**Nanomaterial for Treating Stroke**

**by**

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## Abbreviations and Definitions

**Antiplasmin**: A major protein that regulates the activity of plasmin (1).

**Catalase:** An enzyme found in living things that are exposed to oxygen and catalyzes the breakdown of hydrogen peroxide into water and oxygen to protect the cells form being damaged by reactive oxygen species (2)

**Cell membrane:** A phospholipid bilayer that surrounds the cells and separates the cytoplasm from the extracellular environment (3).

**Exo- catalase:** Exosomes coated catalase

**Exo-catalase-tPA:** Exosomes coated catalase and tPA

**Exosomes:** Nanosized vesicles that are secreted by cells and carry several biomolecules (4).

**Exo-tPA:** Exosomes coated tPA

**Fibrin:** Fibrin is an integral part of the clotting cascade and is formed by polymerization of the soluble plasma protein fibrinogen (5).

**Fibrinogen:** A protein that is responsible for blood clot formation, wound healing, and inflammation (6).

**HBMVEC:** Human brain multivesicular endothelial cells are the major type of cells that make up the blood brain barrier and protects the brain against toxins and immune cells (7).

**Plasmin:** A proteolytic enzyme derived from plasminogen, and it is the primary enzyme that breaks down blood clots (8).

**Plasminogen:** The inactive form of plasmin found in blood plasma (9)

**Thrombin:** A proteolytic enzyme derived from prothrombin and catalyzes the conversion of fibrinogen into fibrin (10).

**Tissue plasminogen activator (tPA):** A serine protease enzyme that catalyzes the conversion of plasminogen into plasmin (11).

## Abstract

Stroke is one of the most lethal diseases worldwide where it is expected that among 23 million cases, 7.8 million patients will die in the coming eight years. Several factors might cause strokes including genetics and lifestyles. Due to the increased risks of mortality, and the drawbacks of current conventional therapies, finding new therapeutical methods for treating strokes is crucial. One approach is nanotechnology that has been proven to be promising in the field of medical research including stroke treatments. Many types of nanomaterials have been involved in drug delivery including gold nanoparticles and polymeric nanoparticles aiming for an early diagnosis of stroke and treating it with minimal side effects and maximal success. In this study, we investigated the effect of using cell membrane and exosomes derived from human brain microvascular endothelial cells to enhance the activity of some drugs that are used in treating stroke and tested the ability of using such biocompatible particles in dissolving a fibrin clot without causing cytotoxicity and ROS.

The objective of this study was accomplished by performing corresponding experiments that studied the activity of the prepared particles and proved their effectiveness. In addition, characterization tools such as flow cytometry, biochemical assays, and dynamic light scattering.

This study reveals that the effectiveness of using cell membranes and exosomes to coat tPA and plasminogen in order to enhance their ability in dissolving a fibrin clot, thus further increase their effectiveness in stroke treatments while reducing their side effects when administered freely.

Furthermore, this study proves the effectiveness of catalase against the toxicity and ROS caused by $H_2O_2$ in addition to showing the efficiency of encapsulating catalase within exosomes, alone and with tPA, to enhance the particles efficacy, first treating a stroke, and second by preventing cytotoxicity and ROS.

# Chapter 1: Introduction

## 1.1 Introduction

Strokes and other cardiovascular diseases are the leading causes of death worldwide. Traditional therapies have been used to treat or prevent strokes such as anticoagulants, antiplatelets, and thrombolytic medications, in addition to some invasive options such as a bypass surgery. However, the side effects of these medications such as life-threatening bleeding, the recurrence of the stroke, toxicity of drugs, shot lifetime and bioavailability, and the side effects of an invasive surgery remains a challenge (12).

There are three main types of strokes, ischemic stroke, hemorrhagic stroke, and transient ischemic attach (13).

An ischemic stroke that is the leading type of strokes, is a medical condition that when a blood clot clogs an artery that supplies blood to the brain, thus the blood supply to a part of the brain gets interrupted or reduced. As a result, the amount of oxygen and nutrients that reach the brain tissues is ceased which leads to the death of cells (13).

A hemorrhagic stroke occurs when an artery in the brain breaks or leaks blood which exerts pressure on the brain cells leading to their death. Moreover, hemorrhagic stroke is two types, intracerebral hemorrhage that occurs when the artery ruptures and covers the surrounding tissues with blood, and a subarachnoid hemorrhage that occurs when bleeding takes place between the brain and the tissues that cover it (13).

A transient ischemic attack, that is also referred to as a mini stroke, occurs when the blood supply to the brain is interrupted for a short amount of time that usually lasts for five minutes (13).

The two most common approaches to treat an acute ischemic stroke are early thrombolysis, known as recanalization, and neuroprotection despite the fact that there is not a well define.therapeutic range for treating ischemic stroke yet although many treatments have been developed since it was reported that most therapies fail clinical trials. These failures are related to the fact that treatments are mainly done outside the therapeutic window of the drug, and most importantly, neuroprotectors used in treatments can't cross the blood brain barrier to reach the ischemic injured area in the cerebral part of the brain. Moreover, some diseases such as arterial hypertension and diabetes mellitus highly lower the effect of neuroprotectors thus decrease the efficiency of a treatment. In addition, the location of ischemic strokes might differ from a patient to the other which requires different types of drugs with different doses to be treated (14,15). Other Traditional therapies have been used to treat or prevent strokes such as anticoagulants, antiplatelets, and thrombolytic medications, in addition to some invasive options such as a bypass surgery. However, the side effects of these medications such as life-threatening bleeding, the recurrence of the stroke, toxicity of drugs, shot lifetime and bioavailability, and the side effects of an invasive surgery remains a challenge (16).

Nanotechnology has become a promising prospective in the field of drug delivery due to the development of engineered nanoparticles such as metal nanoparticles including gold and silver nanoparticles, fullerenes, quantum dots, and dendrimers (17). The importance of these nanoparticles in drug delivery is provided by the ability to manipulate their shape and size, thus their surface properties (18). For instance, fullerenes have high internal volume and a large surface area that give these particles an ability to be loaded with drugs and imaging agents, after undergoing some surface modifications, to be delivered to targeted cells and tissues in a body and reducing undesired off-target effects (19).

Moreover, the branched shape of dendrimers and their empty cavities have made these polymers important particles in the medical filed including gene therapy and drug delivery for many therapeutic reasons such as treating prion diseases, bacterial infections, and viral infections (20). Additionally, due to the importance and success of many nanoparticles in biology and the medical field, some drug delivering nanoparticles have been under clinical studies, where others

have already been approved by the Food and Drug Administration (FDA) to be used by humans (21).

The figure below summarizes some applications of nanoparticles in medicine and biology.

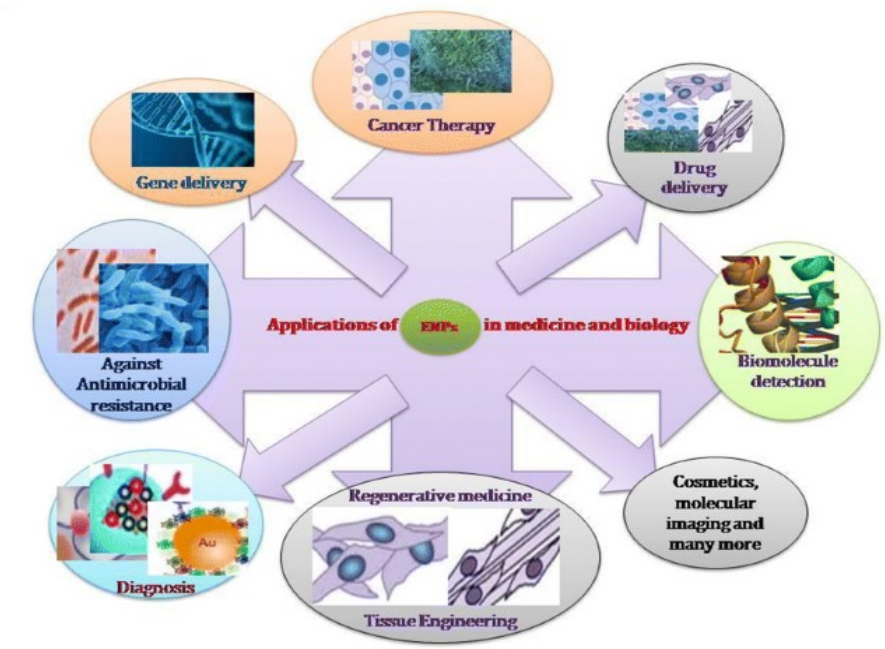



**Figure 1:** The applications of engineered nanoparticles in different medical and biological fields (21).

The design of a nanoparticle is essential in order to fabricate particles that dimensionally and characteristics of biological structures that affect human health and disease. Moreover, the ability to control the characteristics of nanoparticles during synthesis increase the importance of these particles since this gives them the ability to be specifically tuned according to diseases needs on one hand, and individual needs on the other hand, thus different particles could be synthesized to different individuals introducing a so-called personal drug design concept (22,23).

Some nanomaterials that are said to be effective in stroke treatment include CNT and fullerenes that have a drawback in inducing blood coagulation (24), quantum dots that is successful in delivering tPA to the site of thrombosis in ischemic stroke (25), and dendrimers that are good heparin carriers (26).

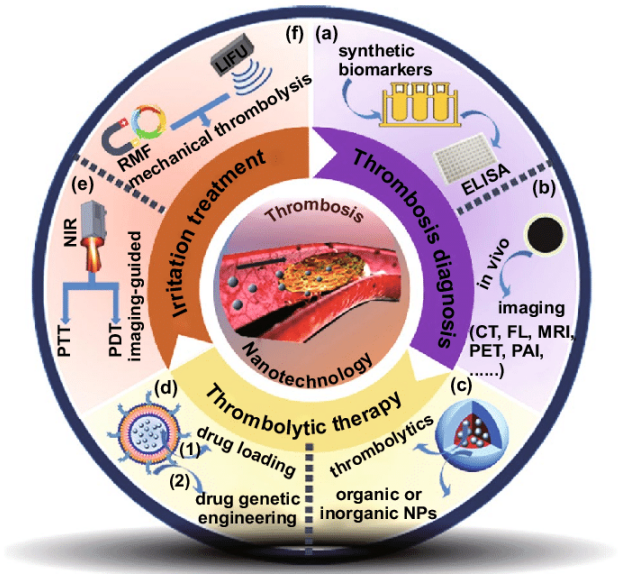



**Figure 2**: A schematic illustration of nano-medicine-based thrombosis diagnosis and treatments (27).

In addition to their special characteristics stated above and there targeting abilities, other advantages of engineered nanoparticles include the ability of crossing biological barriers and long circulating life (28, 29). However, to achieve these important characteristics the correct tool is needed although nanotools varies in size, material, rigidity, and other specifications.

Regarding stroke treatment, nanomedicine is highly promising in improving thrombolysis in ischemic stroke, decrease hemorrhage bleeding, enhance the diagnosis of stroke, and decreasing death. (28, 30)

The following table includes some nanoparticles used for stroke treatment in animal models.

**Table 1**: Nanoparticles used for stroke treatment in animal models (31).

| Carrier | Property | Surface Component | Therapeutic Agent | Animal: Disease Model (Route) |
|---|---|---|---|---|
| LIPOSOME | Stealth | PEG | Hb, fasudil, Xe, luteolin, FK506, CsA, dexamethasone, acetate | Rat: tMCAO (IA, IP, IV), pMCAO (IV), thrombotic stroke (IV), hemorrhagic stroke (IV), global transient ischemia (IV)<br>Monkey: tMCAO (IV) |
| | Stealth, conjugated | PEG, AEPO, T7, SHp, anti-HSP72, anti-CD106, IgG-1, anti-PirB | AEPO, ZL006, citicoline, anti-PirB | Rat: tMCAO (IA, IV), pMCAO (IV)<br>Mouse: thrombotic stroke (IV) |
| | Stealth, charged | PEG, CHOL(+), DOPA(-) | Simvastatin | Rat: tMCAO (IV) |
| | Non-stealth, conjugated | - | baicalin, lycopene, bFGF, OEA | Rat: tMCAO (IN, IV, IG), pMCAO (IV) |
| PLN | Conjugated, loaded | PEG, anti-Fas | 3-n-Butylphthalide | Mouse: tMCAO (IV) |
| NLC | Loaded | - | Ferulic acid | Rat: Global transient ischemia (IV) |
| NANOEMULSION | Mucoadhesion | PEG, chitosan | Quercetin | Rat: tMCAO (IN, IV) |

However, despite the promises that nanotechnology seems to hold in the field of disease control and drug delivery especially that nanoparticles can target specific sites, certain modifications must be done to the nanoparticles to avoid being removed from the circulation to be filtered by the reticuloendothelial system through the liver and spleen thus reducing their therapeutic effect. Therefore, coating the nanoparticles with cell membrane enhances their biocompatibility and extend their circulation time by eliciting their attack by the immune system. moreover, certain proteins on the cell membrane gives the nanoparticles biological properties such as selective adherence and endothelium penetration (32, 33).

Another way that seems to effectively treat and prevent strokes is exosomes therapy since they are unlikely to be attacked by the immune system and have the ability to deliver molecules over large distances in the body (32).

## 1.2 Motivation

Conventional drug delivery methods for treating stroke have limitations and side effect especially when trying to treat a brain stroke due to the blood brin barrier that prevent drugs from reaching the brain. Nanoparticles seem to be a solution. Coating poly-lactic-glycolic-acid-plasmin (PLGA-plasmin) nanoparticles with cell membrane of human brain microvascular endothelial cells (HBMVEC) that are the major cells of the blood brain barrier, and coating tissue plasminogen activator (tPA) with exosomes extracted from HBMVEC is a great potential for treating brain strokes due to the ability of these particles to cross the barrier without being attacked by the immune system and without leading to the side effects that conventional therapies have

## 1.3 Objective

The aim of this research is to verify the ability of cell membrane coated PLGA-plasmin nanoparticles and exosomes coated tPA to treat a stroke as biocompatible nanoparticles. In addition, it is important to prove that these particles do not cause toxicity or ROS to the cells. The third aim is to further strengthen the formulation by oxidative stress analysis.

## 1.4 Technical approach

1. Prepare PLGA-plasmin nanoparticle of desired size
2. Extract cell membrane
3. Coat the PLGA-plasmin nanoparticles with cell membrane
4. Extract exosomes to coat tPA
5. Extract exosomes to coat catalase
6. Do activity assay to determine the desired concentration that is required to treat a fibrin clot
7. Prepare fibrin clots and test the clot lysis with plasmin and tPA formulation
8. Cellular efficacy studies (toxicity and ROS)
9. Improve formulations by oxidative stress analysis

## 1.5 Thesis Organization

This thesis is organized into seven chapters. Chapter 2 is a general background about the use of nanomaterial in the medical field, cell membrane coated nanoparticles, exosomes coated therapeutic drugs, and reactive oxygen species in stroke. In addition, potential therapeutic approaches using nanoparticles for stroke treatment are briefly reviewed. The goal of the literature review is to introduce the contexts of this study and to provide a general idea regarding its goal. Chapter 3 presents the materials that were used and the methods that were followed to perform the experiments. Chapter 4 studies the effect of coating PLGA-plasmin nanoparticles with cell membrane. Chapter 5 studies the effect of coating tPA with exosomes while Chapter 6 investigates the use of exosomes to coat catalase in order to reduce ROS and studies the effect of using exosomes to coat both catalase and tPA at the same time to unite the benefits of both exosomes-tPA and exosomes-catalase particles in one type as exosomes-catalase-tPA. Lastly, Chapter 7 comprises a brief summary and a general conclusion of this work in addition to some future perspectives.

# Chapter 2: Background and Literature Review

## 2.1 Introduction

Strokes and other cardiovascular diseases are the leading causes of death worldwide. Traditional therapies have been used to treat or prevent strokes such as anticoagulants, antiplatelets, and thrombolytic medications, in addition to some invasive options such as a bypass surgery. However, the side effects of these medications such as life-threatening bleeding, the recurrence of the stroke, toxicity of drugs, shot lifetime and bioavailability, and the side effects of an invasive surgery remains a challenge (34,35).

On the other hand, nanotechnology seems to hold success in the field of disease control and drug delivery especially that nanoparticles can target specific sites. However, certain modifications must be done to the nanoparticles to avoid being removed from the circulation to be filtered by the reticuloendothelial system through the liver and spleen thus reducing their therapeutic effect. Therefore, coating the nanoparticles with cell membrane enhances their biocompatibility and extend their circulation time by eliciting their attack by the immune system. moreover, certain proteins on the cell membrane gives the nanoparticles biological properties such as selective adherence and endothelium penetration (36).

Another way to that seems to effectively treat and prevent strokes is exosomes therapy since they are unlikely to be attacked by the immune system and have the ability to deliver molecules over large distances in the body.

## 2.2 Enhancing Thrombolysis in Ischemic Stroke

It is known that the best conventional thrombolysis treatment for acute ischemic stroke is administrating tissue-type plasminogen activator (tPA) intravenously (37) that works by

converting endogenous plasminogen to plasmin. Plasmin, in turn, breaks down the fibrin mesh that surrounds the thrombi thus breaking the clot.

Although this technique is the most well treatment, it is mainly effective to treat early arterial thrombosis (38), and if the treatment persists more than three to four hours, the risks overweigh the benefits since tPA increases the risk of bleeding (39). Therefore, nanomedicine is a promising approach to treat thrombosis safely.

Several types of nanoparticles were developed to carry plasminogen activators (PAs) to the site of thrombosis to target fibrin or platelets as these are the two major components of av blood clot (40).

The figure below shows some polymeric nanoparticles carrying tissue plasminogen activators to treat in vitro thrombosis.

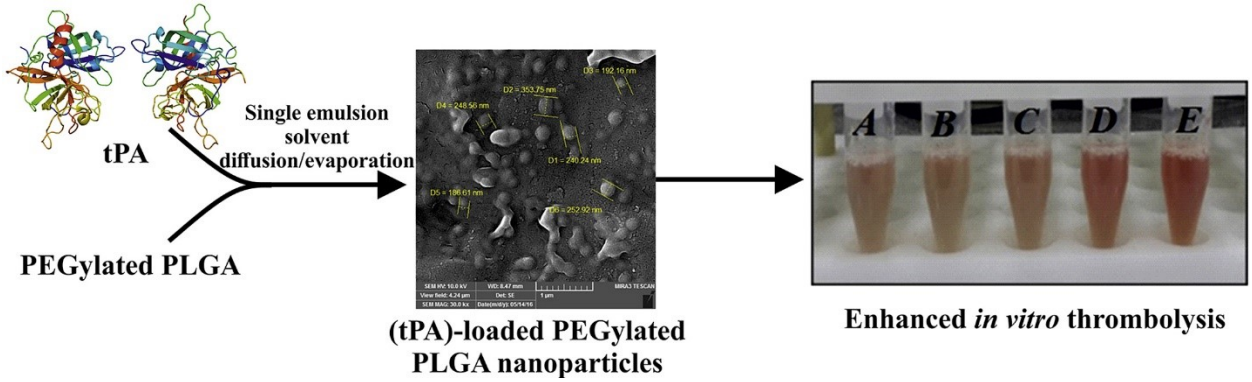



**Figure 3**: Polymeric nanoparticles carrying tissue plasminogen activators for treating thrombosis (41).

Further research showed that coating nanoparticles with tPA and fucoidan, a P-selectin ligand that is expressed when platelets activate, were successive in breaking down platelet-rich blood clots that were unable to lyse using tPA treatment (42,43).

To enhance the specificity of thrombolysis, smart nanodevices were established based on using a blood clot to prompt the release of PA. Moreover, some therapeutic systems such as thrombin sensitive capsules, (44) were designed to utilize the activity of blood clots. In addition, the increase in the oxidative stress and the high shear stress caused by the clot formation are used as triggers (45). For instance, mechanosensitive liposomes were developed to treat thrombosis based on being triggered by the high shear stress at the site of the blood clot. Such thrombosis triggered systems are preferred since they allow a controlled drug release where the amount of drug to be released depends on how severe the blood clot is (46, 47).

Using external stimuli such as using the magnetic field to coordinate the movement of superparamagnetic iron oxide nanoparticles that deliver liposomes to the site of thrombosis and activate their explosion in order to release tPA in a controlled is another approach of controlled drug delivery (48). Other nanosized magnetic particles are based on drilling the blood clot after being delivered to the targeted location (49).

Nanomedicine has been proven to safely deliver therapeutic drugs to the brain to overcome main obstacles that limit the application of neuroprotective drugs (50) such as biodegradable polymeric nanoparticles that deliver therapeutic drugs to the central nervous system in a continuous release (51). Moreover, biodegradable nanoparticles could be synthetic polymeric nanoparticles such as polyglycolic acid, polylactic acid, poly D L-lactide-co-glycolide acid, and polyethylene glycol derivatives, or natural macromolecular polymers such as starch and gelatin (52). Additionally, polymeric nanoparticles are proved to be effective in delivering protective drugs such as hormones and PEGylated epidermal growth factor when tested on animals thus confirming their neuroprotective effect being greatly higher than free drugs.

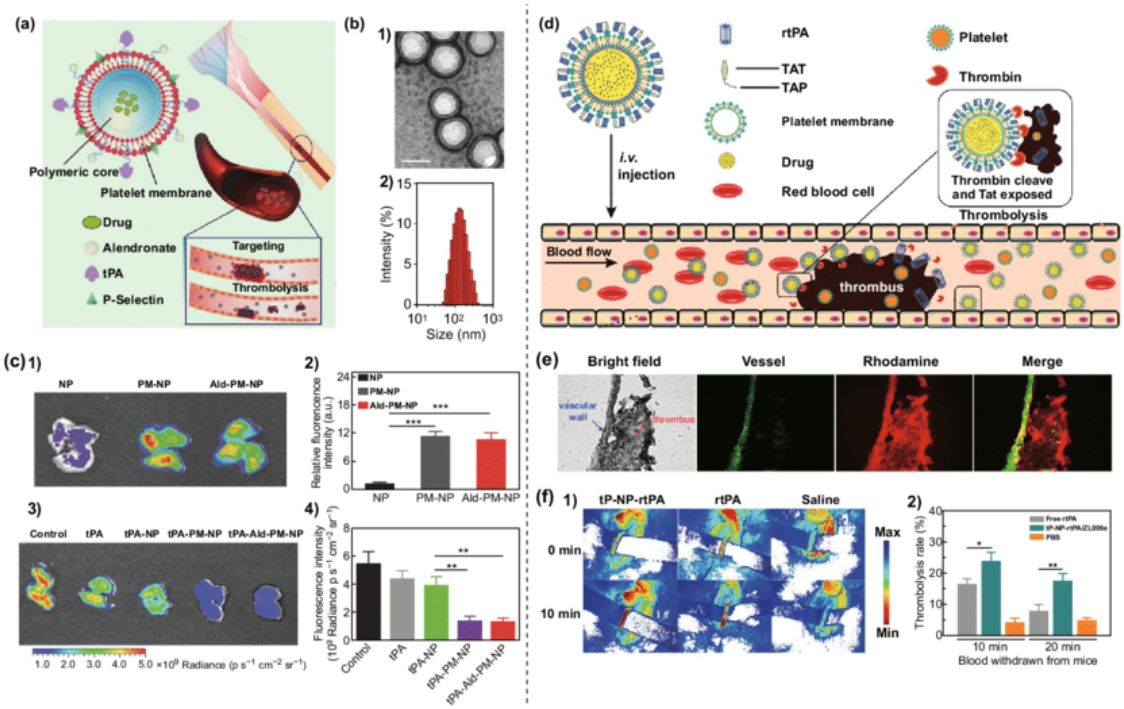



**Figure 4**: Schematic illustration, TEM, and dynamic light scattering of tPA polymeric nanoparticles for treating thrombosis in animal models (53).

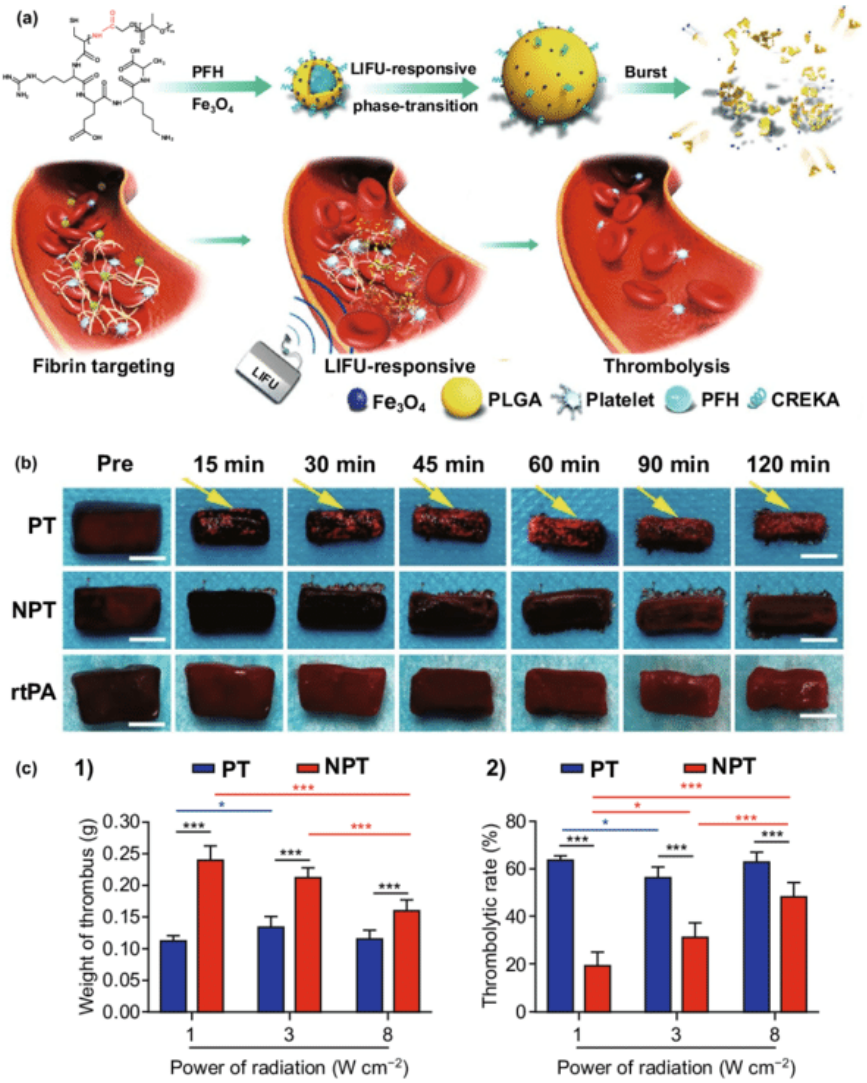



**Figure 5:** a) Schematic illustration of PLGA-based nanoparticles to treat thrombosis, b) thrombolysis using nanoparticles, c) final thrombus weights (53).

To improve their success in clinical application, in addition to reducing toxicity, nanomaterial used in nanomedicine are developed to either undergo renal clearance or to be fully biodegradable (54, 55).

## 2.3 Nanomaterial in Stroke Treatment

Doctor Johann Jakob Wepfer discovered, in 1655, the interruption of blood supply in reaching the brain. When an ischemic stroke occurs, many of the neuron in the brain get lost (56). As to date, there are limited treatment approved by the FDA to treat ischemic stroke and they are restricted to the intravenous administration of tissue plasminogen activator (tPA) four hours after the symptoms appear, although this treatment is supposed to be ineffective due to its therapeutic window which gives rise to the second treatment option that is the mechanical thrombectomy (57). Despite remarkable development of stroke treatments, better treatments are required to identify initial variations in the brain, and to rescue the neuronal milieu that result from ischemic injury which required the research to focus on nanotechnology (56).

A remarkable mission that treatment of ischemic stroke does is delivering the therapeutic drug to the brain by evading the strong and restricting blood brain barrier (BBB).

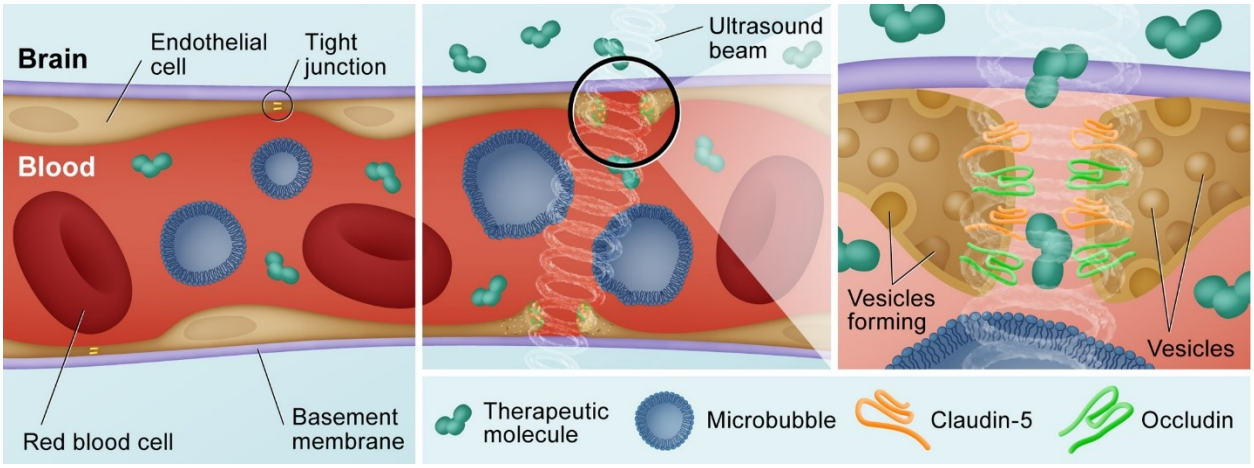



**Figure 6**: Schematic illustration of the blood brain barrier (58).

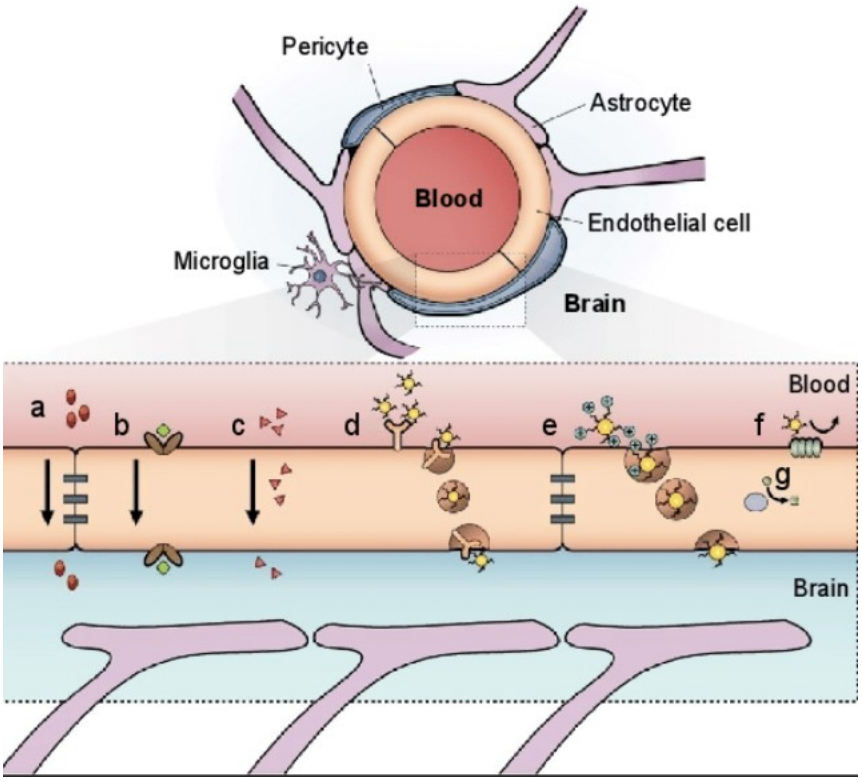



**Figure 7:** Schematic illustration of the blood brain barrier (59).

When an ischemic stroke occurs, the human BBB gets disturbed through two phases. The first phase is the initial phase that occurs few hours after the stroke, and the second phase that is the delayed hyperpermeability phase that occurs two days after the stroke (60, 61).

Nanomaterials could be an important drug delivery system that plays an important role in stroke treatment that can cross the BBB by bypassing the tight junctions of the endothelial cells and the micro-blood vessels that make up this barrier (62). Moreover, using nanomaterial to treat stroke might offer an appropriate interference and a better explanation of ischemic stroke by detecting the initial signs of stress in the cerebro-vasculature might decrease the severity of the stroke or prevent it and by offer the delivery of effective drugs with proper doses thus preventing severe side effects that are one of the drawbacks of current stroke therapies (63, 64).

Using nanomaterial to treat stroke might offer an appropriate interference and a better explanation of ischemic stroke. For example, thoroughly detecting the initial signs of stress in the

cerebro-vasculature might decrease the severity of the stroke or prevent it (65). Moreover, nanomaterial could offer the delivery of effective drugs with proper doses thus preventing severe side effects that are one of the drawbacks of current stroke therapies. This could be done by utilizing nanomedicine that offer a sustained drug release, that in turn, also decrease the clearance (66). Nanotechnology could also offer a drug delivery system that delivers the drug in a timed fashion that targets the foci of the ischemic stroke and despite its limitations, the application of nanotechnology must be designed precisely as to attain an effective and safe therapeutic procedure, therefore different aspects of nanotechnology have been implicated in diagnosing and treating ischemic stroke.

Most strokes that occur are thromboembolic, meaning that when a thrombosis breaks and travels from one vessel to the other, with ischemic stroke accounting for 87% of stroke incidents (67). When a blood vessel in the cerebral is blocked, it becomes hypoxic, perfuse less, and glucose deficient, thus, the part of the brain tissue that is supposed to be nourished by the blocked vessel dies in a non-homogeneous way such that the range of severity indicates the patient's collateral circulation, that is the circulation that occurs around a blocked blood vessel, where the cells in the center of the infract zone undergoes an instance necrosis, while the surrounding region gets partly injured and some cells undergo apoptosis(68, 69). Ischemic stroke could also result from blocked arteries and veins.

As soon as the ischemic stroke starts taking place, the ATP produced by the mitochondria starts depleting thus affecting many molecular and cellular activities and induces an inflammation and vascular modification (70). Moreover, cellular hypoxia-ischemia, with time, causes and increased gain of sodium ions, and increased gain in chloride ions, an increases gain in calcium ions, a decrease in potassium ions, and an overall malfunction in the membrane ion pump and the formation of cytotoxic edema (71). As stated earlier, the BBB is a tightly adjusted barrier made of a single layer of endothelial cells. This barrier controls the transport of material in the brain with its tight junctions being responsible for paracellular material transport. Few days after the occurrence of an ischemic stroke, the tight junctions start failing (72). For this reason, therapeutic drugs can undergo transcellular diffusion after few hours of the stroke occurrence to reach the brain.

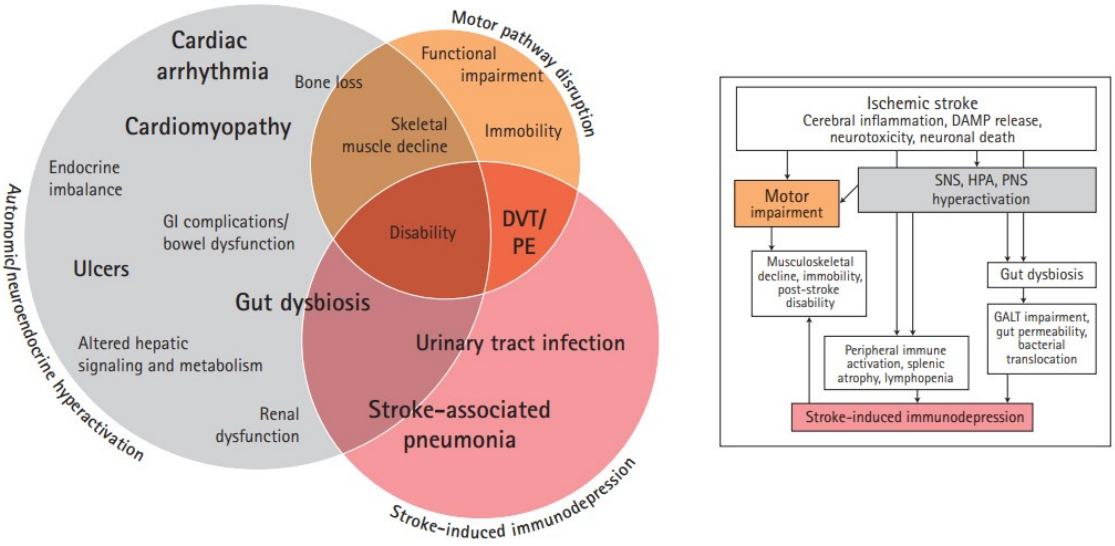



**Figure 8**: The pathophysiological sequelae of an ischemic stroke (73).

The malfunction of the BBB activated different inflammatory responses that in turn release inducible nitric oxide synthase and proinflammatory cytokines that lead to the production of free radicles and reactive oxygen species that disturb the mitochondrial functions. Thereafter, the mitochondria start releasing cytochrome and activates caspase resulting in the lysing of the DNA and cellular apoptosis of nerve cells (74, 75). Moreover, the decrease in cellular energy increases the release of excitatory amino acid neurotransmitters leading to calcium excitotoxicity (76). This the causes demyelination and the denaturation of nerve fibers (77).

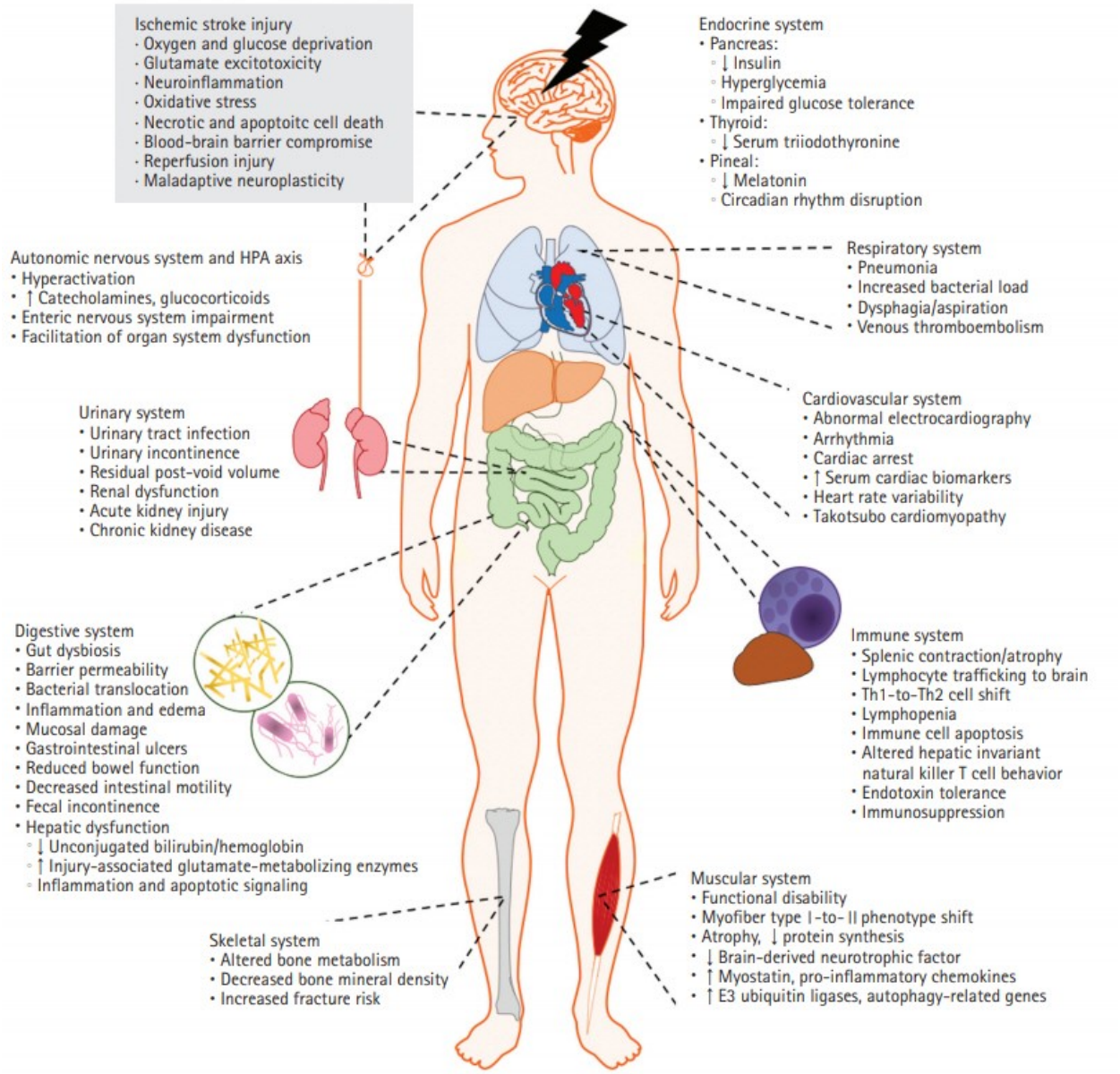



**Figure 9:** The multi-systems effect of ischemic stroke (78).

Reducing the risk of stroke is generally limited to prevention methods such as maintaining a healthy lifestyle, medical interventions, and enhancing the risk factors (79). Moreover, there are two FDA approved treatments that are surgical removal of the thrombosis and thrombolysis (80). However, the disadvantages for these methods, such as narrow time windows for treatment and risk of bleeding, urge the search for new treatments.

Despite that the basic idea behind the approved stroke treatments is restoring the flow of blood in the clogged vessel this could increase the distribution of the blood brain barrier. Moreover, reactive oxygen species and reactive nitrogen species that are released during a stroke further lead to neuro-endothelial damage by inducing endothelial cellular death and a damage to the basement membrane, which increases the breakdown of the blood brain barrier (81). Therefore, an effective therapy must take the properties of the blood brain barrier into consideration and must be able to cross this barrier. However, the thickness of the blood brain barrier is an obstacle that prevent therapeutic drugs from reaching the brain (82). Therefore, nanomedicine offers a longer circulation half-life and a transport through the endothelial to overcome this obstacle.

As mentioned earlier, nanomedicine promises for many modernized technological aspects in treating different disorders of the central nervous system (83) since the surface to volume ration of nanomaterials allow them to carry therapeutic drugs and imaging agents to specific regions allowing targeted delivery, as mentioned earlier. Moreover, nanoparticles are able to enclose particles and shield them from unwanted biological effects (84). For these reasons, nanotechnology has been gaining the attention of many researchers (85, 86). In addition to that, since stroke injury is highly complex and dynamic, it involves specific diagnosis and treatments with advanced technological approaches in order to ensure the safety of the patient and the success of the treatment, that is in turn, the promise of nanotechnology (87).

### 2.4 Nanotechnology in Stroke Diagnosis

Several studies have reported the importance of nanoparticles in diagnosing stroke, including gold nanoparticles, Superparamagnetic iron oxide nanoparticles, PLGA nanoparticles, nanospheres, quantum dots, and metabolizable nanoparticles.

### 2.5 Nanomaterials as Drug Delivery Systems for Stroke Treatment

One of the most used drug delivery nanoparticles that could carry many classes of drugs including drugs for hypertension, hormones, and immunomodulators, in addition to serving as potential carriers macromolecules such as proteins, nucleic acids, and antibodies, are the PLGA nanoparticles (88) because of their biocompatibility and biodegradability, and it is approved by

the FDA to be used in humans with proper morphology, intrinsic properties, physicochemical properties, and method of hydrolysis (89-96).

When hydrolysis occurs to break down PLGA nanoparticles by the action of water molecules, the molecular size of the polymers decreases and voids are created between the polymeric chains, thus accelerates the drug release procedure by the time that the polymer and the hydrolysis products are non-toxic, the fact the make PLGA nanoparticles preferable for drug delivery (97).

Polymeric nanoparticles have been used for the delivery of neuroprotective drugs and antioxidants (98). For instance, PEG nanoparticles have been used to deliver drugs across the BBB to the infracted site (99). Moreover, polysorbate-80 coated nanoparticles bind to low density lipoprotein allowing the nanoparticles to undergo receptor-mediated endocytosis thus enhancing their ability to cross the BBB (100). In addition, due to their natural spontaneous self-assembly, polymeric micelles have attracting researchers' attention for stoke treatment (101).

Some nanoparticles such as liposomes are lipid based nanocarriers (102, 103). Several liposomal drug delivery systems have been described for treating ischemic stroke where animal studies showed positive results where after administration, there was a decrease in the clot size and in the brain edema (104). However, since liposomes undergo a quick clearance from the body due to the surface adsorption of proteins (105), surface modification using PEG is required to increase the circulation time which also enhances the targeted delivery profile (106, 107). Moreover, stimuli-responsive liposomes have been also reported where the release stroke treating drugs is done in a regulated sequence (108).

In addition to polymeric and lipid-based nanoparticles, other types of nanoparticles have been used to treat stroke. For instance, silica-based ceramic nanoparticles have been used due to their highly porous structure and the ability to undergo surface modification to give these particles several desired properties that enhance their targeting properties and drug release (109). For example, studies have shown that silica-magnetic nanoparticles conjugated with tPA could accumulate at the site of the blood clot, meaning that they have an enhanced targeting ability (104).

Another type of biocompatible nanoparticles are the carbon-based nanoparticles in addition to other cell derived nanocarriers such as stem cells and cellular vesicles (110). For example, tagging neural progenitor cells with iron resulted in a successful migration of the particles toward the ischemic stroke site where they reduced neural death.

The important features of nanoparticles give them different functionalities. For instance, in vitro and in vivo studies on nanoparticles have shown success in exhibiting the anti-inflammatory features of nanoparticles against reactive oxygen species by inhibiting these species. This is important since the relation between increased levels of ROS and neural injury in ischemic stroke has been reported. Moreover, polymeric nanomaterials have been FDA approved since, in addition to their biocompatibility, they have a high ability in crossing the blood brain barrier and the double membranes of the mitochondria that allows the creation of an improved therapeutic platform (111).

The safety of nanoparticles to be used in a human body is still debatable since, despite some studies showing that they are safe, other studies have shown that nanoparticles could induce toxic reactions and activate the immune system on one hand, and causing inflammation and ROS, on the other hand (112, 113). Moreover, some nanoparticles such as metal-based nanoparticles could impair the function of endothelial cells and increase the permeability of the BBB thus they are harmful to be used for stroke treatment (114, 115).

Toxicity problems could be prevented by coating nanoparticles with biocompatible substances such as PEG, which also increases the circulation time of the particles and enhances their ability to cross the BBB (116).

An additional side effect of nanoparticles is their ability to returning to the circulation after exiting the BBB increase the risk of anemia caused by hemolysis thus triggering immune response and activated platelets which increase the risk of thrombosis (83).

Therefore, determining the dose of nanoparticles that must be administered and their properties must be modified according to the cell type of the targeted area in addition to determining how would the properties of the nanoparticles be affected by the biological environment (117).

**Table 2**: Summary of targeted delivery of thrombolytics with nanomedicines (118).

| Strategy | Target | Ligand | Nanomedicine | Achievement | Limitation |
|---|---|---|---|---|---|
| Physical targeting | | | Urokinase-loaded NPs | Enhanced clots degradation and BBB protection | No reduction in the risk of HT |
| Ligand-mediated active targeting | Fibrin | Anti-fibrin antibody | Alteplase-loaded NPs | Showed less activity in the absence of embolus | Lack of experimental data *in vivo* |
| | GPIIb/IIIa receptors | cRGD peptide | Target sensitive SK-loaded liposomes | Target sensitive release of SK; enhanced clot dissolution *in vivo* | No data about the risk of HT |
| | GPIIb/IIIa receptors P-selectin | cRGD peptide P-selectin targeting peptide | Phospholipase A2 responsive SK-loaded nanovesicles | Reduced the risk of bleeding | No data about the risk of cerebral hemorrhage |
| Biomimetic targeting | Thrombus | Membrane proteins on platelets | Alteplase-loaded NPs coated with platelets membrane | Achieved responsive response of alteplase in thrombus | No data about the risk of cerebral hemorrhage |

The following figure is a schematic image of polymeric nanoparticles encapsulating drugs.

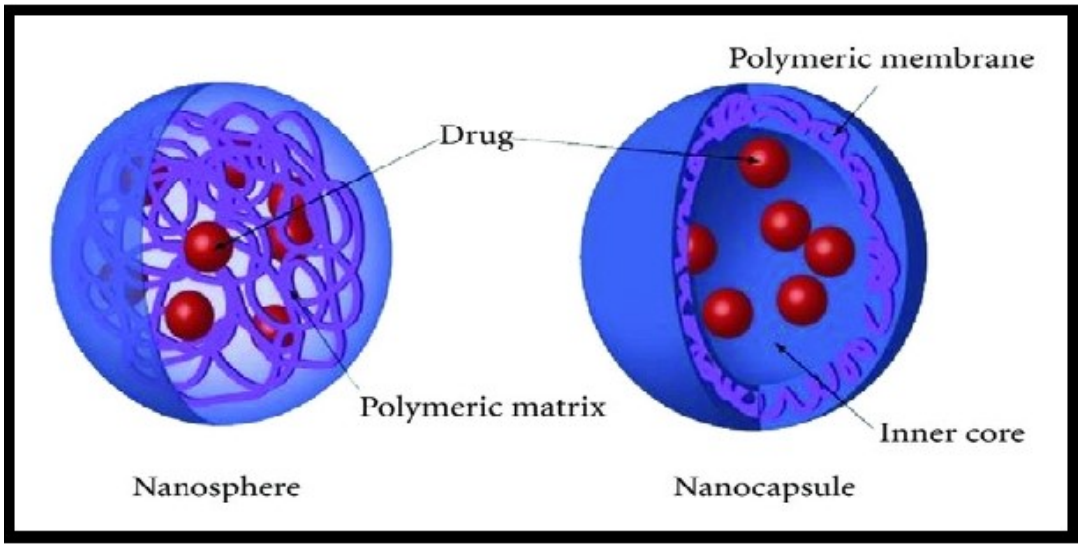



**Figure 10**: Schematic image of polymeric nanoparticles encapsulating drugs (119).

## 2.6 Cell Membrane Coated Nanoparticles for Ischemic Stroke Treatment

### 2.6.1 Introduction

Cardiovascular diseases, including stroke, is the major cause of death worldwide (120). Traditional therapies such as anticoagulants (121), thrombolytic, antiplatelets, and surgery such as bypass and stent (122, 123). However, the probability of disease reoccurrence that is 50 % (124), the side effects of treatments such as bleeding (125), and other drug induced reactions is a challenge.

Nanotechnology has been successfully achieving its goals in the field of biomedicine by introducing its nanomaterials that allow targeted drug delivery with their special properties (126). However, with the lack of a proper surface modification, these nanomaterials could be quickly removed from the circulation to be cleared form the body by the reticuloendothelial system through the liver and then spleen thus decreasing the effectiveness of the therapeutic drug (127).

The use of polymers to PEGylate the surface of nanomaterial for the reason of increasing their biocompatibility and increase their circulation time is a successful procedure on one hand, however, the risk of toxicity caused by this method limits its use (128).

As a more successful alternative, cell membrane coating has been proven to increase the circulation time and prevent the drug carrying nanoparticles from being invaded by the immune system. Moreover, the functional proteins that are found on the cell membrane gives the nanoparticles additional biological properties including selective adhesion, targeting specificity, and endothelium penetration (129).

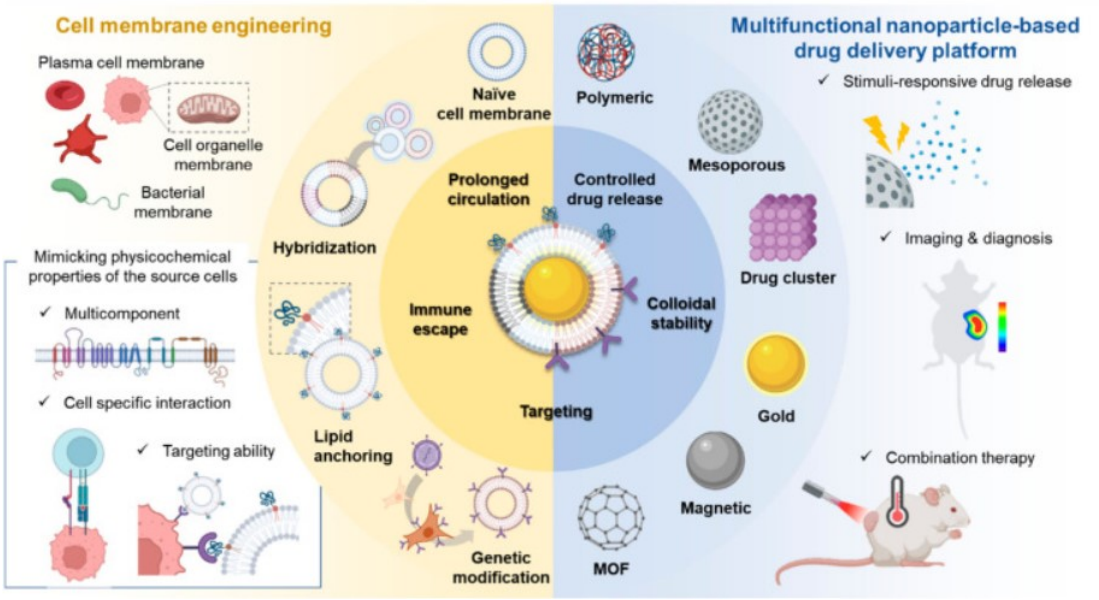



**Figure 11:** Schematic overview of cell membrane-cloaked nanotherapeutics for targeted drug delivery (130).

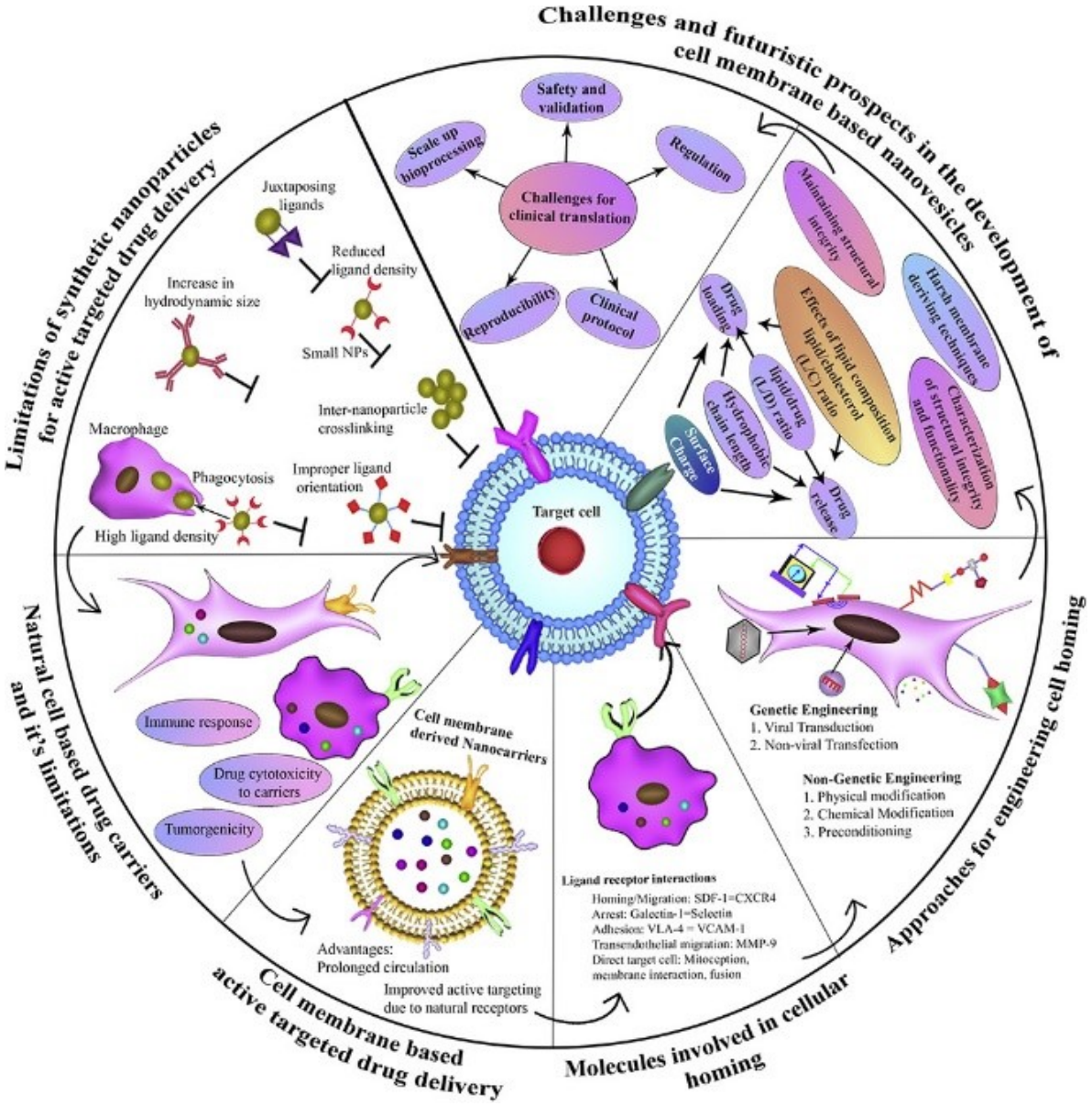



**Figure 12**: Natural and synthetic nanoparticles for drug delivery (131).

Using cell membrane to coat synthetic nanoparticles camouflage the particles with some of the characteristics of intrinsic cells of the body that prevent their uptake by the immune system, on one hand, and gives them several cellular biofunctions presented by different functional proteins of the cells membrane that end up being on the surface of the cell membrane coated nanoparticles, in other words, this mimics the properties of a cell.

Cell membrane coated nanoparticles was fist applied by Hu et at who used the cell membranes of erythrocytes to coat PLGA nanoparticles, which increased their circulation time by 72 hours (124). Many research has been performed to increase the source of cell membrane and the nanoparticles.

Synthesizing cell membrane coating nanoparticles involves three steps including the extraction of the cell membrane, synthesizing the nanoparticles, and finally the fusion step (132).

### 2.6.2 Extraction of the Cell Membrane and Preparing its Core for Loading

Cell membranes are made of phospholipids and several proteins of different functions (133), and they play a role in distinguishing cells, in cellular communications, in signal transduction, and in selectively allow substances to go in and out of the cell.

Methods of cell membrane extraction mainly focus on lysing the cell membrane followed by purification (134). After isolating the cells from the body, they undergo a hypotonic treatment that lyse the cells followed by discontinuous sucrose gradient centrifugation to remove the cytoplasmic contents such as the nucleus and other organelles. After centrifugation, purification is required where several buffers are used to wash the cell membrane, followed by an extrusion via porous polycarbonate membrane in order to get the final pure membrane (135).

The fusion process is the process by which the inner core of the nanoparticles is coated with the extracted cell membrane. This process is achieved either by membrane extrusion, or by a sonication bath. Membrane extrusion is used for small scale production and is done by mixing the extracted membrane with the nanoparticles followed by extruding the mixture through a porous polycarbonate membrane, and this is repeated for a number of cycles (136). On the other hand, the sonication method is done for a large- scale production and requires strictly appropriate sonication powers. One main concern of the sonication method is that high temperatures damage membrane proteins thus affecting the biological activity. Another drawback of the sonication method is the lack of size uniformity of the coated nanoparticles (137).

### 2.6.3 Cell Membrane Coated Nanoparticles for Treating Cardiovascular Diseases

As stated earlier, coating nanoparticles with cell membrane gives the nanoparticles some intrinsic cellular properties such as immune evasion, targeting ability to certain sites, binding affinity to specific receptors. To treat cardiovascular diseases, including stroke, cell membrane coated nanoparticles are manipulated to resemble peripheral cells that have been proven to play important roles in the progression of the disease like red blood cells, platelets, and immune cells (138-140).

### 2.6.4 Cell Membrane Sources

Different types of cells have been used as a source of cell membrane for the purpose of coating PLGA nanoparticles including red blood cells, platelets, macrophages, neutrophils, and stem cells. In all cases, the coated nanoparticles had the functional proteins of the cell membrane on their surfaces and gained an exceptional in vitro stability as compared to the non-coated nanoparticles that allowed the particles to maintain their inner core for a long period of time. Therefore, it was concluded that coating nanoparticles with cell membrane renders the particles the ability to escape the immune system thus increasing their circulation time, which make the coated nanoparticles good nanoplatforms for drug delivery to treat cardiovascular diseases such as stroke and enhanced the therapeutic efficacy in treating ischemic stroke (141-154).

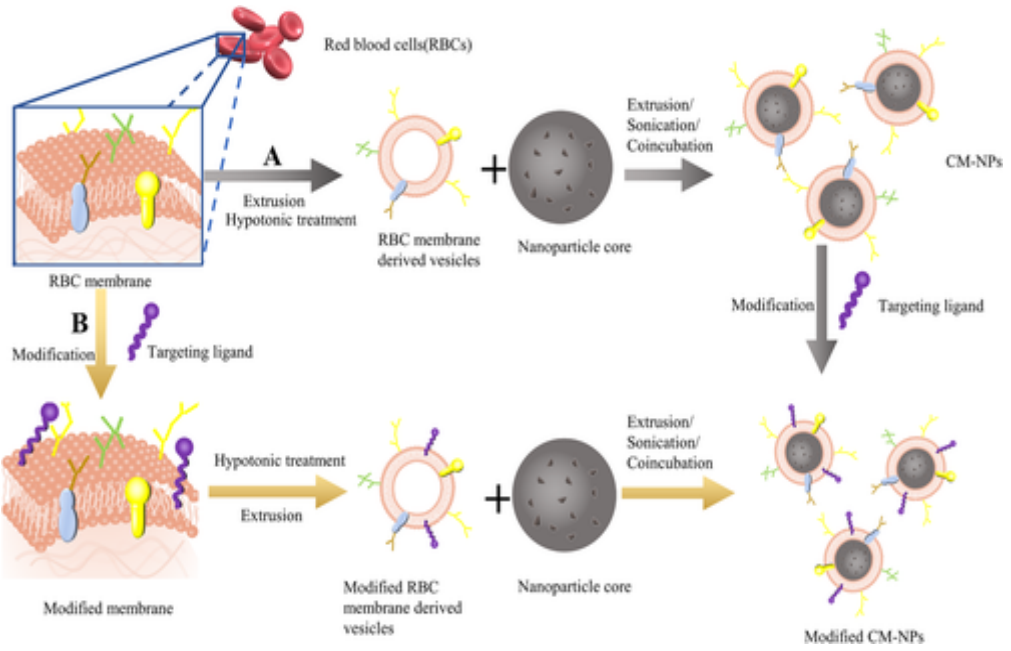



**Figure 13**: Preparation and modification of cell membranes coated nanoparticles (155).

**Table 3**: Unique properties of natural cell membranes (156).

| Cell Type | | Features |
|---|---|---|
| Plasma membrane | RBC | Immunosuppressive effect, long-term blood circulation, and neutralization of toxins |
| | Platelet | Specific binding affinity to injured vasculature, pathogen adhesion, reduced cellular uptake by macrophages, and prolonged blood retention time |
| | Macrophage | Inflammation site-specific accumulation, neutralization of inflammatory cytokines, antigen-homing affinity |
| | Cancer | Homotypic targeting, and low intrinsic immunogenicity |
| Cell organelle membrane | Mitochondria | Selectively binding to mitochondrial membrane ligands, and neutralization of toxins |
| | Nucleus | Improved transfection efficiency in gene therapy |

Since different types of cell membrane could be used to coat different types of nanoparticles to form cell membrane coated nanoparticles, it is good to note that the type of the cell membrane and the physical and chemical characteristics of the nanoparticles used have effects on the drug

release and on the cellular uptake of the coated nanoparticles which must be extensively investigated to enable clinical translation.

### 2.6.5 Cell Membrane Modification

Cell membrane is made of lipids, polysaccharides, and proteins where these functional proteins give the coated nanoparticles their functions.

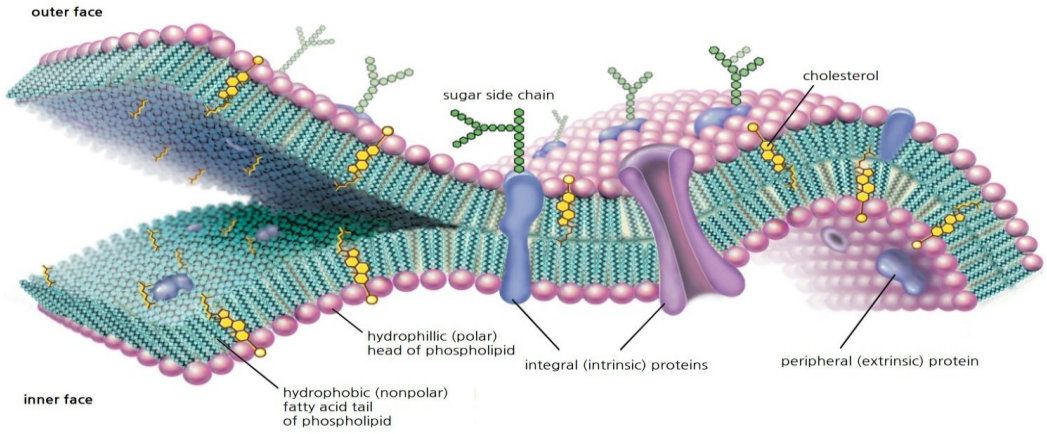



**Figure 14**: Schematic illustration of the cell membrane (157).

As medical science increases, the targeting effectiveness and operations of nanoparticles have been improving. And to enhance the features of membrane coated nanoparticles as to match natural characteristics of the cell membrane including their targeting abilities and enhance their benefits in treatments, cellular membrane has been modified in order to meet specific characteristics (158-165) and these modifications can be divided into three types, physical modifications, chemical modifications, and genetic modifications (166-173).

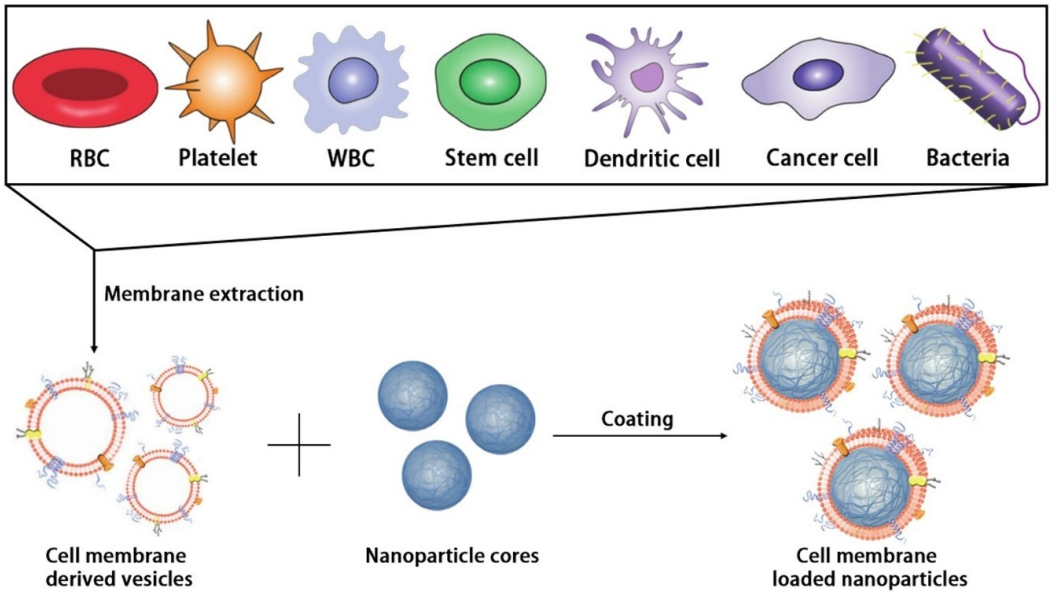



**Figure 15**: Different cell membranes for encapsulating nanoparticles (174).

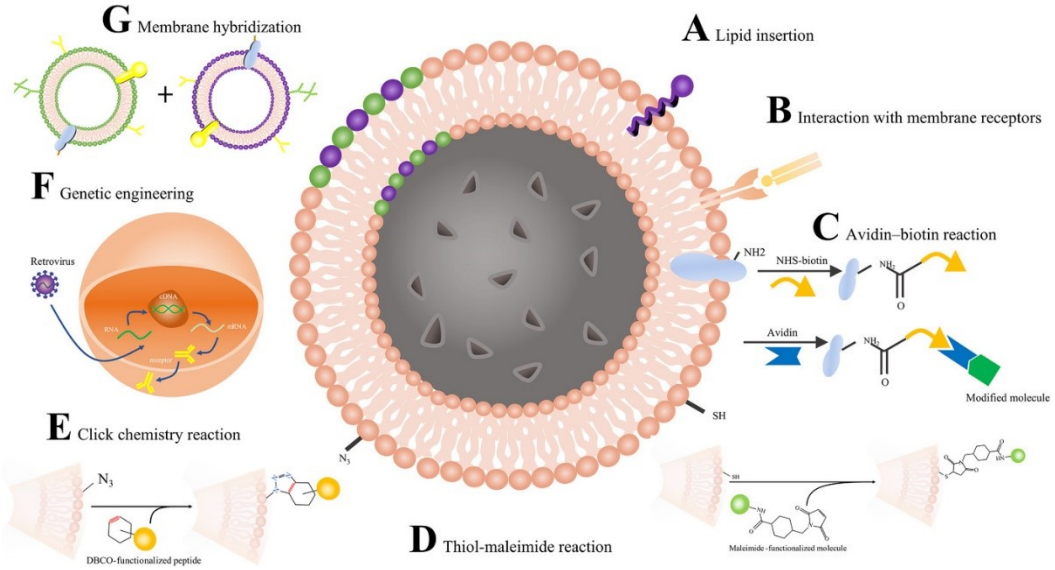



**Figure 16**: Strategies for cell membrane coated nanoparticles (175).

Due to their advantages, cell membrane coated nanoparticles delivery systems are the interest of many researchers and drug delivery

## 2.7 Exosomes in Drug Delivery

### 2.7.1 Introduction

Nanodrug delivery systems have grabbed the attention may researchers in the past decades where many nano-scaled delivery systems have been used to enhance the therapeutic efficiency of therapeutic drugs (176, 177). Nevertheless, cytotoxicity of the material used and the accelerated clearance from the circulation by the reticuloendothelial system and the phagocytic system limits the use of such systems in clinical applications (178), therefore, only few nano-based drug delivery systems have been approved by the FDA for clinical use (179-182).

Many attempts such as PEGylation have been applied to increase the circulation time of nanosized drug delivery systems in the body, however, such applications affect the interaction of the drug with the targeted cell resulting in a decreased the biodistribution of the drug in the tissue (183, 184).

Endogenous based drug delivery systems, like synthetic nano-systems, are promising in drug delivery due to their biocompatibility (185, 186). For instance, exosomes that range in size between 30 to 120 nm, are cell secreted membrane vesicles that function in intracellular communication where they deliver their content from one cell to the other, are one of the promising endogenous drug delivery systems (187).

Exosomes are initially formed as multivesicular bodies that then fuse with the cell membrane to be released out of the cell into the extracellular matrix (188) to allow cellular communication, so cells undergo functional changes or differentiate (189).

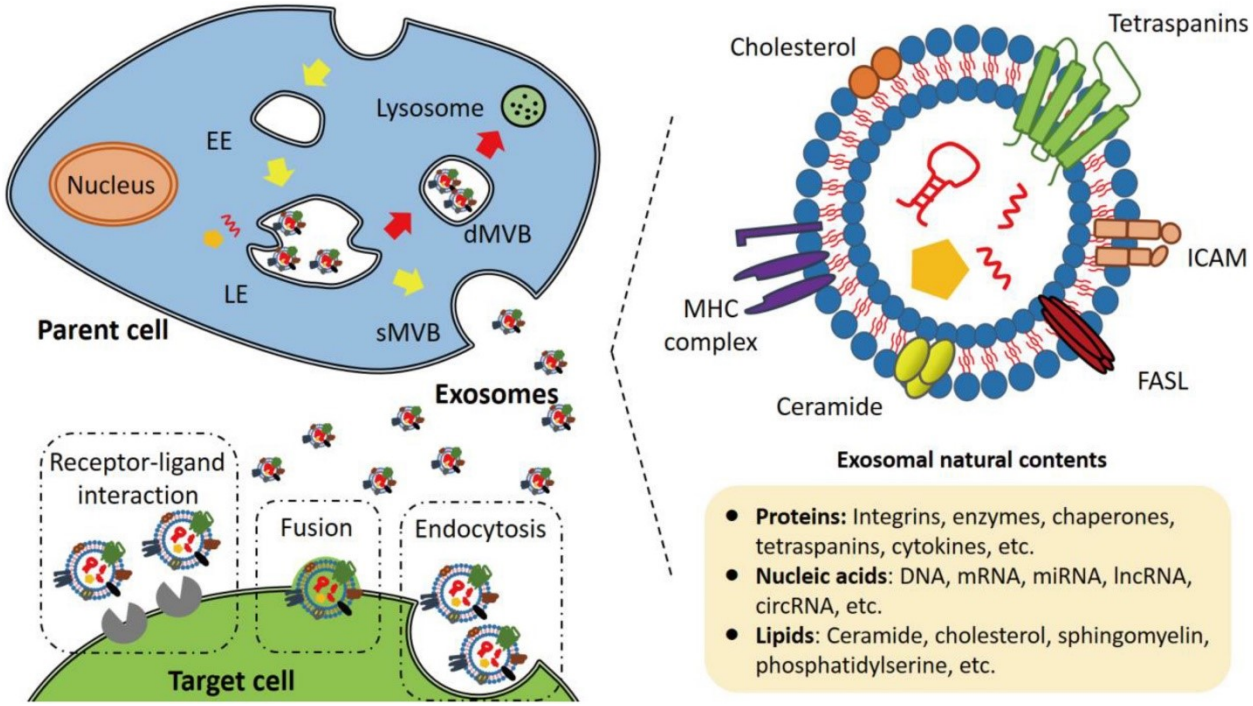



**Figure 17**: The biogenesis, contents, and uptake of exosomes (190).

Exosomes enclose a particular type of mRNA, lipids, regulatory microRNA, and proteins (191), therefore they play an important role in intercellular communication without the need of a direct cell to cell contact (192), as shown in figure 18.

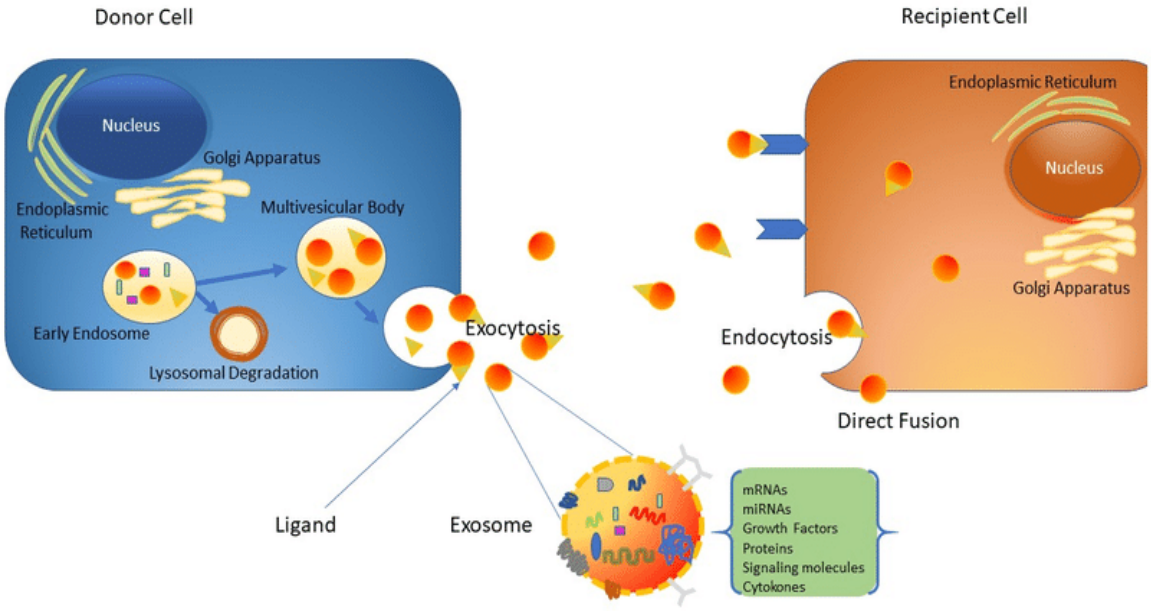



**Figure 18**: Schematic representation of exosome generation, secretion, and cargo transfer from the donor cells to the recipient cells (156).

Several studies have reported the benefits of employing exosomes and nano-carriers (191) since, in addition to their nano-size that allow them to deeply penetrate into tissues, they have a slightly negative zeta potential that allow them to circulate for a long time in the body (192, 193), their cytoskeleton is deformable, and they are similar to the cell membrane (194). Moreover, some exosomes have the ability to escape the immune system where they can circulate without being cleared from the body (195). Therefore, exosomes are considered as natural nano-carriers that are beneficial to be used in clinical applications due to their natural biocompatibility.

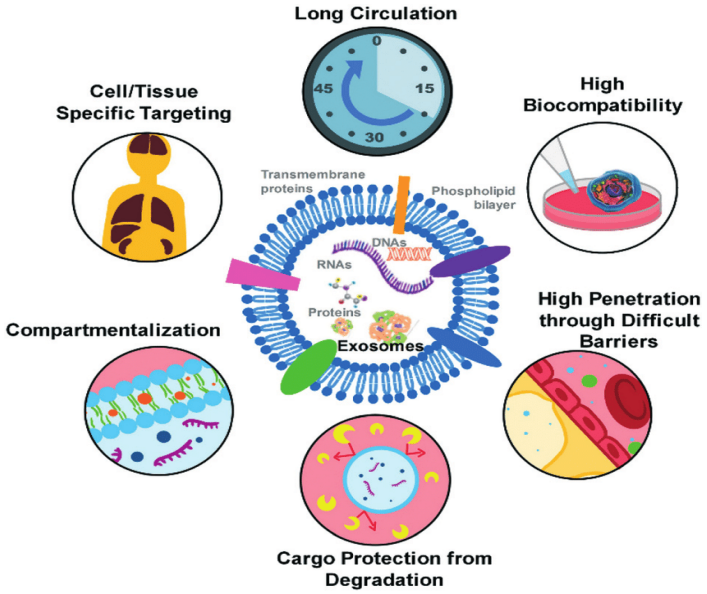



**Figure 19:** Schematic of exosome structures and delivery advancements (196).

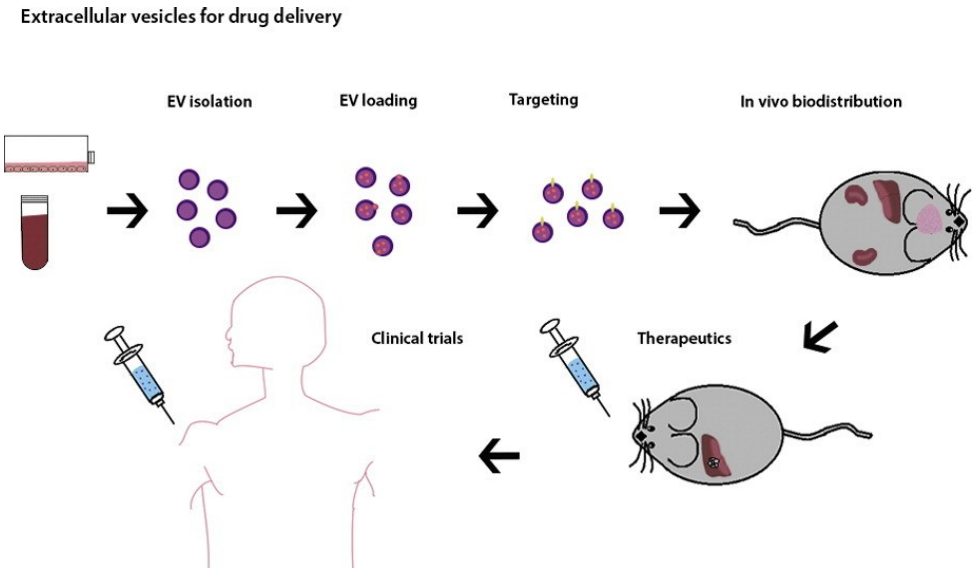



**Figure 20**: Exosomes for drug delivery (197).

Although exosomes are synthesized, under natural conditions, at cellular levels, successful alternations are required (198). When comparing endogenous method that relies on cellular level

and biological approaches, exogenous method results in and enhanced exosomes applications and increase the potential sources of exosomes, thus allow a large production.

After being isolated, exosomes require modification depending on their structural properties and their basic cellular biology in order to enhance their therapeutic and diagnostic abilities. Moreover, exosomes extracted from different types of cells have different physicochemical properties, thus different pharmacokinetics (199). Therefore, since these properties affect the therapeutic effectiveness of exosomes, it is important to properly select the cell from which the exosomes are extracted to be used in a drug delivery system (200-203).

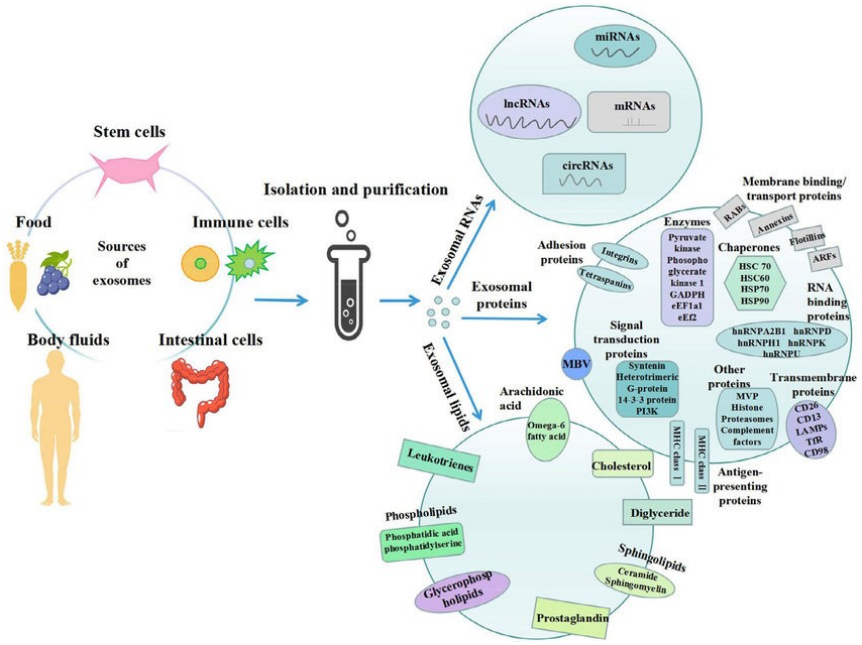



**Figure 21:** Sources and composition of exosomes (204).

### 2.7.2 Engineering Methods for Encapsulation of Therapeutic Agents and Imaging Probes

Exosomes are made of a bilayer of lipid membrane that have the same surface ligands and receptors as the cell of origin. There are two main approaches, active encapsulation and passive encapsulation, to incorporate therapeutic agents into exosomes, and this must be done after understanding the structure of the exosomes and manipulating their function as to be modified

for the therapeutic agent, and each approach results in different loading efficiencies and different stabilities (205).

### 2.7.3 Exosomes for Delivery Therapeutic Agents

Different small hydrophilic and hydrophobic molecules have been encapsulated in exosomes using different loading methods. Most experiments showed that delivering the drugs using exosomes leads to higher accumulation of drug at the targeted cells with an improved stability and a longer circulation time. It was also reported that exosomes could be used to carry therapeutic RNA, therapeutic proteins, and imaging agents that are hard to be delivered in vivo without a carrier (205-206).

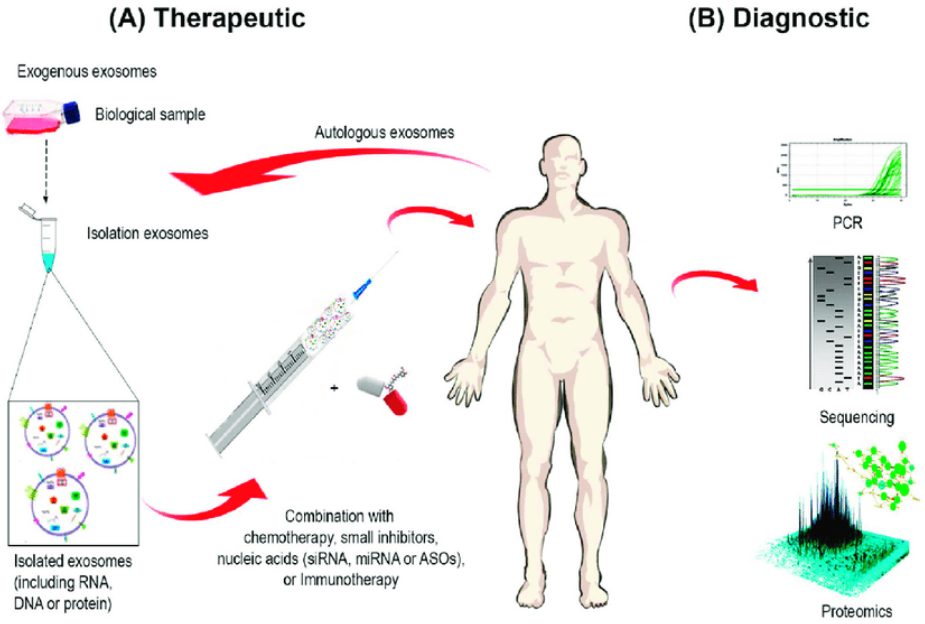



**Figure 22:** Exosome-based diagnostics and therapeutics (207).

### 2.7.4 Exosomes in Ischemic Stroke Treatment

Clinical treatments of ischemic stroke are limited to tissue plasminogen activator and thrombectomy which requires the development of better treatments (208).

Due to their diagnostic and therapeutic abilities, the interest in exosomes as drug delivery systems have been increasing and have gained the attention of stroke research where the underlying therapeutic mechanism of stroke is by transferring the encapsulated molecules such as drugs and microRNA (209, 210).

Ischemic stroke results in an unbalanced ion distribution, metabolism failure, and a reduction in the protein production in the brain cells, thus resulting in an oxidative stress, inflammatory response, and the disruption of the blood brain barrier and increase the brain injury. As a result, the neurons and endothelial cells at the stroke site dye (211, 212).

Moreover, during the recovery time, a repair process is initiated during which recovery takes place via steps such as angiogenesis, neurogenesis, and synaptogenesis, in addition to the formation of new neurovascular units that partly replace the injured tissues (213, 214).

The activation of the immune system and the inflammatory process that take place as a result of an ischemic stroke, in addition to the inflammatory factors and cellular components that get released from dead cells, are factors that accelerate the damage of the blood brain barrier and increase the injury (215).

The blood brain barrier is mainly formed by endothelial cells and tight junctions (216) and it mediates the exchange of molecules and cellular communications between the brain and the peripheral blood, thus sustains the homeostasis of the cerebral microenvironment (217). Ischemic stroke affects the permeability of the blood brain barrier and damages it, thus allowing toxic molecules to reach the brain and result in a secondary injury (218). One week after the ischemic stroke, the blood brain barrier starts to repair gradually after the inflammatory factors start decreasing and the growth factors start increasing (219).

When ischemic stroke occurs, cells such as endothelial cells and neurons undergo necrosis in the ischemic site (212, 219)

#### 2.7.4.1 Biogenesis of Exosomes

Exosomes transport proteins, nucleic acid, and lipids between cells or from a cell to the extracellular matrix (220).

Since exosomes are nano-sized particles and non- toxic, they can cross the blood brain barrier easily without inducing an immune response the fact that make exosomes ideal for treating ischemic stroke and for post injury regeneration, therefore, engineered exosomes that have specific properties are promising for treating ischemic stroke (221).

When an ischemic stroke occurs, brain cells release exosomes that have a profile that differs from that of regular exosomes which allows them to cross the blood brain barrier into the cerebrospinal fluid and the peripheral blood (222, 223). Moreover, as a response to the stroke occurrence, endothelial cells and blood cells also release exosomes. Research has studied the changes that happen to the contents of exosomes, such as proteins and nucleic acids, during stroke.

Some studies have been done to evaluate the changes that occur to the exosomal contents during circulation, such as proteins and nucleic acid and it was suggested that when stroke occurs, the exosomal profile changes where some of these changes could worsen the stroke and increase the risk of other strokes, while others could be helpful as diagnostic tools and recovery (224, 225). For instance, some studies have shown the presence of certain exosomal miRNA in patients with strokes suggesting that this could be used as a potential marker for diagnosing ischemic stroke and distinguishing between its different phases. Other studies have shown that some exosomal miRNA play a role in protecting neurons against apoptosis (226, 227).

### 2.7.4.2 Sources of Exosomes

For the purpose of stroke treatment, data showed that exosomes derived from mesenchymal stem cells, neural stem cells, and adipose stem cells, astrocytes, endothelial cells, and microglia have the ability to reduce the infract size, decrease neural death, and repair the brain (228- 229).

**Table 4**: Exosomes as therapy agents in the treatment of ischemic stroke (230).

| Source | Disease model | Contents | potential target | Assessment standards |
|---|---|---|---|---|
| BM-MSCs | Photothrombosis model in mice | miR-124 | Gli3 and STAT3 in ischemic tissue | Immunohistochemistry of Sox2, Nestin and DCX |
| miR-133b$^{+}$ MSCs | MCAO in rats and OGD model in Primary Astrocyte | miR-133b | Astrocytes | A foot-fault test and a modified mNSS test. Immunohistochemical staining in the IBZ |
| miR-133b$^{+}$ MSCs | MCAO in rats | miR-133b | Connective tissue growth factor and ras homolog gene family member A in the IBZ | The adhesive-removal test and foot-fault test for rats |
| miR-17-92$^{+}$ MSCs | MCAO in rats | miR-17-92 | PTEN Akt, mTOR and GSK-3β | A mNSS and foot-fault tests, histochemistry, immunohistochemistry and Golgi-Cox staining in the IBZ |
| CDCs | Rabbit small-clot embolic stroke model | miR-146a, miR-181b, and miR-126 | Superoxide dismutase-2, | Clinical rating scores and quantal analysis |
| miR-30d-5p$^{+}$ ADSCs | OGD and murine models of MCAO | miR-30d-5p | Microglial | Immunofluorescence and luciferase reporter assay |

**Table 5**: Native exosomes for ischemic stroke (231).

| Exosomes used | Animal model | Target | Results |
|---|---|---|---|
| M2-Exo | Mouse | Stroke treatment | M2-Exo treatment improve neuronal survival, reduce infract volume, improve behavior deficit, Downregulated USP-14 Gene |
| MSCs-Exo | Mouse model/ *in vitro* | Hypoxic ischemia | Exosome treatment decreases neuron apoptosis and neuroinflammation while upregulating miR-21a-5p levels |
| MSCs-Exo | Rats | Stroke recovery | Promote neuroplasticity, functional recovery, inhibiting PTEN expression and upregulation of Exo-miR-17-92 |
| hMSCs-Exo | Mouse Model of EAE | Treat MS | Reduce demyelination, decrease neuroinflammation, upregulated CD4 + CD25 + FOXP + Tregs |
| hMSCs-sEVs | Mouse | Ischemic stroke | Neuroprotective by depleting PMNs, e.g., monocytes, lymphocytes, and reverse post ischemic lymphopenia. |
| EPC-Exo | Mouse | Ischemic stroke | Decreases infract volume, NDS, apoptosis, Upregulate microvessel density miR-126, BDNF, p-TrkB/TrkB and p-Akt/Akt |
| MSCs-Exo | Monkey | Cortical injury | Motor function recovery and neurological dysfunction recovery by shifting inflammatory microglia toward anti-inflammatory. |
| MSCs-Exo | Rats/*in vitro* | Brain Ischemia | Improved motor function, anti-inflammatory cytokines, neurotrophic factors, learning and memory abilities, Downregulated CysLT2R expression and ERK1/2 phosphorylation. |
| MSCs-Exo | Rats | Stroke treatment | Neuritis remolding and Functional recovery, downregulation of connective tissue growth factor and upregulation of mir-133b after exosomes treatment |
| MSCs-Exo | Mice/*in vitro* | Brain Hypoxic ischemia | Exosome treatment confers neuroprotection by reducing neuronal apoptosis and neuroinflammation, upregulated miR-21a-5p and its target gene was Timp3. |
| MSCs-Exo | Rats/*in vitro* | Ischemic stroke | Exosome treatment increased angiogenesis and neurogenesis in a stroke rat model |

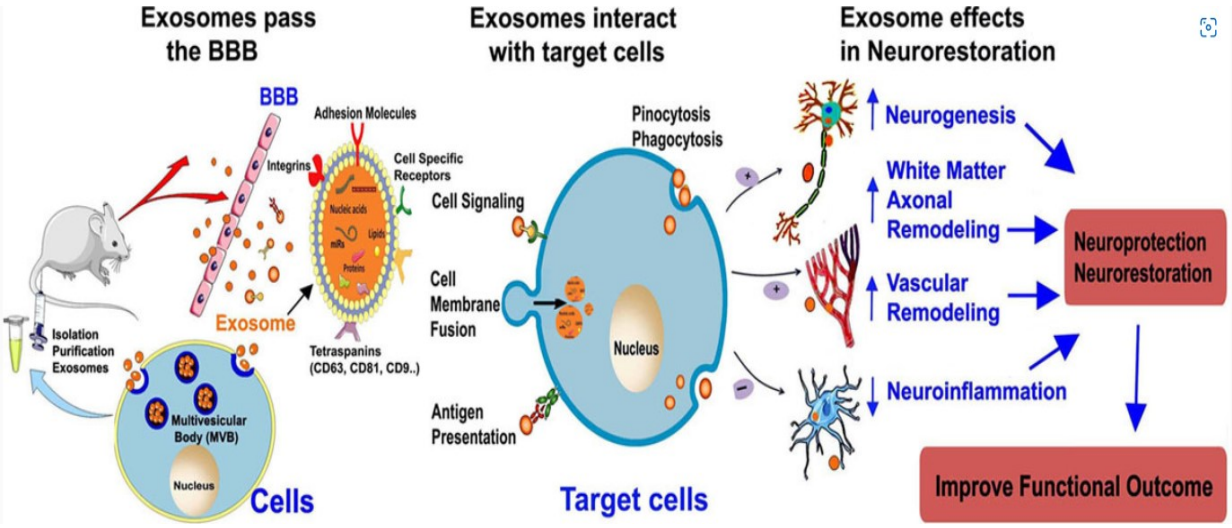



**Figure 23:** Summary of the therapeutic effects of exosome in stroke (232).

## 2.8 Reactive Oxygen Species and its Role in Ischemic Stroke

### 2.8.1 Introduction

According to the World Health Organization, over 15 million people a year, equating to one in every 400 people, suffer a stroke worldwide. The preponderance of people affected are over the age of 65 years, but all ages are at a risk of having a stroke, including children and infants (233). Stroke is a leading cause of mortality after heart disease, equating to 9% of total deaths each year. It is the most common cause of long-term disability, with up to 40% of stroke patients not expected to recover independence (234). Ischemic stroke accounts for approximately 80–85% of all cases, with approximately 30% of the former undergoing hemorrhagic transformation (235).

Research suggests that oxidative stress is related to an excess formation of reactive oxygen species, known as ROS, and this is a basic system that leads to brain damage in stroke. Oxidative stress is a condition that occurs when the cells are exposed to an extremely high levels of oxygen or its chemical derivatives, the ROS, such that the cells lack the ability to counterbalance the high amount of oxygen using antioxidants. Moreover, oxidative stress has been linked to the propagation and pathogenesis of several diseases such as atherosclerosis and stroke (236).

Oxidative stress has an overwhelming effect in the pathogenesis of stroke due to the brain being highly sensitive to the damaged that is induced by the ROS. The brain sensitivity to ROS is related to the fact that is has high concentration of peroxidizable lipids, limited levels of protective antioxidants, high levels of iron, high levels of oxygen consumption, and other oxidation reactions (237).

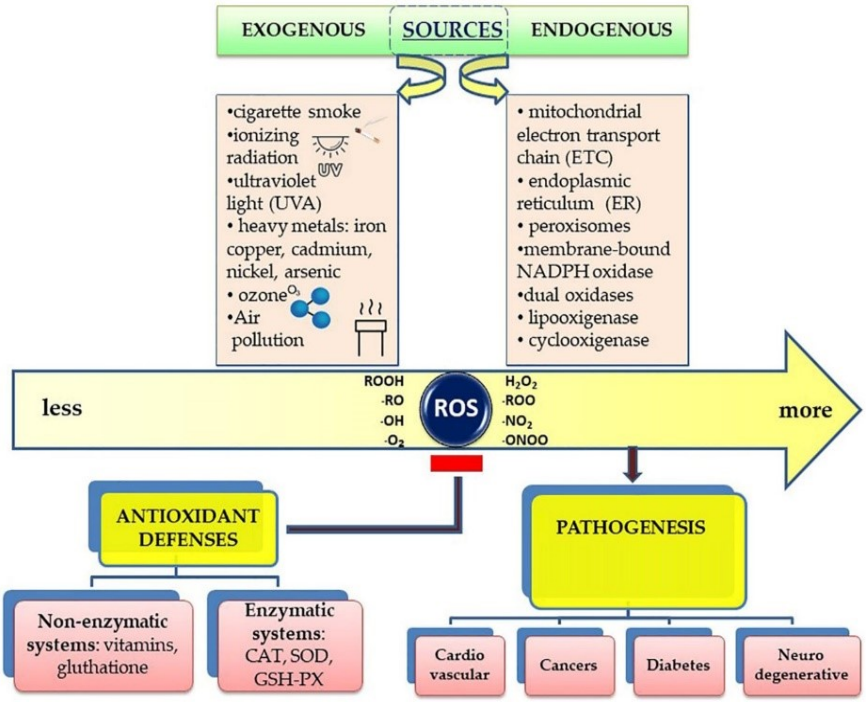



**Figure 24**: Schematic presentation of the sources of free radicals and their effects on the human body (238).

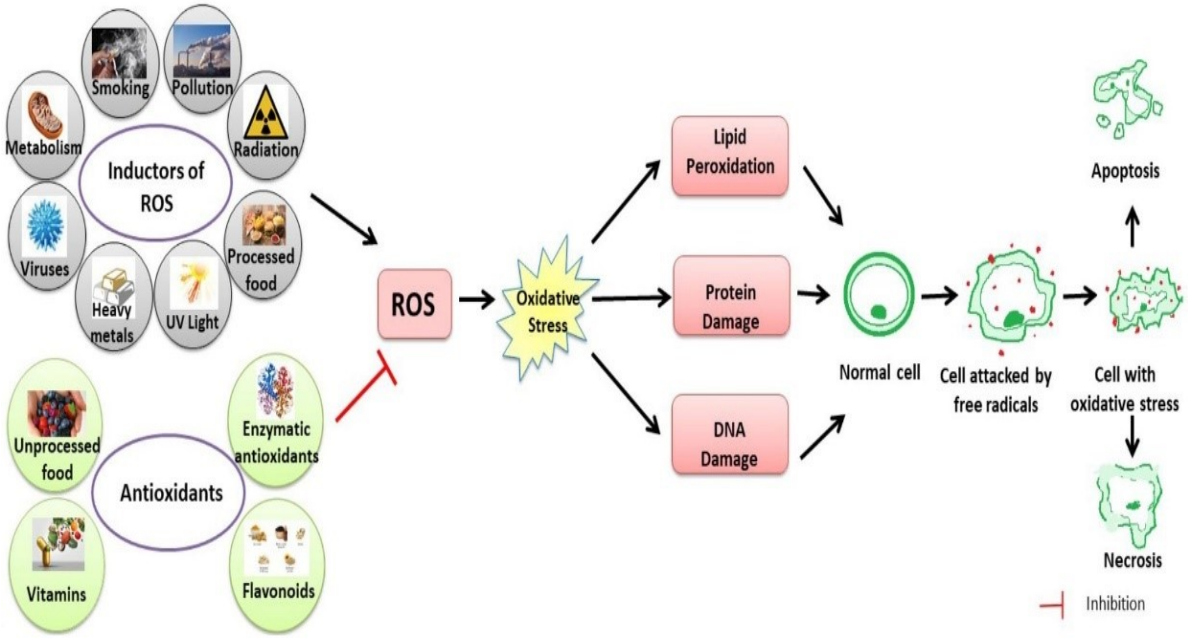



**Figure 25**: Schematic representation of the link between ROS, oxidative stress, and their effects on the human body (239).

Ischemic stroke results form an acute decrease in blood flow and oxygen in cerebral arteries. As a result, ischemia affects two regions, a core where neurons undergo immediate death, and an outer stratum that, although has an unaffected structure, its functions decreases or ceases (240).

Moreover, when an ischemic stroke occurs, the decreases in energy results in lactic acid builds up in neurons thus causing acidosis. Therefore, the acidic environment stimulates a pro-oxidant effect through an increase in the concentration of hydrogen ions which increases that rate at which superoxide anion is converted to hydrogen peroxide ($H_2O_2$) and hydroperoxyl radical ($HO_2$), that is a more reactive species (241).

In addition, ischemia results in several events through different pathways that stimulated the production of ROS. The functions of neurons get affected by the decreases in adenosine triphosphate (ATP) since during ischemia, the decreases in oxygen levels and glucoses substrate prevents the production of ATP resulting in a decrease in energy inactive ion pumps that depend on ATP, de-polarize the membranes, and disturbs the transmembrane ion gradients. As a result, $Ca^{2+}$ gets translocated from the extracellular environment to the intracellular environment resulting in the activation of lipases and cellular proteases in addition to the breakage of cerebral tissues (242).

Reactive oxygen species are the natural byproducts of the metabolism of oxygen, and they include free radicals, oxygen ions, and other peroxides. These species are characterized by their high reactivity since they have unpaired electrons, and when biological stress occurs, they increase to toxic levels that damage the cells (243).

Nevertheless, other that its negative side effects, ROS could play useful roles in cellular signaling, immune defense, apoptosis, and in the breakage of toxic material (244).

Peroxide, $O_2^-$, is an essential ROS that leads to the formation of $H_2O_2$ by dismutation. $O_2^-$ is synthesized in tissues through enzymatic reactions, and it could be obtained from the auto-oxidation of molecules such as hemoglobin and myoglobin, and the oxidation of unsaturated fatty acids (245).

When inflammation occurs, immune cells engulf debris and foreign bodies. This process requires high levels of oxygen thus produces high levels of ROS including $O_2^-$.

ROS play important roles in the immune system where they activate T-cells and stimulate the production of IL-2 (246).

The homeostasis of oxygen is maintained by red blood cells and respiration. moreover, studies have shown that changes in oxygen level are detected by ROS producing proteins cataroid bodies that sense the changes in ROS rates in the mitochondria (247). Moreover, ROS have been proven to play a role as a messenger that regulate some proteins such as tyrosine kinases and a role in apoptosis. $H_2O_2$, for instance, play a major role in some major pathological and physiological stimuli such as inflammatory cytokines and growth factors (248).

Reactive oxygen species play important roles in normal brain physiology. For example, ROS regulate signals from neurons in the central and peripheral nervous system (249). Moreover, ROS, including peroxide and hydrogen peroxide, play a role in modulating the synaptic and the non-synaptic communication between the neurons and the glia. When the neural activity is high, ROS diffuse to myelin sheath of oligodendrocytes resulting in the activation of the protein kinase, that is a structural component of myelin (250).

Moreover, ROS initiates synaptic long-term potential that is needed for the formation of long-term memory. A study showed that the overexpressing of superoxide dismutase improved the learning that depends on the cerebellum and spatial learning that depends on the hippocampus, in transgenic aged mice. Moreover, it was observed that $O_2^-$ plays a role in learning and the formation of memory (251).

### 2.8.2 ROS in Ischemic Stroke

As stated earlier, ROS have substantial effects on cells that result in cellular death and other tissue destruction such as peroxidation of lipids, denaturation of protein, inactivation of enzymes, damage of DNA and nucleic acid, the release of calcium ions form cells, and a damage to the cytoskeleton. These effects result from the excess concentration on ROS to a point that it could not be counterbalanced by antioxidants (252-253). Moreover, the increased level of ROS affects

cellular signaling, blood vascular tone, platelets, and the permeability of endothelial cells which further damages the BBB (254- 256).

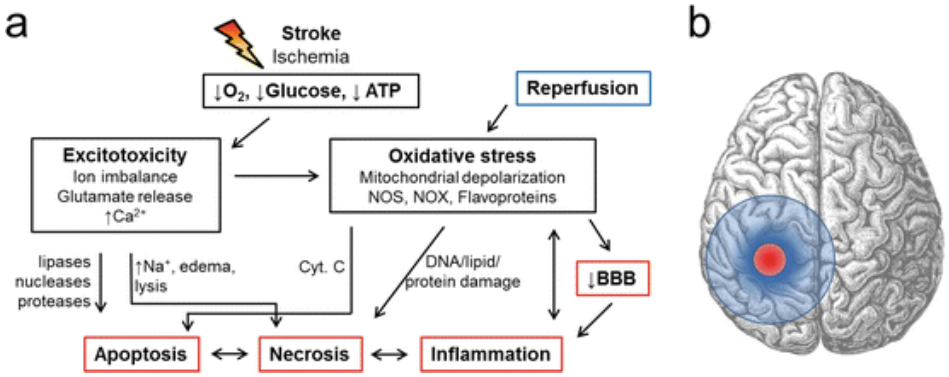



**Figure 26**: Targeting oxidative stress in stroke (257).

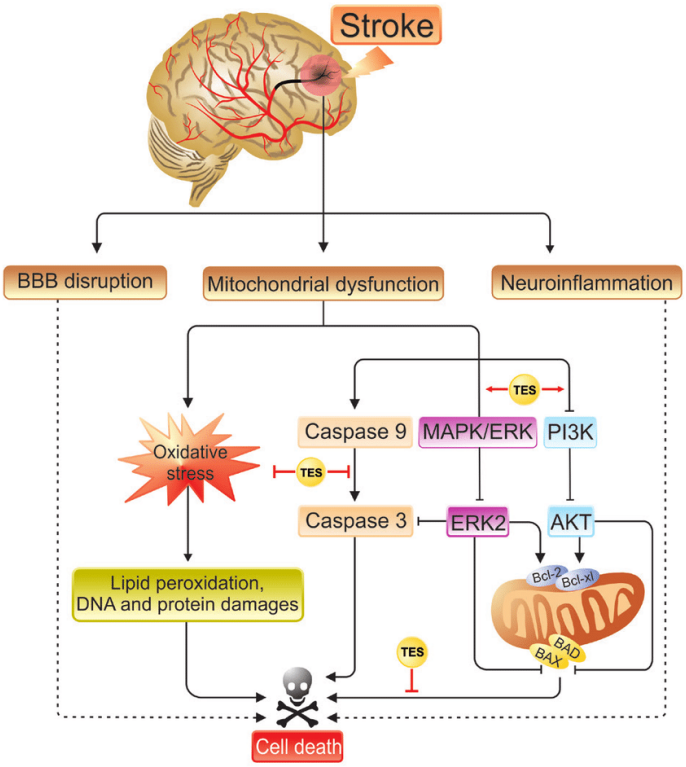



**Figure 27:** Ischemic stroke triggering cascades of complex events resulting in neuronal death in affected area (258).

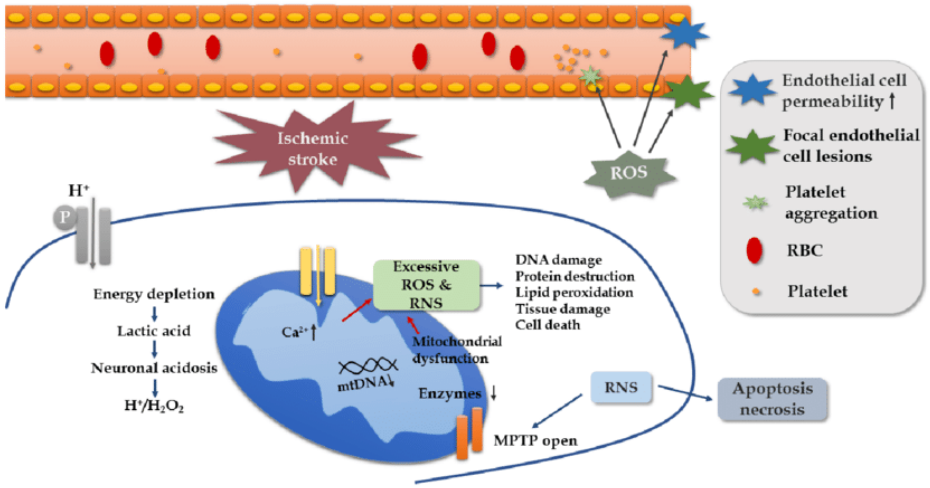



**Figure 28:** Mechanism of oxidative stress in ischemic stroke (259).

### 2.8.3 ROS and Tissue Injury Post-Ischemic Stroke

When reoxygenation occurs, oxidative stress increases rapidly along with an increase in the non-enzymatic reactions that occur in the cytosol and other cellular organelles. The elevation in ROS is said to result from mitochondrial dysfunction, stimulation of certain receptors such as *N*-methyl-D-aspartate, and the free fatty acids (260).

Following an ischemic stroke, the release of glutamate is used to determine the tissue injury. The stimulation of $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors then release $O_2^-$ that then react with NO ending up producing $ONOO^-$. Therefore, it has been suggested that inhibiting $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors could be an effective target for preventing the release of ROS during an ischemic stroke (261).

High $O_2^-$ that is observed when an ischemic stroke occurs as a result to variations in the mitochondrial electron transport functions have been proven to significantly increases the lesion size caused by an ischemic stroke (262).

In addition, the $Ca^{2+}$-dependent enzymes phospholipase $A_2$ and cyclooxygenase produce ROS when subjected to ischemic or re-perfused tissues by regulating protein synthesis and activate

proteins transcriptionally that are highly damaging (263). Studies done on animal using cerebral micro-dialysis showed great release of dopamine that result in cytotoxicity and necrosis of neurons via the production of quinones and ROS that generate lipid peroxidation and lead to protein denaturation (264).

It has been proven that ROS substantially increase the ischemic injury in the brain although that the mechanism by which this contribution occurs and the time during which the ROS get generated during ischemia are unclear. It is believed that an increase in the production of ROS by the mitochondria occurs with reoxygenation that could be blocked by NAD(P)H oxidase inhibitor. This shows that ROS contributes to neural death and promote injury (265).

McCann et al proved that NAD(P)H mediates oxidative stress in rats with endothelial induced stroke where it promotes brain damage beyond the core of ischemia via the activation of catalytic subunits that, along with the levels of $O_2^-$, were significantly high six hours after the occurrence of the stroke and remained high up to seven days (266).

The production of ROS and the time they get produces are believed to depend on the expression of NAD(P)H oxidase, the mitochondrial depolarization rate, the rate of reduction of ATP, and cellular type (267).

It is hard to directly measure ROS in human brain due to their short life. Therefore, biological markers have been used to study the role of oxidative stress in stroke (268).

It has been reported that two days after cerebral ischemia, vitamin C decreases (269). A study showed that high levels of vitamin C improved endothelium-dependent vasodilation in patients with coronary artery diseases by decreasing the production of $O_2^-$ and increasing the level of NO activation, thus decreasing the risk of ischemic stroke (270).

Studies have shown that deficiencies of B vitamins increase the levels of homocysteine, that is similar in structure to the amino acid cysteine and functions in degrading the amino acids cysteine and lysine thus affecting the structure and functions of proteins. Moreover, it was found that lower levels of homocysteine are associated with a decreased risk of stroke (271).

In addition, it was observed that malondialdehyde and HNE, that are by-products of lipid peroxidation, are elevated in patients with ischemic stroke and are related to an increase in the size of infract size and the stroke severity (272).

A study done by Polidori et al showed that the levels of cholesteryl ester hydroperoxide that is the product of an oxidized low-density lipoprotein (LDL) significantly increases after one day of an ischemic stroke and remains elevated for up to ten days (273). Further studies have reported that patients with cerebral infractions have high levels of oxidized (LDL) that is suggested to be oxidized by phospholipids that are released from brain tissues. Therefore, oxidized LDL levels reflect the oxidative stress in a body (274).

When lipids undergo free radical damage, F2-isoprostanes markers are produced as a result of the peroxidation of arachidonic acid (AA). Following peroxidation, F2-isoprostanes are esterified in phospholipids, broken down, and then released into the circulation before being excreted in urine. Some studies have shown that levels of F2-isoprostanes markers, specifically isopentane 8-isoprostaglandin-F-2 (8-iso-PGF-2), increase few days after an ischemic stroke, while other studies do not report this increase (275). In addition, as an antioxidant, levels of vitamin C have been reported to be inversely linked to markers 8-iso-PGF-2 of oxidative stress in stroke patients (276).

Other markers that are considered as biomarkers for oxidative stress in stroke is vitamin A where De Keyser et al showed that high levels of vitamin Ain stroke patients were associated with less severe outcomes and less mortality (277).

### 2.8.4 Potential Therapies and Clinical Trials Using Antioxidants

Till now, acute ischemic stroke could only be clinically treated with thrombolytic agents, however, as stated earlier, the treatment time window and the application of such procedure is limited to less than 5% of patients (278).

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, known as stains, work by lowering the production of cholesterol via increasing the receptors for LDL while decreasing the levels of LDL in the body. Stains have been prescribed to reduce the risk of

morbidity in patients with coronary artery disease. In addition, stains have antioxidant effects that lower the levels of cholesterol by inhibiting isoprenylated proteins such as NAD(P)H, which is a neuroprotective ability in patients with diseases such as ischemic stroke. Inhibiting NAD(P)H oxidase system decreases the catalytic activity of ROS. Moreover, stains decrease leukocyte- induced LDL oxidation while increasing the ratio of $\alpha$-tocopherol to cholesterol and retain the HDL-paraoxonase enzymatic system that get used during the oxidation of HDL (279).

Nevertheless, stains could promote an inflammatory response and an oxidative damage by increasing the production of $ONOO^-$ (280).

Angiotensin-converting enzyme **(**ACE) inhibitors that initiate an inflammatory response at the vascular walls and induce the release of substances that cause vasodilation. Moreover, Angiotensin-converting enzyme inhibitors stimulate the production of NO and prevent the formation of $O_2^-$, $H_2O_2$, and $ONOO^-$ (281). Studies have shown that Angiotensin-converting enzyme inhibitors reduce the risk of stroke by 32% and the risk of death by 61% (282).

Angiotensin receptor blockers are used to treat diseases such as hypertension. A study showed that Angiotensin receptor blockers reduces non-fatal stroke by 28% and total stroke by 24% (283).

In the past years, free radical agents such as edavarone and tirilazad, in addition to a trapping agent known as disufenton have gained the research attention.

For example, the oxygen radical edaravone inhibits peroxidation was proven to be successful in treating patients with ischemic stroke within a window of 72 hours, and the improvement remained three months post treatment (284).

On the other hand, clinical trials that were done on patients with acute stroke didn't show that tirilazad has any effect on the infract volume or on the outcome although animal studies showed successful results, therefore, it was suggested that the dose that was successful in animal was not successful in humans (285).

High levels of ROS play an important role in neural injury that is caused by stroke. Beside their negative side effects, low concentrations of ROS play a role as signaling molecules and regulate the cerebrovascular structure and its tone. Therefore, balanced levels of ROS are needed for normal physiological functions and to inhibit mechanisms that lead to necrosis and apoptosis following an ischemic stroke. Till now, there isn't a specific ROS- targeted stroke therapies where a successful therapy would involve the therapeutic agent to be administered at the time of reperfusion and to maintain a normal level of ROS that don't affect the cerebrovascular structure while maintaining other physiological functions (286-287).

## 2.9 Conclusion

To close, nanotechnology is a promising field in solving major obstacles in treating ischemic stroke. Nevertheless, the development of an effective therapeutic agent requires a clear understanding of brain cells that play a role in the pathology of an ischemic stroke. Good results have been reported in animal studies and clinical trials, when using nanotechnology, and more issues must be overcome to translate such nanomedicine into an effective clinical therapy which requires further research.

Moreover, treating an ischemic stroke depends on many factors including the therapeutic window, the blood brain barrier, and the degree of damage. Nanotechnology promises to cross these barriers by offering an effective stroke treatment using nanomaterials that allow proper diagnosis and therapy allowing a proper targeting ability while having a desired release rate and a long circulation time. Although some challenges exist, the ability to improve and modify nanomaterial has been helping nanotechnology in paving the way toward a better ischemic stroke treatment.

# Chapter 3: Methods and Materials

## 3.1 Materials

Plasmin was purchased from Molecular innovations, and tPA from UM-Ann Arbor medical school. All other chemicals were purchased form Sigma Aldrich and Thermo Fisher Scientific.

## 3.2 Cell Culture

HBMVEC cells were cultured in M199 medium supplemented in 10% FBS and 5% antibiotic and antimycotic.

## 3.3 Cell Membrane Extraction

HBMVEC cells were resuspended in 3ml distilled pbs and centrifuged at 500 x g for 10 minutes. After aspirating the supernatant, the pellet was resuspended in 1 ml tris-KCl-MgCl2 and grinded 20 times then centrifuged at 3200 x g for 5 minutes without discarding the supernatant after being aspirated (this step was done twice). Supernatants were finally combined and centrifuged for 25 min at 17000 x g. the final pellet was resuspended in 100 ul tris-Edta.

## 3.4 PLGA Nanoparticles Preparation

Standard precipitation method was used to prepare nanoparticles. Briefly, PLGA polymer was dissolved in DMF then dripped into stirring water at 0.125 ml/min and the mixture was covered with a holed parafilm, and after 24 hourrs, the mixture was centrifuged and the particles were resuspended in water and stored at 4°C until used.

### 3.5 PLGA- plasmin Reaction

400 µl of 0.3 mg/ml of PLGA nanoparticles were mixed with 3ul of 100 mM of EDC and 3ul of 100 mM NHS and stirred at 4ºC for 20 minutes before adding 10 µM plasmin. After 24 hours, the mixture was centrifuged, and the particles were resuspended in HEPES pH 7.4.

### 3.6 Coating Cell Membrane with PLGA- plasmin Nanoparticles

PlGA-plasmin nanoparticles was mixed with cell membrane at desired ratios (1:1, 1:2, and 1:4) and the mixture was sonicated on ice for 5 minutes. The coated particles were then stored at -80 $^{0}$C until use.

### 3.7 Plasmin Activity Assay

A calibration curve was created by adding 50 µl of 5 different plasmin concentrations, with a 100% being 10 µM, to 50 µl of 2 µM plasmin substrate. 50 µl of PLGA-plasmin nanoparticles and 50 µl of cell membrane coated PLGA-plasmin nanoparticles were also added to 50 µl of 2 µM plasmin substrate, each in a separate well. The activity was then read at 370- 440 nm and plasmin concentration in the samples was calculated based on the calibration curve.

### 3.8 Exosome Extraction

Exosomes from HBMVEC were extracted using ExoQuick-TC Isolation kit according to the manufacturer's protocol.

### 3.9 Exosomes- tPA Reaction

A mixture of 20 µl (1 mg/ml) of tpA 200 µl 0f 50 ug/ml exosomes, 2 µl of 100 mM EDC, and 3 µl of 100 mM NHS centrifuged after being stirred overnight. The particles were then resuspended in 220 µl HEPES pH 7.5.

### 3.10 tPA Activity Assay

A calibration curve was created by adding 50 µl of 5 different tPA concentrations to 50 µl of 2.5 µM tPA substrate. 50 µl of exosomes-tPA particles were also added to 50ul of 2.5 µM tPA substrate. The activity was then read at 370- 440 nm and plasmin concentration in the samples was calculated based on the calibration curve.

### 3.11 Exosomes- catalase Reaction

A mixture of 40 µl (2mg/ml) of catalase, 200 µl 0f 50 ug/ml exosomes, 2 µl of 100 mM EDC, and 3ul of 100 mM NHS centrifuged after being stirred overnight. The particles were then resuspended in 240 µl HEPES pH 7.5.

### 3.12 Exosomes-catalase-tPA Reaction

A mixture of 40 µl of catalase (2mg/ml), 20 µl of tPA (1mg/ml), 200 µl 0f 50 µg/ml exosomes, 2 µl of 100 mM EDC, and 3ul of 100 mM NHS centrifuged after being stirred overnight. The particles were then resuspended in 260 µl HEPES pH 7.5.

### 3.13 Protein Assay

The protein assay was used to create a calibration curve and measure the concentration of cell membrane and exosomes, in addition to measuring the conjugation of catalase in exo-catalase and exo-catalase-tPA, and it was prepared according to the manufacturer's protocol.

### 3.14 Fibrin Clot

Fibrin clots were prepared by mixing 480 µl of 1mg/ml fibrinogen, 6 µl of 1 M $CaCl_2$, 2 µl rhodamine, and 120 µl of 100 units/ml thrombin at $37^0$C for 24 hours after which the clots were washed by pbs and treated with the desired particles.

### 3.15 Cell Toxicity

Toxicity of plasmin, PLGA-plasmin nanoparticles, cell membrane coated PLGA-plasmin nanoparticles, tPA, exosomes, exosomes-tPA, catalase, exosomes-catalase, exosomes-catalase-$H_2O_2$ on HBMVEC was tested by culturing HBMVEC cells in 96 wells plates at a cell density of 10,000 cells/ 100ul media for 24 hours before being treated with desired concentrations of the mentioned particles. The toxicity was then analyzed by measuring the absorbance of alamarBlue at 570- 590 nm excitation and emission. Concentrations used: 1. 1ug/ ml and 2ug/ml plasmin; 2. 10ug/ml and 20ug/ml cell membrane coated PLGA-plasmin nanoparticles; 3. 1ug/ ml and 2ug/ml tPA; 4. 10ug/ml and 20ug/ml exo-tPA; 5. 25 µM exosomes-catalase; 6. 25 µM catalase; 7. 25 µM exosomes-catalase-tPA; 8. 50 µM and 100 µM $H_2O_2$.

### 3.16 ROS

The $H_2$DCFDA assay was used to measure the oxidative stress associated with an increase in the production of reactive oxygen species caused by the addition to plasmin, PLGA-plasmin nanoparticles, cell membrane coated PLGA-plasmin nanoparticles, tPA, exosomes, exosomes-tPA, catalase, exosomes-catalase, exosomes-catalase-$H_2O_2$ to HBMVEC. HBMVEC cells were cultured in 96 wells plates at a cell density of 10,000 cells/ 100ul media for 24 hours before being treated with desired concentrations of the mentioned particles. After 24 hours of treating the cells, the media was replaced by $H_2$DCFDA for 30 minutes then ROS was analyzed by measuring the absorbance at 495- 526 nm excitation and emission. Concentrations used: 1. 1ug/ ml and 2ug/ml plasmin; 2. 10ug/ml and 20ug/ml cell membrane coated PLGA-plasmin nanoparticles; 3. 1ug/ ml and 2ug/ml tPA; 4. 10ug/ml and 20ug/ml exo-tPA; 5. 25 µM exosomes-catalase; 6. 25 µM catalase; 7. 25 µM exosomes-catalase-tPA; 8. 50 µM and100 µM $H_2O$.

# Chapter 4: Cell Membrane Coated PLGA-plasmin Nanoparticles

## 4.1 Results and Discussion

In this chapter, we studied the effect of using cell membrane to coat PLGA-plasmin nanoparticles as compared to non-coated PLGA-plasmin nanoparticles in retaining and enhancing the activity of plasmin.

First, after measuring the concentration of the extracted cell membrane, that is 75.03 µg/ml ± 1.83, the size of the cell membrane, PLGA nanoparticles, and cell membrane coated PLGA-plasmin nanoparticles were measured using DLS.

According to the table below, the average size of the cell membrane is 210 nm that is greater than the average size of the nanoparticles which is 144 nm. However, coating the nanoparticle with the cell membrane didn't have a great effect on the size of the nanoparticles were it only increased by 21.4 nm to become 161.44 nm which is highly acceptable as the cell membrane coated nanoparticles remained within the desired nano scale.

**Table 6:** Size of cell membrane, nanoparticles, and coated nanoparticles.

| | Cell membrane | nanoparticles | Coated nanoparticles |
|---|---|---|---|
| Average size ± SE (nm) | 210 ±29.17 | 144.7± 25.6 | 161.44 ± 51.2 |

*SE: Standard Error

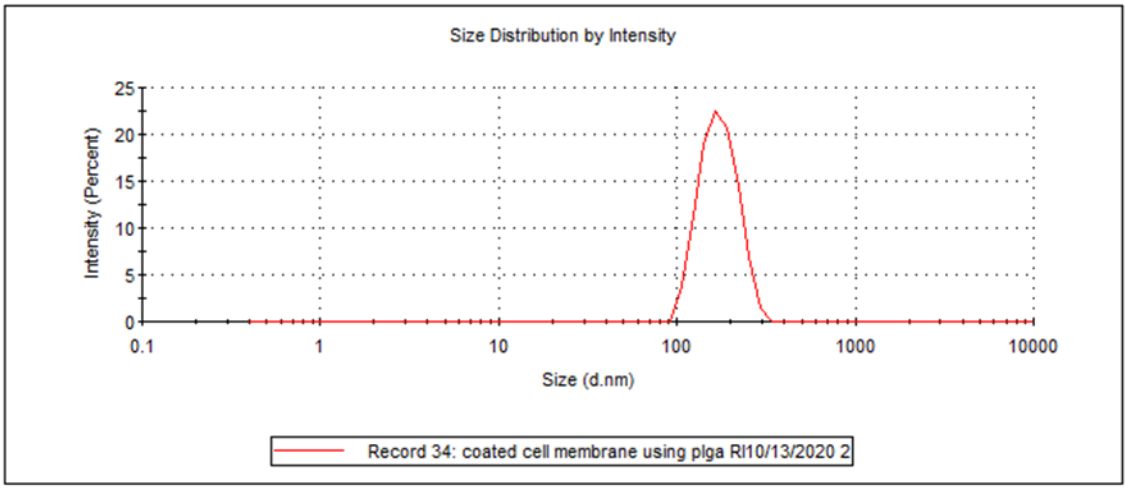



**Figure 29:** Size distribution of cell membgrane coated PLGA-plasmin nanoparticles.

To ensure that the PLGA-plasmin nanoparticles were being coated with the cell membrane, we used the flow cytometry, as an additional characterization method, that showed a difference between the non-coated particles and the coated particles. This is shown in then figure below.

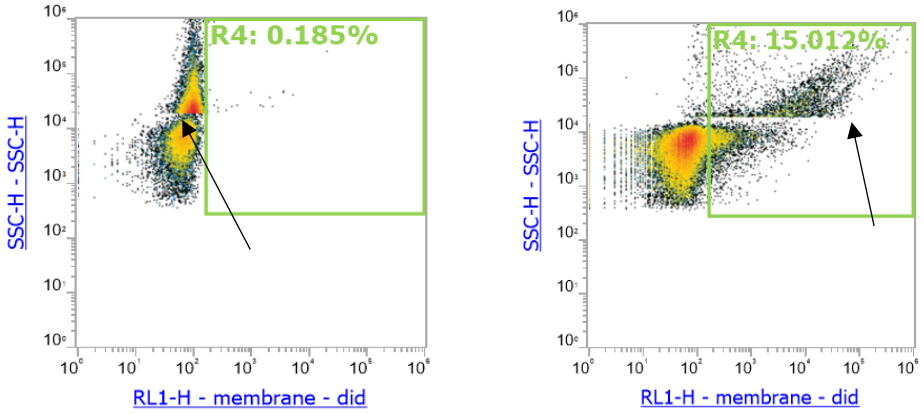



**Figure 30:** Flow cytometry characterization of coated and non-coated PLGA-plasmin nanoparticles. It is obvious that the particles are being coated since the image shows a difference between the coated particles and the non-coated ones.

After measuring the size of the particles, we performed plasmin activity assay in order to determine the amount of plasmin in the coated nanoparticles, and this was determined at 30

minutes using the calibration curve, and this showed that the coated PLGA-plasmin nanoparticles had an average of 30.5% of the added plasmin. Following the plasmin activity assay, anti-plasmin activity assay was performed to determine the effect of antiplasmin on plasmin in both, coated PLGA-plasmin nanoparticles and non-coated pure plasmin. The results showed that the activity of plasmin in the coated PLGA-plasmin nanoparticles was not affected by the antiplasmin, meanwhile the activity the non-coated plasmin was affected. From the figures below, it is obvious that when comparing the activity of plasmin in the presence of antiplasmin, in both coated and non-coated, it was around 10% in the non-coated plasmin whereas it was over 80% in the cell membrane coated nanoparticles. In other words, antiplasmin had a great effect on ceasing the activity of plasmin when the latter was not accompanied by cell membrane coated nanoparticles, however, using cell membrane to coat the PLGA-plasmin nanoparticles was successful in preventing the effect of antiplasmin on the activity of plasmin.

The experiment was also conducted on different cell membrane to nanoparticles ratio to determine whether the ratio has an effect on the results where three different ratios were examined being 1:1, 1:2, and 1:4. Results showed that the ratio has no effect on the results and this is shown in the figures below where in all three cases, the activity of plasmin in the cell membrane coated nanoparticles was retained over 80%.

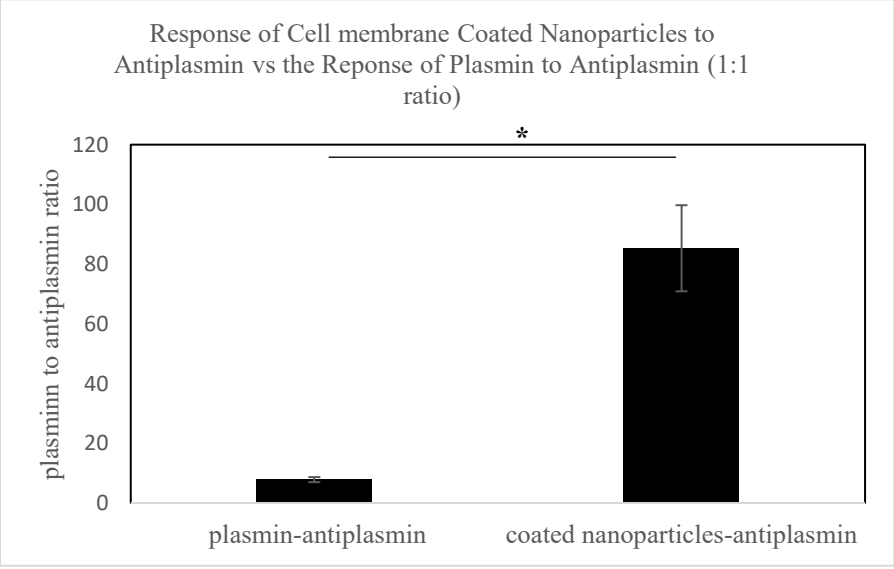



**Figure 31:** Response of cell membrane coated nanoparticles to antiplasmin vs the reponse of plasmin to antiplasmin (1:1 ratio). Data with significance is represented as * $p<0.05$.

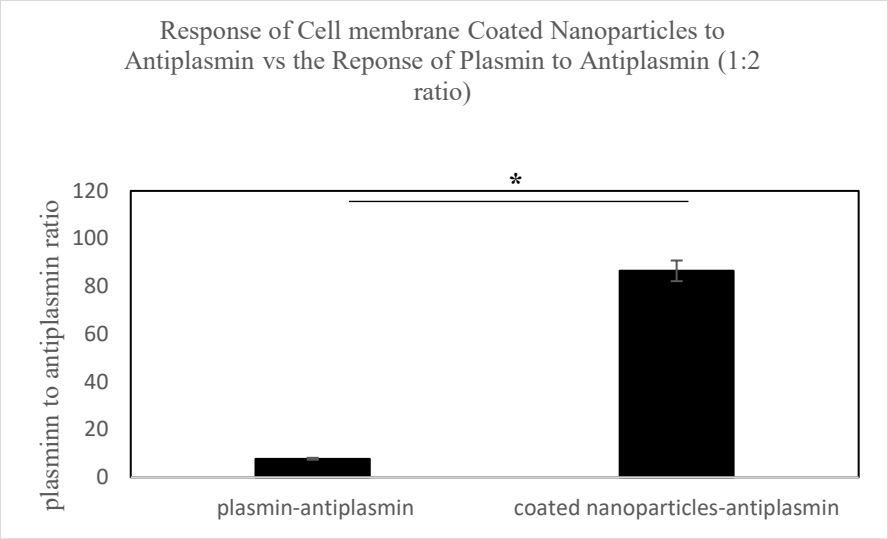



**Figure 32:** Response of cell membrane coated nanoparticles to antiplasmin vs the response of plasmin to antiplasmin (1:2 ratio).

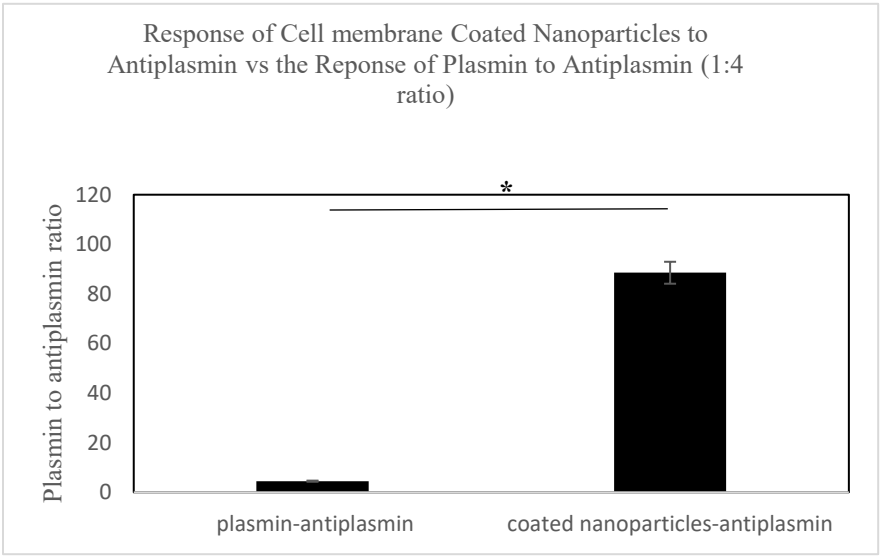



**Figure 33:** Response of cell membrane coated nanoparticles to antiplasmin vs the response of plasmin to antiplasmin (1:4 ratio).

Next, the stability of plasmin in the cell membrane coated nanoparticles was determined by performing a plasmin activity assay and reporting the results at 3 hours and at 24 hours after being incubated at $37^0$ C. It was realized that the stability of plasmin starts to decrease with time

where it decreases from its initial concentration at 100% to reach 67% at three hours and further decreases to reach around 18.5% after 24 hours. On the other hand, the activity of plasmin in the cell membrane coated nanoparticles remained increasing from 100% at 30 minutes till it reached 112% at 3 hours then back to 92% after 24 hours meaning that the activity of plasmin was stable and retained even with time in the coated nanoparticles whereas stability of plasmin was not observed in the non-coated plasmin.

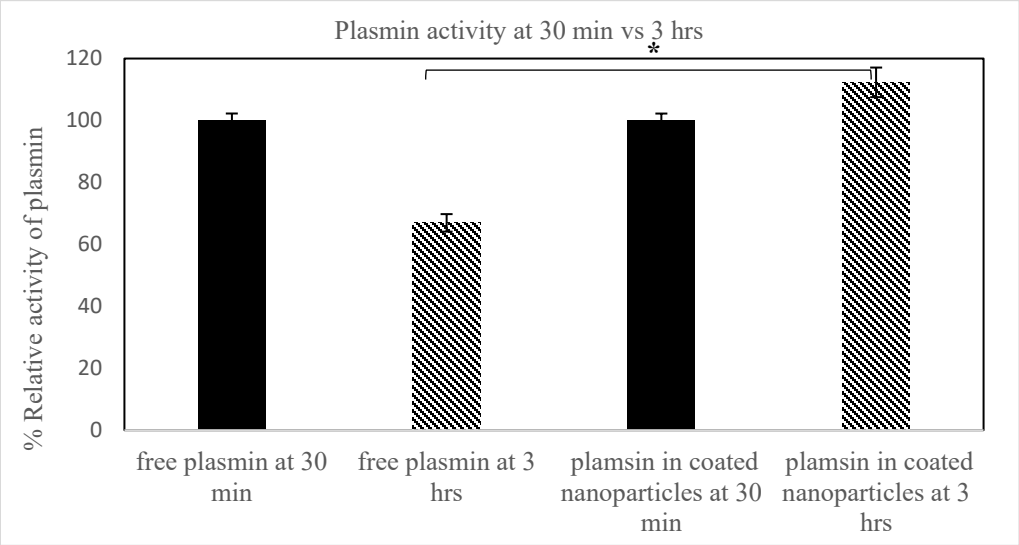



**Figure 34:** Relative activity of plasmin at 30 minutes vs 3 hours in free plasmin and in cell membrane coated PLGA-plasmin nanoparticles. Data with significance is represented as * $p<0.05$

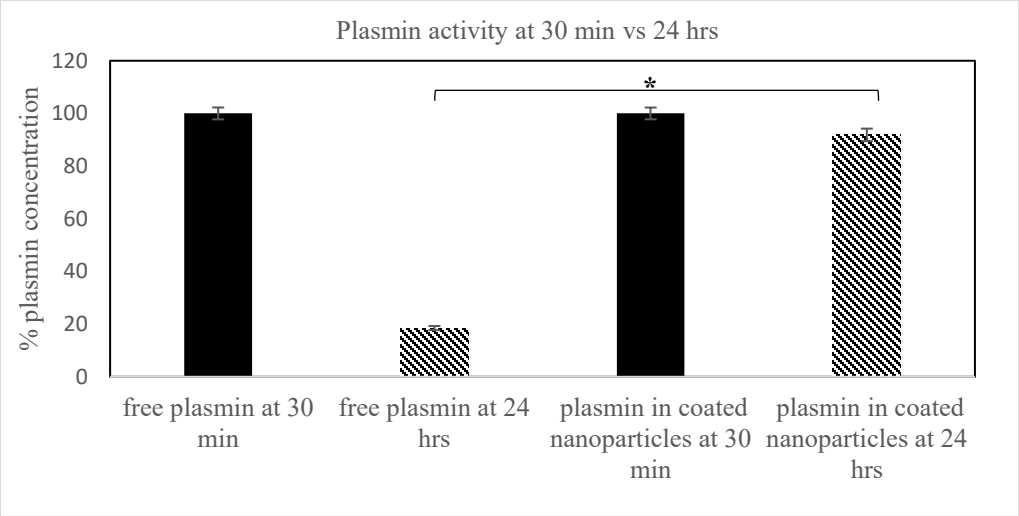



**Figure 35:** Relative activity of plasmin at 30 minutes vs 24 hours in free plasmin and in cell membrane coated PLGA-plasmin nanoparticles. Data with significance is represented as * $p<0.05$.

In order to determine the effectiveness of cell membrane coated PLGA-plasmin nanoparticles on fibrin clots, the clots were prepared, and desired concentration of plasmin, PLGA-plasmin nanoparticles, and anti-plasmin were added to the clots, with a 1:1ratio, with the concentrations being calculated based on plasmin activity assays at 30 minutes. After an incubation time of 24 hours, the rhodamine concentration in the supernatant of the clots was measured using a plate reader. According to the graph below, the amount of rhodamine in the supernatant of the fibrin clots that were treated with plasmin, antiplasmin, and plasminogen was low, around 200%, compared to the concentration of rhodamine in the supernatant of the fibrin clots that were treated with cell membrane coated PLGA-plasmin nanoparticles, antiplasmin, and plasminogen that was around 600% as compared to the control group, that was only treated with HEPES buffer. This shows that the plasmin in the cell membrane coated PLGA-plasmin nanoparticles breaks a fibrin clot effectively, even in the presence of antiplasmin, whereas, when present alone, the activity of plasmin is affected by antiplasmin, thus does not fully dissolve a fibrin clot, therefore, desired results are only achieved by using cell membrane coated PLGA-plasmin nanoparticles.

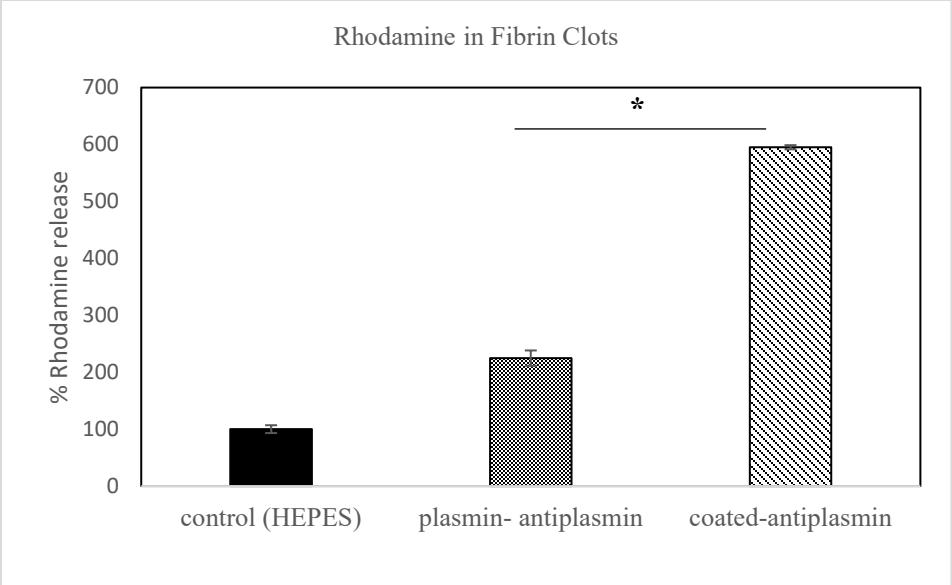



**Figure 36:** Rhodamine release from fibrin clots after being treated with HEPES, plasmin-antiplasmin, and cell membrane coated nanoparticles-antiplasmin. Data with significance is represented as * $p<0.05$.

The figures below show the fibrin clots before and after being treated.

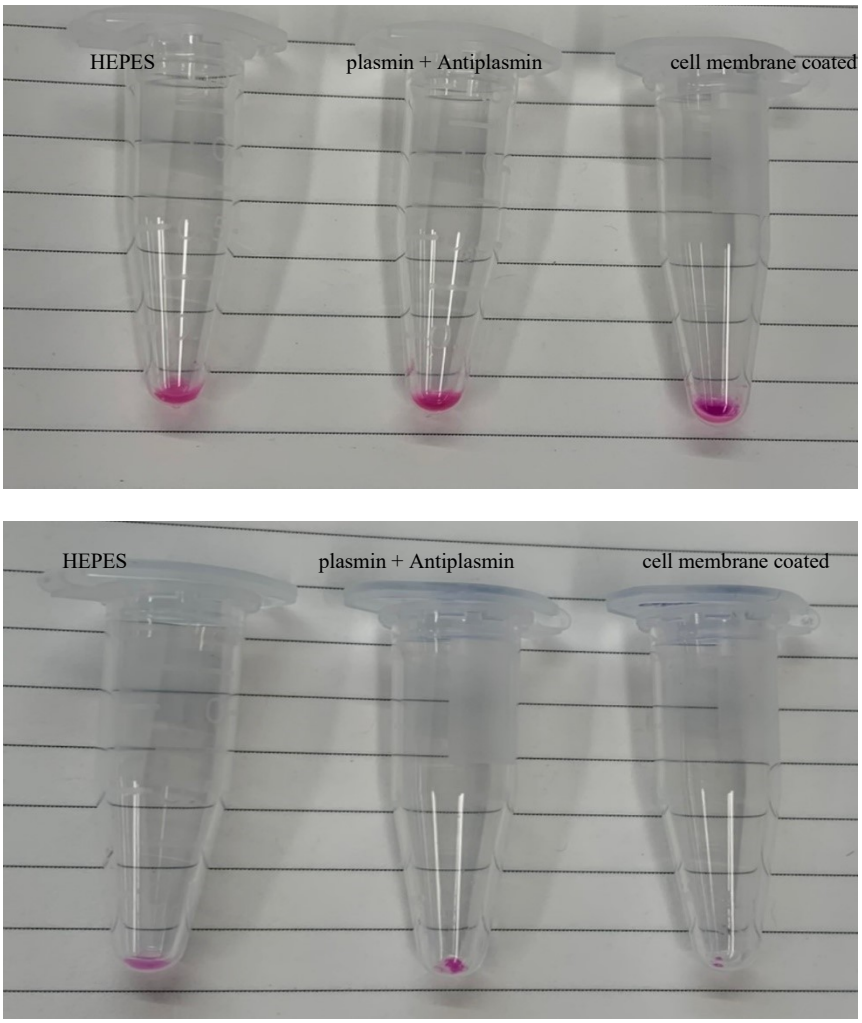



**Figure 37:** Fibrin clots before and after being treated. In the case of HEPES, the clots didn't dissolve or break, in the case of free plasmin, the clot broke down into smaller pieces, and in the case of cell membrane coated PLGA-plasmin nanoparticles, the clot dissolved.

To assess the toxicity effects of cell membrane coated PLGA-plasmin nanoparticles, non-coated PLGA-plasmin nanoparticles, and plasmin, a cellular toxicity study was performed on normal HBMVEC using different concentrations of plasmin. After culturing the cells in a 96 wells plates for 24 hours, they were incubated with cell membrane coated PLGA-plasmin nanoparticles, non-coated PLGA-plasmin nanoparticles, and plasmin for 48 hours after which alamarBlue cell viability reagent was used to determine the viability of the cells by being added directly to the cells that are then incubated at $37^0$C as for the cells to convert resazurin to resorufin, and after an incubation period of two hours, the absorbance was measured using a plate reader. For the figure

below, no significant toxicity was observed from any of the added material as the percentage of viable cells was greater than 90% in all conditions. This means that, PLGA-plasmin nanoparticles, being coated or non-coated with cell membrane, do not exhibit cellular toxicity and could be safely used as therapeutic agents.

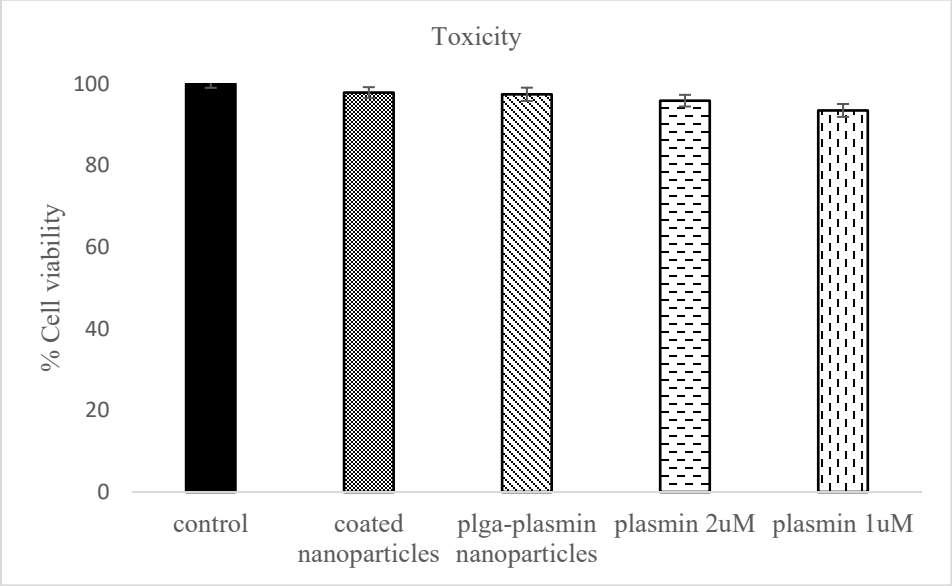



**Figure 38:** Assessment of toxicity effect of cell membrane coated PLGA-plasmin nanoparticles (2 μM), PLGA- plasmin nanoparticles (2 μM), and free plasmin (2μM and 1μM) in human brain microvascular endothelial cells. No significant toxicity observed at the tested conditions.

To determine whether the cell membrane coated PLGA-plasmin nanoparticles induce an increase in the amount of ROS released by the cells, an ROS study was performed using $H_2$DCFDA. After culturing the cells in a 96 wells plates for 24 hours, they were incubated with cell membrane coated PLGA-plasmin nanoparticles, non-coated PLGA-plasmin nanoparticles, and plasmin for 48 hours, the media was removed and substituted by $H_2$DCFDA for 30 minutes after which the absorbance was measured, using a plate reader. It is obvious, by looking at the figure below, that none of the added materials had a negative effect on the cells from the ROS side since in neither case, the relative amount of ROS released by the cells was less than 80%. This means that, PLGA-plasmin nanoparticles, being coated or non-coated with cell membrane, don't increase the ROS secreted by the cells and could be safely used as therapeutic agents.

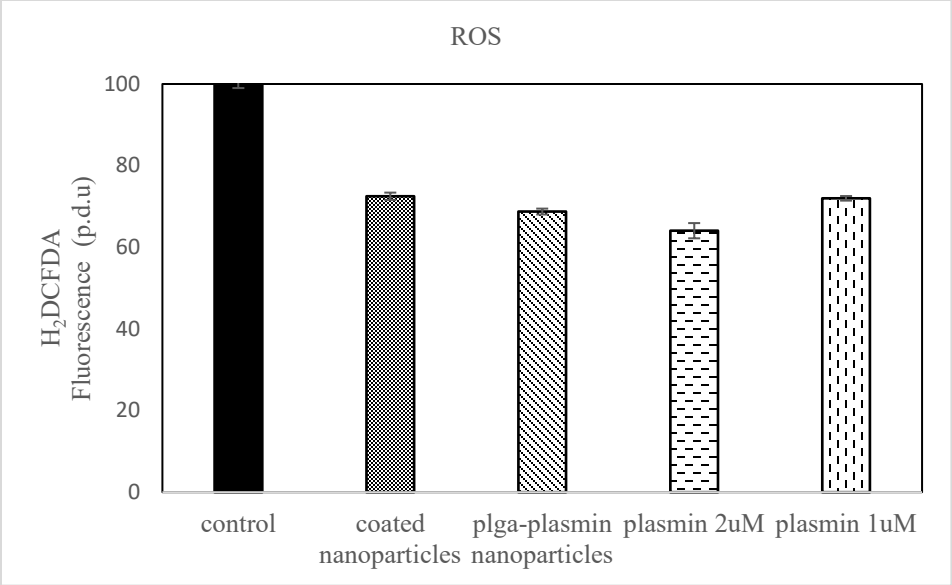



**Figure 39:** Assessment of ROS effect of cell membrane coated PLGA-plasmin nanoparticles (2 μM), PLGA- plasmin nanoparticles (2 μM), and free plasmin (2μM and 1μM) in human brain microvascular endothelial cells. No significant toxicity observed at the tested conditions.

# Chapter 5: Exosomes Encapsulated tPA

## 5.1 Results and Discussion

In this chapter, we studied the effect of using exosomes, of an average concentration of 221.4± 30.73 µg/ml, to encapsulate tissue-plasminogen-activator as compared to free tissue-plasminogen-activator (tPA) in retaining and enhancing the activity of tPA. To do so, first, the size exosomes and exosomes-tPA were measured using DLS.

According to the table below, the average size of exosomes, that is 247.46 nm, increases upon encapsulating tPA to 378.85 nm. However, this increase in size is highly acceptable as the exosome-tPA is still in the desired nano scale.

**Table 7:** Size of exosomes and exo-tPA.

| Particle | Exosomes | Exosomes-tPA |
|---|---|---|
| Average size± SE (nm) | 179.925± 25 | 317.3± 76 |

*SE: Standard Error

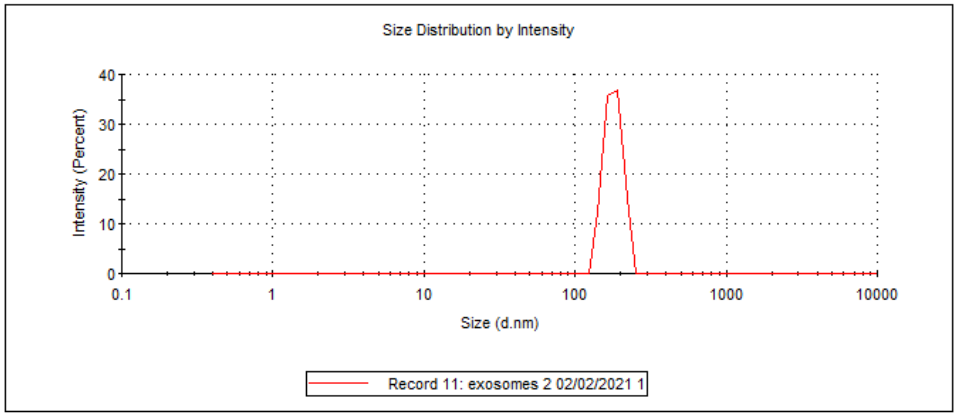



**Figure 40:** Size distribution of exosomes.

To ensure that the exosomes were being extracted properly, we used the flow cytometry, as an additional characterization method, that showed a difference between the beads and exosomes. This is shown in then figure below.

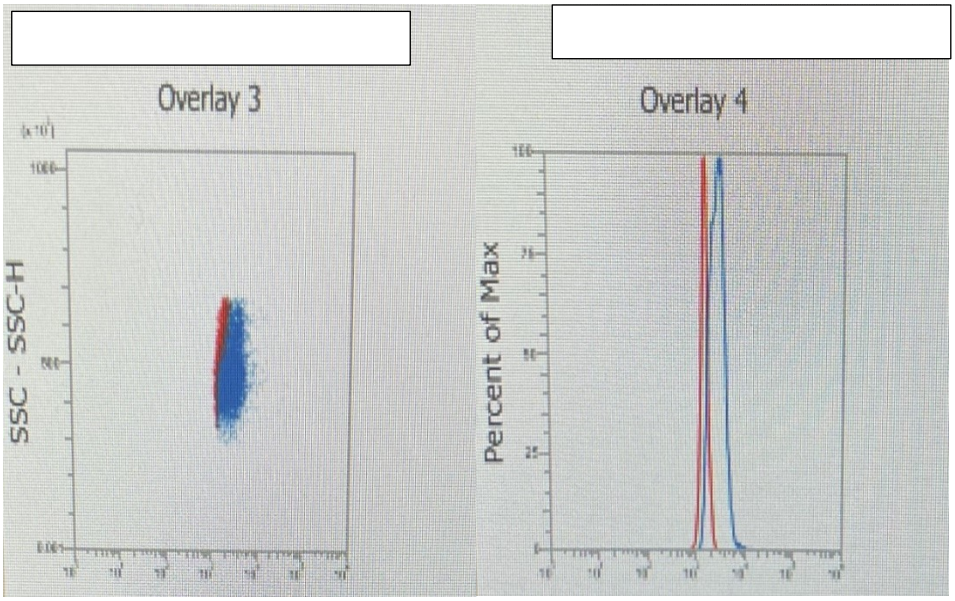



**Figure 41:** Flow cytometry characterization of exosomes. The beads are represented by the red color while the exosomes are represented by the blue color. It is obvious that exosomes are present since, despite the slight overlap, the two curves are separated and, the graph on the left greatly shows a difference between the beads and the exosomes.

Next, we performed tPA activity assay in order to determine the amount of tPA in the exosomes encapsulated tPA, that is referred to as exo-tPA, and this was determined at 30 minutes using the calibration curve to be on average around 40%. Following the tPA activity assay, plasminogen activator inhibitor (PAI) activity assay was performed to determine the effect of PAI on tPA in both, free tPA and exo-tPA. The results showed that the activity of tPA when encapsulated within the exosomes was not affected by the PAI, meanwhile the activity the free tPA was affected. From the figures below, it is obvious that when comparing the activity of both, free tPA and in exo-tPA, it was around 10% in the case of free tPA whereas it was over 50% in exo-tPA. In other words, PAI had a great impact on the activity of tPA when the latter was not encapsulated within exosomes, however, using exosomes to encapsulate tPA was successful in decreasing the negative effect of PAI on the activity of tPA.

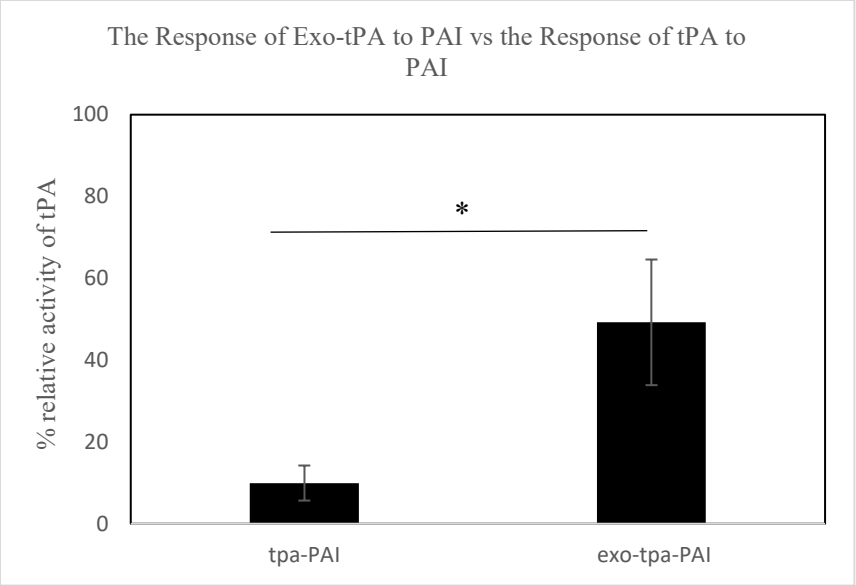



**Figure 42:** Response of free tPA to PAI to antiplasmin vs the reponse of exo-tPA to PAI. Data with significance is represented as * $p<0.05$.

Next, the stability of tPA in exo-tPA was determined by performing a tPA activity assay and reporting the results at 3 hours and at 24 hours after being incubated at $37^0$ C. It was observed that, in the case of free tPA, the stability of tPA starts to decrease with time, at it decreased from its initial concentration at 100% to reach 81% at three hours and further decreases to reach around 16% after 24 hours. On the other hand, the activity of tPA in exo-tPA slightly decreased from 100% at 30 minutes till it reached 96% at 3 hours till it reached 88% after 24 hours, this means that the activity of tPA was stable and was retained even with time in the exo-tPA whereas stability of free tPA was not observed.

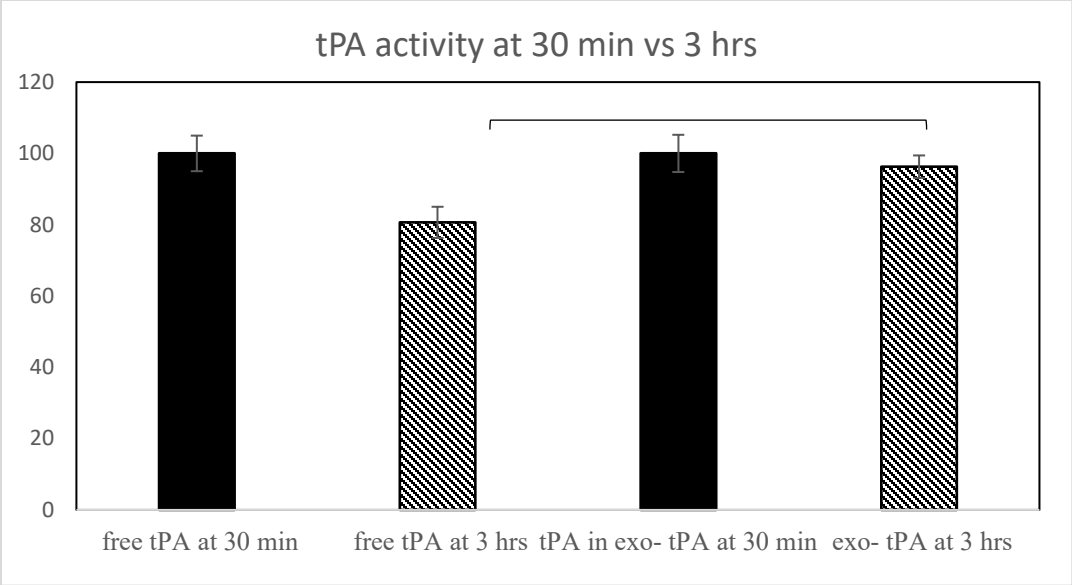



**Figure 43:** Relative activity of tPA at 30 minutes vs 3 hours in free tPA and in exo-tPA. Data with significance is represented as * $p<0.05$.

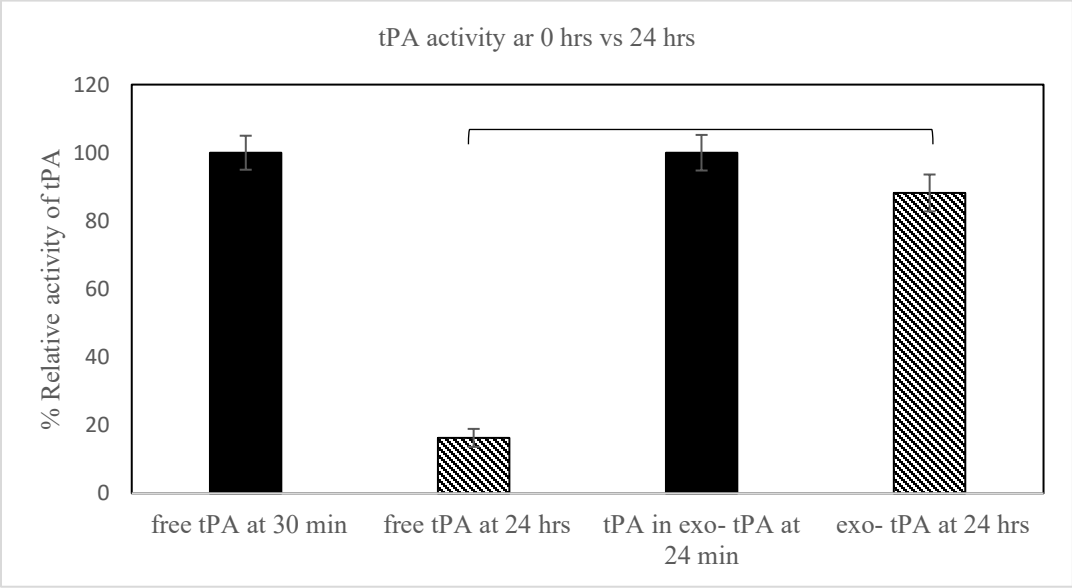



**Figure 44:** Relative activity of tPA at 30 minutes vs 24 hours in free tPA and in exo-tPA. Data with significance is represented as * $p<0.05$.

In order to determine the effectiveness of exo-tPA on fibrin clots, the clots were prepared, and desired concentration of tPA or exo-tPA, along with PAI and plasminogen were added to the clots, with a 1:1:1 ratio, with the concentrations being calculated based on tPA activity assays at

30 minutes. After an incubation time of 24 hours, the rhodamine concentration in the supernatant of the clots was measured using a plate reader. According to the graph below, the amount of rhodamine in the supernatant of the fibrin clots that were treated with tPA, PAI, and plasminogen was low, around 150% as compared to the control group that was treated with HEPES buffer, whereas the concentration of rhodamine in the supernatant of the fibrin clots that were treated with exo-tPA, PAI, and plasminogen that was around 207% as compared to the control group, that was only treated with HEPES buffer. This shows that the tPA in exo-tPA gets successfully activates plasminogen, even in the presence of PAI, and breaks a fibrin clot effectively, whereas, when present alone, the activation of plasminogen by tPA is affected by PAI, thus does not fully dissolve a fibrin clot, therefore, desired results are only achieved by exo-tPA.

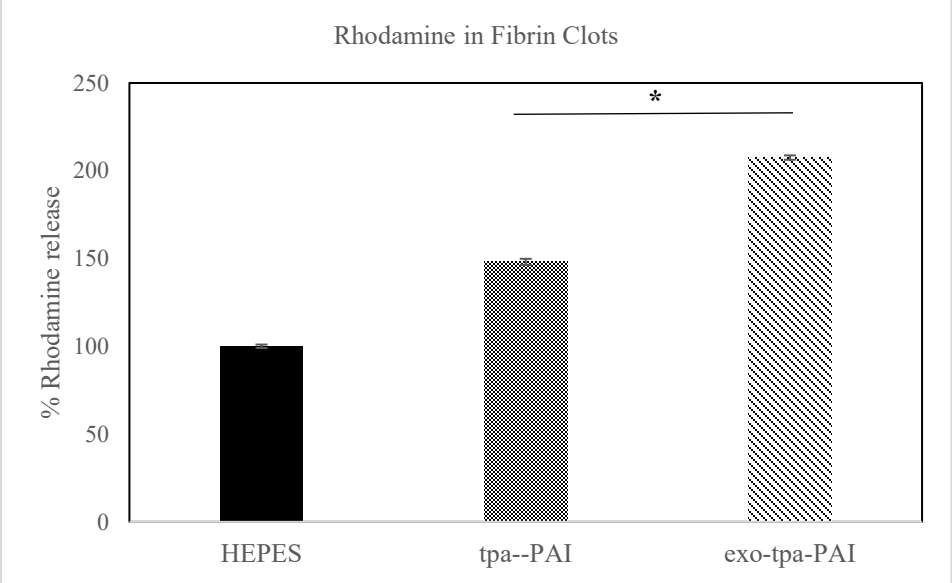



**Figure 45:** Rhodamine release from fibrin clots after being treated with HEPES, tPA-plasminogen-PAI, and exo-tPA- plasminogen- PAI. Data with significance is represented as * $p<0.05$.

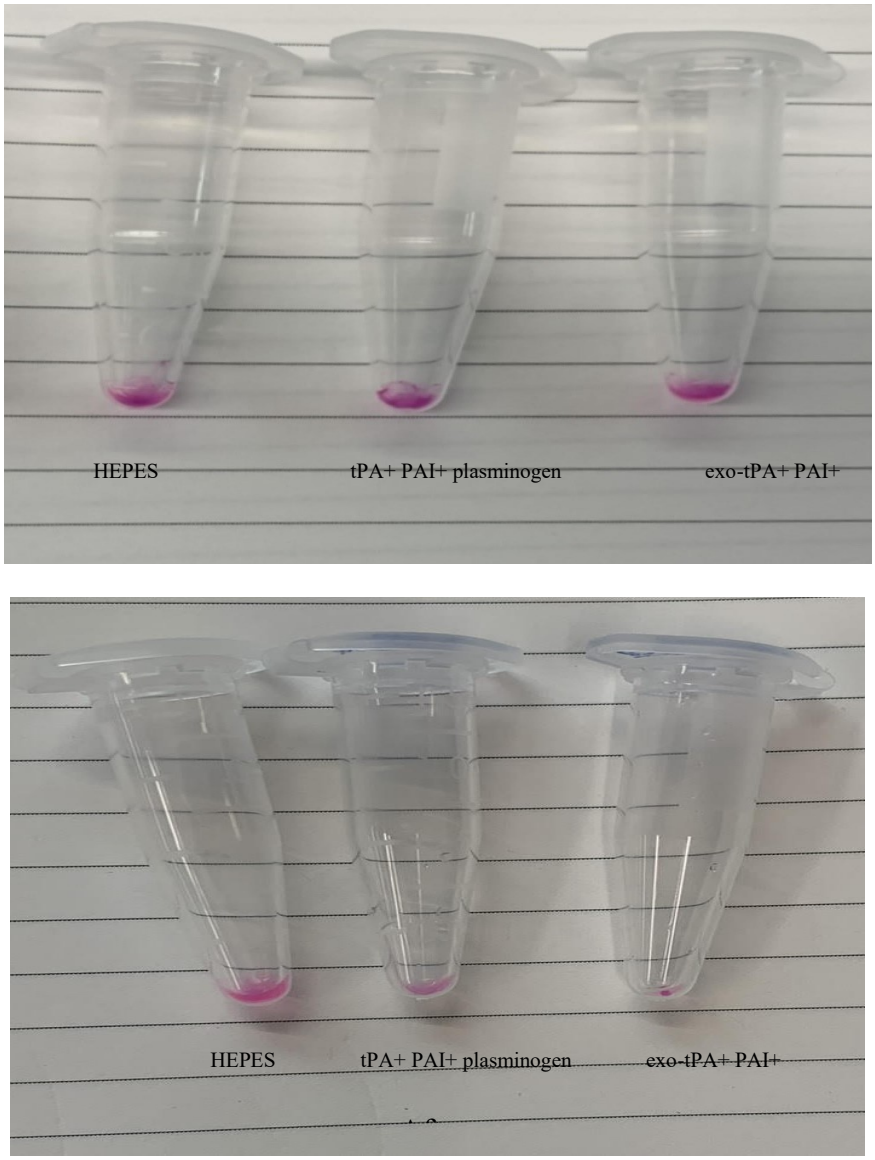



**Figure 46:** Fibrin clots before and after being treated. In the case of HEPES, the clots didn't dissolve or break, in the case of free tPA, the clot broke down into smaller pieces, and in the case of exo-tPA, the clot dissolved.

To assess the toxicity effects of tPA and exo-tPA, a cellular toxicity study was performed on normal HBMVEC. After culturing the cells in a 96 wells plates for 24 hours, they were incubated with different concentrations of exo-TPA and tPA for 48 hours after which alamarBlue cell viability reagent was used to determine the viability of the cells by being added directly to the cells that are then incubated at $37^0$C as for the cells to convert resazurin to resorufin, and after an

incubation period of two hours, the absorbance was measured using a plate reader. For the figure below, no significant toxicity was observed from any of the added material as the percentage of viable cells was greater than 98% in all conditions. This means that, neither free tPA nor exosomes encapsulated tPA exhibit cellular toxicity and could be safely used as therapeutic agents.

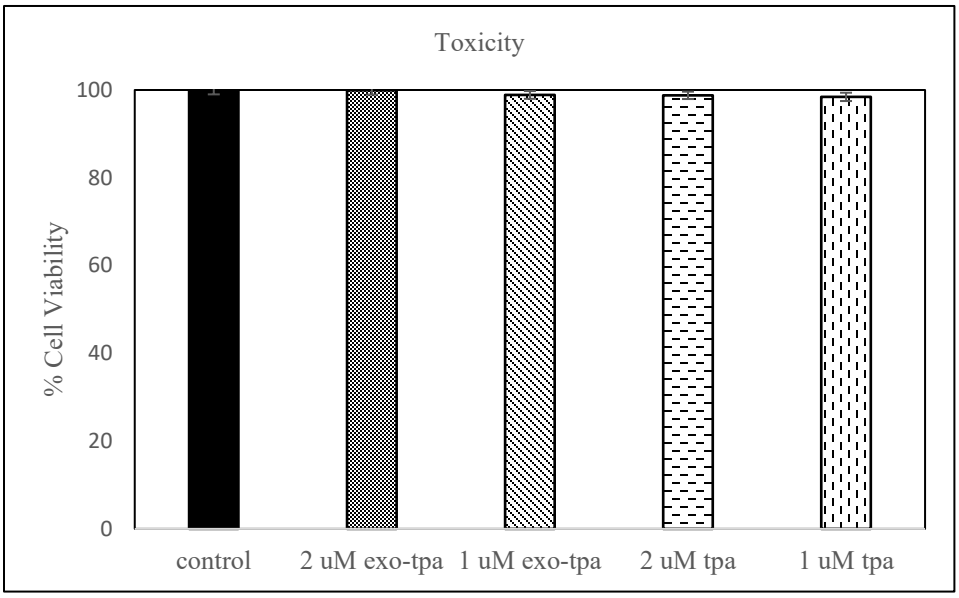



**Figure 47:** Assessment of toxicity effect of exo-tPA and free tPA (2μM and 1μM) in human brain microvascular endothelial cells. No significant toxicity observed at the tested conditions.

To determine whether the exo-tPA induce an increase in the amount of ROS released by the cells, an ROS study was performed using $H_2$DCFDA. After culturing the cells in a 96 wells plates for 24 hours, they were incubated with cell membrane different concentrations of tPA and exo-tPA for 48 hours, then the media was removed and substituted by $H_2$DCFDA for 30 minutes after which the absorbance was measured, using a plate reader. It is obvious, by looking at the figure below, that none of the added materials had a negative effect on the cells from the ROS side since in neither case, the relative amount of ROS released by the cells was less than 86%. This means that, tPA, being free of encapsulate within exosomes, don't increase the ROS secreted by the cells and could be safely used as therapeutic agents.

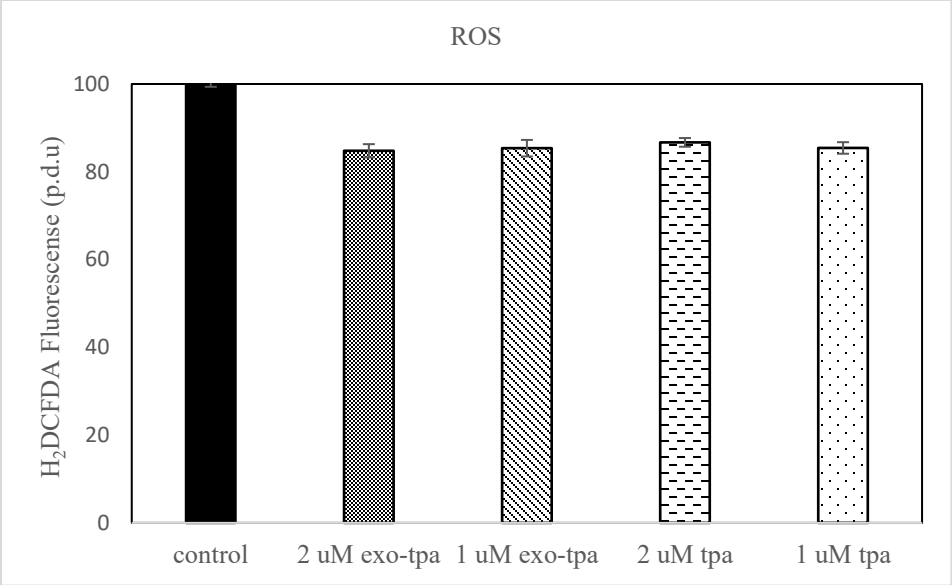



**Figure 48:** Assessment of ROS effect of exo-tPA and free tPA (2μM and 1μM) in human brain microvascular endothelial cells. No significant toxicity observed at the tested conditions.

## Chapter 6: Exosomes Encapsulated Catalase and tPA

### 6.1 Results and Discussion

In this chapter, we studied the effect of using exosomes to encapsulate catalase in order to protect the cells against toxicity and ROS caused by $H_2O_2$. Then we encapsulated exosomes, of an average concentration of 221.4 μg/ml, with both catalase and tissue-plasminogen-activator as to enhance the activity of tPA while, at the same time, protecting the cells against $H_2O_2$. To do so, first, the size exosomes and exosomes-catalase and exosomes-catalase-tPA were measured using DLS.

According to the table below, the average size of exosomes, that is 247.46 nm, increases upon encapsulating catalase to 349.27 nm and increases to 438.905 upon encapsulating catalase with tPA. However, this increase in size is highly acceptable as the exosome-tPA is still in the desired nano scale.

**Table 8:** Size of exosomes and exo- catalase-tPA.

| Particle | Exosomes | Exosomes-catalase | Exosomes- catalase-tPA |
|---|---|---|---|
| Average size± SE (nm) | 179.925± 25 | 349.27± 9.008 | 438.905± 10.06 |

*SE: Standard error

After preparing the exo-catalase and exo-catalase particles, we measured the catalase conjugation in the particles, using BCA protein assay, to determine how much catalase encapsulated within the exosomes. Results of more than three independent experiments showed that 30% of the added catalase reacted. Moreover, in order to measure the amount of tPA that was encapsulated within the eco-catalase particles, we performed tPA activity assay where the results of three

independent experiments showed that the around 25% of the added tPA was successfully encapsulated, and this was determined at 30 minutes using the calibration curve.

Following the tPA activity assay, plasminogen activator inhibitor (PAI) activity assay was performed to determine the effect of PAI on tPA in both, free tPA and exo-catalase-tPA. The results showed that the activity of tPA when encapsulated within the exosomes was not affected by the PAI, even in the presence of catalase, meanwhile the activity the free tPA was affected. From the figures below, it is obvious that when comparing the activity of tPA in both, free tPA and exo-catalase-tPA, it was around 5% in the case of free tPA whereas it was over 93% in exo-catalase-tPA. In other words, PAI had a great impact on the activity of tPA when the latter was not encapsulated within exosomes, however, using exosomes to encapsulate tPA was successful in decreasing the negative effect of PAI on the activity of tPA even when the exosomes encapsulated catalase in addition to tPA.

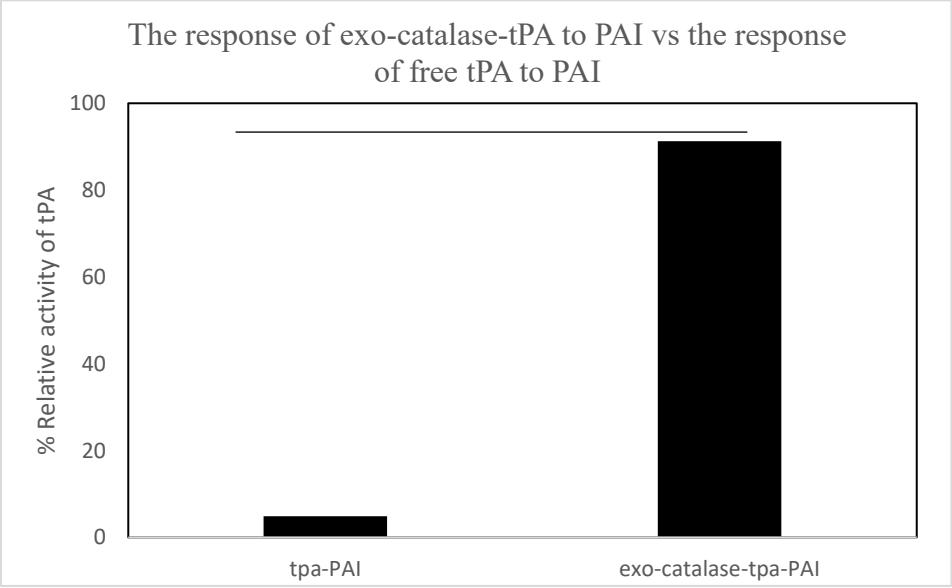



**Figure 49:** Response of free tPA to PAI to antiplasmin vs the reponse of exo-catalase-tPA to PAI. Data with significance is represented as * $p<0.05$.

In order to determine the effectiveness of exo-catalase-tPA on fibrin clots, the clots were prepared, and desired concentration of tPA, catalase, or exo-catalase-tPA, along with PAI and plasminogen were added to the clots, with a 1:1:1 ratio, with the concentrations being calculated

based on tPA activity assays at 30 minutes. After an incubation time of 24 hours, the rhodamine concentration in the supernatant of the clots was measured using a plate reader. According to the graph below, the amount of rhodamine in the supernatant of the fibrin clots that were treated with tPA, PAI, and plasminogen was low, around 243%, compared to the concentration of rhodamine in the supernatant of the fibrin clots that were treated with cell exo-catalase-tPA, PAI, and plasminogen that was around 373% as compared to the control group, that was only treated with HEPES buffer. This shows that the tPA in exo-catalase-tPA successfully activates plasminogen, even in the presence of PAI, and breaks a fibrin clot effectively, whereas, when present alone, the activation of plasminogen by tPA is affected by PAI, thus does not fully dissolve a fibrin clot, therefore, desired results are only achieved by exo-catalase-tPA. Moreover, comparing rhodamine release in the clot that was treated with catalase only to the clot that was treated with HEPES, the release in both cases is almost equal, and this was expected as catalase has no clot dissolving properties.

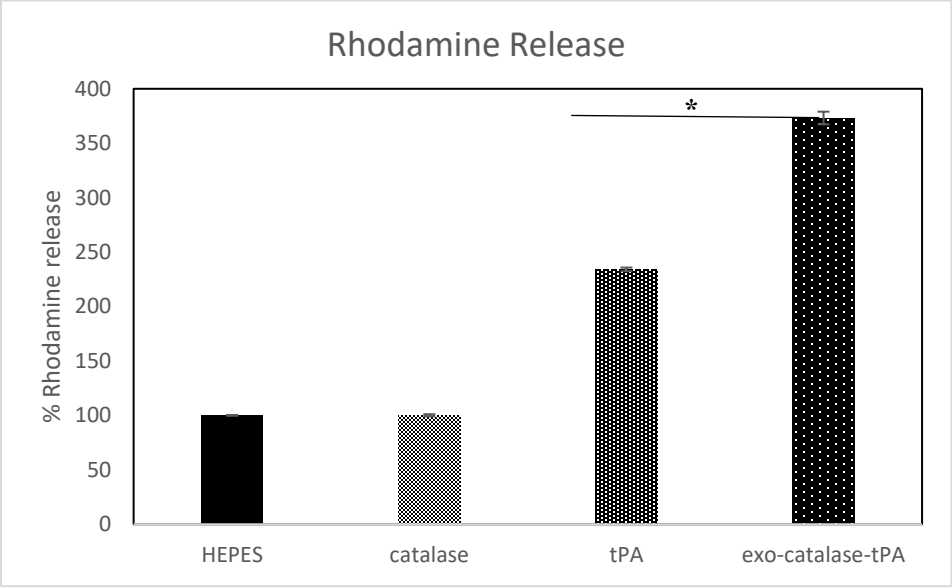
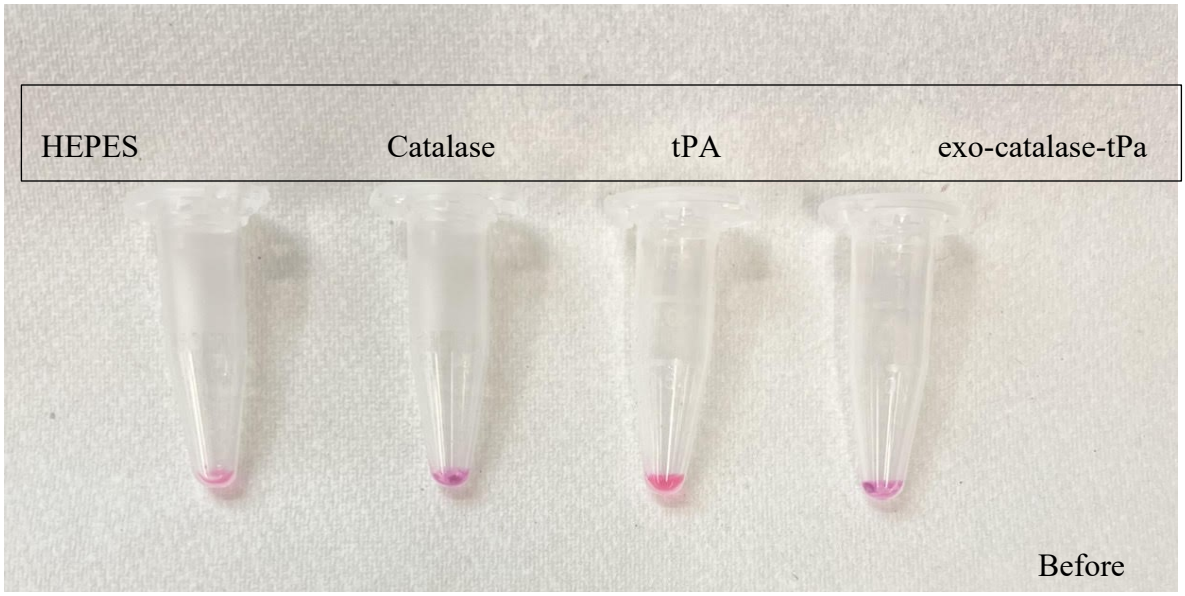



**Figure 50:** Rhodamine release from fibrin clots after being treated with HEPES, catalase, tPA-plasminogen-PAI, and exo-catalase-tPA- plasminogen- PAI. Data with significance is represented as * $p<0.05$.

The figure below shows the fibrin clots before and after being treated.



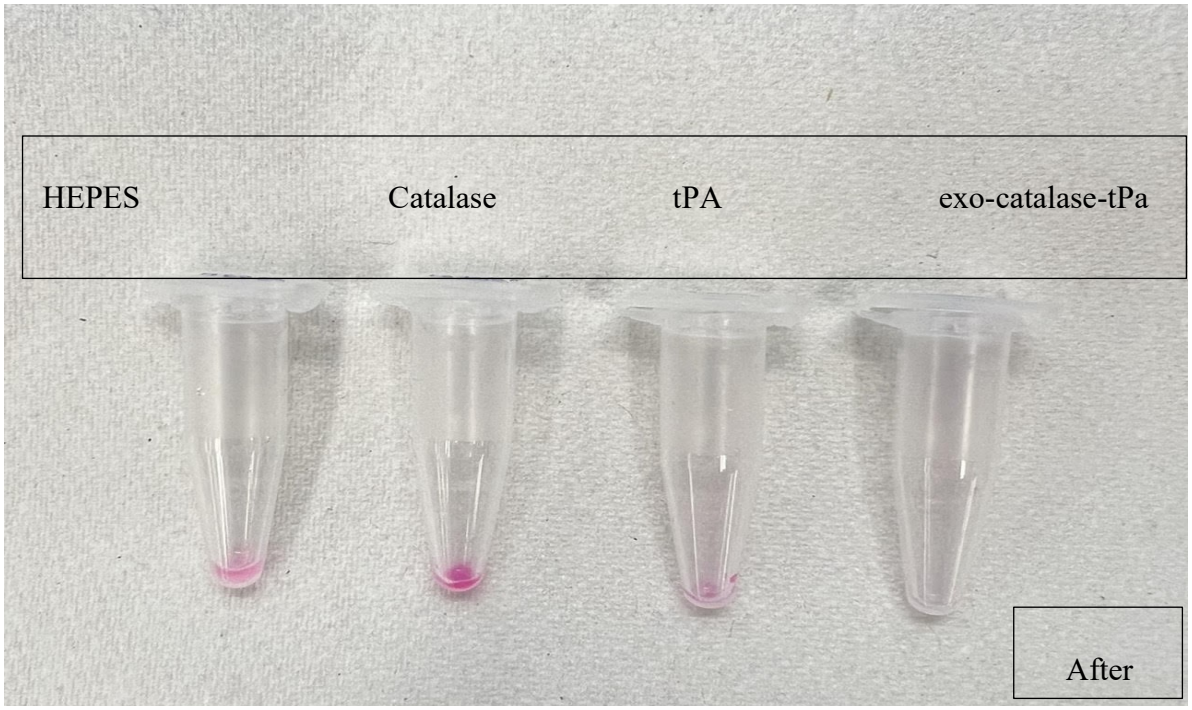




**Figure 51:** Fibrin clots before and after being treated. In the case of HEPES and catalase, the clots didn't dissolve or break, in the case of tPA the clot broke down into smaller pieces, and in the case of exo-catalase-tPA the clot dissolved.

To assess the toxicity effects of catalase, tPA, $H_2O_2$, exo-catalase, and exo-catalase-tPA, a cellular toxicity study was performed on normal HBMVEC. After culturing the cells in a 96

wells plates for 24 hours, they were incubated with different concentrations of catalase, tPA, $H_2O_2$, exo-catalase, and exo-catalase-tPA for 48 hours after which alamarBlue cell viability reagent was used to determine the viability of the cells by being added directly to the cells that are then incubated at $37^0$C as for the cells to convert resazurin to resorufin, and after an incubation period of two hours, the absorbance was measured using a plate reader. For the figure below, this test has proved the toxicity of $H_2O_2$ since the average % viability of cells was 34% in the case of 50 µM $H_2O_2$ and 26% in the case of 100 µM $H_2O_2$, this result is similar to the case where tPA is added to the cells in addition to $H_2O_2$, thus tPA doesn't prevent $H_2O_2$ from causing cytotoxicity. On the other hand, there was no significant toxicity observed from any other added material as the percentage of viable cells was greater than 100% in all conditions. This means that, neither free catalase, free tPA, exo-catalase, nor exo-catalase- tPA exhibit cellular toxicity and could be safely used as therapeutic agents. Additionally, it is obvious that adding catalase and catalase enclosed exosomes reduced cellular toxicity caused by $H_2O_2$ since, comparing the cells with $H_2O_2$ to those with $H_2O_2$ in addition to catalase, exo-catalase, and exo-catalase-tPA, it is clear that toxicity in cells with only $H_2O_2$ is much higher where the % cell viability increased from 26%- 34% to 83-94% when catalase and exosomes enclosed catalase were present.

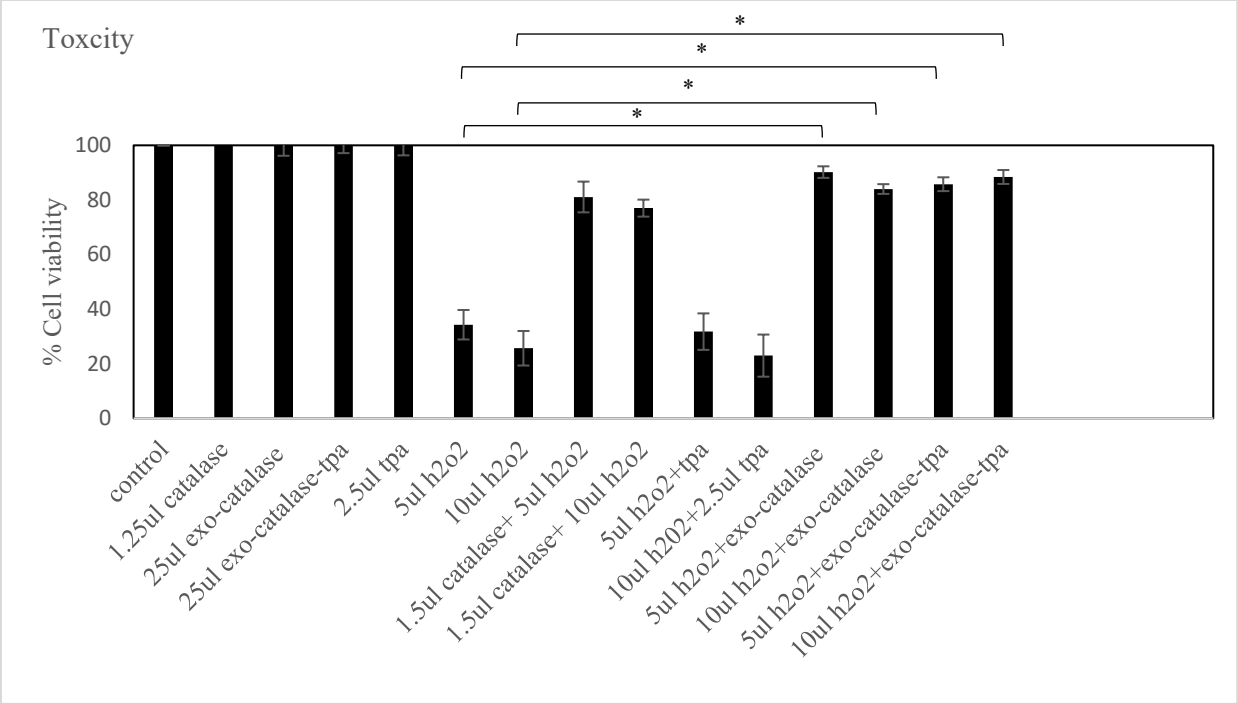



**Figure 52:** Assessment of toxicity effect of catalase, exo-catalase, $H_2O_2$ (50 µM and 100 µM), exo-catalse-tPA and free tPA (2.5µM) in human brain microvascular endothelial cells and the ability to reduce the cytotoxicity caused by $H_2O_2$ using catalase and catalase containing exosomes. No significant toxicity was observed when using catalase and catalase containing exosomes even in the presence of $H_2O_2$. Data with significance is represented as * $p<0.05$.

To determine whether catalase, tPA, $H_2O_2$, exo-catalase, and exo-catalase-tPA induce an increase in the amount of ROS released by the cells, an ROS study was performed using $H_2$DCFDA. After culturing the cells in a 96 wells plates for 24 hours, they were incubated with cell membrane different concentrations of tPA and exo-tPA for 48 hours, then the media was removed and substituted by $H_2$DCFDA for 30 minutes after which the absorbance was measured, using a plate reader. It is obvious, by looking at the figure below, that none of the added materials had a negative effect on the cells from the ROS side other than $H_2O_2$ when present alone or with tPA, since tPA doesn't have antioxidant properties, where, in both cases, the ROS release was 240- 409 p.d.u. . However, adding catalase or catalase enclosed exosomes was able to prevent this high ROS release where an obvious decrease was observed in $H_2O_2$ containing cells reaching 89- 93 p.d.u which is a great decrease compared to the presence of $H_2O_2$ alone (240- 409 p.d.u).

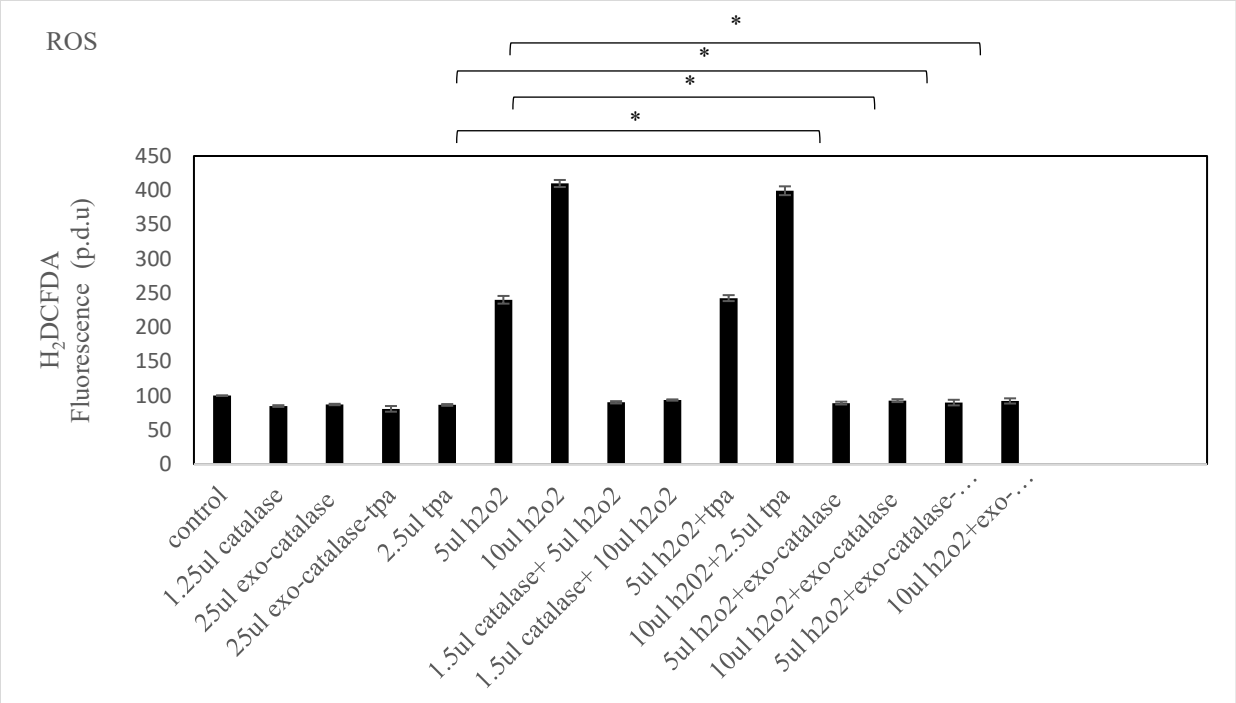



**Figure 53:** The effect of $H_2O_2$ (50 µM and 100 µM) induced ROS on human brain multivesicular cells and the ability to reduce this ROS caused by $H_2O_2$ using catalase and catalase containing exosomes. No significant toxicity was observed when using catalase and catalase containing exosomes even in the presence of $H_2O_2$. Data with significance is represented as * $p<0.05$.

# Chapter 7: Conclusion and Future Perspectives

## 7.1 Conclusion

Although that several studies have been done to test and prove the effectiveness of using nanomaterials to treat stroke, using cell membrane encapsulated PLGA-plasmin nanoparticles and exosomes encapsulated tPA and catalase, that are studied in this thesis, have not been explored yet.

In this research, we studied the effect of using human brain multivesicular cells derived cell membranes and exosomes to coat particles that take place in the dissolution of blood clots. Due to the therapeutic effectiveness of PLGA-nanoparticles in addition to their safety on human cells, instead of coating plasmin alone, we coated PLGA-plasmin nanoparticles with cell membrane as to give these particles biocompatible features and decrease the deactivation of plasmin by antiplasmin. Moreover, the goal of coating tPA with exosomes is to prevent the deactivation of tPA by PAI using a biocompatible delivery system.

This study revealed the success of the examined particles in achieving our goals where cell membrane coated PLGA-plasmin nanoparticles were able to dissolve fibrin clots and retain the stability of plasmin even in the presence of antiplasmin, unlike free plasmin. Additionally, the use of exosomes encapsulated tPA showed successful results in dissolving fibrin clots, prevent the activity of tPA from being altered by PAI, and maintaining the stability of tPA.

In addition, to prevent cytotoxicity and ROS caused by hydrogen peroxide, that accumulates in the brain following as ischemic stroke, we coated the antioxidant enzyme, catalase, with exosomes and this showed and improved success in preventing cytotoxicity and reducing ROS in cells as compared to the use of free catalase. Therefore, we decided to incorporate catalase and tPA in exo-catalase-tPA as to achieve the benefits of both exo-tPA and exo-catalase at the same time, and the results confirmed our expectations where cytotoxicity and ROS were prevented by

catalase upon using exo-catalase-tPA, fibrin clots were dissolved, and the activity of tPA was retained in the presence of PAI.

## 7.2 Future Perspectives

This thesis studies the effectiveness of cell membrane and exosomes in improving the efficacy of PLGA-plasmin nanoparticles and tPA in stroke treatments. However, this study doesn't discuss the effectiveness of the nanoparticles from preventing the drug from crossing the blood brain barrier, therefore, trans-wells could be used to determine the amount of the nanoparticles, being cell membrane coated PLGA-plasmin nanoparticles, exo-tPA, exo-catalase, or exo-catalase-tPA, that cross the blood brain barrier, with non to minimal being the desired goal. Furthermore, the extended circulation time, the method of administration, and the effectiveness of these nano-formulated could be studied in vivo using animal models.

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