosome-induced osteogenic process of response of hBMSCs. These results confirmed the possibility of getting better results combinating exosomes with scaffolds and suggest the potential of exosome/ β-TCP scaffold in bone repairing (Jieyuan Zhang et al. 2016).

Spinal cord injury (SCI) is a severe neurological trauma with high morbidity and mortality, and also have been some researches in using bioscaffolds in its treatment (X. Wang et al. 2019). Combination of growth factors and biomaterials demonstrated affective SCI repair by decreasing lesion cavity, promoting vascular formation and increasing neural attachment and axonal outgrowth (Grulova et al. 2015). Furthermore, not only scaffolds are being testes in SCI but the combination with exosomes too. Beneficial factors of EVs like attenuation of apoptosis, inflammation and promotion of angiogenesis gives EVs a potential role in their use in scaffolds for SCI treatment (J. H. Huang et al. 2017). EVs derived from differentiated PC12 cells and MSCs also showed protective effects by inhibiting the expression of phosphatase and tensin homolog (G. Xu et al. 2019). Due to the inflammatory pathways are characteristic in SCI pathogenesis, EVs, which show inhibition of these processes could improve scaffold treatment in SCI as it is show in Figure 7.

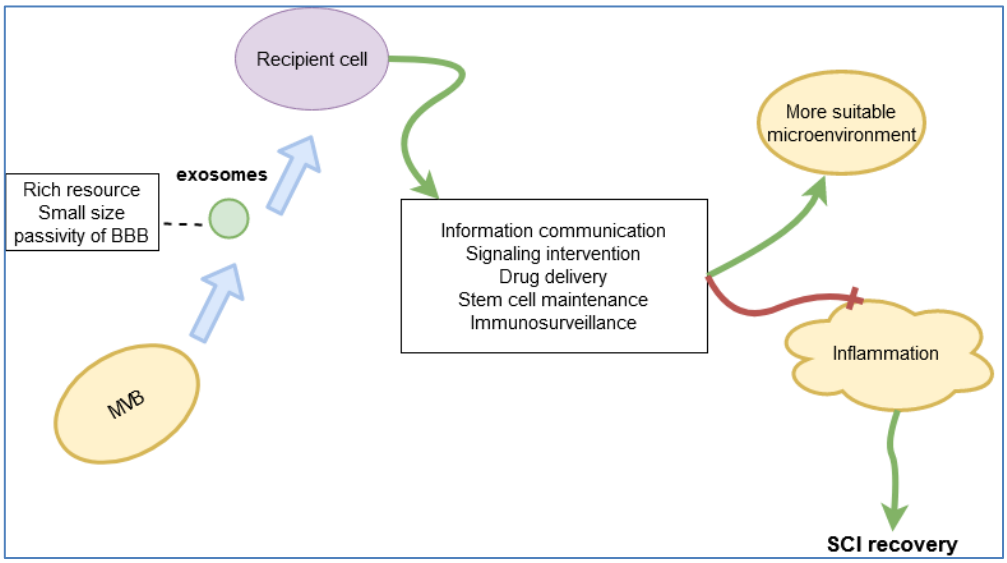

Figure 7. Prospective role of extracellular vesicles (EVs) combined with bioscaffolds in stem cell therapy for SCI.

Coming back to bone regeneration, recently a study about scaffold enriched with exosomes has developed a PLA-based scaffold that was able to uptake and release exosomes and enhance the ostegenic commitment of human adipose-derived mesenchymal stem cells. The innovation in this study is the proposition of building the combination of scaffolds and exosomes in 3D printing, a novel technique and with the success of this scaffold combination of exosomes and scaffolds is still recommended by authors of these researches (Gandolfi et al. 2020), where in some of them are explained strategies of combining these two elements to generate the desired effect in tissues. (Figure 8).

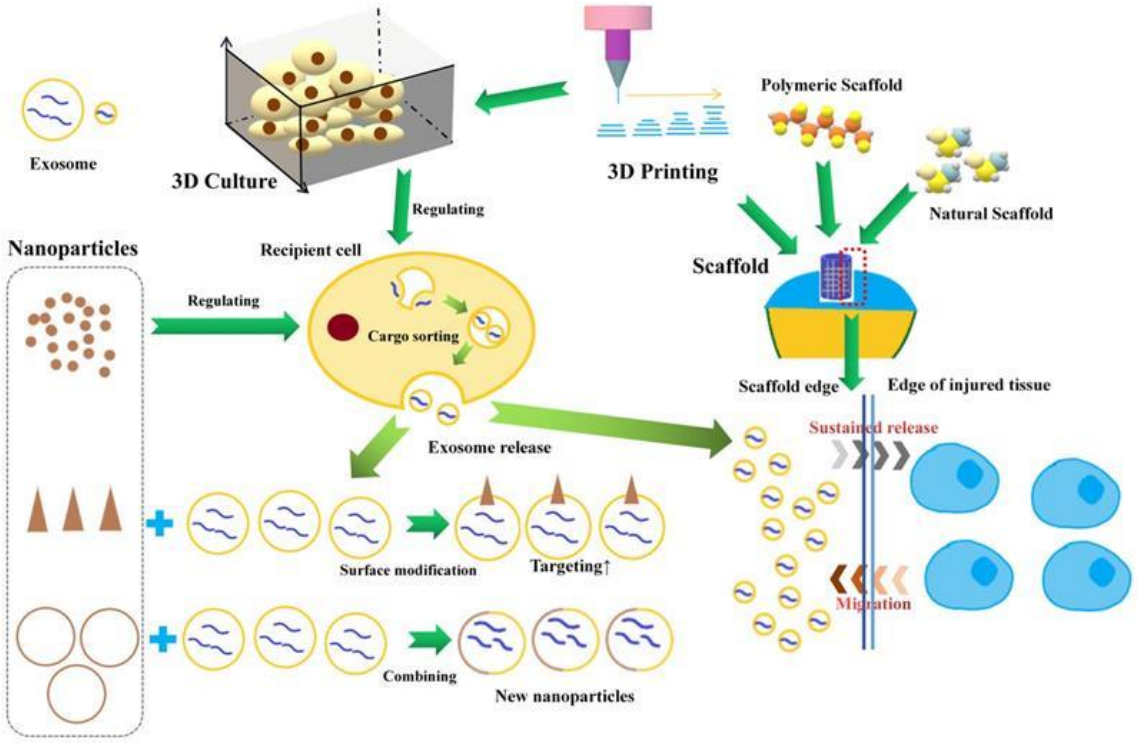

Figure 8. Process of addition exosomes into a 3D printed bioscaffold (Q. Zhou et al. 2020)

4.2 Combination of hydrogels with exosomes

Hydrogels have been recurrently used to create drug delivery systems in many applications searching concrete and desirable therapeutic effects (Caló and Khutoryanskiy 2015). They are hydrophilic polymer networks along the three dimensions after being crosslinked and they form matrices with high water content. Some of the most common polymers used in hydrogels development are hyaluronic acid, gelatin, collagen, chitosan or alginate, which are from natural origin, but there is also common the use of synthetic material such a, poly(lactic-co-glycolic acid) (PLGA), poly(ethylene glycol) (PEG) or poly(hydroxyethyl methacrylate (pHEMA)), and in some cases it is used combination of both (Peppas et al. 2006).

Hydrogels are known for having tunable physical properties that can take advantage of customization of some properties such as the degradation rate of the matrices to release, in this case, exosomes. The use of hydrogels in DDS of exosomes achieves with low amount of exosomes the same therapeutic effect and it is sustained over a period of time depending of the degradation rate of the hydrogel (B. Liu et al. 2018). Using exosomes instead of cells has some advantages: avoid transfer of cells, which can cause immunogenic reactions or damage or mutate DNA (Riau et al. 2019); small size of exosomes allows them to circulate through any organ and also cross BBB (Verweij et al. 2019) and at last, their natural origin from the body imitates cell surface, which has biochemical properties and functions like avoiding phagocytosis, fuse membranes and also bypass lysosomal engulfment (J. Xu, Camfield, and Gorski 2018) apart from the homing properties due to membrane proteins and lipids.

All characteristics from hydrogels mentioned before make hydrogels excellent candidates to encapsulate exosomes in DDS applications. There are three main ways of exosomes encapsulation into the hydrogel matrix (Riau et al. 2019) as they can be seen in Figure 9.

The first method consists on incorporate exosomes into the polymer mixture followed by the crosslikers to shift the composite into gel (Riau et al. 2019). An example of this method is the hydrogel created by Qin and coworkers. This hydrogel was made of a composite substrate made of thiolated heparin, thiolated hyaluronic acid and gelatin. First, BMSC-derived exosomes were incorporated and after that the thiolated polymers that were mixed with exosomes were crosslinked with the admixture of poly(ethylene glycol) diacrylate, known as (PEGDA) (Y. Qin et al. 2016).

The second method is characterized by the addition of exosomes after the polymerization of hydrogel. This method is sometimes known as “breathing” method. According to Riau et al (Riau et al. 2019), “The breathing typically consists of placing the swollen hydrogel into a solvent to remove the entrapped water, followed by soaking the hydrogel in an aqueous solution containing the exosomes that causes the hydrogel to swell and breath in the exosomes.” For the success of this technique pores in hydrogel need to have a greater size than exosomes and for this chitosan/silk fibroin can be used in the hydrogel sponge to encapsulate for example, platelet-rich plasma exosomes (N. Xu et al. 2018). This method is less used in clinic due to the early polymerization of the hydrogel, however it is an easy way to test in vitro.

The third and last method is the most advance, and suggests the incorporation of exosomes at the same time that polymerization is happening. In this method crosslinker and exosomes solution are simultaneously added using dual chamber syringe (Riau et al. 2019). An example of this technique was carried out by Wang and colleagues with a mixture of oxidative hyaluronic acid, polypeptide hydrogel, poly-Ɛ-L-lysine and adipose-derived mesenchymal stem cells (C. Wang et al. 2019).

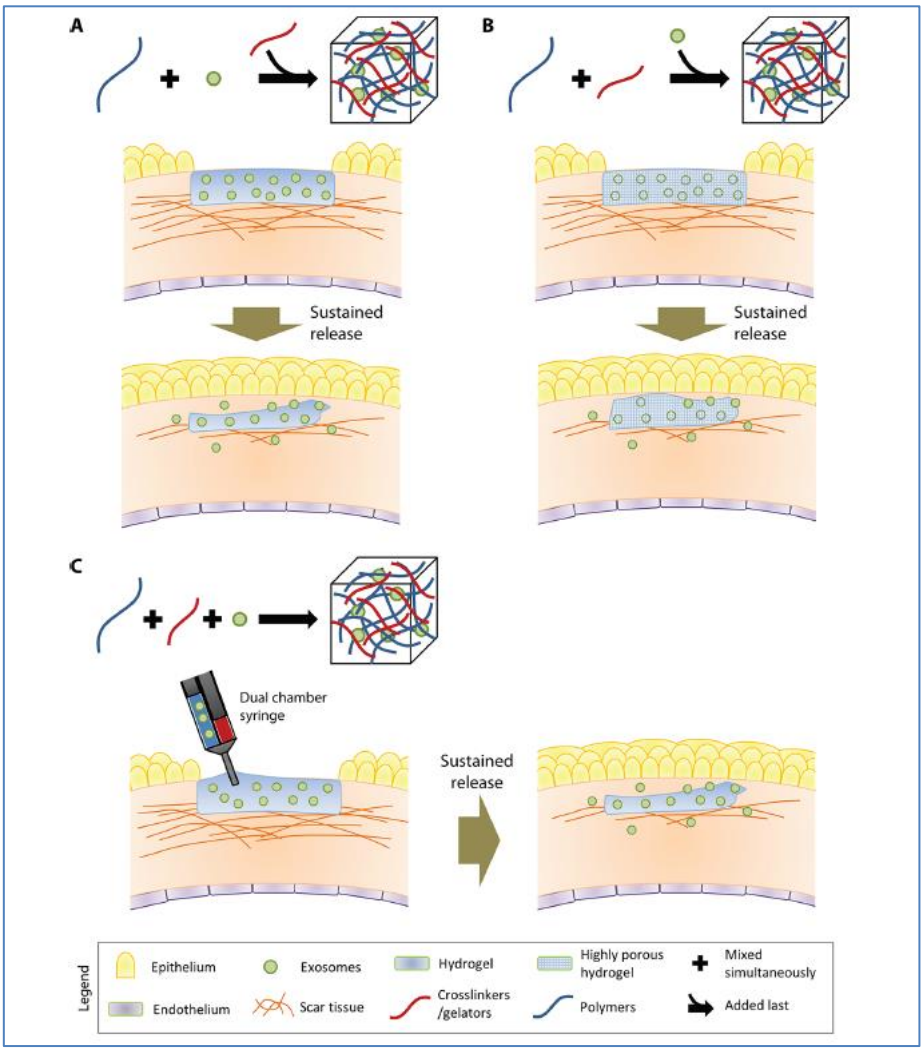

Figure 9. Three different methods of loading exosomes into hydrogels. A)Mix exosomes and then polymerization, B)polymerization and then adding exosomes and C)adding crosslinkers and exosomes simultaneously (Riau et al. 2019)

In Table 11 there are some studies listed where hydrogels from different materials were combined with exosomes encapsulating them in different applications.

The use of hydrogel in combination with exosomes is a huge advance in DDS therapy. It allows by a simple injection a fast circulation of exosomes that would make a backup of them in the lung, liver, spleen, or gastrointestinal tract, where exosomes can be found just 2 hours after injection (Takahashi et al. 2013). Furthermore, exosomes half-life could be shorter due to the speed of the fluid and the exposure to external enviroment. Hydrogels can avoid expending loaded exosomes too soon and they allow a concentrated and time maintained delivery of

content of exosomes in the target site and it can be achieve these effects with a reduced amount of exosomes compared to a DDS with only exosomes treatment. Before, it was told that a main problem of exosomes therapy is low ratio and efficiency of exosomes isolation and purification. With this advantage, exosomes could be used in lower quantities and the production of them could then not to be as higher as before (Riau et al. 2019).

Materials	Cell source of exosomes	Duration of release	Clinical application	Reference
Adamantane and β-cyclodextrin-modified hyaluronic acid hydrogel	Bone marrow-derived endothelial progenitor cells	21 days	Cardiac regeneration in infarcted heart	(C. W. Chen et al. 2018)
Alginate hydrogel	Blood plasma	4 days	Skin regeneration in chronic diabetic wound	(Guo et al. 2017)
Collagen type I Gelfoam® sponge	Cardiomyocyte-derived IPSCs	21 days	Cardiac regeneration in infarcted heart	(B. Liu et al. 2018)
Chitosan hydrogel	miR-125-3p-overexpressing synovium MSCs	6 days	Skin regeneration in chronic diabetic wound	(Tao et al. 2017)
Chitosan hydrogel	Placenta MSCs	Not reported	Angiogenesis promotion in ischemic tissue	(K. Zhang et al. 2018)
Chitosan/silk fibroin sponge	Blood plasma	Not reported	Skin regeneration in chronic diabetic wound	(N. Xu et al. 2018)
HyStem®-HP hydrogel	BMSCs	Not reported	Bone regeneration	(Y. Qin et al. 2016)
pH-responsive polypeptide (Pluronic F127, oxidative hyaluronic acid and poly-ε-L-lysine) hydrogel	Adipose MSCs	21 days	Skin regeneration in chronic diabetic wound	(C. Wang et al. 2019)
Self-assembled peptide amphiphile (C16-GTAGLIGQ-GG-GHRPS) hydrogel	Umbilical cord MSCs	21 days	Self-assembled peptide amphiphile (C16-GTAGLIGQ-GG-GHRPS) hydrogel	(Han et al. 2019)
Chitosan/Silk hydrogel	GMSCs	14 days	Acceleration of wound healing in a diabetic rat skin defect model	(Shi et al. 2017)
Polypeptide-based FHE hydrogel (F127/OHA-EPL)	AMSCs-exo	21 days	Promoting chronic diabetic wound healing and complete skin regeneration	(C. Wang et al. 2019)
photoinduced imine crosslinking hydrogel glue (EHG)	Human induced pluripotent stem cell line iPSS-01	>14 days	Tissue patch for articular cartilage regeneration	(X. Liu et al. 2017)

Table 11. List of some of the studies that combined hydrogels with exosomes

However, encapsulation technique for exosomes is a knowledge field that is still in its early stage but there are appearing more challenges while research is advancing. These challenges include toxicity of residual crosslinkers needed for polymerization of hydrogels premature gelation during injection (there is need of improve of gelling temperature, polymer

concentration and applicator system) or kinetic release profiles in vivo (which often cannot be translated from in vitro profiles). All characteristics, included highly tunable degradability and shape of hydrogels allow customizable application of an exosomes DDS. For all these reasons, and due to their high potential for specific therapy for patients, there are good estimations in the commercialization of exosome-loaded hydrogel products (Riau et al. 2019).

4.3 Artificial exosomes

As it was said before, therapeutic applications of exosomes are under clinical trials and there is no legal treatment based on exosome yet. The reason of this circumstance is not only due to the early step where all researches in this field are, but the lack of scalable production at clinical grade, lack of good normative in characterization which ensures reproducibility while keeping safety, the higher costs for that effectiveness, no standard has been redacted yet and absence of efficient cargo loading strategies (Lu and Huang 2020). However, these promising researches cannot be discarded due to the potential role of exosomes in therapy, in liquid biopsy, in DDS (especially for gene therapy) and in regenetaive medicine in general. For this reason, some studies (Johnsen et al. 2014) developed what they called 'exosome-based semisynthetic nanovesicles (NVs)', which is a kind of exosomes which are exosomes with specific modifications for achieving certain goals, but the same drawbacks are still present: production and isolation. The next step is design and creation of complete synthetic exosome-mimetic bodies using bionanotechnology (García-Manrique, Gutiérrez, and Blanco-López 2018).

According to two trends in nanofabrication, there are two main approaches of developing fully synthetic exosomes: Top-Down and Bottom-up methodologies.

Top-Down methodologies are based on obtaining smaller nanomaterials by disassemble bigger units with higher complexity into smaller ones. One way to start this process is culturing cells for the later use of its membranes to form vesicles (García-Manrique, Gutiérrez, and Blanco-López 2018). Two main strategies in this method are extrusion over polycarbonate membrane filters and specific microfluidic device. The first strategy has the property of reducing average size and reduces the size difference of colloidal systems, making them more homogeneous. Their main applications has been tumor treatments by the encapsulation of some chemotherapeutics (Jang et al. 2013). In the other hand, microfluidics, which consists on pressurization over a device with parallel hydrophilic microchannels, results in a device which can cut cells (alive) at the time they are crossing the microchannels. To enhance the encapsulation, some exogenous material can be added in the cell suspension

Both methods are able to mass production, have target properties which allow true cell interactions thank to expression of receptors and co-receptors with high tuneability and natural immunotoleration. However, these methodologies have some weaknesses. There is no selectivity in the cargo process due to tha passivity of this process, which cargo let membrane fragments encapsulate them without forcing the reactions. This problem is followed by another, which is the need of purification steps by trained personnel and makes these methodologies time-consuming (García-Manrique, Gutiérrez, and Blanco-López 2018). Diferent approaches are listed in Table 12.

Generation approaches	Cells origin	Application	Ref
Manual extrusion over polycarbonate membrane filters with a device for liposome preparation	Human monocytes (U937) and murine mouse macrophages (Raw 264.7) cell lines	Targeted delivery of chemotherapeutics to an in vitro model (TNFα-treated HUVECs) and in vivo-induced malignant tumors (CT26 mouse colon adenocarcinoma cells)	(Jang et al. 2013)
Manual extrusion over polycarbonate membrane filters with a device for liposome preparation	Murine mouse macrophages (Raw 264.7) cell line	In vivo biodistribution ofexosomesand artificial counterparts	(Hwang et al. 2015)
Centrifugal-induced extrusion over membrane filters in a device designed to be used in lab centrifuges	Murine mouse embryonic stem cell line (D3)	Gene delivery to NIH-3T3 fibroblast cells / Enhanced in vitro cell proliferation for regenerative medicine (murine skin fibroblasts)	(Jeong et al. 2014; W. Jo et al. 2014)
Pressurization over hydrophilic microchannels array on a microfluidic device	Murine mouse embryonic stem cell line (D3)	Gene delivery to NIH-3T3 fibroblast cells	(Wonju Jo et al. 2014)
Living cells sliced with silicon nitride blades in a microfluidic device	Murine mouse embryonic stem cell line (D3)	Material delivery to mouse embryonic fibroblasts	(Yoon et al. 2015)
Serial extrusion	Monocytes (U937)	Proof of functional delivery of siRNA	(Lunavat et al. 2016)
Serial extrusion	MSCs	Targeted delivery of paclitaxel	(Kalimuthu et al. 2018)
Serial extrusion	Murine pancreatic β cell line (MIN6)	Therapeutics to facilitate the differentiation of bone marrow cells to insulin-producing cells	(Oh et al. 2015)
Serial extrusion	Natural killer cells	Immunotherapeutic agent for cancer therapy	(L. Zhu et al. 2018)
Alkaline treatment followed by sonication and ultracentrifugation	Monocytes (U937)	Delivery of dexamethasone to mitigate inflamation	(Go et al. 2019)

Table 12. Compilation of approaches of developing artificial exosomes by top-down techniques

Bottom-Up techniques, by contrast, consist on forming units with higher complexity using simple molecular compounds as creation unit, focusing on their chemical and physical features. This appears to be a first step in designing and manufacturing unnatural exosomes. Block assembling allows select membrane proteins as well as modifications in chemical groups with covalent bonds due to the huge capacity of modification and tuneable composition. One method which is often used is hydration over a film, which consists in a process of two steps. First, all compounds are placed over a thin film and then, the film is hydrated by an aqueos medium and this produce the encapsulation (García-Manrique, Gutiérrez, and Blanco-López 2018). This process had success in producing artificial exosomes with a classical liposome formulation (De La Peña et al. 2009). Using chemical bioconjugation procedures and seeking

for interaction with T cell receptors; some proteins, peptides and ligands have been attached to surface of artificial exosomes (Martinez-Lostao et al. 2010). Some ligands reported to show apoptotic behaviour and decreased activation of T cells in autoimmune diseases when they were incorpored into de composition of artificial vesicle (Martinez-Lostao et al. 2010). More recently, a microemulsification method has showed capacity of building artificial exosomes from small compounds. Bovine serum albumins were encapsulated in micelles and worked as artificial exosomes simulating antigen presentation to dendritic cells (Kexin Li et al. 2015).

The main advantages of Bottom-Up techniques are the production of high pharmaceutical grade product which its final composition is fixed by selected formulation, high adaptability for many applications and offers an ideal platform for basic studies of independent elements. However, this is the most complicated method to try as a scaled-up method, due to the cost of time and resources of manufacturing each vesicle. Furthermore, the more surface elements required the more time and resources it would consume and become in a harder challenge (García-Manrique, Gutiérrez, and Blanco-López 2018). There is also a list of approaches of bottom-up techniques in Table 13.

Finally, there are some ways of combining both techniques in a hybrid bottom-up and top-down techniques to increase likeness level of artificial exosomes. Hybrid exosomes contain both manufactured elements and biological compounds derived from natural exosomes and the result share advantages of both techniques making the combination of both the most promising strategy in safety and efficiency. They have been studied for cancer drug delivery (Vázquez-Ríos et al. 2019), chemotherapy in a metastasic lung tumor (Fei Xiong et al. 2019) and DDS in tumor with macrophage-derived exosomes (Rayamajhi et al. 2019).

Strategy	Formulation	Application	References
Thin film hydration method (TFHM) and maleimide-based bioconjugation strategy	Classical liposome: Phosphatidylcholine (PC), Cholesterol (Chol)	Ex vivo and in vivo T cell expansión for immunotherapies	(De La Peña et al. 2009)
TFHM andNi2+/His-Tag protein coordination as bioconjugation strategy	Mimicking exosome lipid composition: PC, Chol, sphingomyelin (SM)	Downregulation of T cell activation in an autoimmune disease animal model (antigen-induced arthritis); immunotherapy for apoptosis induction in hematological tumors	(Martinez-Lostao et al. 2010)
Microemulsion and micelle-assembling method for vesicle formation	Innovative liposomes: PC, chemopor EL (CpEL), dioleoyl-phosphoethanolamine (DOPE)	Proof of concept of artificial antigen presentation to dendritic cells	(Kexin Li et al. 2015)
Thin film hydration method	1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), SM, Chol, 1, 2-dioleoyl-sn-glycero-3-phosphoserine (DOPS), DOPE (21/17.5/30/14/17.5, m/m)	Functional delivery of VEGF siRNA	(Lu et al. 2018)
Ethanol injection methodology	Chol, PC, SM, Ceramide (Cer) (0.9/1/0.4/0.03, w/w); integrin α6β4	Targeted delivery of miR-145	(Vázquez-Ríos et al. 2019)
Cell-free protein synthesis	DOPC/SM/Chol/DOPS/DOPE (21/17.5/30/14/17.5, m/m); Cx43; chitosan nanoparticles	Functional delivery of VEGF siRNA	(Lu et al. 2019)

Table 13. Compilation of approaches of developing artificial exosomes by bottom-up techniques

Conclusion and future perspectives

First of all, it is remarkable the advantages that exosomes have to offer to regeneration therapy and medicine. During past years tissue and cellular regeneration has become one of the major aims in this field and many approaches tried and still are trying to give a solution. The main method was stem cell therapy, which showed potential regeneration due to differentiation property of stem cell. However, side effects appeared and the most important one was the immunogenic response, which created a hostile environment by attacking stem cells, considered foreign bodies and promoting inflammation in the area that was supposed to regenerate. This effect was critical because could lead to not regenerating tissues at all.

Recent investigations tried to search an alternative to stem cell therapy and after some advances in extracellular vesicles, many groups reported exosomes as a viable substitution for stem cell therapy. Not only was a less invasive body than stem cells but offered a high potential in drug cargo and signaling with different membrane proteins or RNA. Studies showed very low immunogenic response and in general, exosomes applications showed better results.

After exosomes therapy began to be a possibility in regeneration, different applications appeared and regeneration in the Central Nervous System was of high importance due to the impossibility of regenerate by itself. There have been many researches in Traumatic Brain Injury due to its high frecuency and be one of the major causes of degeneration of CNS without any other disease causing the problem.

Both Drug Delivery System and Biomarker are approaches with a lot of potential and the main research lines and they have been used in TBI and other pathologies associated to CNS such as Spinal Cord Injury, CNS tumor or stroke. By DDS aplications different drugs and regeneration promoters can be delivered to the affected area passing through the Blood Brain Barrier and the use of exosomes as biomarkers allows to adquire information about certain pathologies as well as target cells of interest.

Especially in DDS application of exosomes therapy it is important to maintain in time the delivery of substances. In order to accomplish this, exosomes have been combined with biomaterials and different combinations resulted mostly in improvements of the performance of the drug delivered. The most used biomaterials in these researches have been bioscaffolds and hydrogels. Both biomaterials showed time-maintained delivery of the substance and also improved the conditions of the environment to enhance drug activity. The most important conditions improved thank to biomaterials was inflammation. The problem with inflammation is the impossibility that generates in the regeneration process and for this reason was an issue which needed a solution. Exosomes itself induces low inflammation due to compability with organisms, which means low immunogenic response to the incorporation of exosomes and as a result lower inflammation than other transporters. However, bioscaffold and hydrogel almost completely inhibits inflammation, so the environment became susceptible to the actuation of all factors than enables metabolic routes to produce grow factors.

In the case of hydrogels is has also demonstrated to use less amount of exosomes for the same result obtained without any biomaterial. This means more feasibility in a clinical application because one of the drawbacks is low efficiency in isolating and production of exosomes at high amounts.

Finally, with the purpose of improve the main disadvantages of therapy with exosomes have been developed artificial exosomes. By two different techniques, Top-Down and Bottom-Up, some research teams are trying to solve the mass production problem and high specifity needed for exosomes respectively. Top-Down techniques are based on creating small bodies from bigger ones, making possible production of large quantities of exosomes at the same time. On the other hand, Bottom-up techniques creates exosomes from smaller structures in a very specific way. This has a low mass production rate but exosomes can have very specific components such as certain proteins and RNA.

For all these reasons exosomes are present and future in tissue regeneration and has many future perspectives in many fields, especially in cancer, where exosomes can be used as diagnostic and treatment agents.

As a final remark, this work was going to be different but with the pandemic situation due to COVID-19 many laboratories were closed and this project needed to change. Originally, this work was aiming another approach of exosomes research. It is known all the advantages that have exosomes in regenerative therapy but as part of natural communication between cells they may have a role in different pathologies. In reunion with a doctor from Hospital Clínico of Madrid, it was suggested exosomes from CNS tumors could be involved in methastasic process carrying tumorgenic factors to other parts of the body. In this research line was based this work before the impossibility of doing laboratory work. It was going to be matching a visit of some days to the Hospital Clínico in Madrid to learn how to isolate and purify exosomes.

As a result of the modification of the work, this project became completely theorical about all the applications of exosomes in regenerative medicine, their advantages and its use in several pathologies, especially in CNS and the benefits of combining them to biomaterials

The main purpose of this work is to be used as a reference of the theory and applications of exosomes, getting faster answers to future researches that seek all this information about exosomes.

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Also I want to thank to Hospital Clinico of Madrid were I was supposed to share some days learning the methodology of isolation and purification of exosomes but in the last moment had to be cancelled due to the circumstances.

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