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Nanocarriers for nose-to-brain delivery: a novel strategy for the treatment of CNS disorders

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Ph.D Thesis

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To my family and Andrea

"Perplexity is the beginning of knowledge"
Khalil Gibran

"I have not failed 700 times. I have not failed once.
I have succeeded in proving that those 700 ways will not work.
When I have eliminated the ways that will not work, I will find the way that will work."
Thomas Edison.

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ABSTRACT

The number of neurodegenerative diseases is estimated to be a few hundred. Despite the high prevalence and incidence, central nervous system (CNS) disorders are still incurable. The current therapeutic approach is based on the administration of symptomatic drugs which reduce the signs and symptoms of CNS diseases but not its causes. The development of effective preventive or protective therapies has been impeded by the difficult to deliver therapeutic agent to the brain.

The blood-brain-barrier (BBB) precludes the delivery of drugs to the brain, preventing the therapy of a number of neurological disorders. In the last 20 years, intranasal (IN) administration has gained great attention in research and has been investigated extensively with regard to its feasibility to serve as a direct drug transport route to the CNS. Drugs can be transported directly from the nasal cavity to the brain through the olfactory epithelium by trigeminal nerve systems and olfactory nerve pathways thereby bypassing the BBB. Even though some studies have demonstrated the transport of therapeutic agents to the brain via nasal route, the quantities of drug transported to the brain are very low, normally less than 0.1%, which is less than the therapeutically effective dose.

The incorporation of drugs into nanoparticles (NPs) might be a promising approach to improve the amount of pharmaceuticals delivered to the CNS, protecting them from the enzymatic activity in the nasal cavity and enhancing their transport across the biological barriers. Taking into account these considerations, the goal of my PhD thesis is to assess the effective molecule delivery to the brain by using a new approach: IN administration combined with the nanotechnology-based carriers.

In particular my aim was to investigate whether polymeric NPs can end up the brain after IN administration; which region of the brain can be reached; how does surface property affect NPs transport. This thesis focuses on designing and exploring novel and different polymeric nanosystems with aim to improve nose-to-brain delivery.

Once NPs translocation to the brain via this route was determined, our nanosystems have been formulated to study their potential application in important neurological conditions: epilepsy and brain cancer. The promising nanosystems were successfully loaded with Oxcarbazepine and the model siRNA. In particular, we investigate PLGA NPs, unmodified and surface modified. PLGA is an FDA-approved biodegradable polymer, it allows the preparation of NPs able to encapsulate both hydrophobic and hydrophilic compounds.

Surface modification of PLGA carrier, such as PEGylation or chitosan coating, would serve as one of the excellent approaches to manage drug delivery properties of formulations by interaction of surface coating with a biological system and to enhance brain delivery.

In **Paper I** we deeply studied PEGylated PLGA NPs, this work was based on several technological analyses aimed at obtaining PEGylated NPs with simple composition and long-term storage suitable for nose-to-brain delivery. To achieve this purpose a screening to select the degree of PEGylation of PLGA (5-10-15%) was performed and the effects of the double function of sucrose as surfactant-like and cryoprotectant agent was evaluated. Mucoadhesive evaluations between NPs and mucin were assessed by the mucin particle method and differential scanning calorimetry. Preliminary *in vitro* evaluation of cytotoxic properties of PEGylated systems was also performed.

Our results suggest the use of sucrose for its double effect. We support the use of PEG 5% to confer a sufficiently hydrophilic and uncharged surface to minimize effectively mucin-NPs adhesive interactions, allowing particles to diffuse rapidly through human mucus and cross respiratory epithelium. Our nanosystems did not show any cytotoxic effects. Therefore, in the present work we propose a new formulation for IN drug delivery.

In **Paper II** we looked at the *in vivo* fate of PLGA NPs and PLGA NPs surface modified with Chitosan (CS) after IN administration in rats. These formulations have been optimized in terms of mean size and stability by photon correlation spectroscopy and Turbiscan AGS, and tested *in vivo*. Both NPs systems were loaded with Rhodamine B and in vitro release study was evaluated by dyalisis bag technique. Biodistribution studies were carried out in healthy rats after IN administration of NPs at different time intervals. Fluorescent microscopy was conducted to value the localization of NPs in the CNS. Our results contribute to the understanding that compounds encapsulated in NPs may have a direct access to the CNS following IN administration. Our findings led us to hypothesize that different pathways were involved in the transport of unmodified and modified NPs, suggesting the involvement of the olfactory transport and the trigeminal nerve pathway respectively.

Furthermore, additional experiments, (**Paper III**, in preparation), were partially reported to confirm our results. In particular, the investigation of DiR-loaded PLGA NPs biodistribution and bioavailability to the brain after IN administration in living healthy mice by Fluorescence Molecular Tomography system.

Once established that our NPs reach different brain areas, we aim to investigate whether NPs can enhance the efficacy of the drugs loaded.

Paper IV (in preparation) is focused on the encapsulation of Oxcarbazepine in PLGA NPs aiming at direct nose-to-brain delivery to improve epileptic therapy, the possibility of using less daily drug amounts to reduce undesirable interactions and toxic effects and to evaluate the possible neuroprotection of this drug against the seizures and brain damage induced by Pentylentetrazole administration.

We also investigate nose-to-brain delivery by using NPs system for gene therapy in **Paper V** (in preparation). The partial negative charge and the susceptibility to degradation by nucleases have hampered nucleic acid use in a naked form. In this study, we investigate the use of "homemade" polymers as potential delivery carriers of siRNA in order to protect it from instability and degradation. The polymer bind to siRNA through electrostatic interaction to form complexes in a non-covalent manner. The nanocomplexes were characterized in terms of size, zeta potential and stability. Cell cytotoxicity of the nanocomplex was determined in A431 cell line. Transfection and silencing efficiency were evaluated *in vitro* and *in vivo* after IN administration in rats by using Western Blot. Our nanocomplexes present mean diameter less than 300 nm, positive surface charge and good stability under destabilizing conditions. Our systems show good *in vitro* transfection and down regulation of the model protein *in vivo*.

LIST OF ABBREVIATIONS

AEDs Antiepileptic drugs

AGO 2 Argonaute 2

AUC Area under the curve

BBB Blood brain barrier

BBBD Blood brain barrier disruption

C_{max} Maximum concentration

CNS Central Nervous System

CS Chitosan

CSF Cerebrospinal fluid

CPP Cell-penetrating peptide

D Daltons

DDS Drug delivery systems

DiR $DiOC_{18}(7)$

DNA Deoxyribonucleic acid

dsRNA Double stranded RNA

DSC Differential scanning calorimetry

DTX Docetaxel

EMA European Medicine Agency

FDA Food and Drug Administration

FMT Fluorescence Molecular Tomography

G Gram

GRAS Generally recognized as safe

h Hour

IC50 The concentration of an inhibitor that is required for 50-percent inhibition of an enzyme in vitro

I.N. Intranasal delivery

I.P. Intraperitoneal administration

I.V. Intravenous administration

kD Kilodaltons

$\mathbf{K_{el}}$ elimination rate constant

mg Miligram

mg.mL^{-1} Miligram per mililiter

min Minute

mM Milimole

miRNA Micro RNA

MPS Monuclear phagocyte system

mRNA Messenger RNA

MRP-1 Multidrug Resistance Protein-1

MRT mean residence time

MTT 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide thiazolyl blue-indicator dye

Mw Molecular weight

NPs nanoparticles

N/P ratio Nitrogen to phosphate ratio

NT Nucleotide

OX Oxcarbazepine

PAMAM Poly(amidoamine)

PASA Poly(aspartic acid)

PBS Phosphate buffer saline

PCL Poly(ε-caprolactone)

PDI Polydispersity Index

PEG Poly ethylene glycol

PEI Poly(ethylenimine)

PGA Poly(glycolic acid)

P-gp P-glycoprotein

PLA Poly(lactic acid)

PLGA Poly(lactic-co-glycolic acid)

PS Polystyrene

PTZ Pentylentetrazole

RISC RNA-Induced Silencing Complex

RNA Ribonucleic acid

RNAi Ribonucleic acid interference

ROI Region of interest

rpm Rotations per minute

RSC Racine's convulsive scale

s Second

SEM Scanning electron microscopy

siRNA Small interfering RNA

TEM Transmission Electron Microscopy

$\mathbf{T_{max}}$ Time of maximum concentration observed

TQ Thymoquinone

TJs Tight junctions

TSI Turbiscan stability index

US Ultrasound

UV Ultra-violet

V Volt

w/v Weight per volume

µg Microgram

µL Microliter

µg.µL^{-1} Microgram per microliter

ºC Celsius degree

% Percent

CHAPTER I

INTRODUCTION

1. NEURODEGENERATIVE DISEASES AND BBB

The term neurodegeneration is a combination of two words "neuro," referring to nerve cells (i.e., neurons), and "degeneration," which refers to, in the case of tissues or organs, a process of losing structure or function. Thus, in the strict sense of the word, neurodegeneration corresponds to any pathological condition primarily affecting neurons (Przedborski et al., 2003). In practice, neurodegenerative diseases represent a large group of neurological disorders with heterogeneous clinical and pathological expressions affecting specific subsets of neurons in specific functional anatomic systems; they arise for unknown reasons and progress in a relentless manner (Nguyen et al., 2014). The number of neurodegenerative diseases is currently estimated to be a few hundred, and, among these, many appear to overlap with one another clinically and pathologically, rendering their practical classification quite challenging.

Increasing age is the main risk factor for developing a neurodegenerative disorder.

Over the past century, the growth rate of the population aged 65 has far exceeded that of the population as a whole. Thus, it can be anticipated that, over the next generations, the proportion of persons suffering from some kind of neurodegenerative disorder will double. This prediction is at the center of growing concerns in the medical community, for the increasing magnitude of emotional, physical, and financial burdens on patients, caregivers and society that are related to these disabling diseases (Przedborski et al., 2003). Despite many studies in this research field, patients suffering from debilitating central nervous system (CNS) disorders, such as brain tumors, HIV encephalopathy, epilepsy, cerebrovascular diseases and neurodegenerative disorders, far outnumber those dying of all types of systemic cancer or heart disease (Nasreen et al., 2015).

There are currently no therapies available to cure neurodegeneration. For each of the diseases, medication can only alleviate symptoms and help to improve patients'quality of life. Furthermore, the chronic use of several drugs is often associated with debilitating side effects, and none seems to stop the progression of the degenerative process.

In keeping with this, the development of effective preventive or protective therapies has been impeded by the limitations of our knowledge of the causes and the mechanisms by which neurons die in neurodegenerative diseases and the difficult to deliver therapeutic agent to the CNS (Przedborski et al., 2003). The brain is a delicate organ and evolution built very efficient ways to protect it. The CNS has developed a series of barriers to protect itself from invading pathogens, neurotoxic molecules and circulating blood cells. These structures with diverse degrees of permeability include the blood-cerebrospinal fluid (CSF) barrier, the blood-brain barrier (BBB), the blood-retinal barrier and the blood-spinal cord barrier (Saraiva et al., 2016). Unfortunately, the same mechanisms that protect it against nosy and inappropriate substances can also hinder therapeutic interventions. Many existing pharmaceuticals are rendered ineffective in the treatment of brain diseases due to inability to effectively deliver and sustain them within the brain (Misra et al., 2003). The clinical failure of potentially effective therapeutics is often not due to a lack of drug potency but rather to shortcomings in the method by which the drug is delivered. This is a major impeding factor to progress in the field. Hence, several active compounds may have been abandoned because sufficient drug levels in the brain cannot be achieved via the blood. In response to the insufficiency in conventional delivery mechanisms, new strategies are being developed and investigated in order to more effectively deliver active compounds to the CNS. Unfortunately, most of them are

still invasive and lack the target specificity (Ladola et al., 2014). These strategies generally fall into the following categories: non-invasive technique, invasive technique and finding alternative routes for drug delivery (Fig.1).

The non-invasive techniques include:

-Chemical approaches rely on chemical structure transformation of drugs to improve their unsatisfactory physicochemical properties (such as solubility or membrane penetration) and therefore change their functionalities (i.e. Lipophilic analogs, Prodrugs);

-Colloidal drug carriers, (i.e. Liposomes, Nanoparticles, Micelles, Dendrimers ect.), can be effectively transported across various *in vitro* and *in vivo* BBB models by endocytosis and/or transcytosis, and have demonstrated early preclinical success for the management of CNS conditions. Particle size, surface affinity, and stability in circulation are the important factors influencing the brain distribution of colloidal particles;

-Biological approaches primarily emanate from the understanding of the physiological and anatomical differences of the BBB transportation (i.e. Receptor/vector-mediated delivery of chimeric peptides, Cell-penetrating peptide (CPP)-mediated drug delivery, Viral vectors).

The invasive techniques cover the disruption of the BBB or administration of the drug directly into the brain tissue (i.e. Intracerebral implants, Intraventricular/intrathecal/interstitial delivery, Convection-enhanced delivery, Osmotic BBB disruption (BBBD) strategy, Biochemical strategy, Ultrasound (US)-mediated BBBD strategy.

The Alternative routes for CNS drug delivery include:

-Intranasal delivery which provides a practical, non-invasive method of bypassing the BBB to deliver therapeutic agents to the brain and spinal cord. This is possible because of the unique connections that the olfactory and trigeminal nerves provide between the brain and external environment;

-Iontophoresis is a method to deliver ionized molecules across the BBB by using an externally applied electric current (Lu et al., 2014).

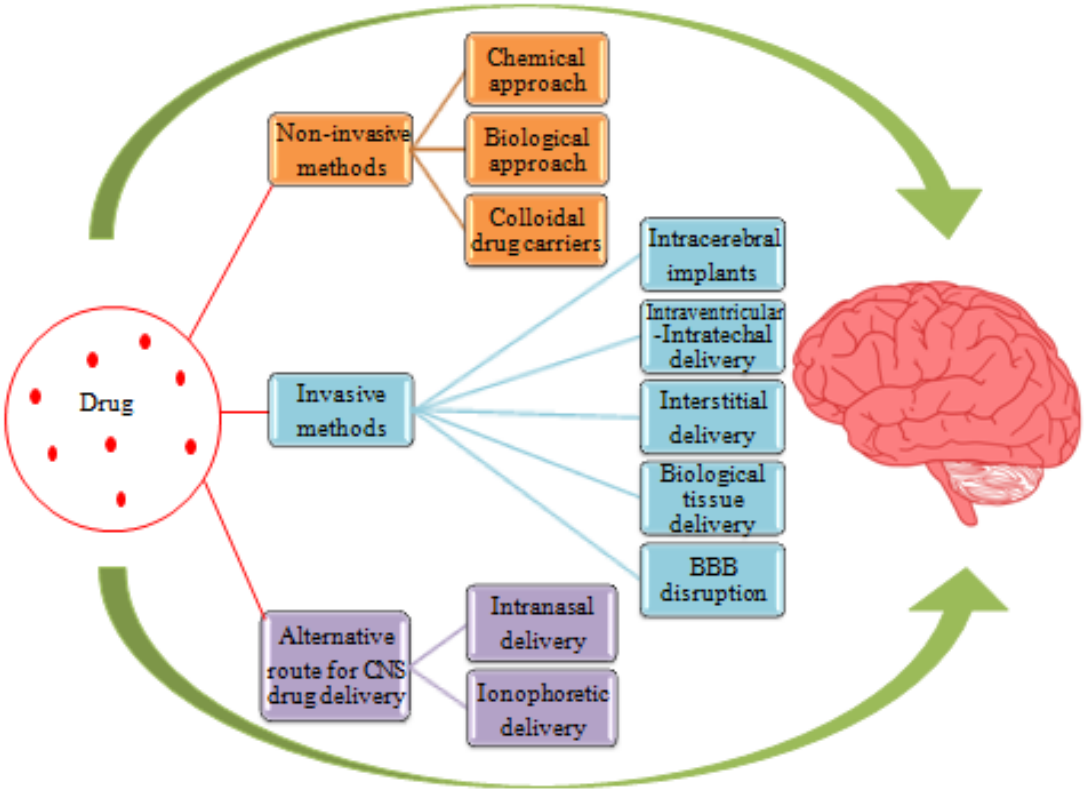

Figure 1. Schematic representation of current strategies for CNS drug delivery.

1.1. Blood brain barrier, general concept and mechanisms of passage

With the endothelium as its central unit, the BBB is a complex multicellular structure separating the CNS from the systemic circulation. The BBB is the most extensive and exclusive barrier among those that CNS has developed to protect itself from invading

pathogens, neurotoxic molecules and control the entry of compounds into the brain, reflecting the brain's critical roles in cognition, regulating metabolism and coordinating the functions of peripheral organs (Pardridge, 2005). Because communicating this information depends on fine control of electrical and chemical signals between neurons, the brain requires a precise and balanced microenvironment. Neuroinflammation and neurodegeneration may develop as a consequence of failure in maintaining any of these components resulting in the breakdown of this specialized multicellular structure. Microvascular endothelium, basement membrane, and glial cells such as astrocytes and pericytes work together to form the BBB (Fig. 2).

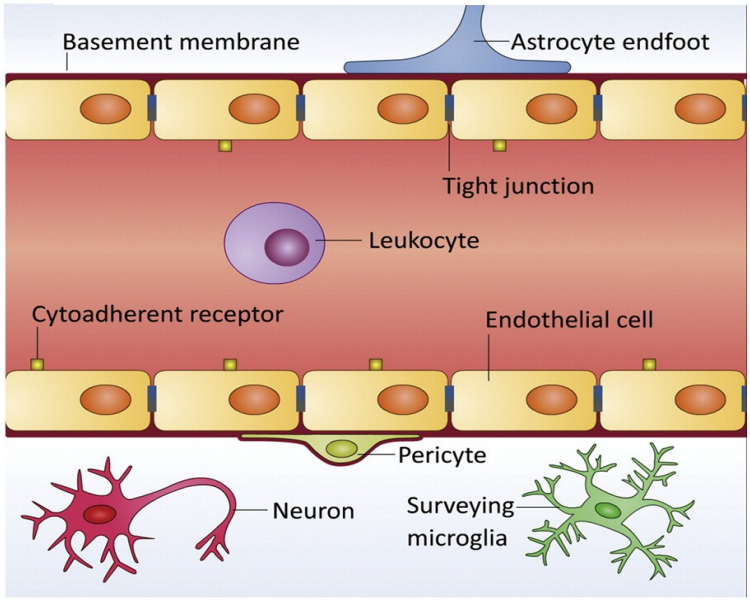

Figure 2. BBB composition. The BBB is mainly composed of vascular endothelial cells, highly connected by adherens and tight junctions (TJs), and a sparse layer of pericytes. A basement membrane and a layer of astrocyte end-foot processes surround the endothelium. Neurons and surveying microglia are also important mediators of BBB integrity in physiological conditions. Adapted from Saraiva, 2016.

To sustain this robust barrier, CNS endothelial cells have properties distinct from endothelial cells in other tissues: the presence of BBB-specific transporter and receptor proteins to control entry and exit of metabolites across cells (transcellular transport); high electrical resistance tight junctions (TJs) to limit movement between adjacent cells

(paracellular transport); low levels of transcytotic vesicles compared to peripheral endothelia and an absence of fenestrae (small pores that allow rapid passage of molecules in peripheral endothelial cells) (Keaney et al., 2015).

The relative impermeability of the BBB results from tight junctions between capillary endothelial cells which are formed by cell adhesion molecules. Brain endothelial cells also possess few alternate transport pathways (e.g., fenestra, transendothelial channels, pinocytotic vesicles), and express high levels of active efflux transport proteins, including P-glycoprotein (P-gp), Multidrug Resistance Protein-1 (MRP-1), and breast cancer resistance protein. The BBB also has additional enzymatic aspects which serve to protect the brain (Gabathuler, 2010). Some small molecules with appropriate lipophilicity, molecular weight (Mw) and charge will diffuse from blood into the CNS. However, the majority of small molecules (mwN500 daltons, D), proteins and peptides do not cross the BBB. It has been reported that approximately 98% of the small molecules and nearly all large molecules (mwN1 kD, kilodaltons), such as recombinant proteins or gene-based medicines do not cross the BBB (Pardridge, 1998). Therefore, to reach the brain, most molecules must cross the BBB through interactions with specific transporters and/or receptors expressed at the luminal (blood) side of the endothelial cells. Crossing the BBB remains a key obstacle in the development of drugs for brain diseases despite decades of research. The minimal BBB transport of the majority of all potential brain therapeutic agents, leads predictably to the current situation, which is that there are few effective treatments for the majority of CNS (Pardridge, 2005).

A schematic representation of different mechanisms used to cross the BBB is shown in Fig. 3.

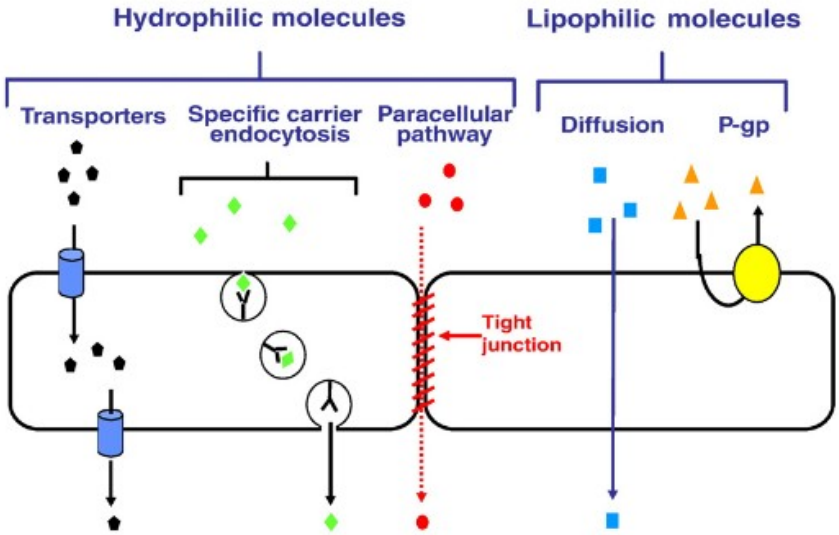

Figure 3. Schematic representation of the transport of molecules across the BBB.
From Gabathuler, 2010.

2. NANOTECHLOGY-BASED DRUG DELIVERY SYSTEMS

"I want to build a billion tiny factories, models of each other, which are manufacturing simultaneously. . . The principles of physics, as far as I can see, do not speak against the possibility of maneuvering things atom by atom. It is not an attempt to violate any laws; it is something, in principle, that can be done; but in practice, it has not been done because we are too big" (Feynman, 1960).

This quote is part of a lecture titled "There's plenty of room at the bottom" by physicist Richard Feynman in 1959, that introduced the concept of nanotechnology as an important field for future scientific researches (Feynman, 1960). Feynman described a process in which scientists would be able to manipulate and control individual atoms and molecules. Over a decade later, Professor Norio Taniguchi coined the term nanotechnology (Nikalje, 2015). Nanotechnology is the study and application of extremely small things and can be used across all the science fields, such as chemistry, biology, physics, materials science, engineering, medicine and pharmaceutics.

Nowadays scientists are finding a wide variety of ways to make materials at the nanoscale to take advantage of their enhanced properties such as higher strength, lighter weight, increased control of light spectrum, and greater chemical reactivity than their larger-scale counterparts (Nano.gov http://www.nano.gov/nanotech-101).

Nanotechnology has been used in medicine for the targeted delivery and/or controlled release of therapeutic agents and the development of treatments for a variety of diseases (Safari et el., 2014). Unfortunately, many drugs, even those discovered using the most advanced molecular biology strategies, have unacceptable side effects due to off-target adverse effects (modulation of unintended targets). Consequently, optimal design of medications for many diseases such as cancer, neurodegenerative and infectious diseases is limited. Drug delivery systems (DDS) present indubitable benefits such as control the rate at which a drug is released and the location in the body where it is released reducing dosing frequency and improving shelf life by enhancing its *in vivo* stability. Several types of nano-sized carriers, such as polymeric nanoparticles, solid lipid nanoparticles, nanostructure lipid carriers, nanocrystals, ceramic nanoparticles, magnetic nanoparticles, polymeric micelles, polymer-drug conjugates, lipid drug conjugates, nanotubes, nanoshells, nanowires, nanocages and dendrimers are being developed for various drug-delivery applications (Mukherjee, 2013; Mostafavi et al., 2013). In figure 4 are shown the most commonly used NPs for biomedical applications.

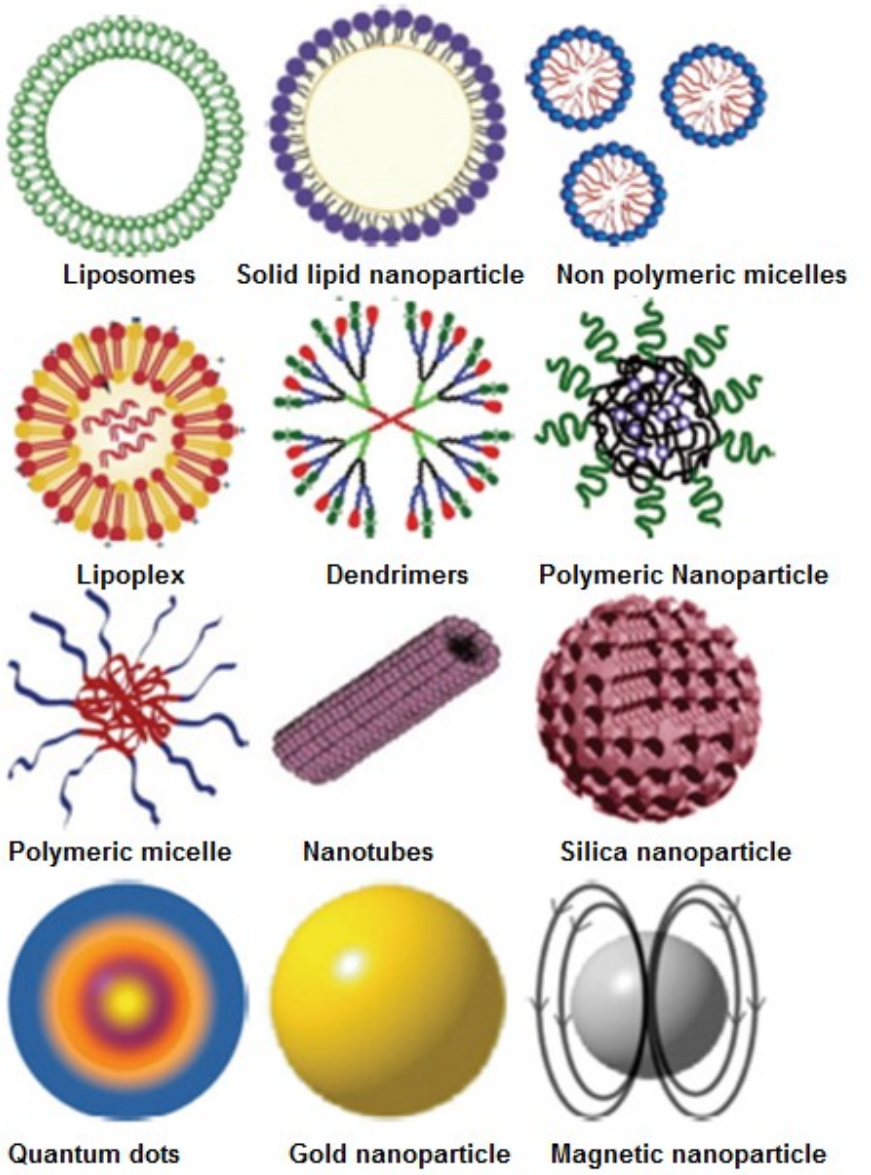

Figure 4. The types of NPs that are more popular for biomedical applications.

The DDS may encourage the use of therapeutic agents that were previously unsafe for disease treatment and targeted carriers may also help to address multi-drug resistant diseases.

This contributes to increase safety, efficacy, patient compliance, extending shelf life of drugs (Saha et al., 2015). Development of a novel drug-carrier system requires considerations of multiple factors. For example, after a drug is selected, a suitable

delivery route, drug release mechanism and kinetics, and proper materials selection have to be taken into account (Park, 2014). The use of biodegradable polymers for biomedical applications is continually increasing and evolving especially their application in nanotechnology systems for drug delivery (Bret et al., 2011; Numata et al., 2013). The main advantages of nanotechnological systems are reported in table 1.

Table 1. Main advantages of nanotechnological systems.

ADVANTAGES OF NANOTECHNOLOGICAL SYSTEMS
Advantages
Particle size and surface characteristics of nanosystems can be easily manipulated to achieve both passive and active drug targeting
Deliver/transport relevant drugs to the brain overcoming the presence of blood–brain barrier
Selective localization in specific tissues
Control and sustain release of the drug during the transportation and at the site of localization, altering pharmacokinetic profile of the active compound, increasing therapeutic efficacy and reducing the side effects
Control release and particle degradation characteristics by choosing the constituents of the matrix
Site-specific targeting by attaching the target ligands to the carrier surface or by using magnetic guidance
Administration by various routes such as oral, nasal, parenteral, intra-ocular etc
Better transmission and retention of the drug in the tumors and inflamed tissues

Nanotechnology has the potential to revolutionize the medical area with new tools for the molecular treatment of diseases, and rapid disease detection. It advances materials with a nanodimension and provides several means for innovative design of nanosize drug delivery systems to overcome biological barriers (Athar et al., 2014).

2.1. Polymers used in preparation of nanoparticles

Nanocarriers based on different engineering hyperbranched polymer types, dendrimers, micelles, hydrogel are a growing area of present-day pharmaceutical research, due to their unique properties and large potential in drug delivery (Athar et al., 2014; Chen et al., 2016). For using polymers in drug delivery, a polymer must exhibit essential characteristics such as biocompatibility, biodegradability, flexibility, minimal side effects and should improve drug release kinetics, as a result of its erosion or degradation in addition to drug diffusion through the polymeric material (Liechty et al., 2010).

Polymer must meet specific quality criteria as reported by Safari and collegues:

a. Biocompatibility backbone of the polymer and its degradation products.

b. Mechanical strength sufficient to meet the needs of specific applications.

c. Degradability with degradation kinetics matching a biological process such as wound healing.

d. Processibility using available equipment.

e. Solubility in various solvents.

f. Chemical, structural and application versatility.

g. Economically acceptable shelf life.

h. Approval by European Medicine Agency (EMA) or Food and Drug Administration (FDA), USA (Safari et al., 2014).

The use of polymers as biomaterials has greatly impacted the advancement of modern medicine. Polymeric biomaterials that are biodegradable provide the significant advantage of being able to be broken down and removed after they have served their function. Their main advantage is that the products of degradation are not toxic/ non-harmful or/and are completely and easily eliminated from the body by natural metabolic

pathways with minimal side effects (Marin et al., 2013). These degradation products define the biocompatibility of a polymer. For example, polymers derived from glycolic acid and from D,L-lactic acid enantiomers are presently the most attractive compounds because of their biocompatibility and their resorbability through natural pathways. Degradation of PLA or PLGA occurs by autocatalytic cleavage of the ester bonds through spontaneous hydrolysis into oligomers and D,L-lactic and glycolic acid monomers (Liu et al., 2006). Lactate converted into pyruvate and glycolate enter the Krebs' cycle to be degraded into CO_2 and H_2O. Bazile and collegues found that after intravenous administration of 14C-PLA radiolabeled nanoparticles to rats, 90% of the recovered 14C was eliminated within 25 days, among which 80% was as CO_2. As reported by the authors, the elimination of the 14C was quick on the first day (30% of the administered dose) but then slowed down. In fact, if the metabolism of the PLA proceeds to lactic acid which is rapidly converted into CO_2 (80% of the total excretion was fulfilled by the lungs), anabolism from the lactic acid may also have taken place leading to long-lasting radioactive remnants, by incorporation of 14C into endogenous compounds (Bazile et al., 1992).

Several parameters influence the degradation rate, including: hydrolysis rate constant (correlated with the molecular weight, the lactic/glycolic ratio, and the morphology), amount of water absorbed, diffusion coefficient of the polymer fragments through the polymer matrix, and solubility of the degradation products in the surrounding aqueous medium. In turn, all these parameters are influenced by temperature, additives (including drug molecules), pH, ionic strength, buffering capacity, size and processing history, steric hindrance etc. (Olivier et al., 2005).

The major mechanisms of degradation for polymers are hydrolysis, oxidation, or enzymatic reactions (Untereker et al., 2009). Numerous natural and synthetic polymers, (Fig.5), have been investigated as candidate for biomedical applications and new materials have been developed to meet new challenges (Ulery et al., 2013).

While natural polymers have been used in the medical field for thousands of years, research into biomedical applications of synthetic degradable polymers is relatively new, starting in the 1960s. In the fifty years since, successes have been numerous, but grand challenges still exist in both the basic and translational elements of biomaterial design.

Natural polymers, materials of both plant and animal origin, are the first option in biomedicine, Since they occur in nature, are often presumed to exhibit enhanced biocompatibility (Russell et al., 2014). However some problem could arise due to batch-to-batch variations in properties or risk of viral infections as reported by Hino et al., showing the transmission of parvovirus B19 by blood products such as fibrin which is widely used as a surgical adhesive, hemostatic agent, and sealant (Hino et al., 2000). Synthetic polymers, on the other hand, have attracted researchers' interest because of their manufacturing flexibility and reproducibility and can be produced using many synthetic methods. Synthesis determines molecular structure with the purpose of achieving product with lower level of impurity. Several of the reactions involved in synthesis of these polymers, include ring opening, polycondensation, bulk synthesis, dehydrative coupling, transesterification, and polymerization.

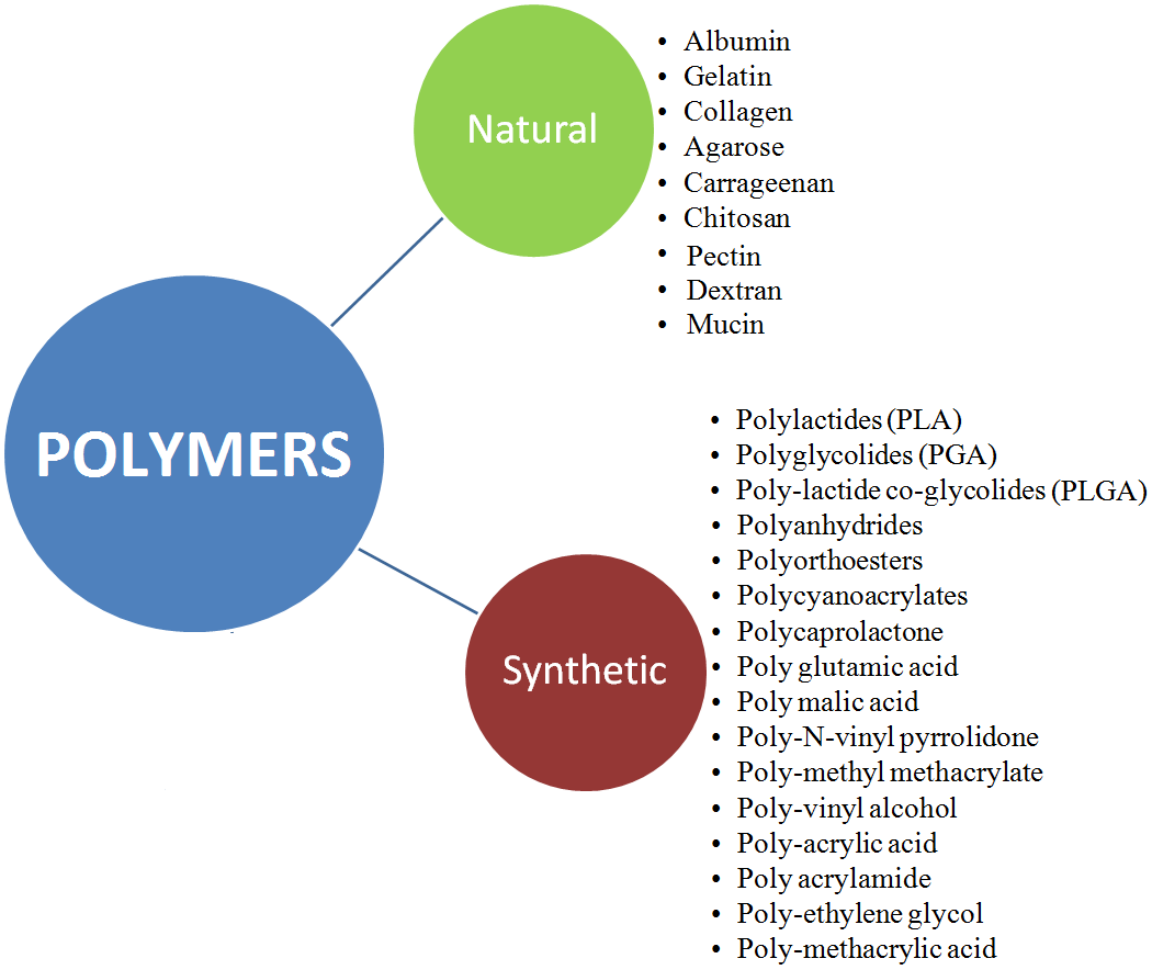

Figure 5. Examples of natural and synthetic polymers.

Controlled degradation of polymers helps to maintain drug levels within a suitable therapeutic window (Siegel et al., 2012). The degradation mechanism of a polymer is essential to achieve an efficient drug delivery, the polymeric matrix has to degrade under physiologic conditions in a controlled manner to allow sustained release of the drug as temporal drug delivery systems (Fig. 6).

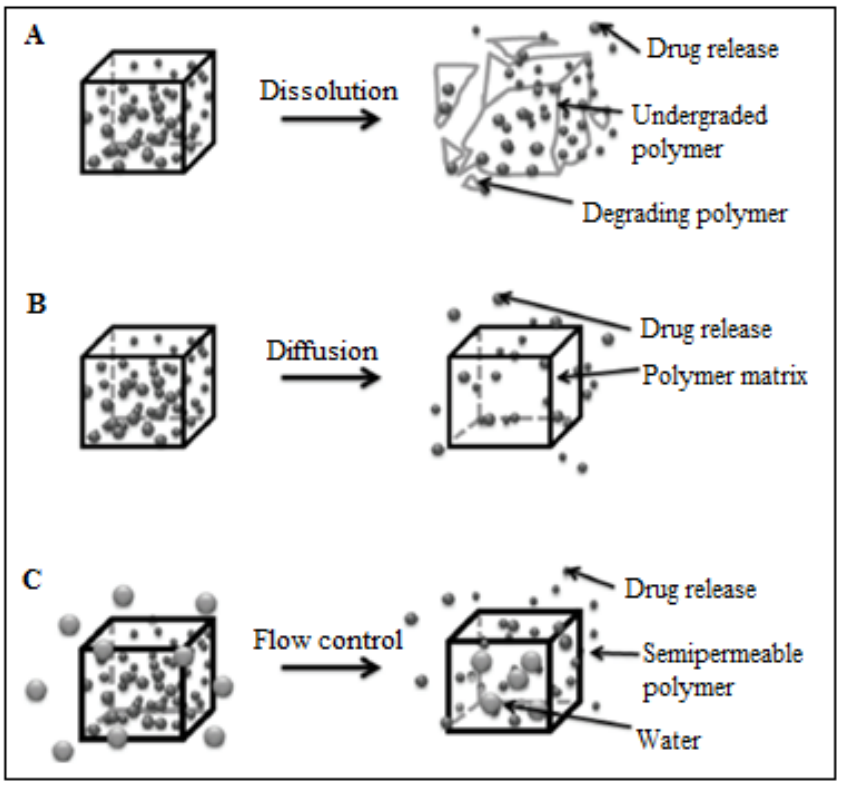

Figure 6. Mechanisms for temporal controlled-release drug systems (A) Dissolution of a polymer with slow break-down that delays exposure of drug to water from the environment of the delivery system. (B) Drug diffusion-controlled release through gaps in insoluble polymeric devices. (C) Controlled flow using osmotic forces on a semi-permeable polymer matrix**.** Adapted from Marin, 2013.

A delivery system promotes the continued release of drug in a specific period of time, which allows to maintain drug concentration in blood or target tissues at the therapeutic level (Uhrich et al., 1999; Lee et al., 2015). As a consequence the frequency of administration can be reduced. Temporal release delays diffusion of the molecule out of the polymeric matrix, inhibiting diffusion or controlling drug flow through the matrix. These strategies involve manipulation of some physicochemical properties of the polymers, e.g., copolymerizing or blending of polymers in order to change the degradation behavior.

2.2. Polymeric NPs

Nanoparticles (NPs) are the solid colloidal particles that can vary from 1 to 1000 nm in size, utilized as career for drug delivery (Saraiva et al., 2016). What makes NPs even more attractive for medical applications is the possibility of conferring on them features such as high chemical and biological stability, feasibility of incorporating both hydrophilic and hydrophobic compounds (in relation to the preparation method), and the ability to be administered by a variety of routes (i.e. oral, nasal, and parenteral). Moreover, NPs can be functionalized by covalent conjugation to various ligands to target specific tissues. Nanosized drug delivery systems can increase drug accumulation in specific tissue and/or reduce drug elimination via passive or active targeting. NPs provide massive advantages regarding drug targeting, delivery and release, and with their additional potential to combine diagnosis and therapy, emerge as one of the major tools in nanomedicine. In figure 7 is reported a strategy to create targeted drug delivery systems.

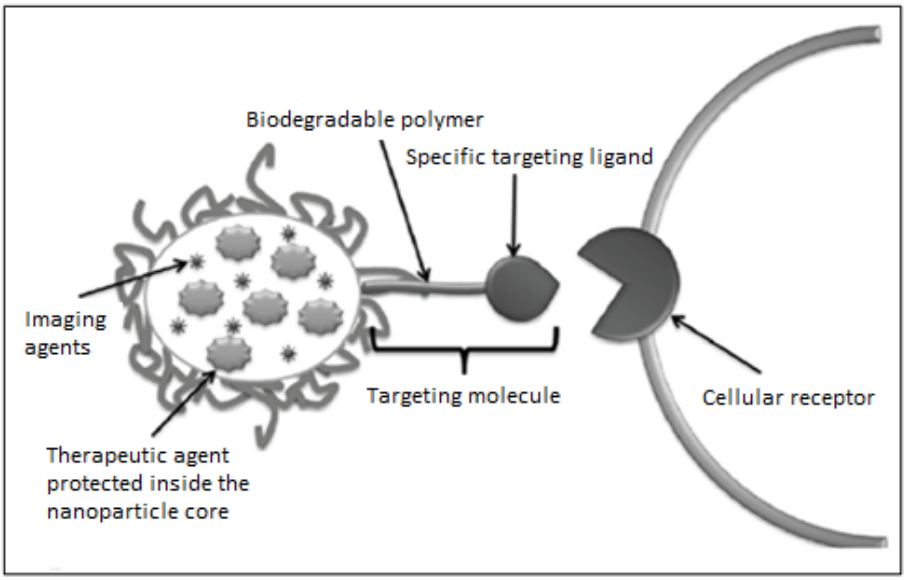

Figure 7. Strategy to create targeted drug delivery systems. Therapeutic tools like genes, proteins, and small drug molecules, as well as imaging tools such as fluorescent probes or magnetic contrast agents are encapsulated inside the nanoparticle core. In parallel, targeting molecules like specific antibodies or recognition peptides are located on the nanoparticle surface. Adapted from Marin, 2013.

A targeted drug delivery system is based on the delivery of a certain amount of drug for a prolonged period of time to a targeted diseased area within the body. This helps maintain the required plasma and tissue drug levels in the body; therefore avoiding any damage to the healthy tissue via the drug and drug loss due to natural distribution in the body (Rani et al., 2014).

NPs can have a natural or synthetic origin. Synthetic NPs may be prepared from polymeric materials such as poly-ethylenimine (PEI), poly-alkylcyanoacrylates, poly-amidoamine dendrimers (PAMAM), poly-ε-caprolactone (PCL), poly-lactic-co-glycolic acid (PLGA), polyesters such as poly-lactic acid (PLA), or from inorganic materials such as gold, silicon dioxide (silica), among others. Inorganic NPs offer advantages over polymeric NPs in terms of control over size and shape and simplicity of preparation and functionalization but also have disadvantages because they might not be degraded or present undesired toxicity (e.g. carbon nanotubes and fullerenes may lead to lipid peroxidation and oxygen radical formation). On the other hand, natural NPs are produced from natural polymers, such as polysaccharides (chitosan, alginate), amino acids (poly-lysine), poly-aspartic acid (PASA), or proteins (gelatin, albumin).

Natural NPs have the advantage of providing biological interaction with specific receptors/transporters expressed by endothelial cells but they have the disadvantage of batch-to-batch variability, and poor tracking capacity by imaging platforms.

These carriers can transport drugs which may be bound in form of a solid solution or dispersion or be adsorbed to the surface or chemically attached (Tiwari et al., 2012).

Depending on the method of preparation, nanocapsules or nanospheres can be obtained differing in their composition and properties such as the ability to encapsulate, deliver and release the active compound (Guterres et al., 2007). Nanocapsules are systems in

which the drug is confined to a cavity surrounded by a unique polymer membrane, while nanospheres are matrix systems in which the drug is physically and uniformly dispersed (Velavan et al., 2015). Knowledge of the physicochemical properties of the drug is crucial in order to select the best method of preparation and starting materials to prepare the carrier system with desired shape, diameter and surface properties and with good entrapment efficiency of the drug.

The essential aspects that NPs preparation methods should have are the use of less toxic reagents, the simple final composition, (minimal number of components/eccipients in the formulation), simplification of the procedure to achieve production scale-up and optimization to improve yield and entrapment efficiency (Nagavarma et al., 2012) .

Preparation techniques are classified according to the initial state of the polymer into two main categories, NPs obtained from polymerization of a monomer or NPs obtained from a macromolecule (Fig. 8).

Figure 8. NPs techniques of preparation: methods for preparation of NPs from dispersion of preformed polymer (red); methods for preparation of NPs from polymerization of monomers (green); other methods commonly used (violet; orange).

It is possible to obtain NPs with the desired properties for a particular application by combining the selection of raw materials and the preparation technique (Dinda et al., 2013). When formulating drug loaded NPs, it should always be kept in mind that in most cases, drug contents are 5-10%wt/wt of NPs weights or even less.

Therefore, generally about 90% of the material administered is NPs excipients with their potential toxicity. Thus, drugs with high intrinsic pharmacological activities should be preferred to avoid the administration of massive dose of NPs material (Olivier, 2005). Other important factors to be considered in designing a successful nanoparticulate system are the drug release and the polymer biodegradation.

The release profile is strictly related to drug solubility, desorption of the surface bound/adsorption of the drug and the diffusion of the drug through the polymeric matrix, degradation rate or erosion profile of the polymer matrix, and combination of erosion/diffusion process (Meena et al., 2011). Moreover, a successful nanoparticulate system should be physically stable without aggregation for prolonged period of time during storage and stable during *in vivo* administration.

Summarizing, the formulation must be scalable and follow cost effective manufacturing process; should be amenable to load small molecules, peptides, proteins, or nucleic acids; should be able to withstand minimal nanoparticle-excipient induced drug alteration, chemical degradation and protein denaturation and should have high drug loading capacity to reduce the quantity of the materials to be needed for its matrix formation and able to interact and overcome with biological barriers.

3. NANOPARTICLES FOR BRAIN TARGETING: CROSSING OR BYPASSING THE BLOOD-BRAIN-BARRIER

Nowadays the development of innovative approaches and new effective treatment for brain diseases is one of the primary goal for pharmaceutical companies and academic research and it is also the most expensive. The process of discovery and development of new drug for CNS disorders, is time-consuming and very expensive.

The average cost of getting a drug onto the market is ever increasing and now approaching US$1 billion before reaching the consumer (Tsaioun et al., 2009). Findings and advances in the field of nanomedicine have generated strategies that improve drug transport across/bypass the BBB, such as the use of NPs, currently under intensive investigation (Saraiva et al., 2016). The current challenges are to design and formulate drug delivery carriers, which must be able to deliver the drug to the brain safely and effectively.

Among different delivery systems, NPs seem to be efficient systems in delivery of conventional drugs, recombinant proteins, peptides, vaccines as well as nucleotides. These last molecules may be advantageously formulated in brain-targeted nanocarriers in order to be protected from their poor stability in biological fluids, rapid enzymatic degradation, unfavorable pharmacokinetic properties, and lack of diffusion toward the CNS. Moreover, the small dose requested for therapeutic activity could easily fit the loading capacity of NPs and would not require the administration of large amount of potentially toxic excipients (Olivier, 2005).

3.1. Crossing the Blood-brain-barrier

NPs are exciting systems for brain drug delivery due to the possibility to modulate them in terms of composition, shape, size, hydrophobicity, coating, chemistry, surface charge and ligands (Fig.9) (Lahkar et al., 2013). Control over these features can enhance the ability of NPs to improve the therapeutic agent stability in biological environment, to control the cargo release into the desired target site, to enhance BBB penetration efficiency and to escape the reticuloendothelial system.

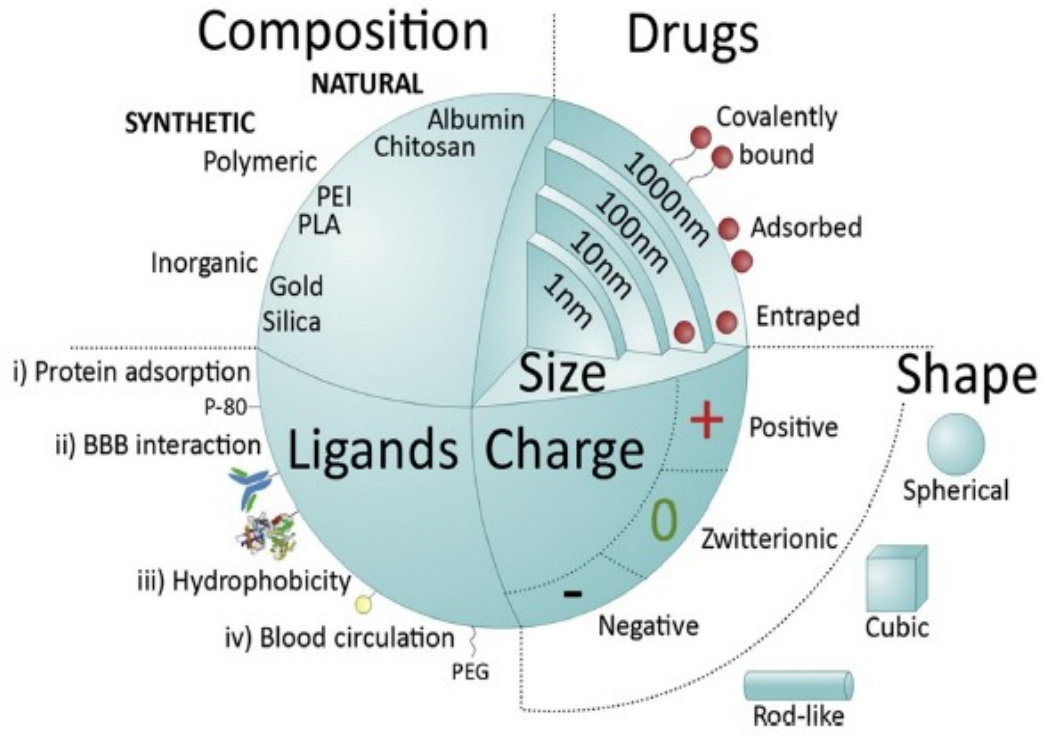

Figure 9. Main NPs features influencing systemic delivery and BBB passage. From Saraiva, 2016.

NPs may be because of its size and functionalization characteristics able to penetrate, overcome and facilitate the drug delivery through the barrier (Tosi et al., 2013). There are different mechanisms and strategies found to be involved in this process, which are based on the type of nanomaterials used and its combination with therapeutic compounds. The use of these nanosystems is expected to reduce the need for invasive

procedures for delivery of therapeutics to the CNS (Dinda et al., 2013). Many strategies are under investigation in order to enhance drug delivery to the brain such as NPs functionalization with different types of ligands. (Fig. 10).

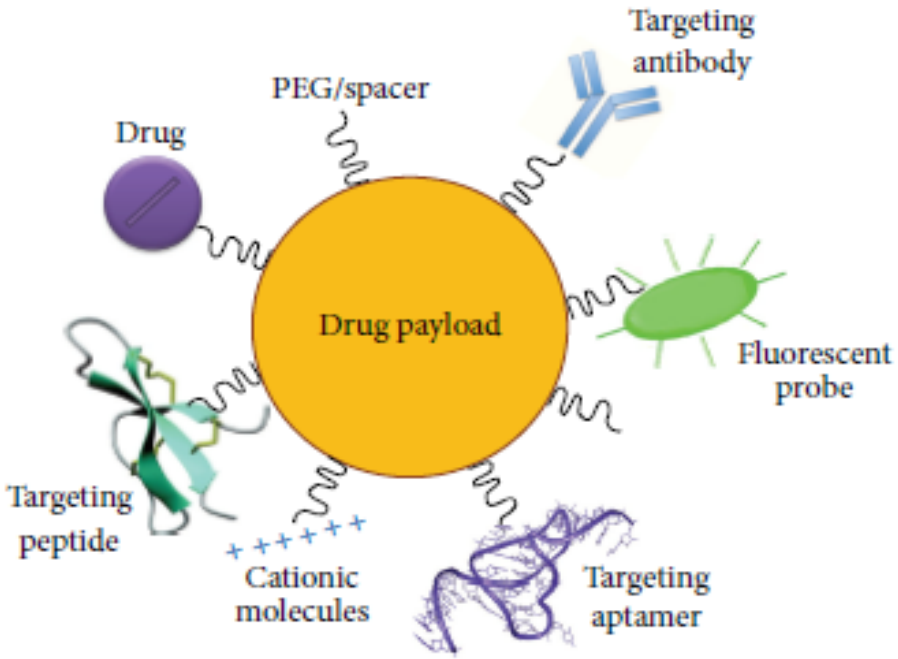

Figure 10. Multifunctionalized NPs. Graphical representation of surface-modified NPs with drugs (incorporated within the core of NPs or coniujated to the surface), targeting molecules (antibodies, peptides, aptamers, and cationic molecules) for brain drug delivery, with PEG for stealthiness and with fluorescent probe as a tracer. From Masserini, 2013.

NPs could play this role at least in two ways: (i) by increasing the drug concentration inside, or at the luminal surface of BBB cells, establishing a local high concentration gradient between blood an brain, higher than that obtainable after administration of the free drug. The gradient should then favor the enhanced passive diffusion of the drug; (ii) by moving themselves into the CNS, together with their drug cargo (Masserini, 2013).

Ligands are distributed into four major categories: i) capable of mediating protein adsorption (e.g. poly-sorbate 80, P-80); ii) able to interact directly with the BBB (e.g. transferrin proteins, antibody or peptides); iii) capable of increasing hydrophobicity (e.g. amphiphilic peptides); and iv) able to improve blood circulation (e.g. poly-ethylene

glycol, PEG). NPs can assume different shapes and charges (negative, zwitterionic, positive).

The shape of NPs also influences body distribution and cellular uptake. The shape of NPs can vary from spherical, cubic, rod-like, among other forms (Fig. 9).

It has been well demonstrated that the size, coating and surface charge of NPs have a crucial impact on the intracellular uptake process as demonstrated by Shilo and collegues which investigated the effect of NPs size on the probability to cross the BBB, using the endothelial brain cell model and found that intracellular uptake of gold NPs is strongly dependent on gold NPs size (Shilo et al., 2015). Another study performed by Georgieva and collegues demonstrated that surface modifications of NPs, (of a fixed sizes), including charge and protein ligands, affect their mode of internalization by brain endothelial cells and thereby their subcellular fate and transcytotic potential. They found that the coupling of a ligand or charge at the surface of a nanoparticle of a given size modifies its entry pathway and processing in human BBB endothelial hCMEC/D3 cells.

Careful analyses suggest that uncoated NPs do not enter in an all-or-nothing or exclusive pathway but following surface modification show preference for a specific pathway(s) and as a consequence NPs are delivered to intracellular compartments that are distinct with regard to their ultrastructural morphology and composition (Georgieva et al., 2011). Therefore, several parameters influence the transport of NPs through the BBB at different extents. So far, NPs conjugated with ligands able to interact with BBB receptors at a relatively low density have the best performance.

NPs brain delivery improvement might require systems that target and cross efficiently the BBB but also systems that are slowly clear from the bloodstream.

Many studies have demonstrated that the surface charge and the morphology of the NPs have a very important effect in the clearance. Neutral and zwitterionic NPs have a longer circulation time after intravenous administration, in contrast to negatively and positively charged NPs (Arvizo et al., 2011). Moreover, as reported by Huang and collegues, short-rod NPs are preferentially retained in the liver and present a rapid clearance rate, while long-rod NPs are caught in the spleen and have a lower clearance rate. If the surface is modified with PEG, retention increases in lung for both formulations (Huang et al., 2011).

3.2. Bypassing the Blood-brain-barrier

Because of the difficulty for drugs or particles to cross the BBB, alternative ways should be considered. “Bypass” rather than “cross” could be one of these.

There is a route to deliver drugs or NPs directly from the nasal cavity to the brain, which is intranasal delivery. This route is considered a promising strategy in brain-targeted drug delivery, it provides a non-invasive method of bypassing BBB to deliver therapeutics into the brain (Illum, 2000).

Delivery of the molecules occurs mainly through olfactory and trigeminal nerve systems in the nasal epithelium to the olfactory bulb and brainstem and further to different parts of the brain. This topic is widely described in section 4. Nose-to-brain.

4. NOSE-TO-BRAIN

Commonly, the nasal route has been used to administer topically acting molecules to treat local diseases, anti-allergic drugs and nasal decongestants are the most typical examples (Djupesland, 2013). During the last decades, intranasal (IN) administration

has gained great attention in research and has been investigated extensively with regard to its feasibility to serve as a direct drug transport route to the CNS as demonstrated by the increasing number of publications in this field (fig.11) (Kozlovskaya et al., 2014).

To identify scientific publications reporting data on drugs, macromolecules, DDS and more specifically polymeric and lipid nanoparticles to the brain via the nasal route, searches in the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed/) have been performed. Screening of the publications was performed, (based on their abstract, and subsequently on their full text), to identify the publications that were suitable for this analysis.

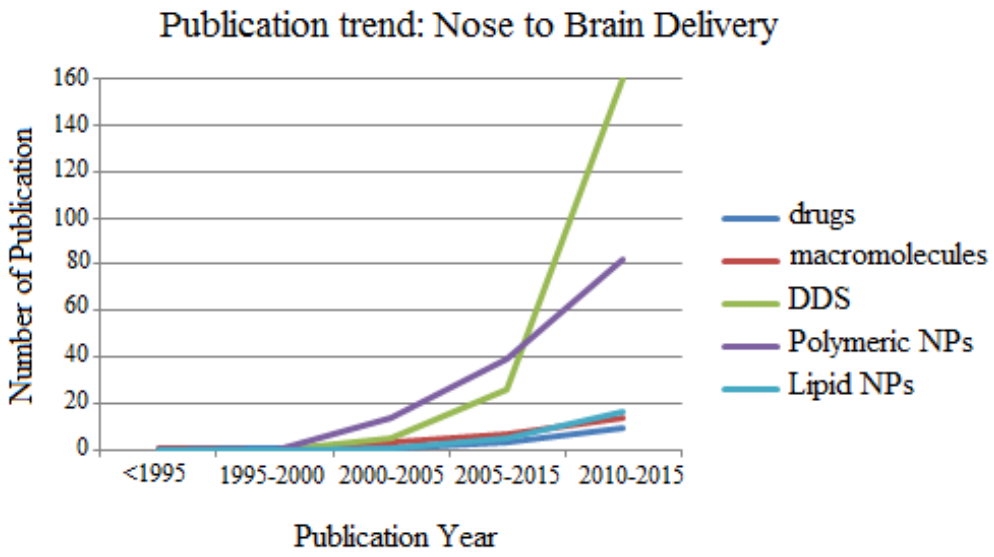

Figure 11. The publication trends in the field of drugs, macromolecules, DDS, polymeric and lipid nanoparticle for brain delivery and targeting via the nasal route. 2015 PubMed database, keys words: intranasal administration to brain macromolecules, drug delivery systems (DDS), polymeric nanoparticles, lipid nanoparticles; Temporal range: From 1995 to 2015.

As shown in Fig.11 the intranasal route has gained interest during the last decade, above all the studies regarding DDS. Among these, polymeric NPs seem to be excellent candidates for brain delivery.

The widespread interest in IN route for therapeutic purposes arises from the particular anatomical, physiological and histological characteristics of the nasal cavity.

"The nose is the only natural corridor where the brain meets the outside world" (cit. by Dr. Gerallt Williams) (Djupsland, 2014).

The olfactory neuroepithelium located inside the nasal cavity is the only area of the body in direct contact with both the CNS and the external environment, which opens up for therapeutic treatments (Chang et al, 2014 ; Sveinbjorn, 2012).

Furthermore, IN administration avoids the gastrointestinal and hepatic metabolism, enhancing drug bioavailability and allowing a lower therapeutic drug dose and fewer systemic side effects (Shabana et al., 2015). Additionally, it also offers several practical advantages either from the viewpoint of patients (non-invasiveness, essentially painless, ease drug delivery and favorable tolerability profile) and pharmaceutical industry (i.e. unnecessary sterilization of nasal preparations) (Pires et al., 2009). Table 2 reports some advantages and limitations of nose-to-brain delivery. Hence, it seems to be an encouraging route for the treatment of acute and also chronic conditions requiring considerable drug exposure.

Although IN route to improve access to the systemic circulation (due to the highly vascularized mucosa) is important for some applications, it is the potential for circumventing the systemic circulation and delivering drugs directly into the brain that represents a particularly novel, attractive and little understood application of IN delivery. Direct transport of active molecule along the olfactory and trigeminal nerves is increasingly considered a promising route whereby drugs delivered to the nose can access the CNS in therapeutic concentrations (Djupsland, 2014).

Table 2. Advantages and limitations of nose-to-brain drug delivery.

ADVANTAGES AND LIMITATIONS OF NOSE TO BRAIN DRUG DELIVERY	
Advantages	**Disadvantages**
Rapid, safe, non-invasive and convenient method	Rapid elimination of drug substances from nasal cavity due to mucocilliary clearance
Avoids drug degradation in gastrointestinal tract, first-pass metabolism and gut-wall metabolism of drugs, allowing enhanced bioavailability	Absorption enhancers used in formulation may create mucosal toxicity
Reduction of systemic exposure of drugs and systemic side effects	Variability in the concentration attainable in different regions of brain and spinal cord
Bioavailability for low molecular weight drugs	Nasal congestion due to cold or allergic condition may interfere with this technique of drug delivery
Rapid drug absorption via highly vascularized mucosa	Suitable for potent drugs since only a limited volume can be sprayed into the nasal cavity
Ease of administration (self-administratiom), Improved convenience, Better patient compliance	Frequent use of this route leads to mucosal damage irritation of nasal mucosa
Convenient route when compared with parenteral route for long term therapy	Mechanical loss of the dosage form could occur due to improper technique of administration
Bioavailability of larger drug molecules can be improved by means of absorption enhancer or other approach	Mechanisms of drug transport are still unclear

4.1. Pathways and mechanisms

Although the exact mechanisms underlying nose-to-brain delivery are not entirely understood yet, an accumulating body of evidences demonstrates that pathways involving nerves connecting the nasal passages to the brain and spinal cord are important (Fig.12). Moreover, pathways involving the vasculature, CSF and lymphatic system have been employed in transport of molecules from nasal cavity to the CNS. It is possible that a combination of these pathways is responsible, even if one pathway may

predominate, depending on the properties of active compounds, the characteristics of formulations and the delivery device used.

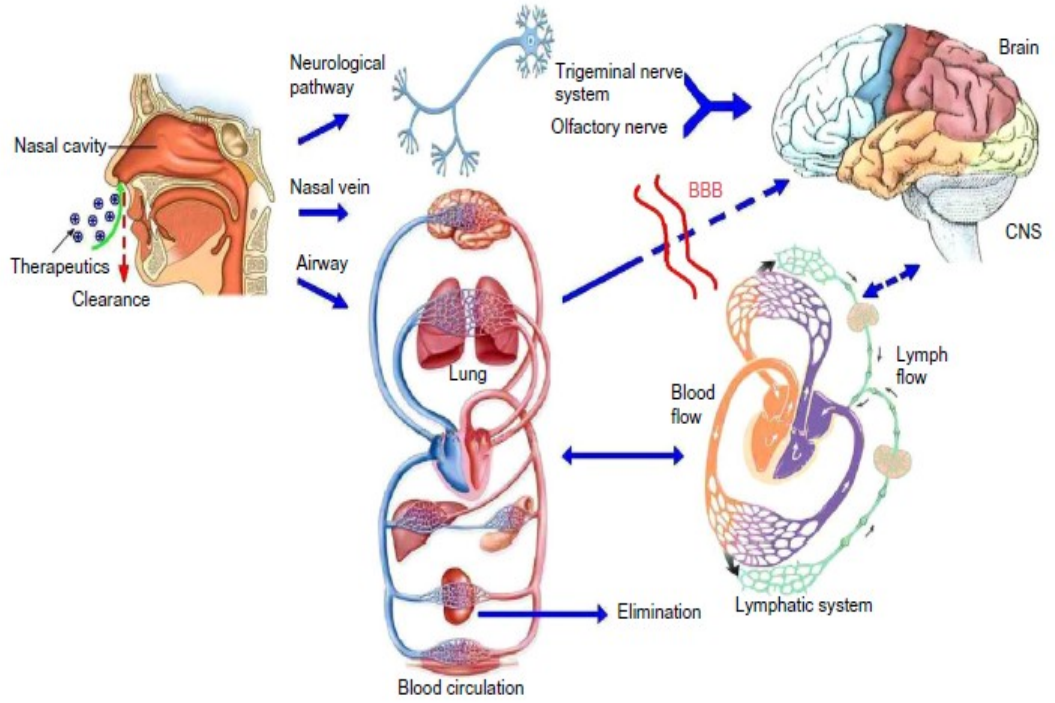

Figure 12. Olfactory and trigeminal pathways to the CNS. From Cui-Tao Lu et al., 2014.

The delivery from the nose to the CNS may occur via olfactory neuroepithelium and may involve paracellular, transcellular and/or neuronal transport. Paracellular pathway through tight junctions between sustentacular cells or the so-called clefts between sustentacular cells and olfactory neurons. This is slow and passive route and it is responsible for transport of hydrophilic drugs and it shows rate dependency on the molecular weight of a drug (Belgamvar et al., 2013). Transcellular process is responsible for the transport of lipophilic drugs that show a rate dependency on their lipophilicity. Expecially across the sustentacular cells, most likely by receptor-mediated endocytosis, fluid phase endocytosis or by passive diffusion. It is mediated rapidly and at a high rate (Pavuluri et al., 2015). Neuronal transport in which drug is taken up into

the neuronal cell by endocytosis or pinocytosis mechanisms and transported by intracellular axonal transport to the olfactory bulb (Mustafa et al., 2016). More recently, the contribution made by the trigeminal pathway to IN delivery to the CNS has also been recognized, especially to caudal brain regions and the spinal cord. The first researchers that clearly demonstrated the involvement of this pathway were Thorne and colleagues which assessed the potential of delivering insulin-like growth factor-I (125I-IGF-I), directly into the CNS following IN administration and they elucidated the mechanisms involved in the transport (Thorne et al., 2004). They found high levels of radioactivity in the trigeminal nerve branches, trigeminal ganglion, pons, and olfactory bulbs, consistent with delivery along both trigeminal and olfactory nerves.

Trigeminal nerve innervates the respiratory and olfactory epithelium of nasal cavity and enters the CNS in the pons and represents another important pathway connecting nasal cavity to the CNS. Interestingly, a small portion of trigeminal nerve also terminates in the olfactory bulbs. The trigeminal nerve-mediated transport pathway also plays a key role in the distribution of intranasally administered drugs to brain areas distant from the olfactory bulbs.

The ophthalmic and maxillary branches of trigeminal nerve are important for nose to brain drug delivery as neurons from these branches pass directly through the nasal mucosa. A unique feature of the trigeminal nerve is that it enters the brain from the respiratory epithelium of the nasal passages at two sites: i) through anterior lacerated foramen near the pons and ii) through the cribriform plate near olfactory bulb, creating entry points into both caudal and rostral brain areas following IN administration. Because one portion of the trigeminal neural pathway enters the brain through the cribriform plate alongside the olfactory pathway, it is difficult to distinguish whether

intranasally administered drugs reach the olfactory bulb and other rostral brain areas via the olfactory or trigeminal pathways or if both are involved.

In addition to these direct pathways, transport may also occur via blood vasculature, lymphatics, and cerebrospinal fluid present in the nasal mucosa tissue (Kozlovskaya et al., 2014). The nasal mucosa is highly vascularized, the relative density of blood vessels is greater in the respiratory mucosa compared to the olfactory mucosa making the region an ideal site for absorption into the blood. The blood vessels with continuous and fenestrated endothelium allow passage of both small and large molecules to enter the systemic circulation following nasal administration (Dhuria et al., 2010). The drug that has been absorbed into the systemic circulation has to cross the BBB in order to reach the CNS. However, many problems may arise with the systemic delivery due to drug elimination via hepatic and renal mechanisms, and some other limiting factors such as: the BBB, drug binding to plasma proteins, degradation by plasma proteases, and potential peripheral side effects (Alavijeh et al.,2005).

4.2. Free molecules delivery via nose-to-brain

Several investigations have been reported concerning the transport of free drug from the nasal cavity to the CNS. Considering the large number of compounds that have been shown in animal models to be directly transferred from the nasal cavity to the olfactory bulb there should be no doubt that this type of transfer occurs (Mathison et al., 1998; Illum, 2000). The question of whether this form of transfer is sufficiently extensive to result in therapeutically effective concentrations at the site of action in humans, however, remains to be answered.

Very interesting experiments were performed by Serrailhero and collegues, using an antiepileptic drug, carbamazepine. They assessed the pharmacokinetics of carbamazepine administered via the IN and intravenous (IV) routes to mice, and investigate whether a direct transport of the drug from nose to brain could be involved.

The similar pharmacokinetic profiles obtained in all matrices following both administration routes indicate that, after IN delivery, carbamazepine reaches quickly and extensively the bloodstream, achieving the brain predominantly via systemic circulation.

However, the uneven biodistribution of carbamazepine through the brain regions with higher concentrations in the olfactory bulb and frontal cortex following IN instillation, in comparison with the homogenous brain distribution pattern after IV injection, strongly suggests the involvement of a direct transport of carbamazepine from nose to brain. Regarding the mean residence time parameter (MRT), higher values were attained for plasma and brain after IN administration comparatively to IV administration, in contrast with the liver, where the highest MRT value was assigned to the IV route (Serralheiro et al., 2014). Westin and collegues investigated whether morphine can be transferred along the olfactory pathway to the CNS. In their study the autors found [^{3}H]-morphine in the CNS surrounding the olfactory bulbs by autoradiography in rats within 5 minutes of IN administration (Westin et al., 2007).

Morphine was found in the olfactory bulb ipsilaterally to the side of the nasal cavity that was administered with the dose after 60 min and a gradient of radioactivity was found also in the brain when higher level of morphine were found closer to the cribriform plate. However, no significant penetration of the radioactivity was detected in deeper brain areas.

Many scientific publications shown that there are evidence of drug directly delivered to the brain after the IN administration but the key finding from all these studies is that the amount of the therapeutic agent into the CNS is normally minimal, less than 1% of the drug administered very low compared to the dose administered (about 0.12% of the administered dose as reported by Jansson) (Charlton et al., 2007; Jansson, 2004; Sakane et al., 2004; Sakane et al., 1991). The optimization of nasal administration using DDS represents a possible strategy to overcome this problem. Moreover, drug should have specific properties to be administered intranasally, as reported in table 3.

Table 3. Summary of drug properties required for nasal delivery.

SUMMARY OF DRUG PROPERTIES REQUIRED TO IMPROVE NASAL DELIVERY
Given the low volume of nasal cavity, the drug's solubility in water must be high enough to accommodate the necessary dose
For high bioavailability, a drug must be resistant to metabolizing enzymes in the nasal environment
Drug residence time in contact with the mucosal membrane is an important factor influencing drug absorption
Potential local toxicities need to be considered in parallel with benefits
Nasal solutions with tonicities ranging from0.6–1.8% NaCl equiv. are well tolerated, 0.9% NaCl equiv. being isotonic.
The nasal cavity can accommodate only a low solution volume, necessitating highly concentrated nasal drug solutions

Drug delivery technologies represent a good strategy to improve drug properties for nasal delivery and can also modify drug release profile, absorption, distribution and elimination for the benefit of improving product efficacy and safety, as well as patient convenience and compliance (Mittal et al., 2014; Kapoor et al., 2016).

Furthermore, DDS can protect the encapsulate compound from degradation because the nasal mucosa retains some enzymatic activity (Mistry, 2009). The pharmacokinetic

parameters reported in some papers supported the superiority of nose-to-brain delivery of therapeutic agents through nanoparticles (NPs) (Bhavna et al., 2014). Higher T_{max} value were obtained in the brain compared to blood suggests preferential nose-to-brain transport following IN administration of drug-loaded NPs (Niyaz et al., 2015; Kulkarni et al., 2015). Alam et al. carried out an interesting study into the delivery of thymoquinone (TQ) into the brain, and they simultaneously investigated the plasma pharmacokinetics and brain distribution profiles of the TQ-loaded NPs in Wistar rats after IV and IN administration in order to assess whether a direct nose-to-brain transport pathway was involved (Alam et al., 2012).

The biodistribution pattern and different pharmacokinetic properties of intranasally administered NPs were evaluated using scintigraphic imaging. The concentrations of 99mTC-loaded TQ-NPs in the liver when administered intravenously was higher compared to IN 99mTC-loaded TQ-NPs and 99mTC solution because of the presence of the reticuloendothelial system. A similar pattern of 99mTC-loaded TQ-NPs distribution was also obtained in the lungs and in kidney.

The higher concentrations of 99mTC achieved in the highly perfused organs, such as liver, lungs, and kidney are probably due to the combined activity of the circulating blood passing through the organs as well as particle uptake by reticuloendothelial system cells. The brain:blood ratio of the drug was found to be higher for the TQ-NPs formulation over the IN TQ solution. Similarly, the brain:blood ratio of the drug were higher for the IN TQ solution compared to the IV TQ solution. Moreover, following IN TQ-NPs, the drug concentrations in the brain were sustained for 2–3 hours, which was lacking in TQ solution (IN and IV). The substantially higher uptake in the brain after IN administration suggests a larger extent of selective transport of TQ-NPs from nose-to

brain. The formulations showed a significant difference in T_{max} (0.5 and 2 hours), C_{max} (242.88, 1717.74, and 2417.17 counts) and K_{el} (0.101, 0.086, and 0.0696 counts/hour) for IV TQ solution, IN TQ solution, and IN TQ-NPs, respectively. Significantly lower C_{max} ($P > 0.01$) and AUC ($P > 0.005$) for the IN TQ solution may be due to the mucociliary clearance under normal circumstances, which rapidly clears the instilled formulation. On the other hand, TQ-NPs which are intrinsically mucoadhesive showed a significant improvement in C_{max} and AUC. This demonstrates the value of the mucoadhesive agent in prolonging the contact time of the formulation with the nasal mucosa. The significantly higher AUC and C_{max} for TQ-NPs compared to the TQ solution is attributed to the nanoparticulate carriers (Alam et al., 2012).

However to date, there are relatively few studies describing the specific qualities or characteristics that a suitable carrier for this route should possess; and the localization of NPs into specific brain regions related to the time after IN administration (Buchner et al., 1987). NPs formulations coupled with the strategy of IN administration may facilitate the transport of a significantly larger amount of drug to the brain. Consequently, these formulations have the potential to create an effective therapeutic response at a lower dose than unencapsulated CNS therapeutics (Piazza et al., 2014). In order to foresee whether IN delivery of NPs could became clinically relevant, technological optimization of the nasal drug formulation, as well as further evidence and pre-clinical investigations are needed to evaluate the therapeutic efficacy attained via this route.

5. EPILEPSY

Epilepsy is the 4th most common neurological problem only migraine, stroke and Alzheimer's disease occurs more frequently (Hirtz et al., 2007). Epilepsy is a chronic neurological disorder that affects approximately 50 million people worldwide (Reynolds et al., 2000). Epilepsy is a CNS condition in which nerve cell activity in the brain becomes disrupted, causing recurrent seizures or/and periods of strange behavior, sensations and sometimes loss of consciousness with highest incidence in seniors and young children, but epilepsy can begin at any age (http://www.ninds.nih.gov/disorders/epilepsy).

Epilepsy can be considered a spectrum disorder because of its different causes, different seizure types, its ability to vary in severity and impact from person to person, and its range of co-existing conditions (Beletsky et al., 2012). Generally, a person is not considered to have epilepsy until he/she has had two or more unprovoked seizures separated by at least 24 hours. There are many possible causes for developing epilepsy, such as genetic factors, developmental brain abnormalities, abnormality in brain wiring, an imbalance of nerve signaling in the brain (in which some cells either over-excite or over-inhibit other brain cells from sending messages), infection, traumatic brain injury, stroke, brain tumors but for about half of those with this condition a cause is not identified. Anything that disturbs the normal pattern of neuronal activity from illness to brain damage to abnormal brain development can lead to seizures (http://www.ninds.nih.gov/disorders/epilepsy).

Seizures can be divided into two major groups: focal (partial) and generalized.

Focal seizures originate and affect a limited area, or focus, of one hemisphere of the brain (and may spread to other regions). About 60% of people with epilepsy have focal

seizures. Different areas of the brain (the frontal, temporal, parietal and occipital lobes) are responsible for controlling all of our movements, body functions, feelings or reactions. So, focal seizures can cause many different symptoms. Partial seizures are split into two main categories; simple partial seizures and complex partial seizures. In simple partial seizures a small part of one of the lobes may be affected and the person remains conscious but may experience motor, sensory, or psychic feelings. Instead, complex partial seizure affects a larger part of the hemisphere than a simple partial seizure and the person may lose or have alteration of consciousness which can produce a dreamlike experience. Some people with focal seizures may experience auras that is an unusual sensations that warn of an impending seizure.

Auras are usually focal seizures without interruption of awareness but some people experience a true warning before an actual seizure (Spencer, 2015). These seizures are frequently described by the area of the brain in which they originate, can often be slight or uncommon, and may go unnoticed or be mistaken for anything from intoxication to daydreaming or can easily be confused with other disorders.

Generalized seizures are a result of abnormal neuronal activity that rapidly emerges on both hemispheres of the brain simultaneously. These seizures may cause loss of consciousness, falls, or a muscle's massive contractions. The many kinds of generalized seizures include:

- ✓ *Absence seizure*s may cause the person to appear to blanking out or staring into space with or without slight twitching of the muscles;
- ✓ *Tonic seizures* cause stiffening of muscles of the body, generally those in the back, legs, and arms.

- ✓ *Clonic seizures* cause repeated jerking movements of muscles on both sides of the body. The movements cannot be stopped by restraining or repositioning the arms or legs.
- ✓ *Myoclonic seizures* cause brief shock-like jerks of a muscle or group of muscles.
- ✓ *Atonic seizures* cause a loss of normal muscle tone, which often leads the affected person to fall down or drop the head involuntarily.
- ✓ *Tonic-clonic seizures* cause a combination of symptoms, including stiffening of the body and repeated jerks of the arms and/or legs as well as loss of consciousness.
- ✓ Secondary generalized seizures, they only become generalized (spread to both sides of the brain) after the initial event (a partial seizure) has already begun (https://www.epilepsy.org).

At this time there are no medications or other therapies that have been shown to prevent epilepsy. In the absence of a specific etiological understanding, approaches to drug therapy of epilepsy must necessarily be directed at the control of symptoms by chronic administration of antiepileptic drugs (AEDs). However, seizures remain uncontrolled in at least 30% of all epilepsies despite adequate AEDs therapy (Schmidt, 2009). This demonstrates a continued need for developing new antiepileptic drugs or new formulations with the aim of creating new concepts and original ideas to effectively prevent epilepsy or its progression.

If, on one hand, the cellular basis of human epilepsy is far from being fully understood, in the other hand thanks to De Lanerolle and collegues clear evidences for located brain damage and epilepsy were demonstrated. They provided the first evidence of such reorganization of a hippocampal seizure focus in human temporal

lobe epilepsy (de Lanerolle et al., 1989). Evidence from several studies demonstrate that seizures may cause neuronal degeneration and cognitive dysfunction as reported by, Mendez and Lim, which found that seizures have been linked to both sporadic and early-onset Alzheimer's disease (Holmes GL, 2016, Mendez et al., 2003). Anti-convulsants, often termed AEDs remain the mainstay of treatment and consist of a group of drugs that are highly susceptible to drug-drug interactions (Halvorsen et al., 2016); however, not all medications work for all types of epilepsy or for every individual (Panayiotopoulos, 2005).

The main goal of AEDs treatment in epilepsies is to achieve seizures freedom with minimal if any drug-related adverse reactions. This is achieved in around 50–70% of patients with a single appropriately selected AED at target therapeutic doses as reported in the Kwan and Brodie study (Kwan et al., 2000).

The appropriate use of AEDs requires a deep understanding of their clinical pharmacology. The mechanism of action of most AEDs can be categorized as either affecting ion channels, augmenting inhibitory neurotransmission, or modulating excitatory neurotransmission. The ion channels affected include the sodium and calcium channels. Augmentation in inhibitory neurotransmission includes increasing CNS concentrations of GABA, whereas efforts to decrease excitatory neurotransmission are primarily focused on decreasing (or antagonizing) glutamate and aspartate neurotransmission (Goldeberg, 2010).

Moreover, awareness of pharmacokinetic properties, side effects, indications, dosage forms, AED-AED interactions, and AEDs metabolic pathway as well as inducer or inhibitory effects on liver can help in the optimization of AEDs therapy.

Pharmacokinetic interactions are a common complicating factor in AEDs selection. Interactions can occur in any of the pharmacokinetic processes: absorption, distribution, or elimination. Caution should be used when AEDs are added to or withdrawn from a drug regimen (Faught, 2001). Careful and rigorous diagnosis and classification of seizure and syndrome type is critical to select the suitable pharmacotherapy. Patient characteristics such as age, comorbid conditions, ability to comply with the prescribed regimen, and presence or absence of insurance coverage also can influence the choice of AEDs. Polytherapy should be avoided if possible, but it is inevitable in approximately 30–50% of patients who fail to respond to single-drug therapy. It is important to underline that special groups of patients with epileptic disorders require particular attention and management. Children, the elderly, women (particularly women in pregnancy) and people with mental and physical disabilities are vulnerable and their treatment is more demanding (Bourgeois, 2000; Camfield et al., 2003; Willmore, 2000; Tallis, 2004; Bruno et al., 2002; Tatum et al., 2004; Brobtkorb, 2004; Derek et al., 2016). Because therapy is continued for many years (often a lifetime), chronic side effects must be considered. Novel agents are helpful because about a third of patients continue to be pharmaco-resistant. Based on these evidence and considering that many active drugs have been already known, the development of new formulations of the known compounds with anticonvulsant activity may help to improve drug activity; reduce or eliminate seizures and the acute and chronic side effects that occur during the treatment.

5.1. Oxcarbazepine

Oxcarbazepine (OX) is the model drug selected in this project. It is a second generation AEDs. OX is used for the treatment of partial seizures as a monotherapy or as an adjunctive therapy in adults and children aged 4 to 16 years. OX is also sometimes used to treat acute mania in adults, as well as bipolar disorder, a disease that causes episodes of depression, episodes of frenzied, abnormal excitement, and neuropathic pain. The mechanism of action for OX is not completely understood. Since oxcarbazepine is one of the most effective and frequently used antiepileptic drugs, we aim to evaluate the possible neuroprotective action of this drug against the seizures and brain damage induced by PTZ administration after IN administration of OX-loaded NPs in rats.

6. GENE THERAPY

"We used to think that our fate was in our stars, but now we know, in large measures, our fate is in our genes" quote by James Watson, well introduces the fascinating and interesting field of genetics and gene therapy. Genes, the functional unit of heredity, are specific sequences bases that encode instructions to make proteins.

Gene alteration significantly impacts on protein functions, resulting in genetic disorders (Misra, 2013).

Many neurodegenerative diseases are caused by genetic mutations, most of which are located in completely unrelated genes.

Gene therapy can help us target the origin of the disorder instead of using drugs to alleviate symptoms. For this purpose, it has drawn significant attention as a promising strategy for specific treatment of numerous gene-associated human diseases ranging

from cancer, hemophilia, hypercholesterolemia, neurodegenerative diseases to autoimmune diseases (Chen et al., 2016). Gene-based therapy is the intentional modulation of the expression of such genes in specific cells to treat pathological conditions. This modulation is accomplished through the introduction of exogenous nucleic acids such as DNA, messenger RNA (mRNA), small interfering RNA (siRNA), microRNA (miRNA) or antisense oligonucleotides. However, naked genetic molecules cannot be internalized efficiently by target cells because of their serum nuclease susceptibility, rapid renal clearance, reduced uptake by target cells, phagocyte uptake and toxic effect arose by immune response stimulation, which seriously inhibits their clinical application.

Moreover, given the big hydrodynamic size the negative charge and low stability of these macromolecules under physiological conditions, their delivery is commonly mediated by carriers or vectors (Hin et al., 2014). The applications and therapeutic outcomes of these nucleic acids may vary depending on their mechanisms of action.

It is difficult to achieve the expected biological effects of nucleic acids by traditional delivery strategies owing to many biological barriers.

Besides instability, several obstacles have to be surpassed before nucleic acids take action at their desired sites. In order to achieve the target site, nucleic acids should be able to translocate across extracellular, cellular and intracellular biological membranes.

If the target site is located in the CNS, the TJs between endothelial cells of CNS vessels (BBB), have to be overcome. Furthermore, these molecules may face many enzymes and proteins during their delivery to the target cells, which may degrade them or trigger immune response (Fig. 13). Finally, the low efficacy is also often related to their non-specific biodistribution to non-target cells and tissues (Zhu et al., 2010).

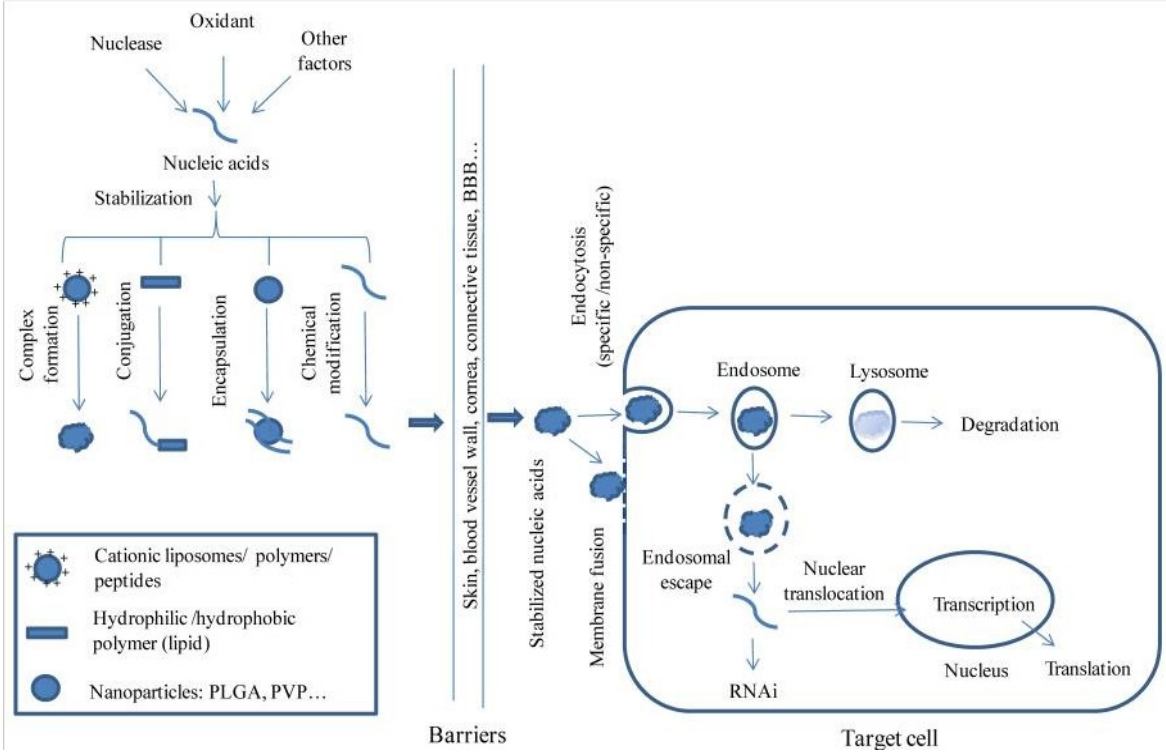

Figure 13. The barriers and strategies of nucleic acid delivery. From Zhu et al., 2010.

For some therapeutic purposes, transient gene expression and silencing are often preferred as it allows for a better control of the therapeutic effect. With the developments of material sciences and the rapid progress of nanotechnology, nanosized materials for gene delivery have attracted worldwide attentions (Lee et al., 2012).

With that said, in this work, a bigger focus was given to RNAi therapies, trying to design a successful *in vivo* delivery strategy.

Whit this in mind, we attempted to satisfy the following major criteria:

(i) the carrier system should protect nucleic acids from degradation by nucleases;

(ii) it should help nucleic acids cross the cell membrane, escape from endosome and finally enter either the cytoplasm;

(iii) it should have no or fewer side effects caused by either nucleic acids or the method itself;

(iv) it should prolong prevent non-specific disposition of nucleic acids to facilitate their delivery to the target cells.

6.1. RNAi mechanism

Since about the turn of this century, scientists have realized that 50 years of focus on DNA had blinded them to the wide range of biological roles held by its chemical cousin, RNA. The old view was that DNA contained life's instructions, proteins carried them out, and RNA served as little more than a go-between. It is now become clear that RNA has vast potential for controlling how cells interpret the instructions embedded in the genome (Check, 2007).

RNA interference (RNAi) can be compared to a light switch because it is able to turn off gene expression (Fig. 14), more specifically, it is the process by which expression of a target gene is effectively silenced or knocked down by the selective inactivation of its corresponding mRNA by double-stranded RNA (dsRNA). Long dsRNAs are cleaved by the RNase III family member, Dicer, into 19-23 nucleotides (nt) fragments with 5' phosphorylated ends and 2-nt unpaired and unphosphorylated 3' ends.

These small dsRNAs are called siRNAs. Each siRNA duplex is formed by a guide strand and a passenger strand.

The endonuclease Argonaute 2 (Ago 2) catalyzes the unwinding of the siRNA duplex. Once unwound, the guide strand is incorporated into the RNA-Induced Silencing Complex (RISC), while the passenger strand is released. RISC uses the guide strand to find the mRNA that has a complementary sequence leading to the endonucleolytic cleavage of the target mRNA (Ryther et al., 2005).

The cleaved mRNA, which is subsequently degraded by intracellular nucleases is no longer available for translation of the corresponding protein.

Therefore exogenous RNA is able to induce gene silencing with very high specificity when introduced directly into the cells, thereby circumventing the Dicer mechanism. RNA interference has proven to be an extremely potent and versatile tool to specifically reduce expression of targeted genes (Mehrotra et al., 2015).

Use of this technology has rapidly moved from *in vitro* cell culture studies to *in vivo* administration.

However, the efficacy of siRNA administered *in vivo* without modification or carriers may be limited by factors governing stability and regulation of uptake of the siRNA into the target cells, where inhibitory effects are exerted. Moreover, since naked siRNA molecules are water-soluble and carry a net negative charge, they are subject to excretion in the mucosa following administration (Akhar et al., 2007; Sharma et al., 2014; van Woensel et al., 2013).

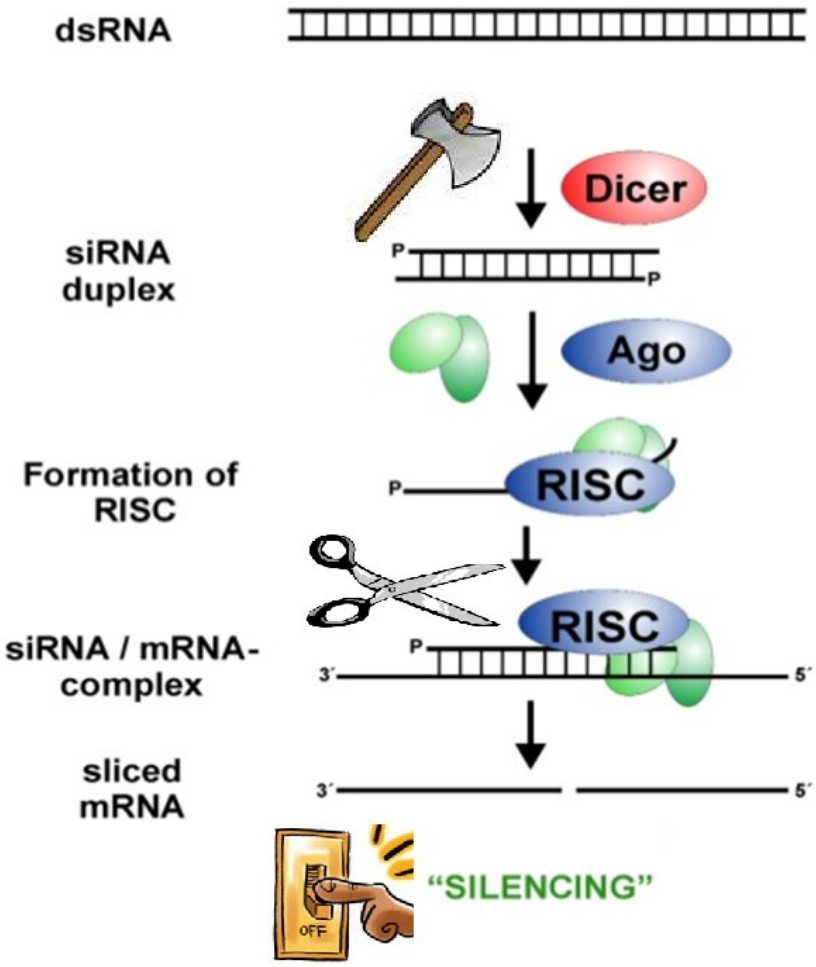

Figure 14. Mechanism of siRNA silencing. Adapted from http://www.gene-quantification.de/si-rna.html

6.1.1. SiRNA delivery

Effective and non toxic delivery of siRNA into cells presents one of the major obstacles that hinder the use of siRNA in the drug discovery process and clinical applications (Liang et al., 2013). Naked siRNA is a high molecular weight molecule having a negatively charged phosphate backbone which causes electrostatic repulsion with the negatively charged cellular membranes and thus limits it diffusivity into the cell (Singha et al.,2011). Naked siRNA's susceptibility to serum nucleases, renal clearance and non-targeted biodistribution, provide hindrance in cell-targeted delivery. Poor stability and short half-life in circulation seriously limits the use of naked siRNA for therapeutics

(Miele et al, 2012). As a result, various vectors as well as chemical modification strategies have been explored for the efficient delivery of siRNA to targeted cells.

The vectors used can be categorized into two broad categories: viral and non-viral vectors (Nayerossadat et al., 2012).

6.1.2. Viral vectors

Several adenovirus, retrovirus and lentivirus have been used as vectors for siRNA delivery systems. Using such viral vectors helps overcome the problem of poor transfection efficiency and poor cell targeting. However, viral vectors have some limitations of their own. Viral vectors have high potential for mutagenesis due to the lack of their insertional predictability, limited loading capacities and may cause adverse immune reactivity. These disadvantages severely limit the use of viral vectors for siRNA delivery (Nayerossadat et al., 2012).

6.1.3. Non-viral vectors

Due to the various limitations of viral siRNA deliver systems, the focus is now on engineered non-viral vectors for safer cell-specific siRNA delivery. Nonviral systems generally include either chemical methods, such as cationic liposomes and polymers, or physical methods, such as gene gun, electroporation, particle bombardment, ultrasound utilization, and magnetofection (Ramamoorth et al., 2015). Efficiency of this system is less than viral systems in gene transduction, but their cost-effectiveness, availability, and more importantly less induction of immune system and no limitation in size of transgenic DNA compared with viral system have made them more effective for gene delivery than non-viral delivery systems to date. An obvious advantage of physical methods is the simplicity.

Physical methods applied for *in vitro* and *in vivo* gene delivery are based on making transient penetration in cell membrane by mechanical, electrical, ultrasonic, hydrodynamic, or laser-based energy so that DNA entrance into the targeted cells is facilitated (Kamimura et al., 2011).

Chemical systems are more common than physical methods and generally are nanomeric complexes, which include compaction of negatively charged nucleic acid by polycationic nanomeric particles, belonging to cationic liposome/micelle or cationic polymers (Hasan et al., 2014). These nanomeric complexes are generally stable enough to produce their bound nucleic acids from degradation and are competent to enter cells usually by endocytosis. Cationic non-viral delivery systems have several advantages such as low toxicity and antigenicity, long-term expression with less risk of insertional oncogenesis (Nayerossadat et al., 2012). For example, chitosan is considered a promising carrier for gene delivery. Many researcher have studied its ability in condensation of nucleic acid and form stable polyplex (MacLaughlin et al., 1998). MacLaughlin et al. studied chitosans of different molecular masses ranging from 7 to 540 kDa and found that the particle size of chitosan/DNA complexes prepared at an N/P ratio of 6:1 with a plasmid concentration of 100 μg/ml increased significantly from 100 to 500 nm along with the increase of chitosan's molecular mass. Huang et al. reported that low-molecular-mass chitosan was less efficient at condensing plasmid DNA, resulting in unstable polyplexes compared with its high-molecular-mass counterparts. Others authors studied chitosan's protonation and its complexation with DNA at different pHs (Liu et al., 2005; Ishii et al., 2001). In addition to charge density, other characteristics also influence the performance of chitosan, including solubility,

degradation and crystallinity. Proper modification of chitosan can improve its performance in the delivery of nucleic acids (Zhu et al., 2010).

7. POLYMERS SELECTED IN THIS PROJECT TO ENHANCE NANOPARTICLES BRAIN TARGETING

The selection of polymers to prepare NPs for specific application is a crucial factor. Polymers, of both synthetic and natural origin, have been selected in this project, to improve NPs brain targeting via intranasal delivery (Fig.15). These materials present very promising and attractive properties as reported in the following paragraphs.

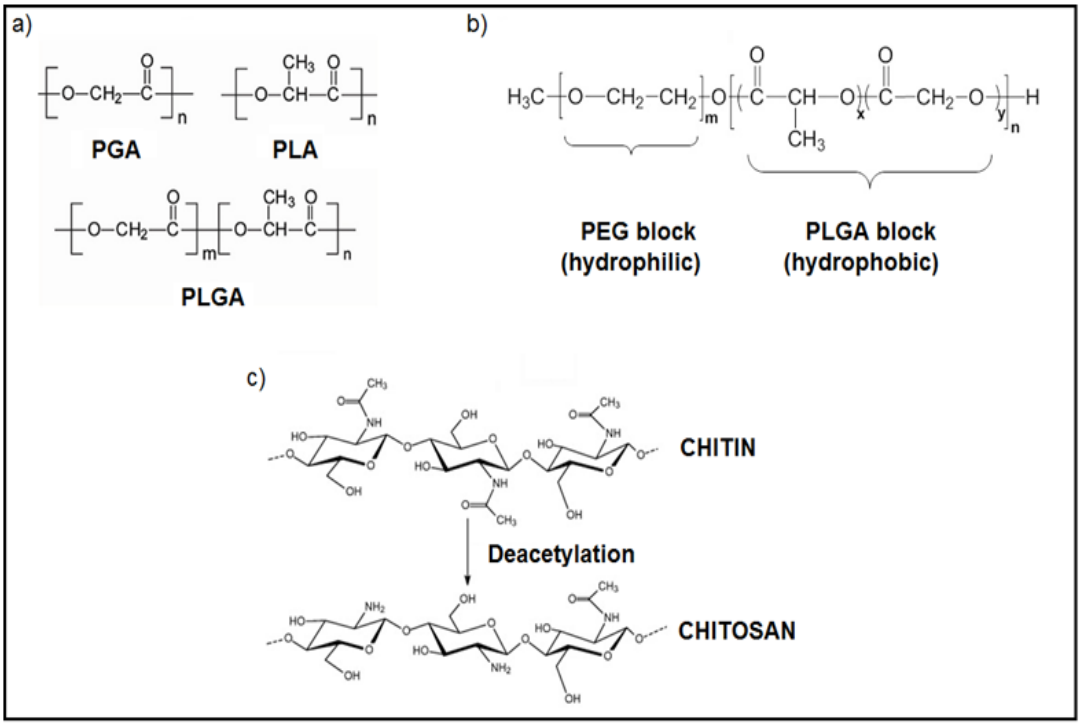

Figure 15. Chemical structure of: a) poly-glycolic acid (PGA), Poly-lactic acid (PLA) and poly-lactic-co-glycolic acid (PLGA); b) PLGA-PEG block co-polymer; c) Chitin and Chitosan.

7.1. PLGA

PLGA, (Figure 15a), are a family of FDA-approved biodegradable polymers that are physically strong and highly biocompatible and have been extensively studied as delivery carriers for drugs, proteins and various other macromolecules such as DNA, RNA, vaccines and peptides for treatment of several importat diseases (Makadia et al.,2011). PLGA is a copolymer of poly lactic acid (PLA) and poly glycolic acid (PGA). It is the best defined biomaterial available for drug delivery with respect to design and performance. PLGA can be processed into almost any shape and size, and can encapsulate molecules of virtually any size.

It is soluble in a wide range of solvents including chlorinated solvents, tetrahydofuran, acetone or ethyl acetate. In water, PLGA biodegrades by hydrolysis of its ester linkages. PLGA physical properties have been shown to depend upon multiple factors, including the initial molecular weight, the ratio of lactide to glycolide, the size of the device, exposure to water (surface shape), temperature and storage (Keles et al., 2015). Mechanical strength, swelling behavior, capacity to undergo hydrolysis and subsequently biodegradation rate of the polymer are directly influenced by the degree of crystallinity of the PLGA, which is further dependent on the type and molar ratio of the individual monomer components in the copolymer chain. Biodistribution and pharmacokinetics of PLGA follows a non-linear and dose-dependent profile. Furthermore, previous studies suggest that both blood clearance and uptake by the MPS may depend on dose and composition of PLGA carrier systems. The degradation of the PLGA carriers is quick on the initial stage (around 30%) and slows eventually to be cleared by respiration in the lung. To address these limitations, studies have investigated the role of surface modification, suggesting that incorporation of surface modifying

agents can significantly increase blood circulation half-life. Surface modification of PLGA carrier, such as pegylation or chitosan coating, would serve as one of the excellent approaches to manage drug delivery properties of formulations by interaction of surface coating with a biological system and to enhance brain delivery.

7.2. PLGA-PEG

PLGA–PEG block copolymer, (Figure 15b), is one of the most promising systems for NPs formation, drug loading, and *in vivo* drug delivery applications, because it can be easily synthesized and it possesses all good qualities of PLGA and also PEG capability (Locatelli et al., 2014). Poly(ethylene glycol) (PEG), FDA approved polymer, is extensively used as a surface modifier of particulate drug carriers to provide important biological properties such as reducing toxicity and extending circulation time (Vonarbourg et al., 2006).

Surface modification of PLGA with increasing degree of pegylation, (PLGA-PEG) NPs, has been reported to enhance passage across the BBB by adsorption mediated endocytosis (Li et al., 2011). Pegylation of NPs is an important strategy to enhance the interaction of NPs with various physiological barriers. Pegylation increases NPs stability in biological media, enhancing their circulating half-life because of reduced phagocytosis and clearance by reticuloendothelial cells and reduced uptake by the liver, thus allowing NPs to reach areas of the body in greater concentrations than those of non-pegylated PLGA (Gref et al., 1995).

The PEG layer may have different roles in a particle biological fate, and all of them depend on the chain coverage-density. PEG coatings are known to prevent aggregation and to stabilize particles and colloidal suspensions in physiological salt concentration

media by steric and hydration repulsions. The resistance to non-specific absorption (opsonization) of plasmatic proteins is the most important determinant of NPs fate once injected in the host. High resistance to protein adsorption leads to a decreased uptake by the MPS, decreased degradation and elimination rate leading to a longer half-life in the blood stream, which in turn influences drug pharmacokinetic parameters. The resistance to protein binding (the so-called "antifouling effect") is dependent on PEG chain coverage conformation and is usually achieved at high coverage-density (Rabanel et al., 2014). The PEG layer on NPs surface can also improve drug encapsulation by providing a physico-chemical barrier to drug escape and it could affect drug release pattern. In addition to the above mentioned biological properties, it has been recently demonstrated that PEG surface coverage controls NPs transport through biological matrices such as the gastrointestinal tract mucus, the cervicovaginal mucus, the pulmonary mucus and the tumor extracellular matrix. Increase in PEG coating density or layer thickness seems to affect NPs cellular uptake as well.

Low cellular uptake can be advantageous since it increases circulation time due to MPS avoidance. On the other hand, it may also decrease drug efficacy if the intended targeted cells are not internalizing efficiently the drug carriers. This step is critical for the efficacy of several types of drugs with intracellular target such as siRNA or drugs subjected to efflux pumps (Rabanel et al., 2014). Thus, surface modifications with PEG add new physicochemical properties to existing polymers.

As demonstrated by Lai et al. conjugating a 2 kDa homopolymer PEG to the surface of 100 and 200 nm polystyrene (PS) NPs, diffusion coefficient of NPs through human cervicovaginal mucus increased by 20 and 381 times, respectively (Lai et al., 2007). Similar findings were obtained by Tanga and collegues, which discovered that densely

coating non biodegradable latex particles with PEG, effectively minimizes adhesive interactions between NPs and mucins, thereby allowing NPs to rapidly penetrate highly viscoelastic human mucus by moving through openings between mucin mesh fibers (Tanga et al, 2009). Biodegradable polymers such as PLGA with PEG have been commonly used to form core–shell structured NPs to encapsulate a variety of therapeutic compounds (Cheng et al., 2007; Gu et al., 2008). PLGA-PEG NPs have a number of appealing features: their hydrophobic core is capable of carrying highly insoluble drugs with high loading capacity, while their hydrophilic shell provides steric protection and functional groups for surface modification (Chan et al., 2009).

7.3. Chitosan

Chitosan is one of the most commonly used polymers in the scientific research dealing with a wide range of biopharmaceutical and biomedical applications including food science and technology (Bellich et al., 2016). Chitosan is a molecule with a carbohydrate backbone structure similar to cellulose, which consists of two types of repeating units, *N*-acetyl-d-glucosamine and d-glucosamine, linked by (1-4)-β-glycosidic linkage. It is a biopolyaminosaccharide cationic polymer that is obtained from chitin, which is the second most ubiquitous natural polysaccharide after cellulose on earth, by alkaline deacetylation and characterized by the presence of a large numbers of amino groups on its chain (Figure 15c). A common method for chitosan synthesis is the deacetylation of chitin, usually derived from the shells of shrimp and other sea crustaceans, using excess aqueous sodium hydroxide solution as a reagent.

Chitosan is insoluble in water but soluble in dilute acidic solutions of acetic, citric, and tartaric but not phosphoric or sulfuric at pH less than 6.5 (Roberts, 1992). Chitosan is

available in low and high molecular weights, ranging between 3,800 and 20,000 Da, and with different grades of deacetylation degree. The molecular weight and degree of deacetylation strongly affect chitosan properties, particularly during the development of micro- and nanoparticles. Chitosan is often claimed to be GRAS (Generally Recognized As Safe) and bioabsorbable. Chitosan exhibits interesting chemical (i.e reactive amino groups, reactive hydroxyl groups available, chelates many transitional metal ions) and biological (i.e.biocompatibility, hemostatic, fungistatic, spermicidal, antitumor, anticholesteremic, accelerates bone formation) properties, (Dutta et al., 2004).

Although chitosan has revealed all these therapeutic activities it is widely used as a polymeric drug carrier owing to its biocompatibility, biodegradability, and non-toxic characters. Furthermore, Chitosan is characterized by mucoadhesive properties owing to the electrostatic interaction between the positive charge on ionizable R-NH_3^+ group and the negative charge on the mucosal surfaces (Kockisch et al., 2003). The interaction of the protonated amine groups with the cell membrane results in a reversible structural reorganization in the protein-associated TJs, which is followed by opening of these TJs. The "tunable" aspect of chitosan allows its optimization to give appropriate biomaterials for therapeutic applications, in principle enabling also the optimization of its biological profile. Another advantage that makes chitosan superior to other polysaccharide polymers is the ease of chemical modifications in the structure, especially in the C-2 position, which provide derivatives with different characteristics, with potential use in different applications.

Drugs of different classes such as anticancer, anti-inflammatory, cardiovascular, antibiotics, antihistaminic, anti-thrombic, steroids, antiosteoporotic, antidiabetics, CNS acting, opioid analgesics, corticosteroids, antihyperlipidemic, antiemetics, proton pump

inhibitors, enzymes, toxoids, DNA, hormones, growth factors, proteins, and amino acids have been loaded or encapsulated into chitosan particulate systems (Sinha et al., 2004; Dasha et al., 2011). In general, the mucoadhesive nature, which increases the time of attachment at the absorption site, the easy availability of free amino group for cross-linking, ease of fabrication of polymeric particles without using hazardous solvents, the cationic nature that permits ionic cross-linking with multivalent anions, and finally the ability to control the release of the administered drug makes chitosan the polymer of choice for developing the polymeric particle and a good candidate for pharmaceutical formulations through several routes of administration (Lavertu et al., 2006; Ahmed et al.,2016). Mistry studied the effect of chitosan coating on *in vitro* uptake and transport of 100 nm polystyrene (PS) NPs over porcine olfactory epithelium mounted in Franz's diffusion cell. It was found that PS NPs surface modified with chitosan were retained in greater numbers in the mucus layer compared with unmodified equivalents (Mistry, 2009). They also observed that increasing the cationic charge on chitosan-modified particles, by reducing the pH of the buffer from pH 6.0 to 4.5 in porcine model, increased the particle association with mucus from 10 ± 3% to 39 ± 4% of administered dose. This demonstrated that the mucoadhesion potential was primarily controlled by electrostatic interactions between mucus and chitosan-coated NPs. Another important observation was carried out by Kumar and collegues, in their study the direct nose-to-brain transport of risperidone in simple nanoemulsion and chitosan-modified nanoemulsion formulation, was evaluated in rodents (Kumar et al., 2008). It was found that the highest concentration (78%) of risperidone in the brain was obtained with a chitosan-modified mucoadhesive nanoemulsion formulation, compared with a simple nanoemulsion formulation (57%) and simple risperidone solution (62%).

8. AIM OF THE STUDY

CNS drug delivery is inhibited by the restricted transport of drug candidates across the BBB. Over the last few years, intranasal route has emerged as a promising approach for brain delivery of drugs.

Despite its advantages, the nasal drug administration presents some limitations that must be considered during the development of nasal formulations. Rapid elimination of drug substances from the nasal cavity due to mucociliary clearance, it is the most suitable route for potent drugs since a limited volume can be sprayed into the nasal cavity. Consequently, particular problems may appear if nasal delivery of high doses of poorly water-soluble drugs is necessary.

Nevertheless, these drawbacks are often overcome making use of pharmaceutical technology-strategies involving the synthesis of prodrugs, the use of enhancers or bioadhesive polymers to increase drug permeability and residence time in nasal cavity. NPs are considered one of the most promising and versatile DDS into inaccessible regions like the brain, being able to provide protection to drugs while efficiently delivering them into the brain.

Taking into account these considerations, the goal of my thesis is to investigate an innovative approach that combines nanotechnology-based systems with IN administration for brain targeting.

An improved understanding is needed to determine the NPs systems suitable for nose-to-brain delivery and the influence of NPs physico-chemical and surface properties on CNS delivery and localization.

To achieve this purpose we studied NPs based on PLGA and its surface modification with CS and PEG to obtain systems negatively, positively, and neutrally charged and NPs based on chitosan derivatives.

The questions that we tried to answer with this work are:

- ✓ Can NPs end up the brain after IN admnistration?
- ✓ Which region of the brain can be reached?
- ✓ How does surface property affect NPs transport?
- ✓ Can NPs improve the efficacy of the therapeutic agent?

This study will lead to the identification of different pathways involved in NPs direct translocation from the nasal cavity to the CNS related to their properties and will therefore be helpful for a better understanding of the potential use of intranasal administration for its feasibility to serve as a direct drug transport route to the CNS.

The study was performed in a sequential manner in accordance with the points listed below.

(i) Design drug delivery systems different in physico-chemical and surface properties to investigate the influence of these variables on their potential direct transport from nose-to-brain;

(ii) Select and formulate PEGylated PLGA NPs with simple composition and long-term storage to reduce the number of excipients in the formulation. Study the nanosystem mucoadhesive power by evaluating the type and intensity of its interactions with mucin. Assess the influence of a highly lipophilic drug (model drug docetaxel, DTX) on NPs properties. Perform preliminary cytotoxicity evaluation of NPs on HT29 cell line;

(iii) Optimize PLGA and Chitosan-PLGA NPs formulations, evaluate Rhodamine loadead NPs translocation to the brain after IN administration in rats and detect their localization into specific brain areas;

(iv) Confirm our results with additional experiments, DiR loaded PLGA NPs biodistribution and bioavailability to the brain after IN administration in healthy mice;

(v) Formulate Oxcarbazepine-PLGA NPs to evaluate the possible neuroprotective action of this drug against the seizures and brain damage induced by PTZ administration;

(vi) Develop a novel, efficient brain delivery system composed of homemade chitosan derivatives-siRNA nanocomplexes via nose-to-brain to evaluate potential down-regulation of the model protein in rats.

CHAPTER II

Revisiting the role of sucrose in PLGA-PEG nanocarrier for potential intranasal delivery

In press, Accepted Manuscript, Pharmaceutical Development and Technology

Revisiting the role of sucrose in PLGA-PEG nanocarrier for potential intranasal delivery

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Abstract

The efficient design of nanocarrier systems is a major challenge and must be correlated to the route of administration. Intranasal route is studied for local, systemic or cerebral treatments. In order to develop nanocarriers with suitable properties for intranasal delivery, to achieve brain targeting, and to market the product, it is extremely important the simplification of the formulation in terms of raw materials. Surfactants and cryoprotectants are often added to improve structuration and/or storage of polymeric nanoparticles. Thus, PLGA-PEG nanocarriers were prepared by nanoprecipitation method evaluating the critical role of sucrose as surfactant-like and cryoprotectant, with the aim to obtain a simpler formulation compared to those proposed in other papers. Photon Correlation Spectroscopy and Turbiscan analysis show that sucrose is a useful excipient during the preparation process and it effectively cryo-protects nanoparticles. Among the investigated nanocarriers with different degree of PEG, PEGylated PLGA (5%) confers weak interaction between nanoparticles and mucin as demonstrated by thermal analysis and mucin particle method. Furthermore, in vitro biological studies on HT29, as epithelium cell line, does not show cytotoxicity effect for this nanocarrier at all texted concentrations. The selected nanosystem was also studied to load docetaxel, as model drug, and characterized by a technological point of view.

Keywords: intranasal administration; nanoparticles; DSC; mucoadhesion; PLGA-PEG; docetaxel.

Running Head: Sucrose: stabilizer and cryoprotectant effects

Abbreviations

BBB – blood brain barrier

BCSF – blood cerebral spinal fluid

CNS – central nervous system

DSC – differential scanning calorimetry

DTX – docetaxel

EE- encapsulation efficiency

EMA- European Medicines Agency

FDA- Food and Drug Administration

IN – intranasal administration

LC- loading capacity

MTS–(3-(4.5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)

N2B – nose to brain

NPs – nanoparticles

PBS- phosphate buffer solution

PCS – photon correlation spectroscopy

PDI – polydispersity index

PEG – polyethylene glycol

PES- phenazine ethosulfate

PLGA – poly lactic-co-glycolic acid

PLGA-PEG- poly(d,l-lactide–*co*–glycolide)–*block*–poly(ethylene glycol)

SEM – scanning electron microscopy

Sf/Si- (final mean size/initial mean size)

TSI- (Turbiscan stability index)

Introduction

The advancement of pharmacological treatments to the brain is constrained due to the existence of protective barriers (blood brain barrier, BBB and blood cerebral spinal fluid, BCSF), which restrict the passage of exogenous molecules into the brain.[1] In the last 20 years, intranasal administration (IN) seems to be a promising approach to Central Nervous System (CNS) treatments.[2,3] Exploiting IN, drugs can be directly transported from the nasal cavity to the CNS via the olfactory epithelium and the trigeminal nerve bypassing the BBB.[4,5,6] The optimization of nasal administration using nanoparticles represents a promising strategy to enhance brain delivery of molecules. Instead, a suitable nanocarrier should have proper properties to be transported directly to the brain exploiting nose to brain delivery (N2B), with the advantage of reducing and/or avoiding the systemic pathway.[7,8] Nowadays, small colloidal carriers, especially polymeric nanoparticles (NPs) are being widely used and many "conventional free drugs" can improve their pharmacological activity when loaded into nanocarriers, such as polymeric, lipidic, or phospholipidic nanoparticles.[9,10] In order to achieve market access long-term stability and quality data are mandatory as recommended by regulatory agencies (e.g. Food and Drug Administration, F.D.A., in USA; European Medicines Agency E.M.A., in Europe). Furthermore, simplifying and minimizing nanocarrier composition is preferred to achieve regulatory approval.[11] The efficient design of innovative nanocarriers should be the major challenge in drug delivery development both for academic and industrial researchers.[12] Currently, literature reports some

consolidated preparation methods, in which stabilizers are commonly included in the formulation to obtain a well-structured nanosystem and to reduce the mean nanoparticles' diameter. Usually, stabilizer agents do not prevent aggregation phenomena observed during storage. Thus, freeze-drying process is necessary to convert the formulation to a solid dosage form. Unfortunately, freeze-drying process modify the physicochemical properties of colloidal systems negatively affecting particle size, polydispersity index and early drug release.[13] For these reasons cryoprotective agent is required during this process.[14,15] As widely reported in literature, the most useful cryoprotectants are sugars because they affect the glass transition temperature (Tg' and Tg), which is important to obtain a freeze-dried cake with a stable amorphous form, a high redispersion speed, an appropriate residual moisture content and stabilization upon storage.[13,14] Although a different number of polymers have been investigated for the preparation of NPs, poly-lactic-co-glycolic acid (PLGA) is one of the most used as a result of its biocompatibility and safety profile for human use.[16, 17] Surface modification of PLGA NPs with polyethylene glycol (PEG) can modulate the interfacial properties of the system with the biological environment.[18]

As previously reported by Wang[19], sucrose could be used as stabilizer. The aim of this study is to evaluate the critical role of sucrose as surfactant-like and cryoprotectant agent (one component- two functions), to obtain a simpler PLGA-PEG formulation to minimize the number of components ensuring long-term storage of nanocarriers. We firstly evaluated the effect of Tween® 80 on different degrees of PEGylation (5% di-block, 10% di-block and 15% di-block) of PLGA NPs in terms of mean size and size distribution, also surfactant-free formulation were studied. Long-term stability of the samples was assessed by high performance stability analysis using the Turbiscan AGS and

Photon Correlation Spectroscopy (PCS). The selected nanosuspension (NPPEG5) was additionally investigated for mucoadhesive property by using conventional and unconventional techniques, "mucin particle method" and differential scanning calorimetry (DSC) respectively. Cytotoxicity evaluation of NPPEG5 at different concentrations was also performed on HT29 as epithelial cell line. The nanosuspension was loaded with Docetaxel (DTX) and a physicochemical, morphological and technological evaluation was carried out.

MATERIALS AND METHODS

2.1 Materials

PLGA 50:50 and PEG copolymers RESOMER® RGP d 5055 (PLGA-PEG 5%), RESOMER® RGP d 50105 (PLGA-PEG 10%), RESOMER® RGP d 50155 (PLGA-PEG 15%), and PLGA, RESOMER® RG 502 H were purchased from Boehringer Ingelheim (Ingelheim am Rhein-Germany). Polyoxyethylene Sorbitan Monooleate (Tween® 80), chitosan low molecular weight (molecular weight 50,000-190,000 Da, based on viscosity) and docetaxel were purchased from Sigma-Aldrich S.r.l. (Milan-Italy). Sucrose was purchased from Farmalabor S.r.l. (Bari, Italy). Mucin from porcine stomach type II was purchased from Sigma-Aldrich (St Louis, MO,USA). All other chemical reagents, solvents used and deionized water are of analytical grade.

2.2 Preparation of nanoparticles

NPs were prepared by the "*nanoprecipitation method*" as previously reported.[14] PLGA-PEG (30 mg) was dissolved in the organic phase

(8 ml acetone). The aqueous phase (water/ethanol 1:1 V/V) was composed of Tween® 80 (0.1; 0.25 or 0.5 % w/V), also "surfactant-free" NPs were prepared. The organic

phase was added dropwise under constant stirring at room temperature into the aqueous phase (volume ratio 1:2) until a milky suspension had formed. The organic solvent was removed under vacuum (Büchi R 111), (38°- 40° C and 450-500 bar). The excess of surfactant was removed by three cycles of ultracentrifugation at 12000 rpm, 8°C 1h, followed by pellet re-suspension in water (~ 10 mg/ml) [Beckman (Fullerton, CA) model J2-21 centrifuge equipped with a Beckman JA-20.01 fixed angle rotor]. The same procedure was also used for surfactant free NPs (one-step of centrifugation) to remove the aqueous water/ethanol phase. Pellets were re-suspended and analyzed.

For long term storage the freeze-dried study, the prepared samples were re-suspended in water and in aqueous solution containing different concentrations of sucrose (1; 2; 5% w/V) as cryoprotectant. The samples were frozen and freeze-dried for 24 h (EDWARDS MODULYO).

2.3 Preparation of NPPEG5 with new performance of sucrose

In order to investigate the ability of sucrose to act as a stabilizer, NPPEG5 was selected and was prepared through nanoprecipitation method in which the aqueous phase (water/ethanol 1:1 V/V) was composed of sucrose (0; 0.5; 1; 1.5; 2 % w/V), (Supplementary data, Fig.1). Nanoprecipitation occurred as previously described. The final selected NPs was loaded with DTX (3% w/w; drug/polymer), dissolved in the organic phase (acetone).

2.4 Particle Mean Size Measurement

Dynamic light scattering (DLS) analysis was performed using NanoZS90 (Malvern Instruments Ltd, UK, ZETASIZER), to determine NPs mean size and polydispersity index before and after freeze-drying step. The experiments were conducted using a photodiodes laser (wavelength 670 nm) in solid phase having a nominal power of 4.5

mV. PCS measurements were determined at an angle of 90°. For the fitting of the data for the decay of scattering of light, it was applied the method of cumulants of third order.

2.5 Differential Scanning Calorimetry (DSC)

The thermal properties of NPs were evaluated by DSC analysis. Experiments were performed using a DSC of the Mettler type DSC 12E, connected with a Thermo cryostat (Lauda ECOLINE RE 207). Indium was used to calibrate the instrument. The detection system was a Mettler Pt100 sensor. The reference was an empty aluminum pan. The thermotropic values were calculated using a system "software" Mettler TA89E and FP89 version 2.0, installed on a PC IBM PS/2 type 57SX computer, having 8MB RAM memory. After freeze-drying process, each sample was submitted to heating and cooling cycles in the temperature range 30-200 °C at a scanning rate of 5°C/min (heating) and at a scanning rate of 10°C/min from 200-20°C (cooling).

DSC analysis was also performed to evaluate interaction between mucin and polymer (PLGAPEG5) or NPs (NPPEG5). [20,21] NPs were mixed with 1 mL of mucin solution (1% w/V pH 5.8), and the blend was incubated at 37°C for 8h; then, it was freeze-dried and subjected to DSC measurement within 25–300 °C at a heating rate of 20 °C/min. The same procedure was carried out to prepare the samples made with polymer and mucin. The calorimetric analysis was also performed on mucin (1% w/V), NPs and polymer; all suspensions were prepared in phosphate buffer pH 5.8. The thermograms of mucin/NPs and mucin/polymer blends were compared to those of mucin and polymer and NPs respectively. Tests were performed in triplicate for each sample.

2.6 Mucin Particle Method

In order to establish the interaction between mucin particles and NPPEG5, "Mucin particle method" was performed. [22,23,24]

We evaluated the mucoadhesion properties by measuring the change in particle size and zeta potential of the mixed suspension mucin/NPPEG5 by Zetasizer NanoZS90 (paragraph 2.4). Test was performed on NPPEG and mucin particles according with Takeuchi et al.[24] Briefly, mucin particles (1% w/v) were suspended in a buffer solution (pH 5.8) and then mixed with an appropriate ratio (1:1, 1:5, 1:20; V/V) of nanosuspension (NPPEG5). The samples were stored at 37 °C and, at different time intervals, PCS analysis was performed.

2.7 Turbiscan analysis

Turbiscan™ AGS (Formulaction, l'Union, France, a robot and a storage station integrated TurbiscanLAB) was used to examine the dispersion stability of NPPEG5 with 0, 0.5, 1, 1.5, 2% w/V sucrose. This instrument allows to use a simple technique to observe reversible and irreversible destabilization phenomena in the sample without the need of dilution.[25,26] Turbiscan™ AGS is useful to detect destabilization phenomena much earlier and also in a simpler way than other methods. The suspension (10 mL) was placed in a flat-bottomed cylindrical glass tube and was placed in the instrument.

The transmission of light from the suspensions was then measured periodically (1 h) along the height at 25 and 60°C. The experiments were performed until 48h. The sedimentation behavior of the suspensions was monitored by measuring the backscattering and transmission of monochromatic near infrared (λ = 850/880 nm).

2.8 Physico-chemical and morphological analysis of DTX loaded NPPEG5

SEM analysis

Scanning electron microscopy (SEM) was performed to evaluate the surface morphology of NPs using a SEM XL-30 (Philips, Eindhoven, the Netherlands). NPs were fixed by means of bi-adhesive tape on a glass disk applied to an aluminum stub (TAAB, Laboratories Equipment, Berks, UK) and evaporated under vacuum overnight. Before the SEM analysis, the samples were metallized under argon atmosphere to 10 nm gold palladium thickness (EMITECH-K550 Sputter Coater, Houston, Tex., USA).

Drug Encapsulation Efficiency and loading capacity, *in vitro* DTX release

The drug encapsulation efficiency (EE) and loading capacity (LC) of DTX NPPEG5 was determined. The amount of DTX was measured by the HPLC analysis. The encapsulation efficiency (EE) was calculated as EE = Drug encapsulated in nanoparticles/Total drug added × 100% and drug loading capacity (LC) as LC = Drug encapsulated/Total materials × 100%. The entrapment of DTX PLGAPEG5 nanoparticles was expressed as loading capacity. Five hundred milligrams aliquots of freeze-dried DTX loaded NPPEG5 were poured in screw-capped tubes and suspended in 5 ml of buffer (pH 5.8). The tube was placed under magnetic stirring in a water bath maintained at 37±0.5 °C. At fixed time intervals (1, 4, 6, 24, 48, 72, 240 h) the tubes were taken out from the water bath and centrifuged at 12,000 rpm, 8°C for 1 h.

The pellets were re-suspended in 5 ml of fresh buffer and placed back into the water bath to continue release measurement. The collected supernatants were extracted three times with 5 ml of dichloromethane. The extraction solvent was evaporated and DTX residue was solubilized in 500 µl of acetonitrile. The amount of DTX was determined

using a Varian Prostar model 230 liquid chromatograph (Varian, Milan, Italy), equipped with an autosampler Varian Model 410 and a Galaxie software for data elaboration.

A Gemini-NX C-18 column (4.6 mm × 250 mm, pore size 5 µm; Phenomenex, Torrance, CA, USA) was used for the analysis. A mixture of water/CH_3CN (40:60 v/v) was used as mobile phase.

The column effluent at a flow rate of 0.5 ml/min was continuously monitored at λ= 230 nm. The linear regression coefficient (r^2= 0.9995) was determined in the range 0.5-40 µg/ml (*n* = 5). No interference of the other formulation components was observed.

2.9 Cell lines and culture conditions

The study was performed on a colorectal adenocarcinoma cell line: HT29 (ATCC® HTB¬38™), as epithelial cell line. Cells were grown at 37°C in a 5%CO_2 atmosphere in McCoy's 5a Medium Modified, (ATCC) supplemented with 10% heat-inactivated fetal calf serum (Euroclone) and 100U/ml penicillin-streptomycin (Sigma).

2.10 MTS assay and cell viability.

Cell viability was determined using a colorimetric method, the CellTiter 96 Aqueous One solution Cell Proliferation Assay (TB245, Promega Corporation). This assay uses a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent, phenazine ethosulfate (PES) which is combined with MTS to form a stable solution. MTS is chemically reduced by cells into a colored formazan product that, as measured by the amount of 490 nm absorbance, is directly proportional to the number of living cells in culture. Briefly cells were seeded into 96-well plates at 5x103 cells/200 µl /well and allowed to adhere to the plate overnight. The next day the cells were treated with

unloaded NPPEG5 with a polymer concentration of 1 - 0.005 mg/ml. Dilutions were made using the culture medium from stock solution of the drug in ethanol or NS suspensions, respectively. After 24-48-72h hours of incubation the culture medium was aspirated and cells were washed with PBS (pH 7.4); 100 µl of fresh culture medium without drugs and 20µl of MTS were added to each well and cells were incubated for 3 hours. The plates were read on a Microplate Reader (Synergy HT, BIO-TEK). Cell viability was expressed as the percentage of viable cells in treated sample relative to non-treated control cells. All the experiments were repeated three times in triplicate.

2.11 Statistical analysis

Statistical analysis was performed using Prism 6 (Graphpad Software, US). Each experiment was performed at least three times with triplicate measurements. Unless otherwise stated, data points represent mean of triplicate ± SD. Statistical analysis used ONE-WAY ANOVA. Statistical significance was taken as $P < 0.05$.

3. RESULTS AND DISCUSSION

The combination of intranasal administration and drug delivery using nanocarriers represents a good strategy to enhance drug availability to CNS. Moreover, NPs surface properties could influence their interactions with biological substrates.[27] As previously demonstrated by our research group, chitosan/PLGA NPs take a lag time to reach the brain, probably due to a strong interaction between chitosan and the site of administration (nasal cavity) (Bonaccorso et al., in preparation). PEGylation of NPs is an efficient strategy to enhance the interaction of NPs with various physiological barriers[28], due to its ability to promote the diffusion and/or penetration through mucus

and epithelium.[29] Based on these considerations, PEGylated PLGA polymers were selected to prepare our nanocarriers.

3.1 Effect of PEGylation degree and amount of Tween® 80 on NPs physicochemical properties

Nanoprecipitation method was selected according to polymer properties. In the nanoprecipitation method, a colloidal suspension formed when the organic phase is slowly added to the aqueous phase under moderate stirring. Some authors suggest the "Marangoni effect" as the molecular mechanism involved in the formation of NPs due to interfacial hydrodynamic phenomena and turbulence between two miscible liquid phases. Other studies promote the "ouzo effect" as a driving force for the formation of NPs.[30] When a hydrophobic solute is rapidly brought into the so-called "ouzo region" the local supersaturation can lead to a spontaneous nucleation of small particles that subsequently grow or aggregate to form nanoparticles.[31] Ouzo region is the metastable region between miscibility limit curve and the stability limit curve. There is an overlap in physical parameters involving Marangoni and ouzo effect such as the concentration gradient, the diffusivity, the temperature and the interfacial tension. The variation of interfacial tension and concentration causes changes in the global balance avoiding the formation of NPs, according with Marangoni effect.[32] According to the theorists of ouzo effect the spontaneous emulsification could be achieved with or without surfactant in a region far away from the critical point of the phase diagram. Starting from these theories we evaluated the influence of two variables, such as the degree of PEGylation of PLGA and the concentration of surfactant used, up to the complete omission of this component to prepare NPs defined "surfactant-free", on both the formation and the properties of NPs in terms of mean size and PDI.

Figure 1 should be inserted here

As shown in figure 1, the properties of NPs with lower degree of PEG (5%; NPPEG5) were influenced by the use of surfactant. The mean size of surfactant-free NPs were less than 300 nm, which is an important requirement for the selected route of administration. The mean size of NPPEG5 decreased with the increase of Tween® 80 concentration. The mean size of NPPEG10 e NPPEG15 was influenced by the PEGylation degree rather than Tween® 80 amounts. According to Kirby and colleagues, the degree of PEGylation influenced NPs physicochemical properties. PEGylated NPs prepared using nanoprecipitation method showed a lower mean size compared to those obtained by modified solvent evaporation previously reported by Kirby and colleagues.[33] Moreover, polydispersity index values were affected by PEG degree of copolymers. In fact, an homogeneous distribution of NPs was promoted by a low PEG degree (PDI < 0.3). Imaging the hypothetical structure of these systems, it is conceivable to suppose that the chain of PLGA collapses in the aqueous phase (non-solvent) and the portion of PEG is oriented outside. The nature of these copolymers supports this hypothesis, the hydrophilic PEG chain covalently linked to the portion of the hydrophobic PLGA, could lead to the separation of closely related components because the two polymer blocks are not miscible. PLGAPEG polymers allow to obtain NPs without surfactant.

3.2 Effect of sucrose as cryoprotectant on surfactant-free NPs

Nanoprecipitation method was used to prepare surfactant free NPs. The colloidal nanosuspensions were characterized by low storage stability, in fact, aggregation phenomena occurred after one month of storage (increase of mean size and PDI >0.4). Freeze-drying is a very useful process to obtain long storage stability of nanocarriers. Several studies report the use of disaccharides for their cryoprotective effect on NPs.

Among these, the most used are trehalose and sucrose because of their capability of stabilizing a wide range of colloidal systems.[13] We choose sucrose for some advantages compared to the other: i) easy rehydration; ii) good price/quality ratio; iii) potential use as "surfactant-like".[19,34] Furthermore, oppositely to trehalose, sucrose does not increase the viscosity, even at higher concentration, as described by Sola- Penna and Meyer-Fernandes.[35]

As reported in table 1 the formulations cryoprotectant-free showed a macroscopic and irreversible aggregation (mean size = nd; PDI= 1). In order to evaluate the suitable sucrose concentration to achieve cryoprotective effect, we used Sf/Si parameter (ratio between the final and initial particle sizes).[36] Ratio close to 1 indicates a good cryoprotective action, values > 1 are not acceptable.

Table 1 should be inserted here

According to our results, the cryoprotective effect of sucrose on surfactant-free NPs was deeply correlated to the PEGylation degree, since sucrose was able to preserve the initial mean size of NPs when the polymer had a low PEG amount, as easily evidenced by Sf/Si values.[14] After the rehydration of NPPEG5 containing sucrose a visually acceptable suspension with no aggregation was achieved.[37] Otherwise, sucrose did not provide its cryoprotective effect in the formulation with higher PEG content (NPPEG10 and NPPEG with 1, 2, 5 % w/V of sucrose). In fact, these samples showed higher Sf/Si values and were not homogeneous as confirmed by PDI (PDI > 0.3). This phenomenon is probably due to the presence of sterically hindered PEG chain, which hamper the adhesion of sucrose onto NPs surface, or to the increase in solution viscosity, which might affect the turbulence at the interface.[38]

Sugars are chemically innocuous and can be easily vitrified during freezing. They are also attractive as excipients due to their influence on the glass transition temperature (Tg).[15] The thermotropic analysis of NPs formulations presented characteristic sucrose melting peak (Tm) at 180°C and NPs peak (Tg) in the range from 43 to 53°C (table 1, Fig 2. Supplementary data). The presence of sucrose determined a shift of NPs peak, until the disappearance when higher concentration of cryoprotectant were used due to the polymeric chains immobility. Cryoprotectants vitrify upon freezing to form an amorphous glassy matrix that inhibits NPs mobility and thus aggregation.[39] The immobilization of NPs in the glassy cryoprotectant matrix preserved them against the mechanical stress of ice crystals or the dehydration process.[40] The excipient forms an amorphous mass and maintains the integrity of NPPEG5 suspension, that was selected for further investigation.

3.3 The effects of the double function of sucrose

It has been shown that it is possible to replace surfactants with other molecules with stabilizing effect such as PVA and, more recently, sugars for the formation of NPs. So the use of the disaccharides may have a dual function: stabilizer and cryoprotectant agent at the same time. In order to investigate the potential double function of sucrose, NPPEG5 was selected among all formulations tested because showed the ideal properties for long-term storage when sucrose was added as cryoprotectant. As observed in our previous studies, PEG content of 5% was selected because increasing PEGylation degree tends to reduce the loading capacity of lipophilic molecules. The concentration, as well as the nature of the steric stabilizer (surfactant), plays an important role in NPs production and protection during freezing and/or freeze-drying process. Moreover, it has been demonstrated that the combination of steric stabilizer and typical

cryoprotectant excipients is a critical factor in formulation development, due to their possible interaction and competition.[40] Thus, the effect of sucrose as "surfactant like" and cryoprotectant on NPPEG5 was evaluated. For this purpose, we modified the nanoprecipitation method to obtain NPs prepared and stabilized by sucrose (Figure 1, Supplementary data). As shown in figure 2, NPs prepared with sucrose at different concentrations showed mean size ~270 nm, the presence of sucrose did not affect this parameter respect to the selected surfactant free NPs (NPPEG5). Conversely, PDI was affected by sucrose, demonstrating the effect of this excipient in the formation of NPs with homogeneity improvement.

Figure 2 should be inserted here

The nanocarriers were analyzed by PCS after 10 days of storage at room temperature.

NPs with sucrose as "surfactant-like" showed unchanged mean sizes and PDI. This result should be due to the presence of sucrose that lead to variation in the viscosity of the external phase compared to the surfactant-free nanosystems. This phenomenon could increase storage stability reducing particles aggregation. Turbiscan analysis was performed at 25 °C and 60°C (accelerated stability test) to verify our hypothesis.

Our results (figure 3a) demonstrated an increase of TSI (Turbiscan Stability Index) values at 60 °C the occurance of instability phenomena for all formulations independently to the presence of sucrose. TSI parameter is a statistical factor useful to easily rank the stability of the sample in a kinetic way or in a data table. This parameter is calculated as the sum of all the variations detected in the samples in terms of size and/or concentration. The higher value of TSI corresponds to a lower stability of the product. [41,42]

Moreover, aggregation phenomenon occurred due to the reduction in viscosity probably caused by the increase in temperature. These results confirmed PCS analysis performed for both set of samples (25 and 60 °C) (figure 3b).

Figure 3 should be inserted here

Thus, to achieve long-term stability freeze-drying process is required. We investigated the ability of sucrose, used as surfactant-like, to protect nanosuspension during the freeze-drying process. Our results indicate that an aggregation phenomenon occurred after the rehydration of freeze-dried NPs (Table 2).

Table 2 should be inserted here

We further investigated the average diameter of our systems after the supplementary addition of sucrose before freeze-drying process to evaluate the potential increase of its cryoprotective effect. Sf/Si values decreased proportionally as the concentration of sucrose increased.

The use of sucrose showed again enhancement of the nanocarrier homogeneity, as confirmed by the reduction of the PDI values (< 0.1) with the increasing of sucrose concentration.

3.4 Effect of NPs preparation method on mucoadhesive property.

Preparation methods can influence nanoparticles surface properties, in fact different structures can be described when nanoparticles are prepared starting from PLGA-PEG polymers (micelles, brush, mushroom, etc…).[43] In particular, our aim was to examine the influence of nanoprecipitation technique on the mucoadhesive properties of NPPEG5, obtained adding sucrose exploiting its double function. For this purpose, we exploited two different tools, DSC and PCS. DSC is an analytical instrument that could

be used to provide information on the chemical interactions between substances studying thermotropic parameters.

Figure 4 should be inserted here

Figure 4 shows the thermograms of NPPEG5-mucin and PLGAPEG5-mucin blends and the raw materials (mucin, PLGAPEG5). As expected, the copolymer thermogram show characteristic peaks at 38–48 °C.[44] The NPPEG5 thermogram (fig. 4-a) presents two thermotropic phenomena: the glass transition temperature (Tg) at 38°C, a prominent exothermic transition peak at 190 °C corresponding to a crystallization process.[44] Mucin is characterized by two thermotropic events: the first one, glass transition, is a second order transitional peak and may be related to the amorphous nature of mucin; the second endothermic peak (200°C) represents its melting point (fig.4-b).[45] The DSC thermogram of PLGA-PEG and mucin blend (figura 4d) demonstrates that interactions between raw materials occurred. The characteristic peak of the copolymer has a completely different shape, (one peak instead of two) while the mucin peak is affected by a weak shift. The shift or the disappearance of the endothermic peak of mucin confirms the interaction of substances.[46,47] As shown in figure 4-c (NPPEG5-mucin blend) mucin shows a broad peak and NPPEG5 peak shifts left, also, the interaction between NPs and mucin occurred.

The study was carried out at a specific pH value according to the administration route (pH 5.8). The break of electrostatic interaction of mucin at pH < 6 can produce a conformational change from a random coil to a rod by exposing hydrophobic regions, which were folded and sequestered in the interior at neutral pH. This is a favorable condition for the interaction between mucin and other entities.[48] Our findings are in accordance with previous studies demonstrating the influence of pH on mucin

behavior. In order to confirm these results, we performed the "Mucin Particle Method", which is a general test for the evaluation of mucoadhesive properties of materials and/or nanocarriers.[24] According to Wang et al. when mucin particles were mixed with NPs, the particle mean size changes, due to high carrier affinity to mucin.[49] We carried out the study testing different mucin/NPs ratios incubated at 37°C after 1 and 24 hours of incubation. As reported in figure 5 (A-B) the size distribution of NPPEG5/mucin blends of the suspension presented very heterogeneous distribution of particles, PDI close to one. These samples show two and/or three different peaks of size distribution, one due to the mean size of free NPPEG5 and the others due to mucin particles and mucin-NPs aggregates. In figure 5A, we report the mean size of two principle particle peaks at different time of incubation. In particular, the mean size of NPPEG5, which is ~300 nm, increase up to 500 and then 600 nm after 1 and 24 hour of incubation, respectively (volume ratio 1:5). The interaction is found to be dependent on the ratio between NPs and mucin. Takeuchi et al. carried out mucoadhesive studies on chitosan evaluating zeta potential values, great change in surface charge indicates the strong mucoadhesive properties of the polymer.[43] In our study, this parameter remains almost unchanged, NPPEG5 showed a slight zeta potential changed from neutral to low negative values (from 0 to -6 mV). Thus, our findings led us to hypothesize that weak interaction occurred between NPPEG5 and mucin (figure 5C).

Figure 5 should be inserted here

Nanocarriers designed to target the brain via nasal administration should prevent drug loss by reducing the adherence to the mucus and the residence time in the nasal cavity and to promote transport into the tissue.[50] Cytotoxicity at different NPs concentrations was assessed using MTS test on HT29 cell line. The results showed that NPPEG5 do

not have any significant cytotoxic effect on HT29 cells at all tested concentrations (figure 3-supplementary data).

3.5 Effects of DTX on properties of NPPEG5

NPPEG5 with sucrose as surfactant-like and cryoprotectant, was selected to load DTX, as the ideal carrier. The average particles diameter of DTX loaded NPPEG5 was 147.30 ± 4.5 nm while unloaded NPs showed 330.90 ± 17.53 nm. The encapsulation with DTX reduced the mean particle size probably because of the drug deposition onto the surface of NPPEG5 by reducing the nucleation process.

This phenomenon influenced also zeta potential values. Slight difference was observed for zeta potential values, from -6.81 ± 6.19 mV for DTX NPPEG5 to -12.1 ± 4.75 for the unloaded nanoparticles. The nanoparticles show a good polydispersity and displayed spherical shape with moderate uniform size as confirmed by SEM images, in agreement with dynamic scattering technique (figure 6).

Figure 6 should be inserted here

The drug loading percentage and the entrapment efficiency were determined indirectly by estimating the unentrapped drug and was found to be low (LC% 0.05 ± 0.15; EE% 3.20 ± 0.30). The low values found for both parameters could be probably due to a diffusion process of DTX away from the polymer matrix because of the high affinity of the drug for the organic solvent used during the preparation and low affinity for PEG portion of polymer. [51] The release behavior of DTX from the NPs exhibits a biphasic pattern, it consists of an initial burst during the first 24h (approximately 60%), followed by slower sustained release until 240 h (data not showed). The initial burst release of drug could be explained by diffusion of drug molecules.

CONCLUSIONS

This work allows to revisit the role of sucrose in the preparation of PLGA-PEG nanoparticles; it highlights the importance of the selection of starting materials and methods to produce nanosystems for specific application. Our findings demonstrate that the degree of PEGylation of PLGA significantly influences the physicochemical properties of nanoparticles and the effect of sucrose as cryoprotective agent. Here, we suggest that the physico-chemical characteristics of NPs, such as their mean size and PDI, are significantly affected by the PEG amount rather than surfactant concentrations. Furthermore, our studies demonstrate that sucrose is a suitable cryoprotectant agent that prevents variation in particle size after the reconstitution of freeze-dried NPPEG5 prepared without the addition of surfactant.

It was found that the addition of sucrose, as surfactant-like, (≤ 2% w/V), during the preparation process, could be useful to form NPs with mean size lower than 300 nm, but any cryoprotectant effect was observed. However, a successful cryoprotectant effect occurred when a further portion of sucrose was added before the freeze-drying process. The use of one-component with double action may represent a novel approach to reduce undesirable interactions between excipients and molecules in the formulation of NPs, to simplify and speed up nanoparticle preparation for intranasal delivery.

As revealed by thermotropic analysis and “mucin particle method”, PEGylated PLGA nanoparticles could avoid the entrapment of nanoparticles in mucus. This could be due to the repulsive interactions between neutrally and negatively charged groups of PLGA-PEG and sialic acid residues of mucus glycoprotein. In addition also the interpenetration of PEG chain into mucus could affect this phenomenon.

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DECLARATION OF INTEREST

The authors report no conflict of interest. The authors are responsible for the content and writing of the article.

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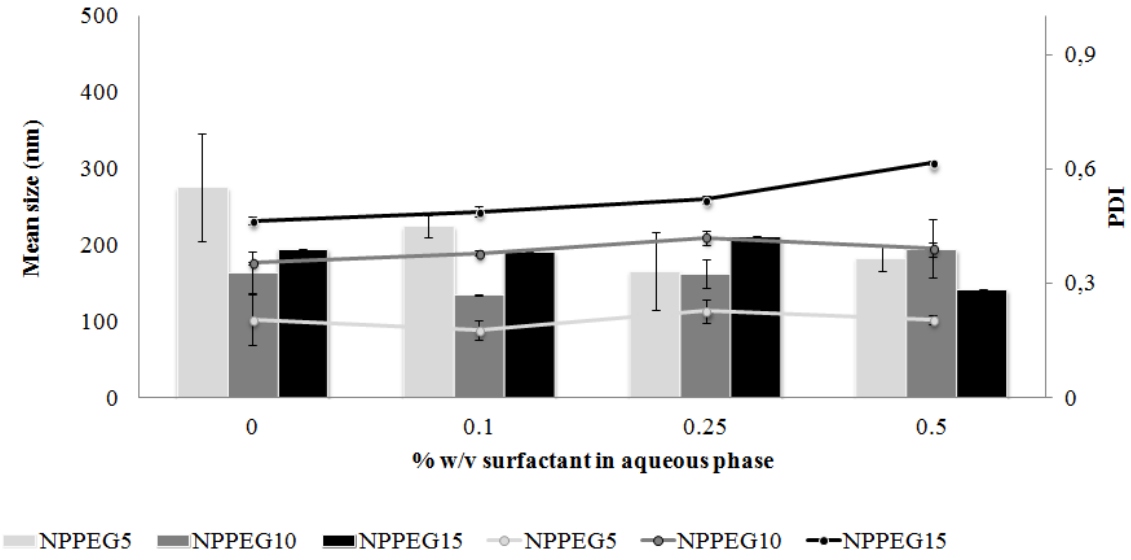


Figure 1. Mean size (histograms) and polydispersity index (P.D.I.; lines) values (± Standard deviation) of nanoparticles prepared with PLGA-PEG with different degree of PEGylation (5, 10, 15 %) surfactant free and with Tween® 80 at different concentrations (0.1; 0.25; 0.5 % w/V).

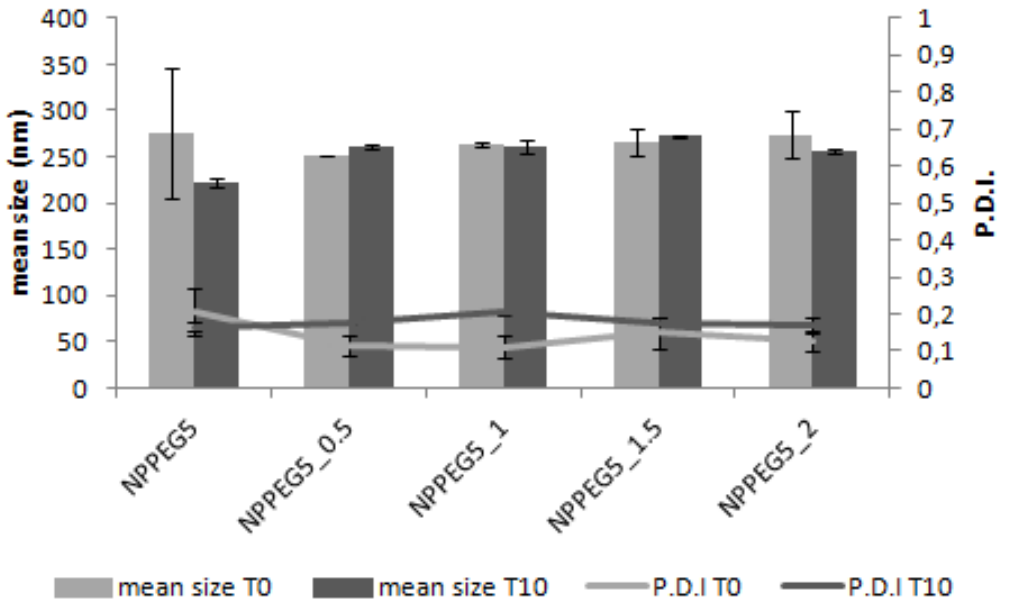


Figure 2. Mean size and polydispersity index (P.D.I.) (± Standard deviation) of NPPEG5 with different concentration of sucrose (0.5; 1; 1.5; 2 % w/V) as surfactant like.

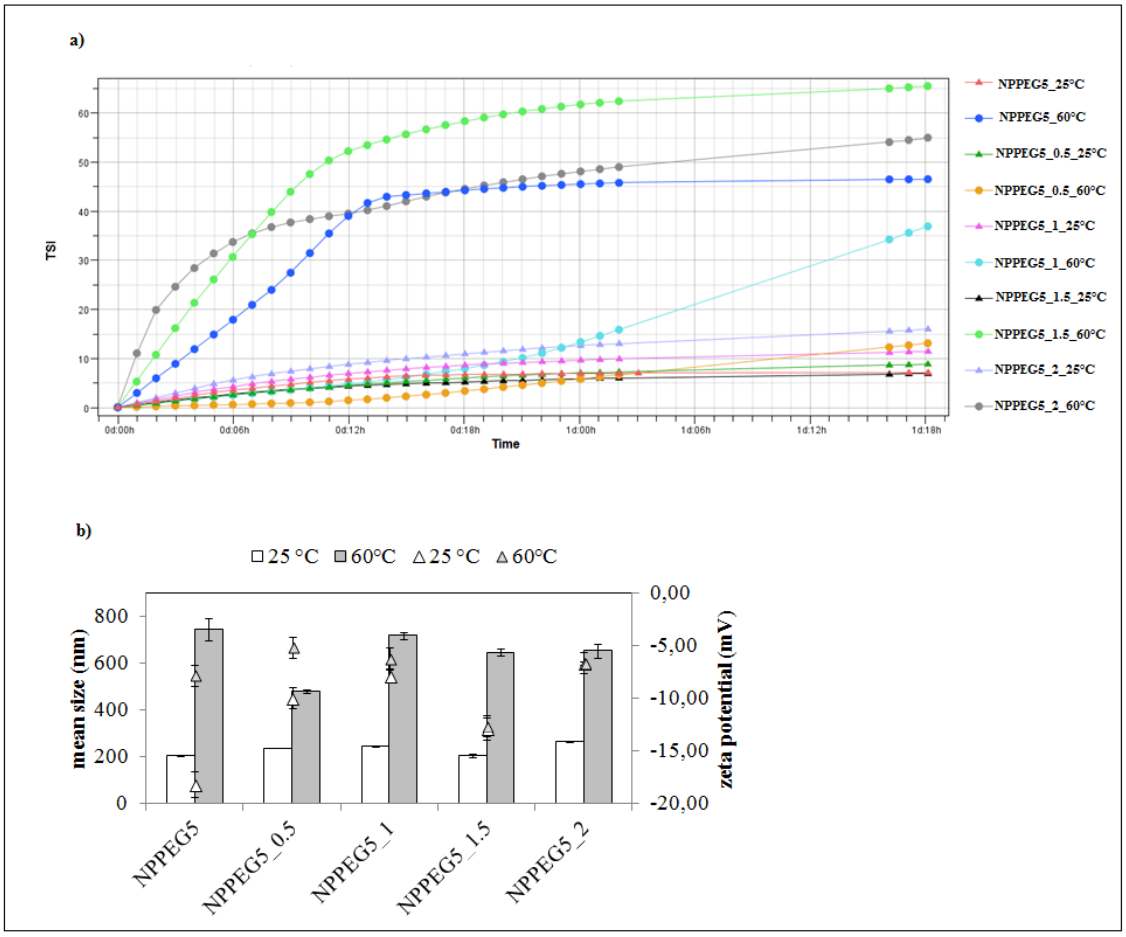


Figure 3. NPPEG5 at different degree of temperature (room and 60 °C) with different concentration of sucrose (0.5; 1; 1.5; 2 % w/V) as surfactant like by: A) Turbiscan, comparison of TSI values; B) PCS, mean size and zeta potential values (± Standard deviation).

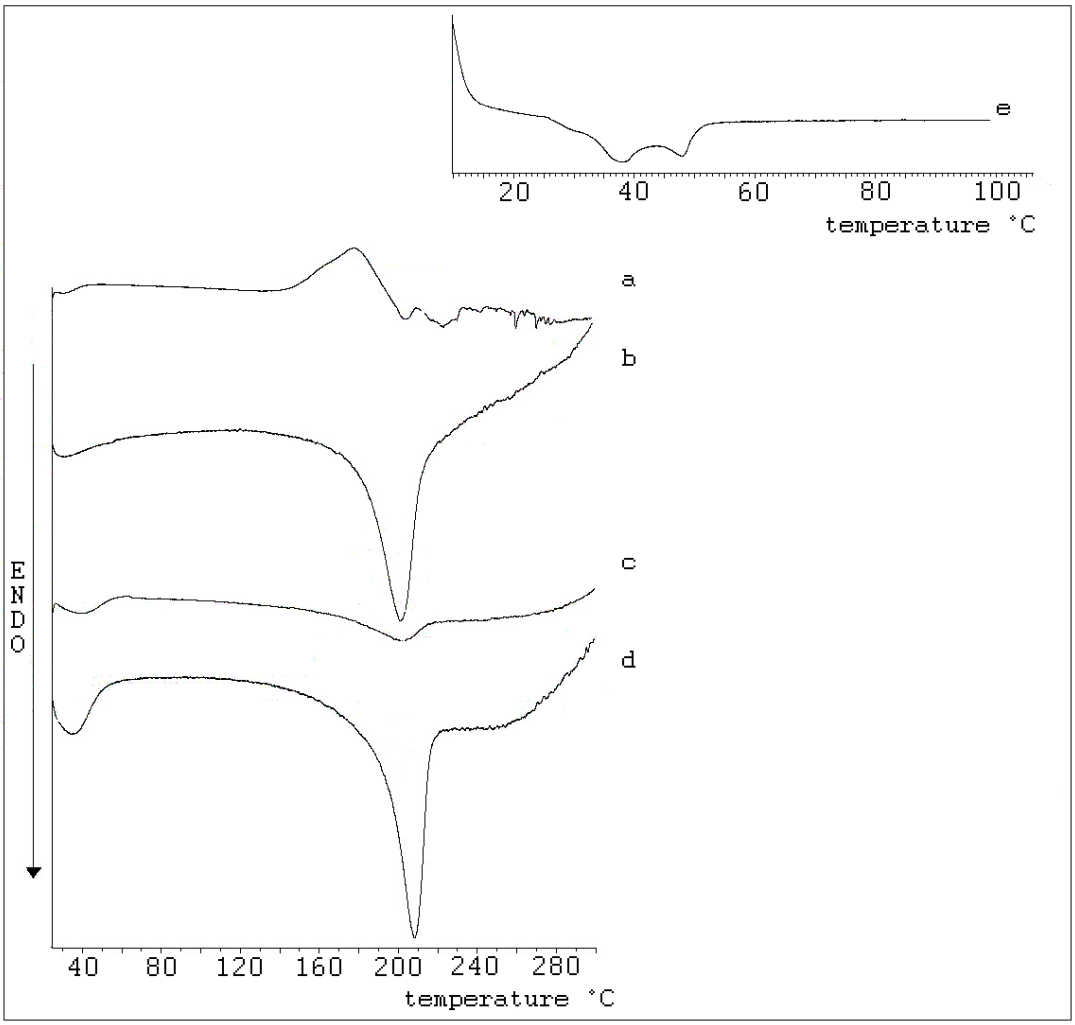


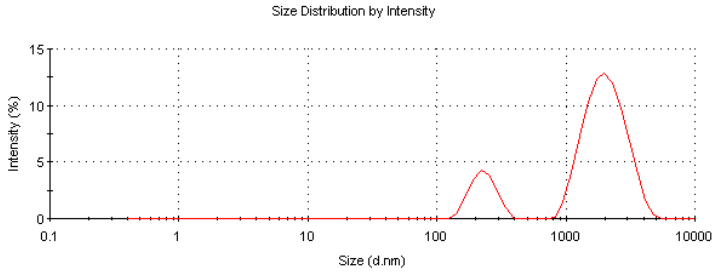
Figure 4. DSC thermograms of NPPEG5 (a), mucin (b), NPPEG5 and mucin (c), PLGAPEG and mucin (d), and PLGAPEG (e). (All samples were analyzed after freeze-dried process and were rehydrated with phosphate buffer at pH 5.8).

(A)

time	Mucin	NPPEG5	mucin_NPPEG5 ratio1:1 V/V		mucin_NPPEG5 ratio1:5 V/V		mucin_NPPEG5 ratio1:20 V/V	
			population 1	population 2	population 1	population 2	population 1	population 2
1	1708.0 ± 304.3	275.90 ± 7.25	**523.0 ± 2.8**	**5123.0±4.9**	277.0±60.0	1879.0±15.4	1324.0±149.3	4754.0±276.1
24	2652.0 ± 142.2	278.52 ± 3.02	598.3± 4.9	4870.0 ± 142.9	572.2 ± 3.5	3629.0 ± 1.5	724.6 ±23.8	3540.0 ± 0.9

275.90

(B)



(C)

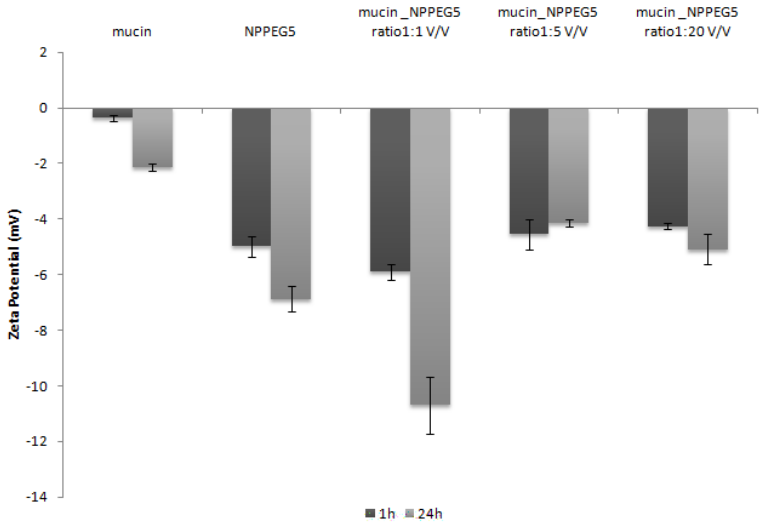


Figure 5. A) Mean size (nm) of NPPEG5, mucin particles and particles obtained by NPPEG5/mucin mixture suspensions at different ratio at 37 °C and at different time of incubations (1; 24h- pH 5,8) . Each value was the mean of three experiment ± Standard Deviation; B) Size Distribution by Intensity of NPPEG5/mucin mixture suspension at 1:1 ratio, after 1h of incubation at 37°C; C) Zeta Potential (mV) of NPPEG5, mucin and NPPEG5 and mucin suspension (pH 5.8) at different ratio: a) NPPEG5; b) mucin; c) (1:1); d) (1:5); e) (1:20). Samples were analyzed at two time intervals (1-24h).

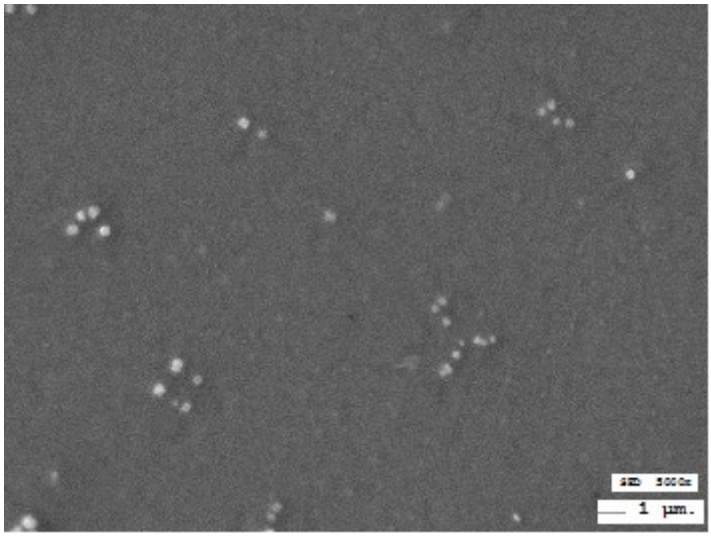
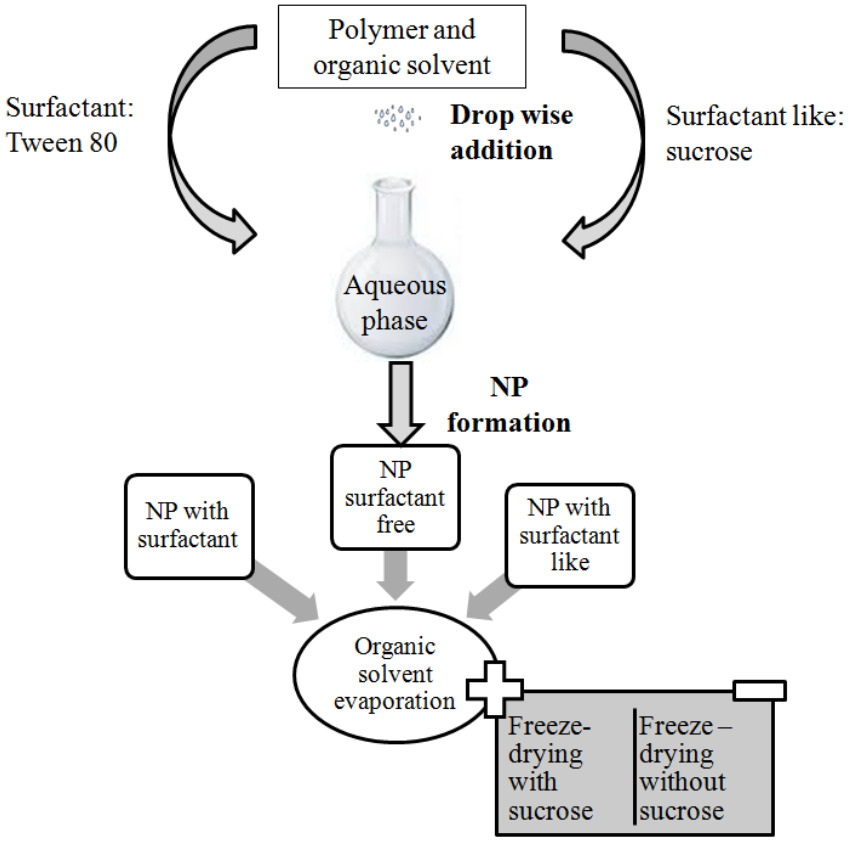
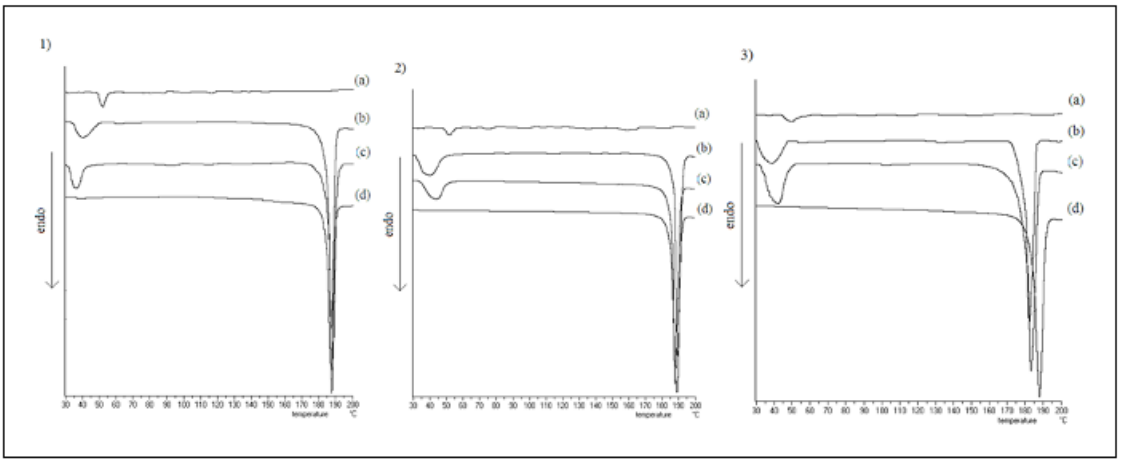


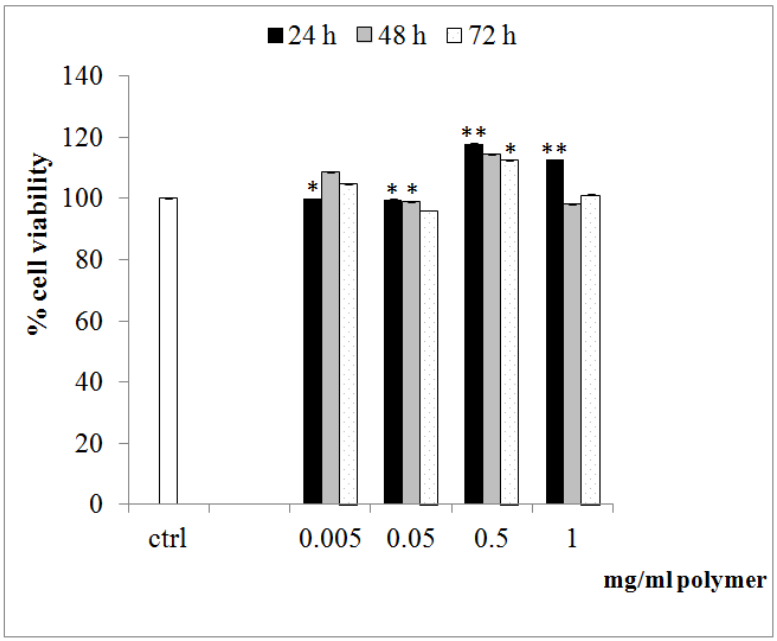
Figure 6. SEM image Docetaxel loaded NPPPEG5 using a SEM XL-30 (Philips, Eindhoven, the Netherlands).



Supplementary 1. Description of the preparation methods used.



Supplementary 2. Thermal analysis of NPs surfactant free with different degree of PEGylation: 1) NPPEG5; 2) NPPEG10; 3) NPPEG15. The curves referred, respectively, to: a) freeze-dried NPs; b) freeze-dried NPs with sucrose 1% w/V; c) freeze-dried NPs with sucrose 2% w/V; d) freeze-dried NPs with sucrose 5% w/V.



Supplementary 3. Cell viability of HT29 cells treated with NPPEG5 at different concentrations assessed by MTS after 24, 48, 72 h of incubation. Each point represents the mean of three independent determination.

Table 1. Physico-chemical values of surfactant-free NPs before and after freeze-drying process with and without different concentration of sucrose as cryoprotectant agent. Values are presentend as th mean of three measurement ± SD (n=3)

NPs	SUCROSE (%w/V)	MEAN SIZE ± S.D[a]. (nm) Before Freeze-Drying	MEAN SIZE±S.D[a]. (nm) After Freeze-Drying	Sf/Si[b]	Tg[c] (°C) ±S.D[a].	PDI[e]±S.D[a]. After Freeze-Drying
NP PEG5	0	275.90 ± 7.25	1000.00 ± 60.19	3.6	52.7	1.000 ± 0.340
	1		219.50 ± 3.45	0.8	39.1	0.148 ± 0.040
	2		283.70 ± 6.89	1.0	36.5	0.423 ± 1.060
	5		253.90 ± 2.30	0.9	-	0.327 ± 0.650
NP PEG10	0	164.40 ± 0.48	nd	nd	52,6	nd
	1		772.00 ± 10.67	4.7	40.2	0.944 ± 1.320
	2		156.09 ± 8.79	1	45.4	0.364 ± 0.890
	5		226.07 ± 4.35	1.4	-	0.29 ± 2.450
NP PEG15	0	195.70 ± 2.05	nd	nd	42.8	nd
	1		190.80 ± 0.09	nd	39.1	nd
	2		nd	nd	37.2	nd
	5		570.40 ± 2.78	2.9	-	0.779 ± 2.560

[a] S.D. - standard deviation.
[b] Sf/Si - final mean size/initial mean size.
[c] Tg – glass transition of freeze-dried powder.
[d] nd- not determined
[e] PDI – polidispersity index

Table 2. Mean size, PDI and Sf/Si values of surfactant-free NPs with 2% of sucrose as surfactant like before and after freeze-drying process with and without an additional amount of sucrose (the to obtain cryoprotectant effect. Values are presented as the mean of three measurements ± S.D. (n = 3).

NPs	SUCROSE Surfactant like (%w/V)	MEAN SIZE ± S.D[a]. (nm) Before Freeze-Drying	MEAN SIZE±S.D[a]. (nm) After Freeze-Drying	Sf/Si[b]	SUCROSE Additional amount (%w/V)	MEAN SIZE±S.D[a]. (nm) After Freeze-Drying	Sf/Si[b]
NPPEG5	0	275.90 ± 71.13	n.d[c]	n.d[c]	0	n.d[c]	n.d[c]
	1	263.80 ± 3.25	n.d[c]	n.d[c]	1	550.00 ± 0.28	2.08
	2	274.30 ± 26.58	n.d[c]	n.d[c]	2	400.70 ± 14.84	1.45
	5	330.90 ± 17.53	n.d[c]	n.d[c]	5	254.10 ± 17.54	0.76

[a] S.D. - standard deviation.
[b] Sf/Si - final mean size/initial mean size.
[d] nd- not determined

CHAPTER III

Nose-to-brain delivery: localization and time-course of polymeric nanocarriers on different brain regions of brain rats

Submitted to Colloids and Surfaces B: Biointerfaces

Nose to brain delivery in rats: effect of surface charge of rhodamine B labeled nanocarriers on brain subregion localization

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Abstract

Nose to brain delivery and nanotechnology are the combination of innovative strategies for molecules to reach the brain and to bypass blood brain barriers. In this work we investigated the fate of two rhodamine B labeled polymeric nanoparticles (Z-ave<250 nm) of opposite surface charge in different areas of the brain after intranasal administration in rats. A preliminary screening was carried out to select the suitable positive (chitosan/poly-l-lactide-co-glycolide) nanocarrier through Photon Correlation Spectroscopy and Turbiscan. Physico-chemical and technological characterizations of poly-l-lactide-co-glycolide (negative) and chitosan/poly-l-lactide-co-glycolide (positive) fluorescent labeled nanoparticles were performed. The animals were allocated to three groups receiving negative and positive polymeric nanoparticles via single intranasal administration or no treatment. The localization of both nanocarriers in different brain areas was detected using fluorescent microscopy. Our data revealed that both nanocarriers reach the brain and are able to persist in the brain up to 48 h after intranasal administration. Surface charge influenced the involved pathways in their translocation from the nasal cavity to the central nervous system. The positive charge of nanoparticles slows down brain reaching and the trigeminal pathway is involved, while the olfactory pathway may be responsible for the transport of negatively charged nanoparticles, and systemic pathways are not excluded.

Keywords: intranasal delivery, chitosan nanoparticles, PLGA, brain, fluorescent microscopy, *in vivo* study, rat

Abbreviations:

BBB- Blood Brain Barrier

CNS- Central Nervous System

CS- Chitosan

CSF-Cerebral spinal fluid

DSC- Differential Scanning Calorimetry

%DTE- drug targeting efficiency percentage

%DTP- direct transport percentage

E.E. - Encapsulation Efficiency

FDA- Food and Drug Administration

GRAS- Generally Recognized As Safe

IN- IntraNasal

LMW- Low Molecular Weight

MPS- Mononuclear phagocyte system

N2B- Nose to Brain

NPA- PLGA NPs

NPBC–chitosan/PLGA NPs (NPBC3; NPBC5; NPBC10; NPBC20)

NPs- nanoparticles

P.D.I.-polidispersity index

PBS- Phosphate Buffer Solution

PCS- Photon Correlation Spectroscopy

PFA-Paraformaldehyde

PLA- Poly-lactide

PLGA- Poly-lactide-co-glycolide

SEM- Scanning electron microscopy

TSI- Turbiscan stability index

ZP- Zeta potential values

ΔH- enthalpy changes

ΔBS- Delta Backscattering

ΔT- Delta Transmission

Introduction

The intranasal (IN) route is commonly used for local disease treatments and systemic delivery of drugs; furthermore, this route is considered to be a potential alternative way of administrating drugs to the brain, specifically to bypass the blood–brain barrier (BBB). The nose to brain route serves as a direct delivery route of molecules to the brain (Chapman et al., 2013). Novel delivery devices have been developed for this purpose (ViaNase ID^{TM}; OptiNose; POD-Precision Olfactory Delivery-device) (Djupesland et al., 2014). These devices can transport nanosuspensions or molecules in solution to specific regions of the nasal cavity in man (olfactory region).

IN administration is a non-invasive method and could represent an alternative strategy for drugs commonly administered orally or intravenously and that have difficulty moving across the BBB (Chapman et al., 2013; Tosi et al., 2016). As reported by Kozlovskaya et al. (2014), several papers describe drug accumulation in the brain following IN application of the drug solution or suspension, but some free drugs could achieve low therapeutic concentration in the brain using this route, as previously demonstrated (Piazza et al., 2014, Meredith et al., 2015, Zhang et al, 2014). Among the different types of drug carriers, due to their mean size, nanoparticles (NPs) used for drug delivery, may increase the therapeutic dose of drugs in the brain when administered intranasally. In fact, these carriers could protect against local enzymatic degradation, increase IN residence time by avoiding mucociliary clearance, and possibly achieve prolonged release at the target site. Shadab et al. (2014) have shown that NPs transported drugs into the brain suggesting a direct transport to this site that bypasses the BBB (Shadab et al., 2014, Kozlovskaya et al., 2014). The selection of polymers requires a preliminary evaluation related to the site of administration and the

hypothetical fate of NPs (Kumari et al., 2010). Chitosan (CS) NPs are the most commonly investigated nanocarriers for nose to brain delivery and are suitable for the transport of hydrophilic molecules (Casettari et al., 2014). CS is a natural polysaccharide that is capable of increasing the NPs residence time in the nares due to its mucoadhesive properties and this polymer may also act as a penetration enhancer; furthermore, it is generally recognized as safe material (GRAS) (Charlton et al., 2007; Duttagupta et al., 2015). Currently, hybrid nanocarriers are prepared using two different types of polymers, and chitosan could be used to confer a positive charge to a PLGA nanoparticle surface (Bharali et al. 2015). Poly-lactide-co-glycolide (PLGA), co-polymer of poly-lactide (PLA), is a suitable material to prepare NPs for lipophilic or amphiphilic molecules for its tunable physico-chemical properties and it is a Food and Drug Administration (FDA) approved polymer (Kapoor et al., 2015). In our previous *in vitro* study, we demonstrated the influence of surface charge on the uptake of rhodamine B labeled NPs via olfactory glial cells (Musumeci et al., 2014), according to other authors for a different type of cells. The proof of concept of nose to brain delivery of NPs is claimed by different authors; for example, some described toxicological effects in the brain because of inhaled ultrafine particles (van Berlo et al., 2014). Others demonstrated the nose to brain direct transport through the evaluation of the "drug targeting efficiency percentage (%DTE)" or "direct transport percentage (%DTP)" values or through *in vivo* behavioral studies. In particular, authors compared the results obtained after IN administration of the drug loaded NPs and other routes (Kozlovskaya et al., 2014; Phukan et al., 2016).

Despite the promising results, some questions have not been answered. First of all, is there a tropism for particular brain sub-regions after IN administration of NPs? If so, is

this tropism influenced by anatomical-physiological conditions? Could surface properties of NPs influence the localization in different brain sub-regions? Literature data show that NPs reach the brain prevalently in the olfactory and/or trigeminal region after IN administration, but no correlation was made between these results and the surface properties of NPs investigated during a time course.

In this work, we carried out *in vivo* studies on rats in order to correlate opposite surface (positive and negative) charges of the selected rhodamine B labeled NPs with the regional brain localization after IN administration up to 48 h. Chitosan-PLGA (*poly*(lactic-*co*-glycolic acid) NPs were selected after a preliminary screening using photon correlation spectroscopy (PCS) and Turbiscan analyses. *In vivo* brain localization analyses were carried out in healthy rats after IN administration of PLGA and Chitosan-PLGA fluorescent NPs through fluorescent microscopy.

Materials and methods

Materials

Low molecular weight (LMW: molecular weight is 50,000-190,000 daltons based on viscosity; viscosity 20-300 cps, deacetylation degree 75-85%) water-soluble chitosan derived from crab shells, Tween®80, rhodamine Band glacial acetic acid (d 1,049 g/ml a 25°C) were purchased from Sigma-Aldrich (Milan, Italy). Sodium sulfate, sodium hydroxide and all other chemicals were analytical grade, purchased from Analyticals, Carlo Erba. The polymer Resomer® 502 H poly-(D,L-lactide-co-glycolide) (50:50, molecular weight is 30,000-60,000) were purchased from Boehringer Ingelheim Pharma GmbH&Co. KG (Boehringer Ingelheim, Germany). Ultrapure water was used throughout this study. For biological studies, we used adult male Wistar rats (200-250 gr

b.w., Envigo s.r.l. - ex Harlan, Italy), Zoletil 100 and Dexdomitor anesthetics, paraformaldehyde and other chemicals from Sigma-Aldrich (Milan, Italy).

PLGA nanoparticle preparation as negative surface charge nanocarriers

PLGA NPs (NPA) were prepared using the solvent displacement method followed by polymer deposition as previously reported (Musumeci et al., 2013). Briefly, the chosen polymer (75 mg) was dissolved in acetone (20 ml). The organic phase was added drop by drop to a 40 ml water/ethanol solution (1:1, v/v) containing 0.5% (w/v) Tween® 80 under magnetic stirring, obtaining a milky colloidal suspension. The organic solvent was then evaporated off under high vacuum at 40° C. Fluorescent-labeled NPs were prepared by co-dissolving rhodamine B (5 µg/ml) with the polymer in the organic phase. The different formulations were purified through ultracentrifugation (15000×g) for 1h at 10°C, using a Beckman (Fullerton, CA) J2-21 model centrifuge equipped with a Beckman JA-20.01 fixed-angle rotor. After washing, the obtained NPs were re-suspended in 5 ml of filtered water (0.22 µm Sartorius membrane filters). This procedure was repeated three times. The obtained samples were characterized according to mean size, size distribution and surface chemistry.

Chitosan/PLGA nanoparticle preparation as surface charge positive nanocarriers

Chitosan/PLGA NPs were prepared using the nanoprecipitation method, "In situ coating method",as described by Sanna et al. (2012) with little modification (modified by Sanna et al., 2012). Chitosan/PLGA NPs, identified as NPBC, were prepared.

The influence of the amount of polymer was evaluated on physico-chemical characteristics. Briefly, PLGA at different concentrations (3, 5, 10, 20 mg/ml;

respectively identified as NPBC3, NPBC5, NPBC10, NPBC20) was dissolved in acetone and was added dropwise into a chitosan (CS) solution (0.1% w/v solubilzed in acetic acid solution 0.1% v/v, 5 mL). The resulting milky colloidal suspension was evaporated at room temperature to remove a portion of the organic solvent (1 h). Then the organic solvent was evaporated off under high vacuum at 40°C. The different formulations were purified through ultracentrifugation (11000×g) for 1h at 8 °C, using a Beckman (Fullerton, CA) J2-21 model centrifuge equipped with a Beckman JA-20.01 fixed-angle rotor. After washing, the obtained NPs were re-suspended in 5 ml of filtered water (0.22 μm Sartorius membrane filters). This procedure was repeated three times. The obtained samples were characterized according to mean size, size distribution and surface chemistry. Also Turbiscan analysis was carried out. After the selection of the suitable nanosuspension (NPBC3), fluorescent-labeled Chitosan/PLGA NPs were prepared by co-dissolving rhodamine B (5 μg/mL) with the PLGA polymer in the organic phase.

Particle size, size distribution and Zeta potential analyses

The particle size and the polidispersity index (P.D.I.) of nanoaggregates were measured performing photon correlation spectroscopy (PCS) with a Zetasizer Nano S90 (Malvern Instruments, Malvern, UK) at a detection angle of 90°, at 25°C with a 4mW He-Ne laser operating at 633 nm. Each value was measured in triplicate. The results are shown as mean ± standard deviation. The samples were analyzed using a disposable cuvette, "DTS 0012 Disposable sizing cuvette", withdrawing 700 μl of suspension. The Zeta potential values (ZP), which reflect the electric charge on the particle surface, were determined at 25°C using the same equipment described previously. For the measurement, samples were diluted appropriately with ultra-purified water.

Turbiscan study

Turbiscan™ AGS (Formulaction, l'Union, France, a robot and a storage station integrated TurbiscanLAB) was used to examine the dispersion stability of NPs. This instrument allows for the use of a simple technique to observe reversible (creaming and sedimentation due to fluctuation on particle size and volume) and irreversible (coalescence and segregation due to particle size variation) destabilization phenomena in the sample. Turbiscan™AGS is useful to detect destabilization phenomena much earlier and also in a more simple way than other methods. Each suspension (15 mL) was placed in flat-bottomed cylindrical glass tubes, which were placed in the instrument and the transmission of light from the suspensions was then measured periodically (1h) along the height at room temperature and at 25 °C for up to 48 h. The sedimentation behavior of the suspensions was monitored by measuring the backscattering and monochromatic transmission near infrared (λ = 850/880 nm). The sedimentation rate was evaluated from the change in backscattering intensity at the top portion of the sample.

Physico-chemical, morphological, calorimetric analyses and *in vitro* release study of labeled NPs

Differential scanning calorimetric analysis

A Mettler Toledo DSC 1 STAR^e system equipped with a PolyScience temperature controller (PolyScienceIllinois, USA) was used to perform calorimetric analyses.

The detection system was a HSS8 high sensitivity sensor (120 gold-gold/palladium-palladium thermocouples) and the ceramic sensor (Mettler Full Range; FRS5) with 56 thermocouples. The signal time constant was 18 s and the digital resolution of the

measurement signal was less than 0.04 μW. Calorimetric resolution and sensitivity, determined through the TAWN test, is respectively 0.12 and 11.9. The sampling rate is 50 values/second. The sensitivity was automatically chosen as the maximum possible through the calorimetric system, and the reference was an empty pan. The calorimetric system was calibrated, in temperature and enthalpy changes, by using indium by following the procedure of the DSC 1 Mettler TA STAR^e instrument. NPs were sealed in an aluminum pan and submitted to DSC analysis to determinate the influence of coating on thermotropic parameters of NPs. Each sample was submitted to heating and cooling cycles in the temperature range 10-200 °C at a scanning rate of 5°C/min (heating) and at a scanning rate of 10°C/min (cooling). Transition temperature and enthalpy changes (ΔH) were calculated from peak areas with the MettlerSTAR^e Evaluation software system (version 13.00) installed on Optiplex 3020 DELL.

SEM analysis

Scanning electron microscopy (SEM) was performed to evaluate the surface morphology of NPs using a SEM Philips mod 500. NP samples were dried for 24 h before the analysis. A small amount of NPs was stuck on a double-sided tape attached on a metallic sample stand, then coated, under argon atmosphere, with a thin layer of gold, using a POLARON E5100 SEM Coating Unit.

In vitro release studies

The *in vitro* release studies of rhodamine from PLGA NPs and chitosan/PLGA NPs were performed using cellulose membrane dialysis tubing (Spectra/Por® 3 Dialysis Membranes, MWCO 3.5kD, Flat width 18 mm, diameter 11.5 mm, Vol/Lg 1.1 ml/cm, Length 15 m/ 50 ft; Spectrum® Laboratories). The samples (1 ml) were enclosed in dialysis bags and incubated in 19 ml PBS (pH 7.4; pH 5.8) with constant shaking, 100

rpm, in water bath at 37 ± 0.5 °C. At predetermined time intervals (1, 2, 3, 4, 5, 24, 48 h), a fixed volume of release medium was withdrawn and replaced with an equivalent amount of release media. These samples were analyzed using UV spectrophotometry at rhodamine λ-max (555.2 nm in PBS pH7.4; and pH 5.8) (UV-VIS 1601 spectrophotometer, Shimadzu Italia, Milan, Italy). Calibration curves for the quantitative evaluation of the probe were linear in the following ranges:(i) 3.24–0.66 µg/ml of rhodamine (r^2 = 0.9984) for analyses in PBS pH 7.4; (ii) 7.05–0.56 µg/ml of rhodamine (r^2 = 0.9991) for analyses in PBS pH 5.8.

***In vivo* experiments**

All the *in vivo* experiments were performed following the Guidelines for Animal Care and Use of the National Institutes of Health. The study was approved by Italian Ministry of Health (permit number 183). Two lots of fifteen adult male rats weighing 200-220g were acclimatized for one week before the study with free access to water and food. The rats were anaesthetized with Zoletil 100 (100 mg/kg, i.p.) and placed on a heated working surface to prevent hypothermia. Both lots respectively received an IN injection of 50 µl of positive or negative rhodamine labeled NPs. The IN injection was given according to Dyer et al. (2002). Briefly, the rats were placed in a supine position and 25 µl were administered in each opening nostril (alternating) using a microliter syringe attached, via a needle, to a short polyethylene tubing, inserted approximately 0.7 cm into one nostril. The procedure was performed slowly in about 1 minute. Each lot was divided into three groups of five rats each and the three groups were sacrificed at different time intervals (8, 24, 48 h) and no treat. A control group of five rats was treated with 25 µl of rhodamine B solution in each nostril. The animals were deeply anaesthetized with an intraperitoneal injection of Zoletil 100 (100 mg/kg) and

Dexdomitor (20-30 μg/Kg) and perfused trancardially with a 4% paraformaldehyde (PFA) solution in 0.1 M phosphate buffer solution (PBS, pH 7.4). The brains were removed, post-fixed overnight in the same 4% PFA and then transferred into a 30% sucrose cryoprotective solution in PBS at 4°C for2-3 days. Serial 25 μm frozen sections of the brain were cut along the sagittal plane, mounted on slides, and air-dried. The fluorescence was examined on a fluorescence microscope (Nikon Eclipse 80i) equipped with filters for the visualization of rhodamine (excitation 530– 560 nm). Images were captured using a digital camera (Nikon) and adjusted for contrast with Adobe Photoshop without compromising data integrity.

Results

The hydrodynamic diameter is one of the most important parameters for the development of suitable nanocarriers for nose to brain (N2B) delivery. Only small particles can be transported to the brain via the olfactory or the trigeminal nerves (Mistry et al., 2009, Mistry et al. 2015). PLGA was chosen as a primary polymer to prepare negative surface nanoparticles. PLGA is widely used as a suitable polymer to prepare nanocarriers and it is a well-known material for our research group (Li Volti et al., 2012; Vicari et al., 2008). Chitosan was chosen to modify PLGA nanoparticles and to obtain positive nanoparticles in order to compare two nanosystems having opposite surface charge. The influence of different PLGA/CS ratios on the physico-chemical characteristics of colloidal carriers was evaluated to select the nanosuspension with the most suitable properties in order to perform *in vivo* studies for N2B delivery.

Microparticles could release molecules in the nasal cavity determining diffusion of free molecules to the brain. Migration may occur through the nasal olfactory and respiratory

mucosa, as well as through the capillaries, lymphatic, and cerebrospinal fluid present in the nasal mucosa (Rassuet al., 2015). Once released in the nasal cavity, the free drug can be affected by biological environment degradation and the drug therapeutic concentration may consequently be achieved. Nanoparticles can be transported via olfactory epithelium and/or trigeminal nerve system and the encapsulated molecule can be released directly to the brain (Kozlovskaya et al., 2014; Mistry et al., 2015).

The effect of polymer amount on mean size, surface properties and stability of CS/PLGANPs

The nanoprecipitation method was successfully applied to prepare CS/PLGANPs. Nanocarriers were obtained through the precipitation of PLGA in the CS polyelectrolyte solution which introduces positive charges on the NPs, allowing us to obtain NPs with a mean size ranging from 200 to 800 nm and a polydispersity index ranging from 0.2 to 0.4. As shown in figure 1, only the formulation having the higher level of polymer produced a heterogeneous particle population. NPs smaller than 250 nm in diameter were obtained at a low polymer concentration (from 3 to 5 mg/ml). All the nanocarriers presented a positive charge about +70 mV and the values were not influenced by PLGA amount (data not reported).

Figure 1 should be inserted here

To support PCS results even Turbiscan analysis was performed. We determined the nano-dispersion stability as a function of PLGA amount. NPs were analyzed immediately after preparation with an appropriate dilution. Taking into account that the nanosuspensions were destabilized through a sedimentation and/or aggregation phenomenon, TSI (Turbiscan Stability Index) was examined. Figure 2A showed the TSI values for all the nanosuspensions at 25 ° for up to 48 h. The nanosuspension with the

lowest polymer amount is the most stable nanoparticle formulation (lowest values of TSI). In figure 2B, ΔTransmission (ΔT) and Δ Backscattering (ΔBs) data showed no variations in the middle part of the graph, so no aggregation occurred. The variation at the bottom may represent a sedimentation phenomenon of a re-dispersible nanosuspension. All NPs appeared sufficiently stable for up to 48 according to the high values of zeta potential.

Figure 2 should be inserted here

The properties of positive and negative rhodamine labeled NPs

We focused our attention on two types of polymeric NPs for *in vivo* studies, in order to investigate their fate in the sub-regions of the brain after IN administration in healthy rats. NPs were characterized using thermal analysis, which represents a useful technique to detect NP properties after the production process (Gill et al., 2010).

DSC thermograms of the raw materials and selected NPs are shown in Figure 3. PLGA sample (C) presented a glass transition temperature (T_g) of about 40 °C. Chitosan (D) presented an exothermic peak at 40°C due to the loss of water molecules. In the thermograms obtained through PLGA NPs (B) the vitreous transition is also present. After the addition of chitosan, the exothermic peak of NPBC3 (A) disappeared and the T_g is rather weak (Latif et al., 2015).

Figure 3 should be inserted here

As depicted in the SEM images (figure 4 A and B), both nanosuspensions are spherical and with smooth surfaces. While NPA appeared well separated, NPBC3 presented some aggregates confirming the different values of P.D.I.

In figure 4 physico-chemical properties are also shown. Both nanosuspensions presented mean size ≤ 230 nm (figure 4 C and D) with positive surface charge for CS/PLGA NPs and negative for PLGA NPs (figure 4 E and F).

The mean size distribution (PDI), as also shown by SEM, highlight a more homogeneous particle population for NPA respect to NPBC3.

Figure 4 should be inserted here

Even a rhodamine *in vitro* release study was performed in order to evaluate that the fluorescence observed through microscopy analysis is due to the probe loaded into the nanocarriers and/or to the probe released from the nanocarriers (figure 1, supplementary data). As shown, 5% of rhodamine was released after 24 h; a slow and prolonged release was observed for up to 48 h where 15% of rhodamine was released (maximum *in vivo* experiment time).

Sub-region localization in the rat brain of fluorescent labeled polymeric NPs

The localization of negative surface charge (NPA) and positive surface charge (NPBC3) NPs were investigated in brain sub-regions at 8, 24 and 48 h after IN administration. We investigated the brain sub-regions in rats that are the principle targets for the different neurological diseases (figure 2, supplementary). The sections were prepared according to the sagittal illustration of the rat brain in *Paxinos* and *Watson* (Paxinos, 1982). Preliminary study demonstrated that no or only very few cells presented fluorescence after 2 and 4 hours of the nasal administrations (data not reported).

No fluorescence is depicted in non-treated brain rats (figure 5A) and slight and homogenous rhodamine distribution is shown in rat treated with rhodamine solution (figure 5B). The uptake of NPs in the brain was significantly increased 8-24 and 48

hours after IN administration and the labeled NPs were found in some brain areas. All NPs were prevalently localized in the cytosol of neural cells (figure 5C). NPs were also observed outside the cells, but were found with reduced frequency when compared to the level found in the cytosol.

Figure 5 should be inserted here

A summary of the labeling found in the various brain regions with negative and positive NPs was reported in Table 1.

Table 1 should be inserted here

The areas, where we found the NPs localized, are the following: *Cerebral cortex,* the frontal and parietal cortex, prevalently, while only a few NPs were found in the occipital cortex. The cerebral layers involved in NPs distribution were layer V and only a few in layers III, IV, VI and none in the superficial layers; *Basal ganglia,* in the caudate-putamen nucleus, prevalently; *Hippocampus,* in CA1-CA3 regions and in the granular layer of the dentate gyrus, few NPs were also observed in the entorhinal cortex and subiculum; *Basal forebrain,* the fluorescent label was observed in Piriform cortex and amigdaloid nuclei. In the *Thalamus* NPs were found in the reticular nucleus, central complex, anterior and dorsal nuclei; *Mesencephalon,* in the tegmental and pretectal nuclei. In the *Cerebellum* NPs were found in the Purkinje cell as well as in the cerebellar and vestibular nuclei; *Myelencephalon,* the reticular substantia, as well as spinal, trigeminal and facial nuclei. In the other brain sub-regions, here not mentioned, no or very scant negative and positive NPs were found (data not shown).

Regarding the trend of each nanosuspension we could describe a localization time – dependent on the surface charge of NPs. In fact, after 8 h of IN administration a decrease in fluorescence for negative NPs from the rostral to caudal area was found.

An opposite trend was observed after 48 h, where the caudal region showed a higher fluorescence compared to the caudal, while after 24h a uniform distribution in all the cerebral structures was found (figure 6). The localization of CS/PLGA NPs had a different trend compared to negative NPs (figure 7). Since we found a weak labeling in all areas after 8 h and 24 h of IN administration, except in the cerebral cortex where the signal was moderate after 24 h, only after 48 h did we observe an intense/moderate labeling in some cerebral structures.

Figures 6 and 7 should be inserted here

Discussion

Preliminary screening is important to select the ideal carrier to perform an *in vivo* study. According to Sanna et al. (2012), the combination of PLGA with chitosan leads to an increase of NP mean size strictly correlated with the increment of PLGA concentration. As shown in figure 1, the increase in polymer concentrations significantly increases the mean particles size. This variable did not influence the surface charge values largely governed by chitosan adsorption on the surface of the NPs. In this work we demonstrate that a reduction of the ratio between PLGA and CS used for the preparation of NPs, allows for the formation of a nanocarrier with a positively charged surface and a mean size below 250 nm. A positive charge of NPBC NP series is due to the protonation of amine groups of CS, the possible electrostatic interaction with carboxylic groups of PLGA masking negative charges. Zeta potential is an important parameter because it allows for the prediction of physical stability and, according to the "electrostatic theory of mucoadhesion", even nanosphere/IN mucoadhesion to increase residence time (Shaikh et al., 2011). The highest Zeta potential values, either positive or negative in

absolute value, produce stable dispersions due to particle electrostatic repulsions. The electrostatic repulsions between particles with the same electrical charge prevent particle aggregation. The highly positive Zeta potential has the potential to produce a formulation with good stability (Honary et al., 2013b). Turbiscan and PCS taken together allowed us to select the NPBC3 sample for the following investigations. NPBC NP series were stable against sedimentation, as evaluated by the Turbiscan analysis. NPBC3 demonstrated a good stability for the total duration of the experiment compared to the other samples, as elucidated by TSI values (figure 2A). ΔT and ΔB data showed no variations in the middle part of the graph, which means there was no flocculation/aggregation of the sample, thus confirming its higher stability in terms of particle size. As previously observed by PCS, this phenomenon is correlated with high zeta potential value (Bru et al., 2004). NPBC3 nanosuspension is affected by the sedimentation process; in fact, the increase of backscattering level that occurred at the bottom indicates a decrease in the concentration of particles consecutive to the sediment formation. The obtained cake was easily re-dispersible (Dihang et al., 2005).

NPBC3 was selected as the positive formulation to perform *in vivo* studies along with NPA (negative). The NP samples selected (NPA and NPBC3) were further characterized (figure 3 and figure 4). Figure 4 summarizes some physico-chemical properties of both nanocarriers. Slight morphological differences were observed between the two samples (figure 4,A and B). The aggregate particles observed through SEM are due to the dried process necessary to perform analyses.

In order to detect the location/distribution in the brain, rhodamine was encapsulated into the nanocarriers as a fluorescent marker. Moreover, cellular uptake of both NPs was investigated. For both NPs the encapsulation efficiency (E.E.) of rhodamine was

assessed and it resulted in ~10%. The selected nanocarriers showed a mean size < 250 nm and opposite surface charge. As previously reported by Mistry et al. (2015), there are three routes that allow NPs to achieve brain delivery after IN administration (systemic, olfactory and trigeminal pathway). We could consider nose to brain transport when the amount of NPs that reaches the brain through the systemic pathway is negligible when compared to trigeminal and olfactory pathways. In the last two years, several authors have examined the influence of NP surface chemistry on the biological environment, in order to increase the therapeutic efficacy of NPs (Honary et al., 2013a; De Jong et al., 2008). However, there are relatively few studies that describe the influence of the surface charge of NPs on the localization in different brain sub-regions (rostral and/or caudal brain regions) during a time course after IN administration (Buchner et al., 1987). Knowing where and when NPs arrive in the brain is very important for drug delivery. Our *in vivo* experiments demonstrate that the fate of negative and positive NPs in neural cells was strictly correlated with their surface properties. Our data could not exclude the involvement of the systemic pathway.

The localization of NPs in some brain sub-regions after 8 h suggested the occurrence of a probable direct N2B transfer. In fact, no PEGylated NPs are removed through a mononuclear phagocyte system (MPS) after 4/6 hours of their presence in the body (Suk et al., 2016). Besides, NPs without suitable ligands on their surface are not able to cross the blood brain barrier (Tosi et al., 2016).

At this point the question is: why did NPs arrive in the brain after a lag time, and different sub-regions are involved? The answer is important not only to explain the potential application of nanocarriers but also the implication in the toxicological phenomenon of these colloidal carriers.

First of all, to better understand our statements, it is important to introduce some results obtained by other authors in this field and the possible mechanisms involved.

Some authors described the pathways and mechanism by which free molecules pass the nasal region to reach the brain if not absorbed via the systemic pathway and not drained by nasal lymphatic (Lockhead et al., 2012). There are two possibilities: the drug is transported to the brain encapsulated in the NPs through the neural or trans-epithelial pathways or the drug is released on the surface of the nasal epithelium or inside the tissue and then transported across the membrane (Mistry et al., 2009). Some experimental evidence demonstrated that the pathways involved nerves (olfactory and trigeminal) connecting the nasal passages to the brain and spinal cord (Dhuria et al., 2010). Analyzing our data, we could hypothesize that negative NPs arrived at the rostral sub-regions and then they were transported to caudal regions. This transport involved subcortical areas. Positive CS/PLGANPs were slower in transferring to the brain parenchyma than negative NPs and presented a prevalent localization in the caudal sub-regions. Two different pathways were used by the two types of NPs tested an intra-neuronal pathway for positive NPs, because the intra-neuronal pathway involves axonal transport and requires hours to days for drugs (and assumed nanoparticles) to reach brain regions. While the negative NPs may reach the brain areas through the extra-neuronal pathway, which probably relies on bulk flow transport through peri-neural channels that deliver drugs/particles directly to the brain parenchymal tissue and/or cerebral spinal fluid (CSF) (Chen et al., 2012). Therefore, we suggested that the surface differences between the nanocarriers tested, deeply influence the time course. In particular, we could affirm that chitosan enhanced the NPs residence time, due to electrostatic interactions and, at the same time for the same reason, it increased the time

to reach the brain for NPs. The appearance of the fluorescence signal in rostral brain regions at early time points for negative NPs suggested the olfactory transport considering that the olfactory pathway may provide both intra-neuronal and extra-neuronal access to the brain (Hadaczek et al., 2006); on the contrary, the transport of positive NPs was different, since we found a weak fluorescent signal in all areas at early time points and a strong fluorescence intensity in caudal brain after 48h suggesting the involvement of the trigeminal nerve transport for positive NPs.

Conclusion

In this work nanotechnology systems and nose-to-brain delivery were investigated in order to provide new, patient-friendly solutions to delivering therapeutic agents to the brain. A specific ratio between PLGA and CS is needed to obtain a carrier having good stability at 25°C. Our data demonstrated that NPBC3 is the more suitable positive nanocarriers to perform *in vivo* study comparing its localization in respect to NPA (negative nanocarrier). Surface charge is a critical parameter to determine the site of localization of NPs. Our *in vivo* studies demonstrated that both NPs can reach the brain, even if the uptake of negative carriers seems prevalent in rostral sub-regions, suggesting that NPA were possibly transported via olfactory pathway while NPBC3 seems to involve the trigeminal pathway.

Our data also demonstrated differences in the localization of NPs in the brain areas dependent on the NPs physico-chemical properties in function at the time after IN administration. These findings raised the hypothesis that therapeutic agent loaded NPs may have a direct access to the CNS following IN administration. Not only NPs can reach the brain but it is possible to obtain a prevalent localization in caudal or rostral

area depending on the NP surface charge. The results observed suggested that not only the mean size, as previously demonstrated by other authors (Mistry et al, 2015), is an important parameter but also the surface properties. On the basis of the neurological disease and in order to obtain a prevalent localization in the brain region affected, the accurate selection of the polymeric matrix to formulate nanocarriers is important for influencing surface charge.

Contributors

All authors gave substantial contributions to the conception or design of the work. In particular, Teresa Musumeci, the corresponding author, Serapide Francesca and Rosalia Pellitteri participated in the acquisition, analysis, or interpretation of data. Angela Bonaccorso participated in the development of experimental section. All authors participated in the drafting of the work and the critical revision of its content.

All authors approved the final version to be published.

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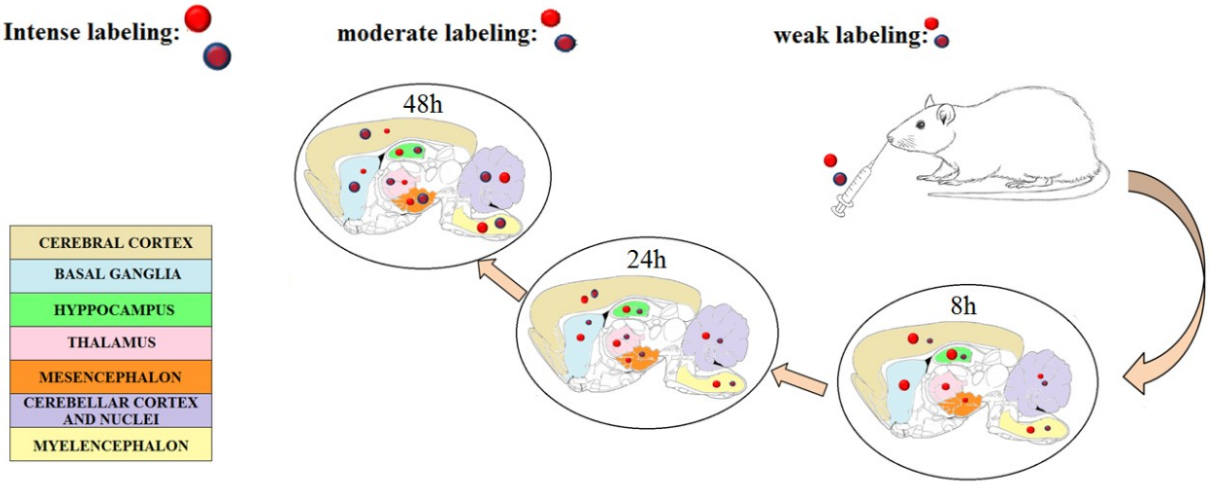
Table 1. Labeled brain regions with unmodified (NPA) and CS surface-modified nanoparticles(NPBC3) at different time. The number of samples used was four. Microscope observation was used to highlighted the labeling of fluorescent in the tissues.

Intense labeling: +++ ; moderate labeling: ++ ; weak labeling: + ; no labeling: -

Brain regions analyzed	after 8 h		after 24 h		after 48 h	
	NPA	NPBC3	NPA	NPBC3	NPA	NPBC3
Cerebral cortex (Frontal, Parietal and Occipital Cortex)	+++	+	++	++	+	+++
Basal ganglia (Caudate and Putamen Nuclei)	+++	-	++	+	+	+++
Hippocampus (Dentate gyrus; CA1-CA3 regions)	++	+	++	+	++	++
Amygdala	++	-	++	+	+	++
Thalamus (Reticular, central complex, anterior, dorsal nuclei)	++	-	++	+	+	++
Mesencephalon (Tegmental, pretectal nuclei)	+	-	+	+	++	+++
Cerebellum (Purkinje cell layer; deep nuclei)	+	+	++	+	+++	+++
Myelencephalon (Facial, spinal trigeminal nuclei, lateral reticular substance).	++	+	++	+	+++	+++



Graphical abstract: Schematic representation of rhodamine-labeled NPs distribution, 8, 24 and 48h after IN administration in rat. Red spot indicates NPA; Blue-red spot indicates NPBC3. The intense, moderate and weak labeling correspond to large, medium and small spots respectively. The brain areas investigated are: cerebral cortex (beige); basal ganglia (light blue); hyppocampus (green); thalamus (pink); mesencephalon (orange); cerebellar cortex and nuclei (violet); myelencephalon (yellow).

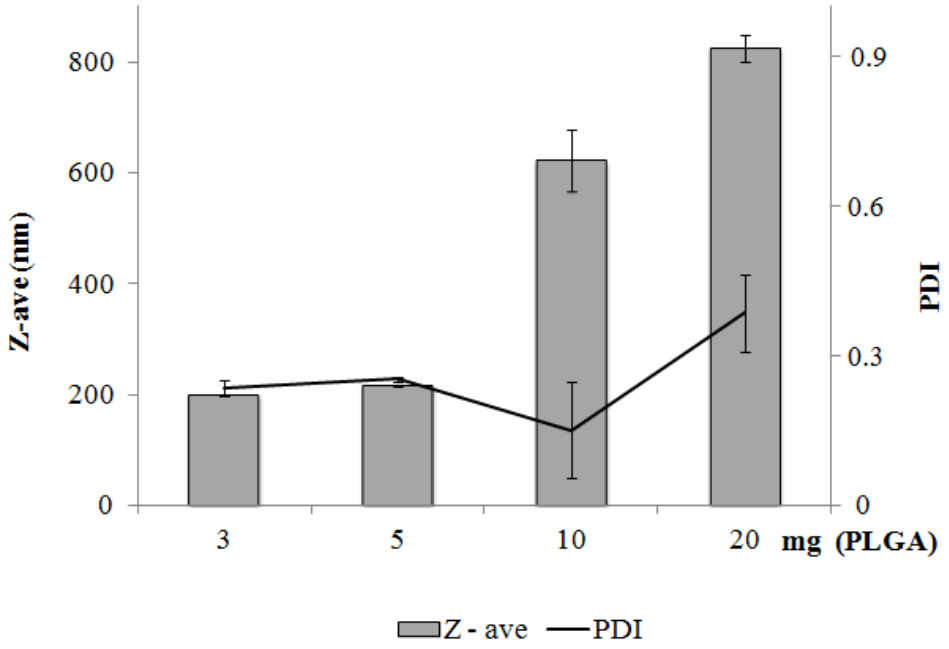


Figure 1 Mean size and polidispersitivity index of positive CS/PLGA nanoparticles obtained with a different amount of PLGA at an invariable amount of surfactant and chitosan. Values are presented as the mean ± S.D. (n = 3).

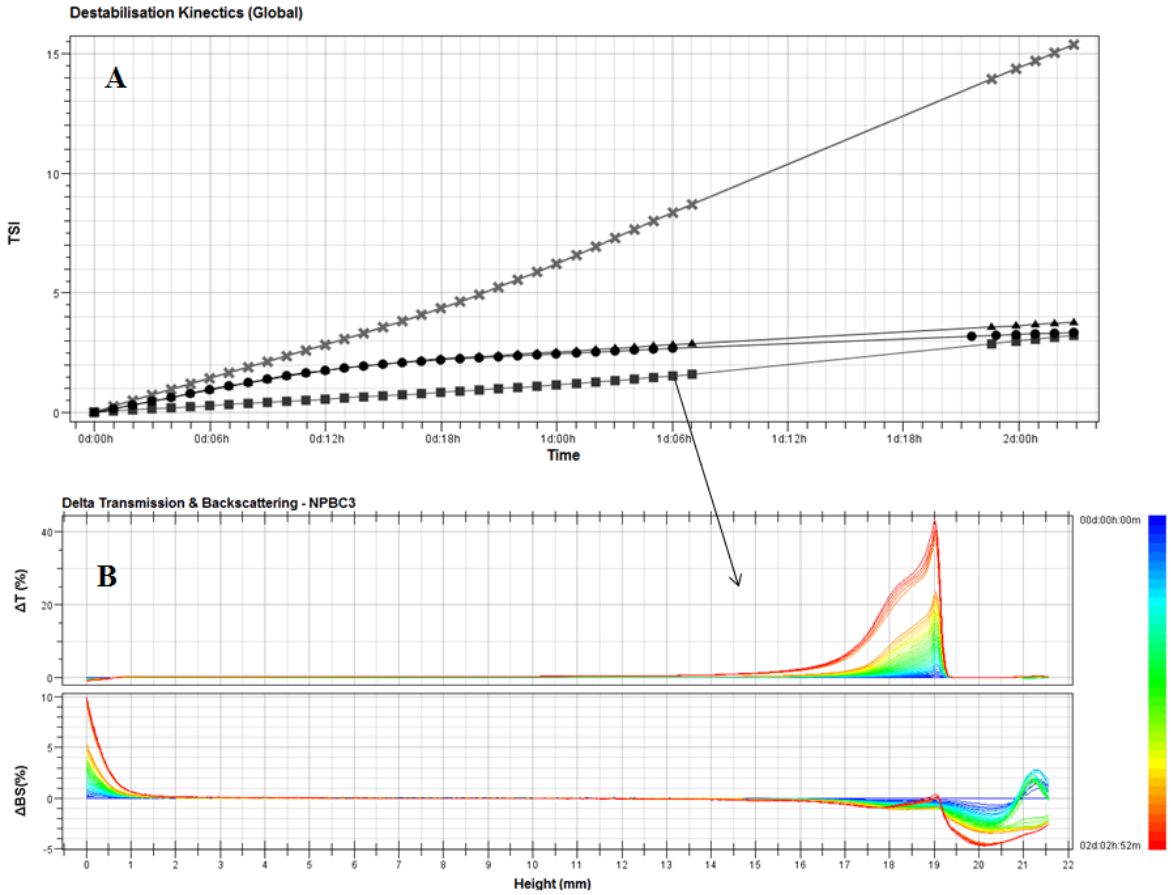


Figure 2. Turbiscan Stability Index (A) as a function of an initial amount of PLGA polymer in positive CS/PLGA NPs at 25°C (-■- NPBC3; -▲- NPBC5; -●- NPBC10; -×- NPBC20); (B) **Δ**-Backscattering (**Δ**Bs) and **Δ**-Transmission(**Δ**T) of NPBC3 at 25 °C.

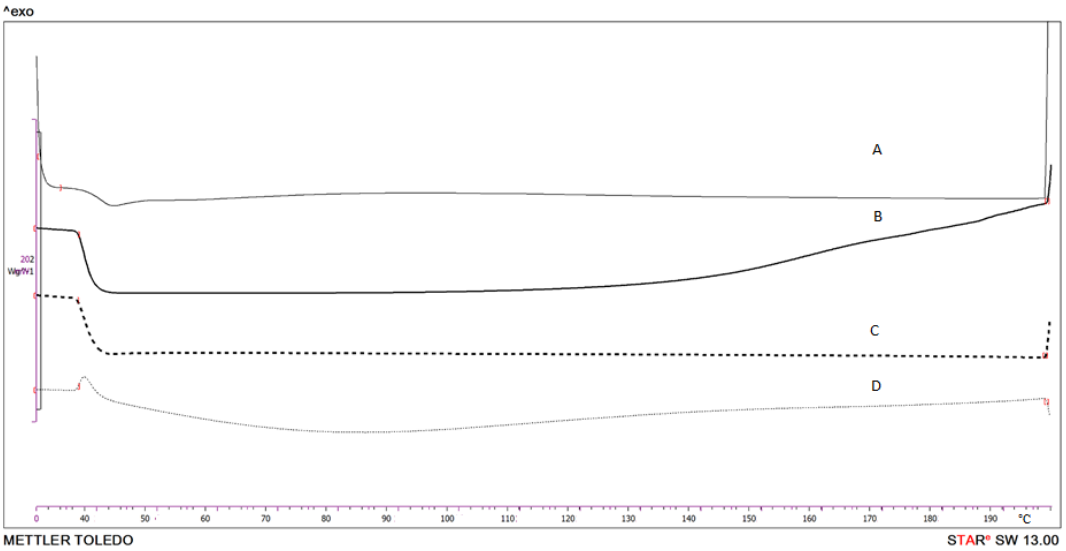


Figure 3. DSC Thermograms of A) NPBC3 (CS/PLGA NPs); B) NPA (PLGA NPs); C) PLGA; D) chitosan.

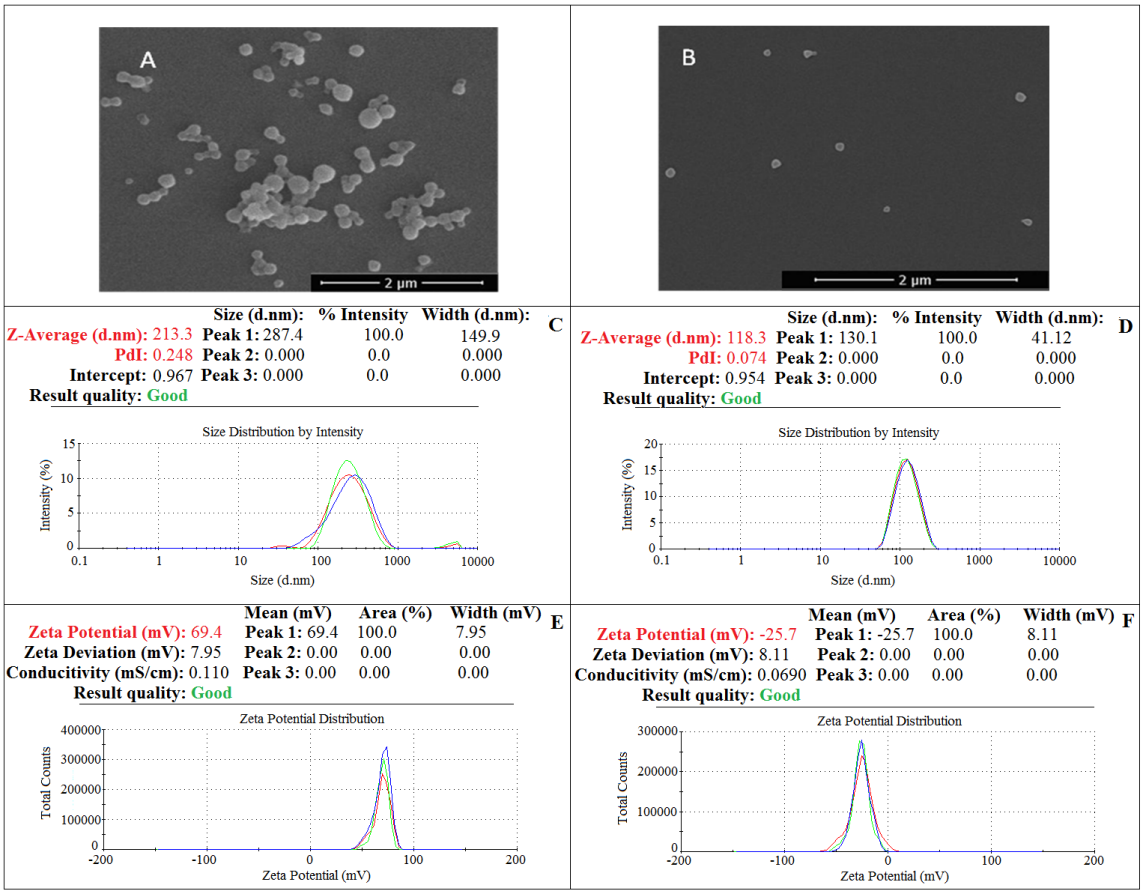


Figure 4. Physico-chemical and morphological properties of the rhodamine labeled nanocarriers (NPA and NPBC3) selected for *in vivo* studies. A) Scanning electron micrograph of NPBC3 (CS/PLGA NPs); B) Scanning electron micrograph of NPA (PLGA NPs); C) Mean size and PDI of NPBC3 (CS/PLGA NPs); D) Mean size and PDI of NPA (PLGA NPs); E) Zeta potential of NPBC3 (CS/PLGA NPs); F) Zeta potential of NPA (PLGA NPs).

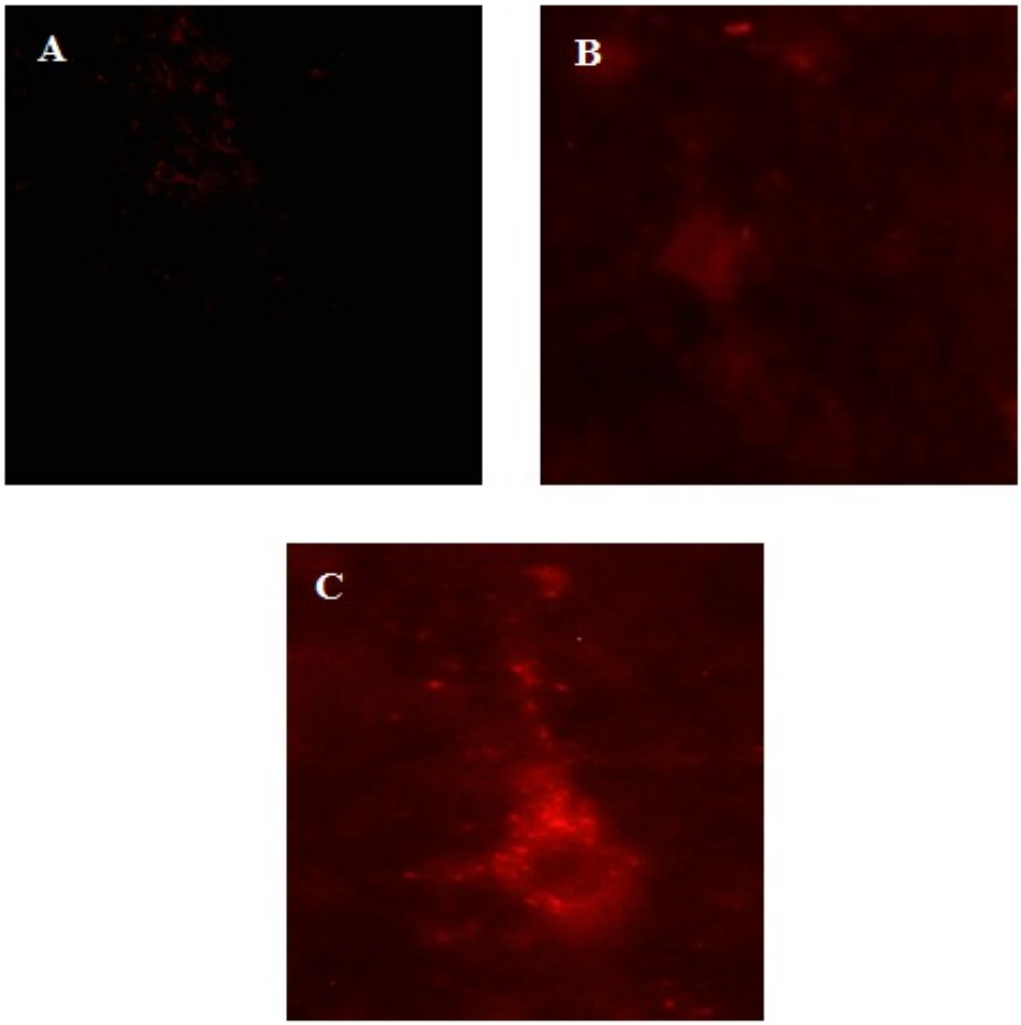


Figure 5. A) Photograph of rat brain not treated; B) Photograph of rat brain treated with rhodamine B solution; C) Photograph of rhodamine labeled PLGA NPs into cytosol of neural cells of the rostral region of the brain after IN administration (24 h).

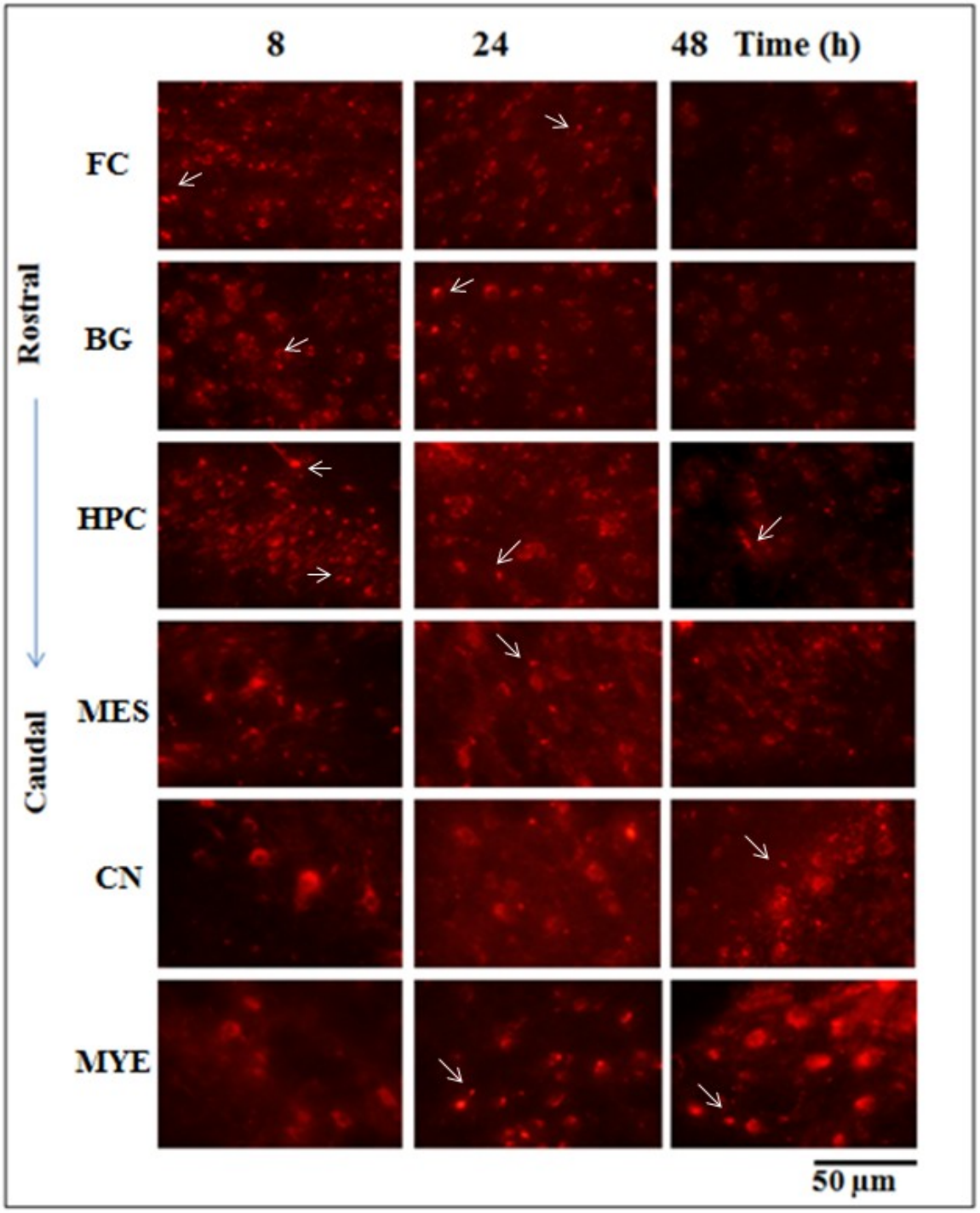


Figure 6. Photomicrographs illustrating the localization of the NPA (negative NPs) in relation to the brain sub-regions during the time-course (8, 24, 48 h) after IN administration. Rostro-caudal sequence of the brain sub-regions investigated: Frontal cortex (FC); Basal Ganglia (BG); Hippocampus (HPC); Mesencephalon (MES); Cerebellar nuclei (CN); Myelencephalon (MYE). The white narrow indicates the presence of rhodamine-labeled NPs. Scale bar=50 µm

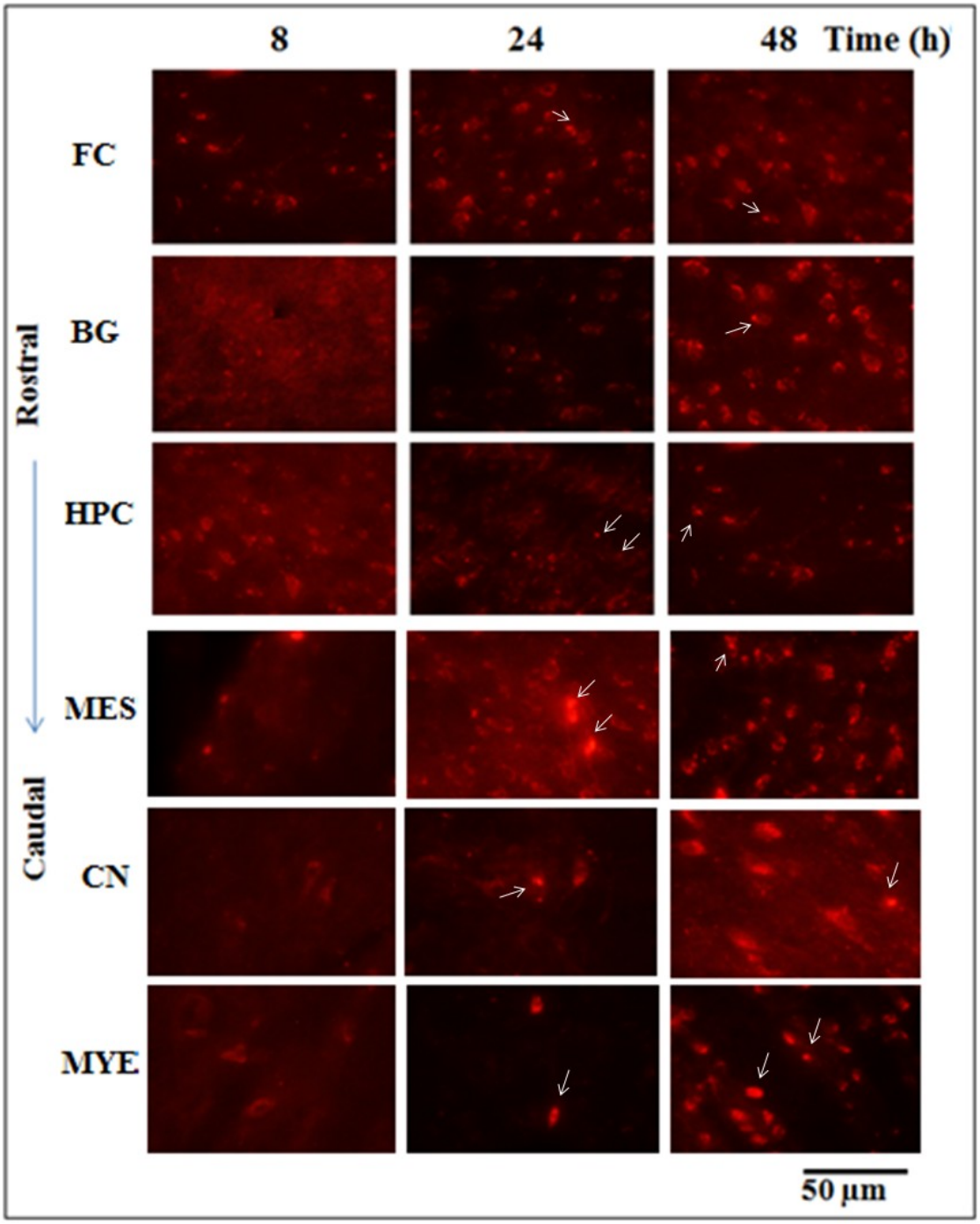
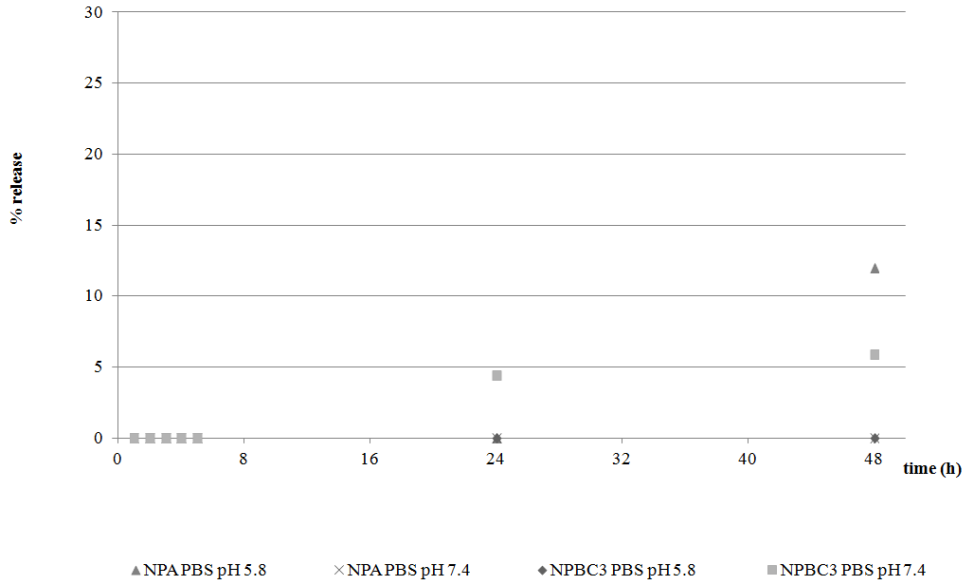
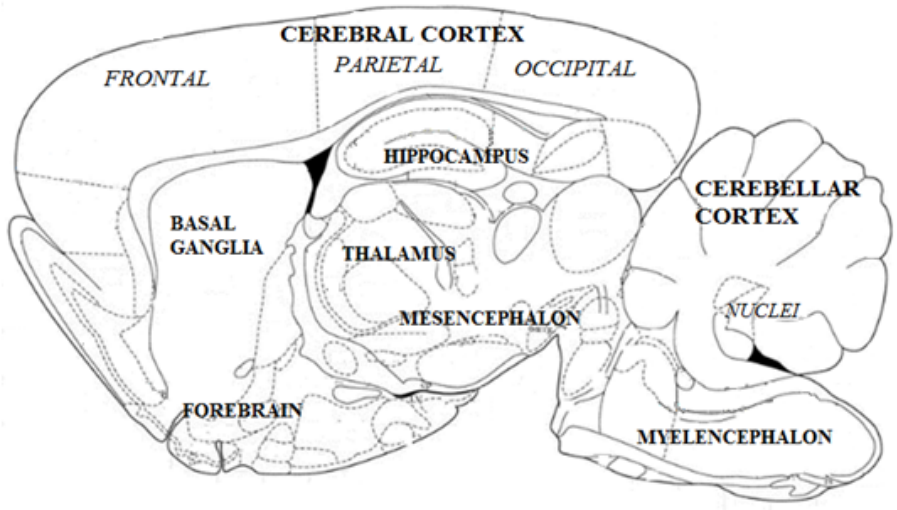


Figure 7. Photomicrographs illustrating the localization of the NPBC3 (positive NPs) in relation to the brain sub-regions during time-course (8,24,48 h) after IN administration. Rostro-caudal sequence of the brain sub-regions investigated. Frontal cortex (FC); Basal Ganglia (BG); Hippocampus (HPC); Mesencephalon (MES); Cerebellar nuclei (CN); Myelencephalon (MYE). The white narrow indicates the presence of rhodamine-labeled NPs. Scale bar=50 µm.



Supplementary Figure 1. Release profile of rhodamine from negative and positive NPs in PBS pH 5.8 and pH 7.4 at 37 °C until 48 h.



Supplementary Figure 2. Modified sagittal section of the rat brain according to Paxinos and Watson.

CHAPTER IV

Nose-to-brain delivery of DiR–loaded PLGA Nanoparticles

In preparation.

Nose-to-brain delivery of DiR–loaded PLGA nanoparticles

Our findings in the preceding chapter clearly demonstrated the brain distribution of Rhodamine B labeled PLGA NPs and CS-PLGA NPs. Additional experiments carried out in collaboration with Prof. Giulio Sancini and Dr Roberta Dal Magro (Department of Health Sciences, University of Milan-Bicocca) were performed in order to confirm our results and were partially reported in this chapter. In particular, PLGA NPs were loaded with the carbocyanine $DiOC_{18}(7)$ (DiR). Biodistribution and bioavailability to the brain was evaluated after IN administration in healthy mice by Fluorescence Molecular Tomography system (FMT).

FMT offers the ability to perform *in vivo* imaging directly in the living animal. Thus, this system allow to capture time course data by avoiding sacrifice of animal models. Moreover, animals are unaffected by the experiment, meaning that they can be imaged repeatedly for days, weeks, or months, allowing to look at changes due to treatment or any other manipulation over time in the same animal. DiR-PLGA NPs were prepared by nanoprecipitation method (Musumeci et al., 2014). Fluorescent-labelled NPs were prepared by co-dissolving the dye DiR with the polymer in the organic phase.

DiR-PLGA NPs were purified by ultracentrifugation. After washing, the obtained NPs were re-suspended in filtered water (0.22 μm Sartorius membrane filters) and freeze-dried using trehalose as cyoprotective agent (Figure 1). This is a very important and crucial step as volume is one of the biggest limitation for IN dosing. Freeze-drying process allow us to obtain a stable and concentrate formulation that can be easily resuspend in a specific small volume.

DiR-PLGA NPs were characterized for zeta potential and size distribution by photon correlation spectroscopy and surface morphology by scanning electron microscopy.

DiR-PLGA NPs with average diameter less than 150 nm were obtained and with a polydispersity index of 0.090 ± 0.030. These systems were negatively charged and with DiR concentration of ~10 µM.

Figure 1. Representative image of DiR-PLGA NPs preparation.

Freeze-dried DiR-labelled NPs were resuspended in 1 mL of sodium chloride physiologic solution and dispersed by sonication for 30 min at 25°C, prior to *in vivo* administration.

Male CD-1 mice, 6-8 weeks old, were used for this study. Mice were anesthetised using a mixture of isoflurane 2.5% -O2 70% -NO2 30% before the treatment and during the whole fluorescence detection procedure. 20 µL of DiR-labelled NPs were instilled into the nose of mice, 10 µL in each nostril, with the help of micropipette. The animals were held in slanted position during the IN administration, in order to avoid swallowing of the formulation. Eight mice underwent a single IN instillation and the biodistribution of DiR-labelled NPs was analysed 3, 24, 48 and 72 h after the administration by means of

FMT system (FMT1500, Perkin Elmer). Additional five mice were treated two times with DiR-labelled NPs by IN administration, 24 h apart, 20 µL of NPs each time and the fluorescence was detected and quantified 3, 24, 48, 72 h after the second instillation.

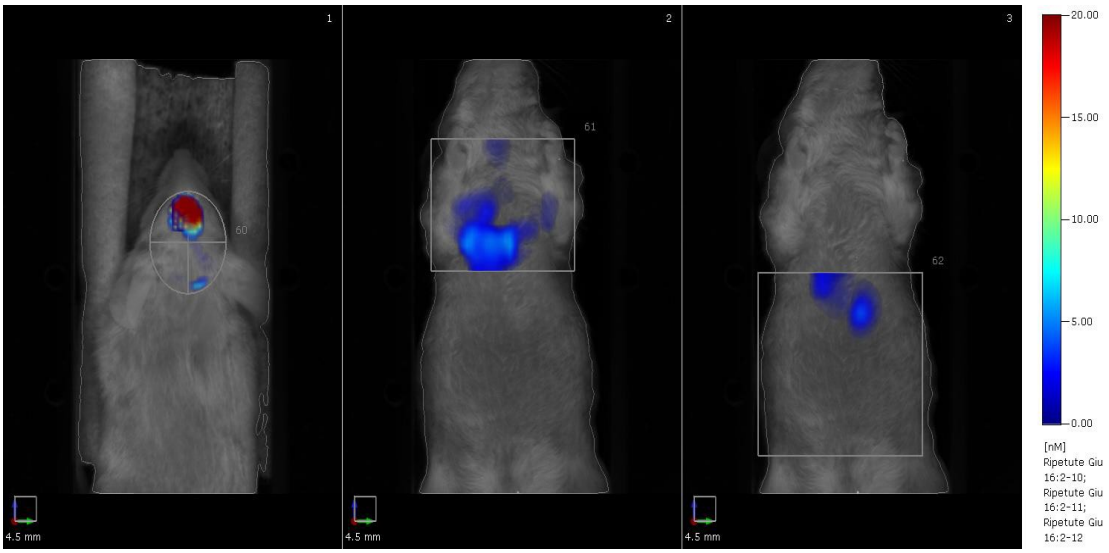


Figure 2. Representative image of DiR-labelled NPs biodistribution after administration by IN instillation. The fluorescence was detected by means of FMT1500 and the amount of fluorophore in the regions of interest (ROI) was quantified using TrueQuant Software.

Our results show that 3 h after a single IN administration, more than 5% of the injected dose of NPs was detectable in the brain. Repeated IN administrations provided a significant increment of NPs-associated fluorescence in the brain (Figure 2 and 3).

Indeed, more than 8% of the injected NPs was measured in the brain 24 h after the second IN instillation. This amount slowly decreased to 4.9% at 72 h from the last IN administration (Figure 3).

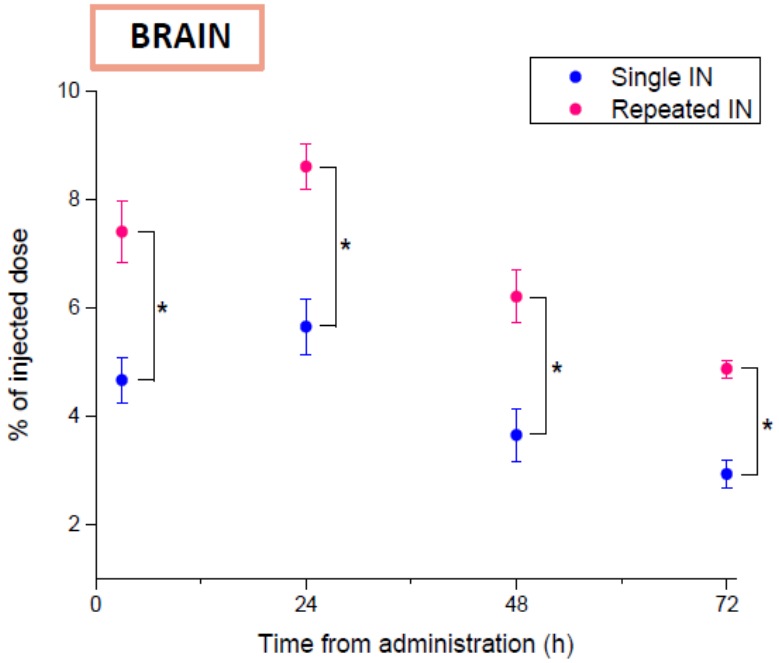


Figure 3. Quantification of NPs-associated fluorescence in the brain of CD-1 mice after single (blue dots) and repeated (pink dots) IN administrations. Values of repeated IN are related to the % of fluorescence measured in the brain 3, 24, 48 and 72 h after the second instillation. Data are expressed as % of the injected NPs. * $p<0.001$ single VS repeated IN at each considered time point (Student's t test).

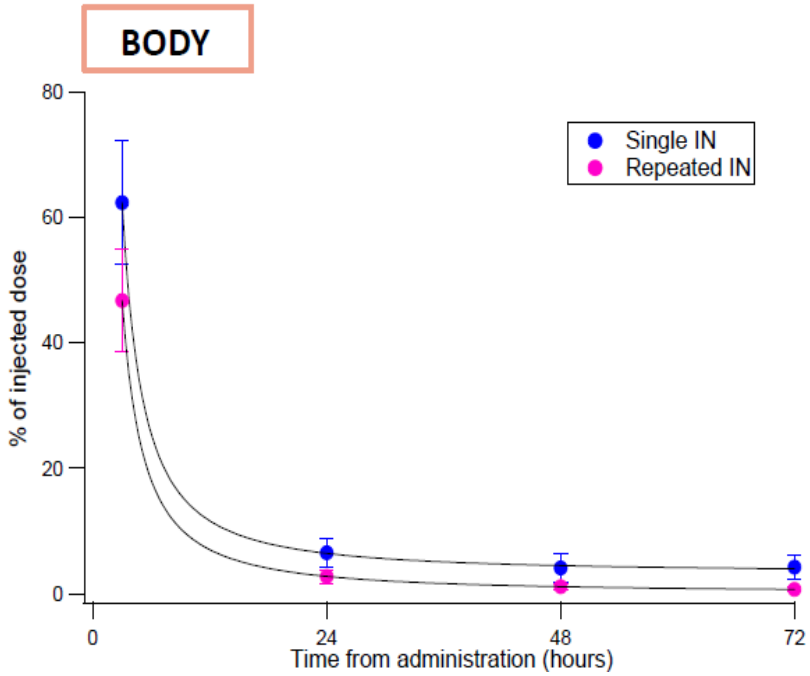


Figure 4. Fluorescence measured in the tissues/organs of both thorax and abdominal cavity after administration of NPs by single (blue dots) and repeated (pink dots) IN instillations. For repeated administrations, the fluorescence was calculated in the suitable ROI at 3, 24, 48 and 72 h after the second treatment.

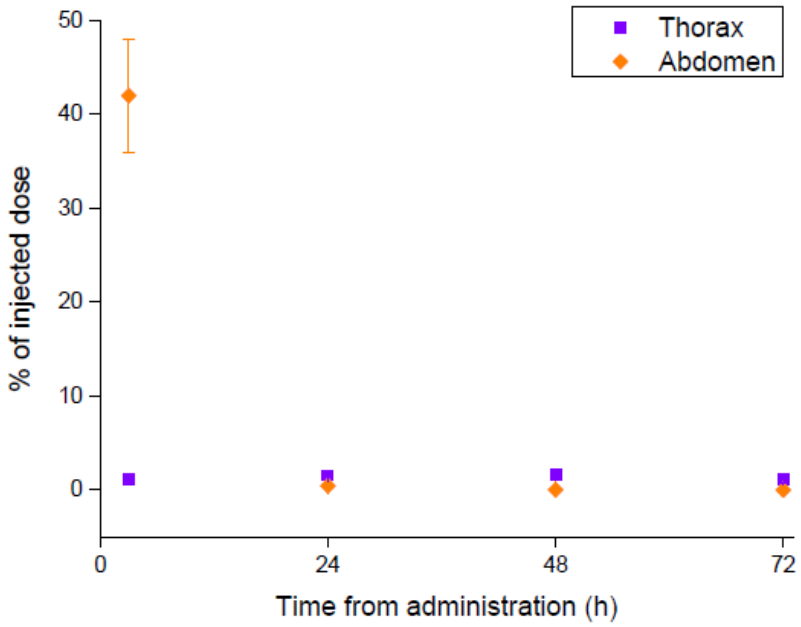


Figure 5. Distribution of DiR-labelled NPs in the thorax (purple spots) and in the abdomen (orange spots) at 3, 24, 48, 72 hours after the second IN administration.

Repeated IN administrations did not affect NPs accumulation in other organs and tissues (Figure 4). Indeed, NPs were quickly cleared from the thorax (mainly the lungs) and from abdominal cavity (Figure 5). Less than 10% of the injected dose was found in extracerebral organs 24 h after single and repeated instillations. These findings support this route as a non-invasive strategy to enhance the bioavailability of therapeutics to the brain. Here we confirm again the translocation of PLGA NPs from the nasal cavity to the brain after IN administration. These findings support this route as a non-invasive strategy to enhance the bioavailability of therapeutics to the brain. We cannot exclude a role for systemic pathway involved in this transport but we claim that the neural pathways are predominant.

CHAPTER V

Controlling seizures by intranasal delivery of oxcarbazepine-loaded nanoparticles in rats

In preparation.

Controlling seizures by intranasal delivery of oxcarbazepine-loaded nanoparticles in rats

Oxcarbazepine (OX) is a new drug chemically related to carbamazepine, it is a compound assigned for the treatment of mono- and adjunctive therapy in partial and generalized tonic-clonic seizures in epileptic adults and children (Wellington and Goa, 2001). Following oral administration, OX shows high systemic distribution to non-targeted tissues and consequently undesirable peripheral pathological conditions may occur (e.i. hematologic, hepatic and renal dysfunctions) (El-Zaafarany et al., 2016). Furthermore, drug-drug interactions can arise and OX may be associated with induction of some enzymes responsible for the metabolism of 25- hydroxyvitamin D, thus, patients receiving high doses of OX have an increased risk of bone loss over time (Mazza et al., 2007). As reported in several studies, profiles of toxicity express on different cell lines treated with OX (Pavone and Cardile, 2003; Aktas et al., 2009).

Moreover, OX like other AEDs, crosses human placenta resulting a major risk for congenital malformation. Therefore, there is a need for a drug delivery system that can provide beneficial therapeutic outcomes for patients by improving the transport of therapeutic agents to the brain and simultaneously limit their transplacental passage for pregnant women (Loapalco et al., 2015). Based on these considerations, our goal is the encapsulation of OX in stable PLGA NPs aiming at direct nose-to-brain delivery to improve epileptic therapy, the possibility of using less daily drug amounts to reduce undesirable interactions and toxic effects and to evaluate the possible neuroprotection of this drug against the seizures and brain damage induced by pentylenetetrazole (PTZ) administration.

In this work, we have encapsulated OX into biodegradable and biocompatible NPs composed of 50:50 poly(lactic-*co*-glycolic acid) (PLGA) by nanoprecipitation method. The NPs were characterized for morphology, particle size, zeta potential, encapsulation efficiency and release profile by dyalisis bag technique. Dose-response curve was studied to evaluate drug efficacy, pharmacokinetics of OX in CSF and blood following intravenous (IV) and IN administration in rats was investigated in order to compare the delivery routes. We further investigated the effect of intranasally administered OX-loaded PLGA NPs against seizures induced by PTZ in rats.

Monodisperse nanoformulations were prepared with average particle sizes about 200 nm and polydispersity index below 0.2. Fair encapsulation efficiency values around 85% were obtained, and differential scanning calorimetry (DSC) measurements demonstrated the amorphous form of the drug in the nanoformulations (Fig. 1).

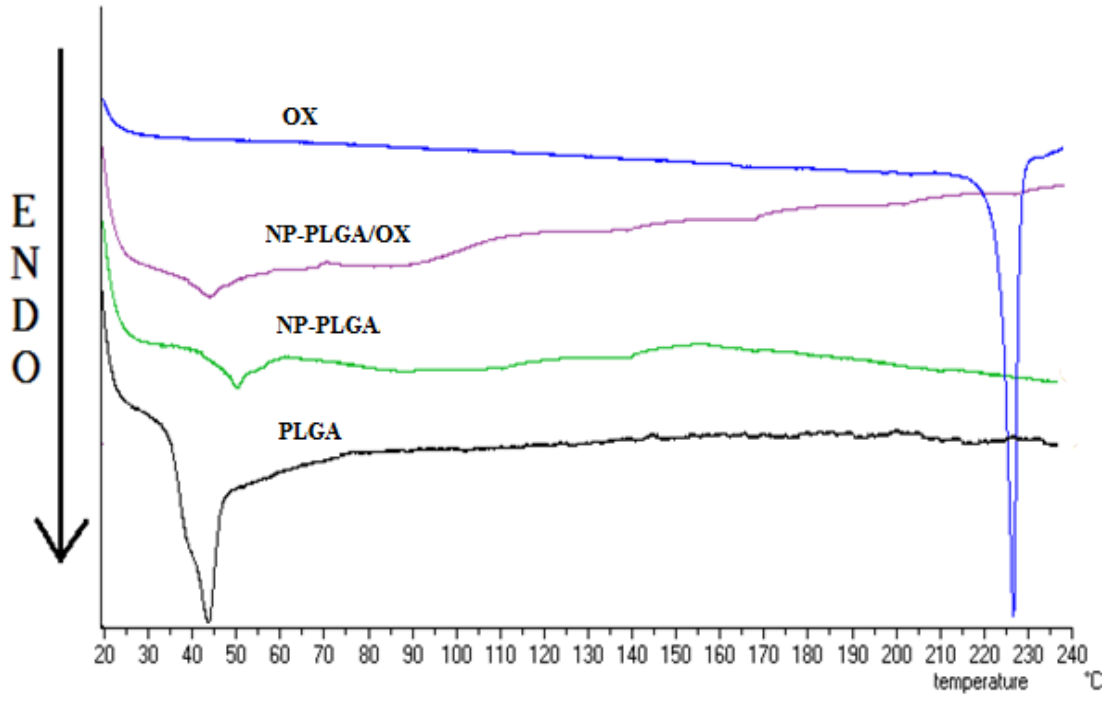


Figure 1. DSC thermograms of Ox; Ox-loaded PLGA NPs; PLGA NPs and PLGA.

The scanning electron microscope (SEM) images shown that NPs formulation appeared to be uniform with almost smooth and spherical shape (Fig. 2)

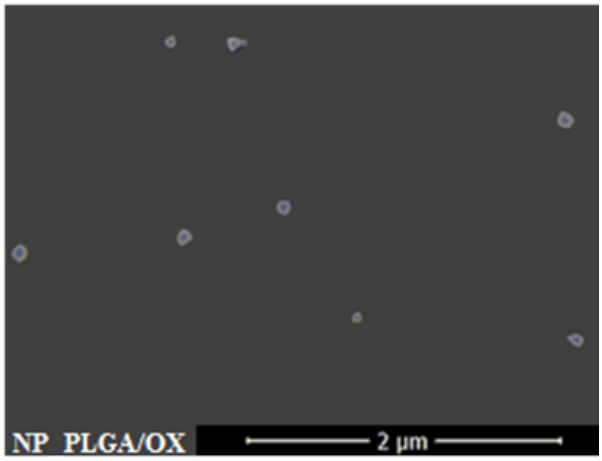


Figure 2. SEM photograph of Ox-PLGA NPs.

Our results from Pharmacokinetics study showed that no OX amounts were detected in the CSF within 180 min after IV administration of the drug while IN administration produced detectable amounts of OX in the CSF of the rats. In particular, the analysis of CSF samples indicated that OX was not detectable 45 min after the nasal administration of the suspension, whereas after 120 and 180 min the different drug amounts were detected. No OX amounts were detected in the bloodstream within 180 min after nasal administration of the drug suspension.

The epileptic model was established by intraperitoneal PTZ injection of rats. PTZ is a selective blocker of the GABA-A receptor, it induces chemical kindling characterized by dose-dependent subconvulsions and generalized tonic-clonic seizures.

For the experimental epilepsy model rats were divided into groups and were treated with different doses of PTZ to induce seizures in rats. The rats were observed for 60 min after PTZ injection for the evaluation of Racine's Convulsion Scale (RCS).

The concentration of PTZ selected to induce persistent generalized tonic-clonic seizure was 50mg/Kg. (Fig. 3).

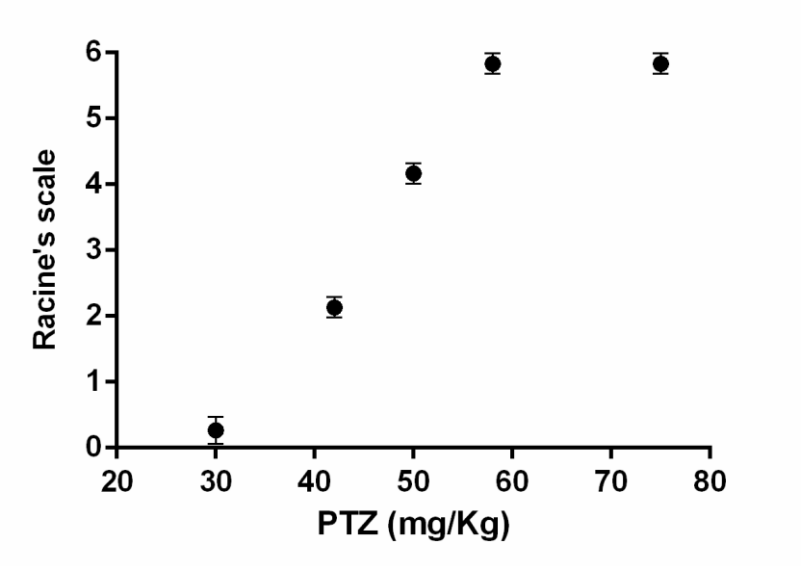


Figure 3. Rats were treated with different doses of pentylenetetrazole (PTZ), monitored for observable seizures, and classified on the Racine Scale. Values are presented as the mean±S.D. (n = 3).
Racine's scale:0: no convulsion; 1: twitching of the vibrissae and pinnae; 2: motorarrest with more pronounced twitching; 3: motor arrest with generalized myoclonic jerks; 4: tonic-clonic seizure while the animal maintained posture; 5: tonic-clonic seizure with loss of the righting reflex; 6: lethal seizure.

Different groups of rats were used in the behavioral studies. Animals were treated with PTZ alone as negative control and with different OX preparations, in particular OX solution by i.p. injection, OX solution by IN administration in two different doses and OX-loaded NPs at the lower dose.

The time required for the onset of seizures, from the time of injection of PTZ, their duration, and seizure scores were taken as the evaluation parameters. The rats were observed for 60 min after PTZ injection for the evaluation of RCS.

OX i.p. failed to induce protection; in these experimental animals, the number and the sings of convulsive episodes were similar to that observed in animals receiving PTZ alone; OX IN at the higher dose offered prologation of the onset of PTZ-induced seizure, reduction in seizure stage and symptoms duration; OX IN at the lower dose is

the minum concentration of the drug required to produce some protective effect against seizures, in these experimental animals the number of convulsive episodes was similar to that observed in animal receiving OX at the higher dose, while the mean duration increase. Several enzymes that are present in the nasal mucosa might affect the stability of drugs, which are often subjected to degradation and consequently the concentration of drug available decreases (Kushwaha et al., 2011).

Animals treated with OX-loaded NPs show mild symptoms related to first stage and with short duration. Moreover, to protect animals from convulsive behavior free OX was given 3 times a day via IN administration. While OX-loaded NPs were given once daily for 3 days. Therefore, OX IN administration by using NPs significantly reduces the frequency of administration, the symptoms appearance and the duration. Furthermore, we also investigate NPs chronic administration, for 2 weeks. Rats experience the same behavioral tendencies but it seems that they did not express any evident side effects. These data have been confirmed by immunohistochemical analysis to evaluate potential OX neuroprotection from brain damage induced by PTZ.

Therefore, IN administration of OX could be a useful strategy to improve epileptic therapy as the oral or IV administration to the patients suffering epilepsy might be impractical or inconvenient.

CHAPTER VI

Nose-to-brain delivery and chitosan derivatives nanocomplexes for siRNA delivery to the brain

In preparation.

Nose-to-brain delivery and chitosan derivatives nanocomplexes for siRNA delivery to the brain

As the population ages, brain pathologies increase their incidence, being the need to find successful treatments of main importance. However, biological barriers, mainly the BBB, are the key obstacles that prevent the effectiveness of possible treatments due to their highly restrictive nature (Masserini, 2013). Over the past decades, new approaches towards overcoming the BBB and its efflux transporters have been proposed (Gomes et al., 2015). One of these approaches is through small interfering RNA (siRNA), which is capable to specifically target one gene and silence it in a post-transcriptional way.

Gene silencing mediated by siRNA has gained significant attention in the last years, the therapeutic potential of siRNAs has been demonstrated in the treatment of many diseases including cancers and neurodegenerative disorders. However, it cannot be used in a naked form due to the partial negative charge and hydrophilicity and its low *in vivo* stability and susceptibility to degradation by nucleases. siRNA is incapable of crossing the biological membrane on its own to exert the gene silencing effect in the cytosol (Draz et al., 2014). Therefore, a carrier that improves stability as well as targeted delivery is highly desired. siRNAs offer the advantages of being highly potent and able to act on targets not easily reached as they can be designed to affect any gene of interest. Therapeutic approaches based on siRNA involve the introduction of a synthetic siRNA into the target cells to induce RNAi, thereby inhibiting the expression of a specific mRNA, and consequently the inhibition of protein synthesis (Lam et al., 2015).

As important as the effective silence is the way to delivery siRNA to its site of action. Effective and safe delivery of siRNA into cells presents one of the major limit that hinder the use of siRNA in the drug discovery process and clinical applications,

a delivery carrier is therefore required. Nanotechnology-based systems can help, by protecting circulating siRNA, providing cell/tissue-targeting and intracellular siRNA delivery (Chiarelli et al., 2015). In this study, IN administration was investigated as alternative and innovative approach for siRNA transport directly to the brain. Biodegradable NPs have been extensively studied for nose-to-brain drug delivery, polymeric NPs are ideal candidates to deliver DNA, RNA, proteins and chemotherapeutic compounds with high specificity (Jivan et al., 2015). Chitosan is one of the mostly used natural polymers, it is a linear biopolyaminosaccharide obtained by alkaline deacetylation of chitin (Argarwal et al., 2015). The use of chitosan for the encapsulation of active components has attracted interest in recent years due to its versatile properties, such as muco-adhesiveness, low-toxicity and biodegradability (Acosta et al.,2015). The potential of chitosan nanocarrier technology has been reported for a variety of applications, the presence of free amino groups is responsible for the interaction of chitosan with biological systems, and the distribution of deacetylated groups along the chitosan molecule may regulate these interactions (Garcia-Fuentes et al., 2012). The wide variety of products that can be obtained as a result of the chemical modification of chitosan can enhance its already valuable properties and can improve the gene transfer activity of the polymer while still retaining the good properties of the material. With this goal in mind, and knowing the beneficial properties of chitosan we synthesized chitosan derivatives, differing in the molecular weight and in the quaternization and palmitoylation degree, for designing new nanocarriers with potential

application for nose-to-brain delivery. This is a very interesting investigation because in some cases high molecular mass cannot guarantee high gene transfection and silencing because the strong interaction force inside polyplexes might hinder the release of loaded nucleic acids (Huang et al., 2005). These facts emphasize the importance of a fine balance between extracellular protection and intracellular release to obtain expected biological effects.

The chitosan derivative self-assembles into micelles and it is capable of solubilising hydrophobic compounds. The cationic character of our polymer, along with the presence of reactive functional groups, provides particular possibilities for utilization in controlled-release technologies. The purpose of the present study was to investigate the use of chitosan derivatives (CP1 and CP2), as potential delivery carriers of siRNA.

The nanocomplexes were prepared and optimized on the basis of various physicochemical characteristics. The morphology of the nanosystems has been studied with a transmission electron microscopy (TEM). Our systems showed a regular shape, an average size less than 300nm, positive surface charge and a good stability under destabilizing conditions as demonstrated by photon correlation spectroscopy and Turbiscan analysis (Fig. 1 and 2).

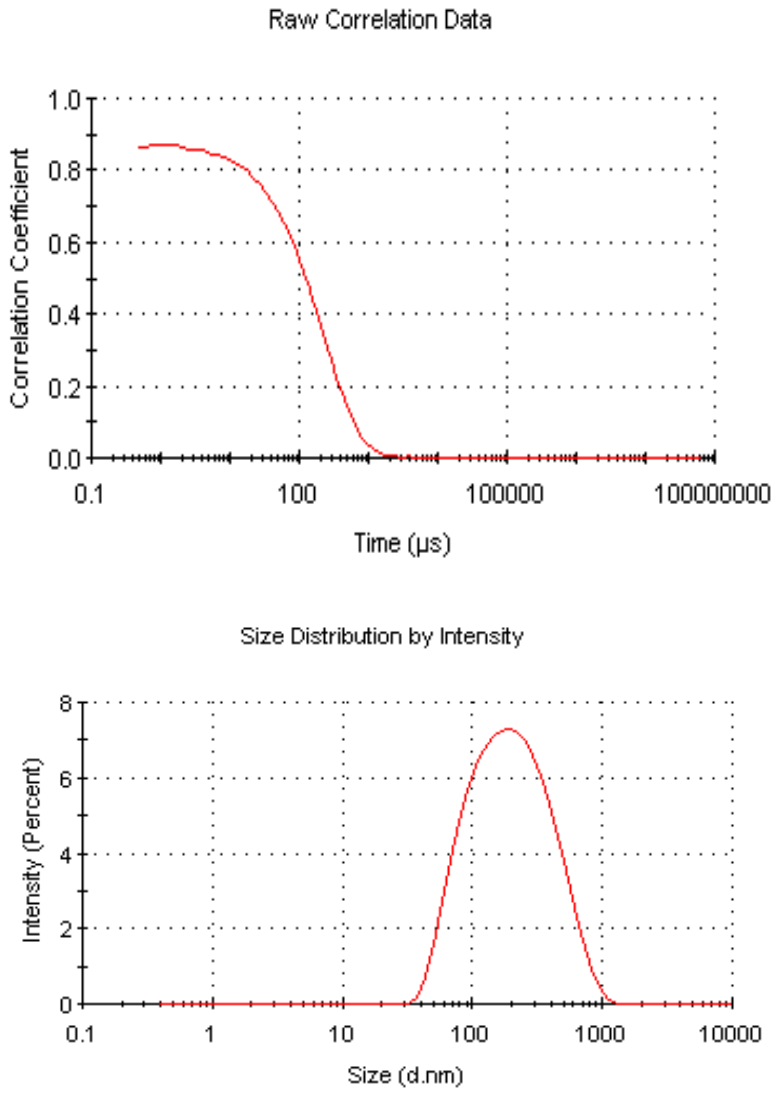


Figure 1. Size Distribution by Intensity of siRNA-CP2 complexes stored at 5°C after 4 weeks.

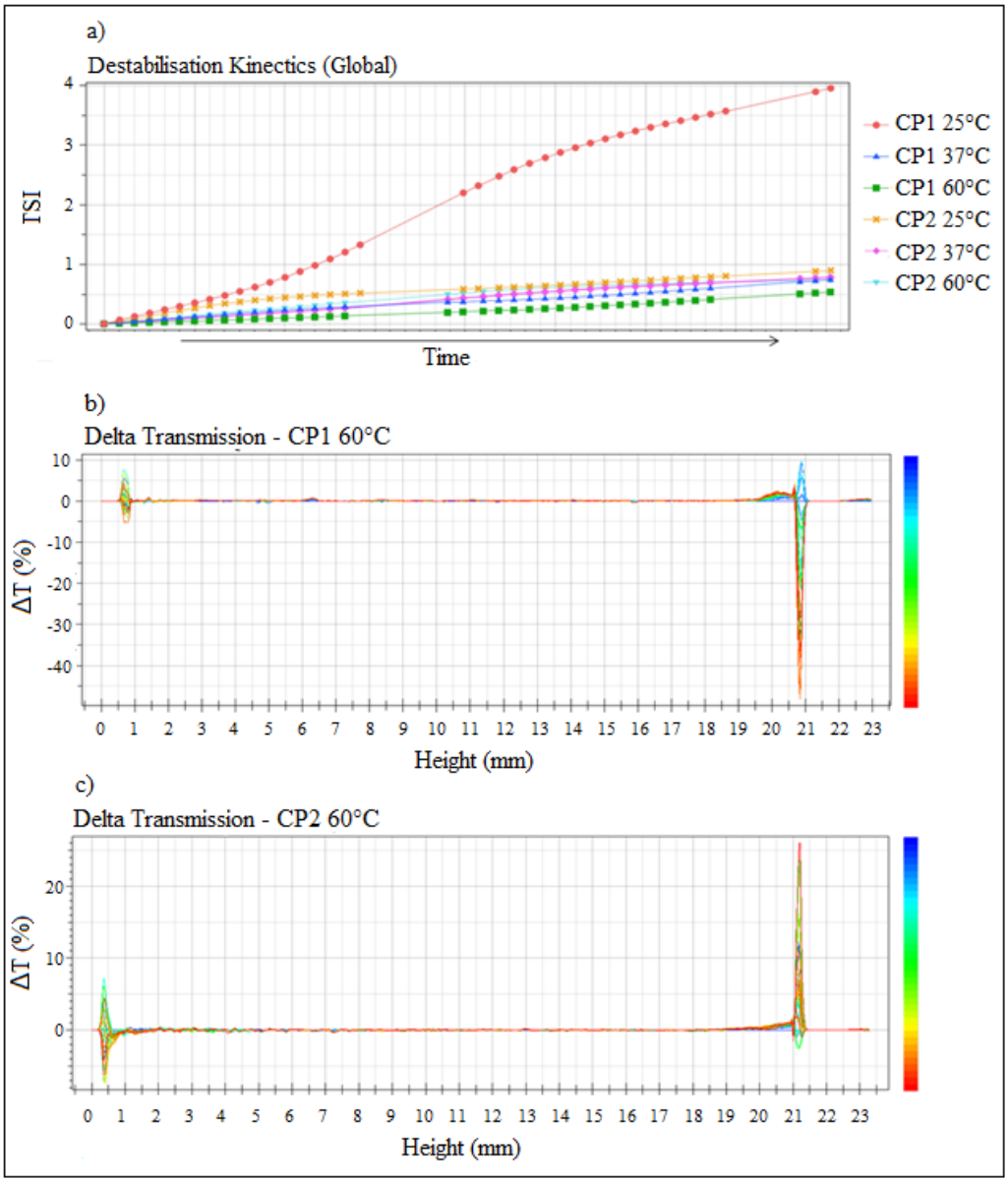


Figure 2. The Turbiscan stability index (TSI) of CP1 and CP2 nanosystems (a) stored at 25, 37 and 60 °C. The progression of the analysis over time is indicated by the arrow. Transmission profiles (ΔT) of CP1 nanosystems (b), and CP2 (c) stored at 60° C is also presented. The data are represented as a function of time (0–10 days) and sample height (0–23 mm)**.**

The cytotoxicity of the polymers was assessed by the measurement of the IC50 with MTT, and also the cytotoxicity of the complexes was achieved in order to select the most suitable formulation for cell delivery. The nanosystem, at the most suitable N/P

ratio, was selected to transfect A431 carcinoma cell line. The good transfection efficiency *in vitro* encouraged us to move forward with *in vivo* studies.

Our nanosystems were intranasally administered in rats to assess siRNA silencing activity in the brain (Fig.3).

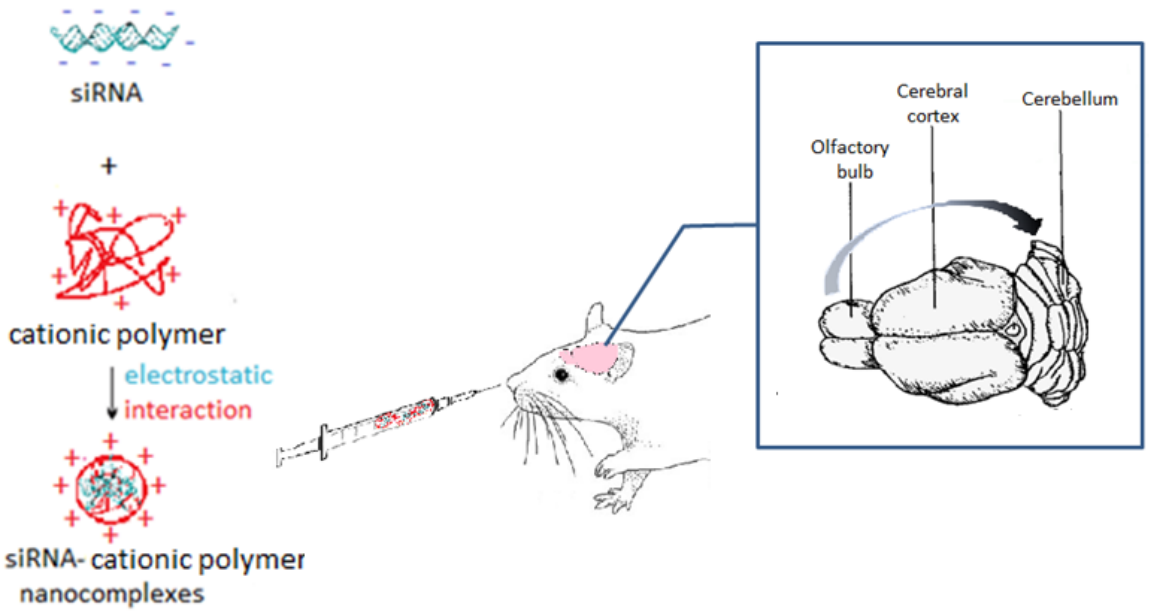


Figure 3. Summary of the *in vivo* study: intranasal administration of the model siRNA-CP nanocomplex in rats. Silencing effect of the model protein in different brain areas.

Based on the obtained results, we developed a new approach that combines nose-to-brain delivery with siRNA- polymer nanocomplex. Production of our systems was achieved by a simple method, that requires a few steps and without the use of solvents. Our nano-formulations showed good physico-chemical properties in terms of mean size, PDI and zeta potential suitable for siRNA delivery. The selected system showed good transfection efficiency *in vitro* and down-regulation of the model protein *in vivo*.

In future this system could be tested in an *in vivo* animal model of solid tumors.

CHAPTER VII

GENERAL DISCUSSION AND CONCLUSION

Neurodegenerative diseases represent one of the most important challenges for therapeutic intervention. Despite the high prevalence and incidence, CNS disorders are still incurable.

The current therapeutic approach is based on the administration of symptomatic drugs which reduce the signs and symptoms of CNS disease, for the comfort and well-being of the patient but not to treat or cure its causes. Furthermore, often the therapeutic treatment is ineffective due to the presence of the BBB which restricts the passage of substances from the bloodstream into the neural tissue. Multiple advances are being investigated to overcome this important limit such as transformation of drug structure by chemical approaches, disruption of the BBB or administration of the drug directly into the brain tissue but all these strategies are invasive and lack the target specificity or could change drug functionality (Ladola et al., 2014).

On the basis of this knowledge, we considered to explore scrupulously the use of colloidal drug carriers, which could effectively transport active molecules to the brain thanks to their exceptional physico-chemical properties. These nanosystems have attracted considerable interest because they can be easily modified in terms of particle size, surface affinity and stability which deeply influence their brain distribution and can be easily manipulated to achieve both passive and active drug targeting.

In order to achieve CNS delivery, the use of nanocarriers was associated with a non-invasive technique which is IN administration. IN delivery was studied as a direct communication route between nose and brain. This seems to be possible because of the unique connection that the olfactory and trigeminal nerves provide between the brain and external environment. Several studies have shown that nose-to-brain delivery of

free drugs could be achieved but at concentrations lower than those needed for the therapeutic level.

For this purpose, nanotechnologies may help to protect the drug from hydrolytic and enzymatic degradation and to improve and control drug release during the transportation and at the site of localization which would lead to the altering of the pharmacokinetic profile of the molecule encapsulated, increasing therapeutic efficacy and reducing side effects. All these properties could be controlled by choosing the constituents of the nanosystem matrix. Based on these considerations we wanted to focus and investigate the role and the influence of NPs surface modification on their fate and behavior after IN administration in experimental animals.

The PLGA polymer was selected for the preparation of NPs due to its tunable mechanical properties, it is physically strong, biocompatible, biodegradable, it exhibits a wide range of erosion times, and most importantly, it is an FDA approved polymer (Makadia HK and Siegel SJ, 2011). To overcome some of its limits such as quick blood clearance and uptake by MPS, studies have investigated its modification such as PEGylation or chitosan coating. These surface modifications of PLGA carriers would enhance interaction with biological systems and brain delivery.

Therefore, the optimization of nasal administration using NPs represents a promising strategy to enhance brain delivery but a suitable carrier should have proper surface properties and an average diameter smaller than that of the olfactory axons to be transported directly to the brain, reducing and avoiding systemic pathway.

Furthermore, nanosystems based on chitosan derivatives for gene therapy were also investigated to prepare stable, safe and efficient carrier systems for targeting the brain via nasal route. The potential of chitosan nanocarrier technology has been reported for a

variety of applications. Chitosan is often claimed to be GRAS (Generally Recognized As Safe) and bioabsorbable. We synthesized chitosan derivatives, differing in the molecular weight and in the quaternization and palmitoylation degree, to enhance chitosan already valuable properties and improve the gene transfer activity of the polymer while still retaining the good properties of the material.

In **Paper I** we pointed out a deep study on the PLGA NPs surface modified with PEG in order to select a new nanocarrier formulation suitable for nose-to-brain delivery.

With this in mind, this work was based on several technological analyses aimed at obtaining PEGylated NPs with simple composition and long-term storage.

Our work highlights the importance of the selection of starting materials and methods to produce nanosystems for specific application. Our findings demonstrated that the degree of PEGylation of PLGA significantly influences the physicochemical properties of NPs in terms of mean size and the effect of sucrose as a cryoprotective agent.

The use of sucrose as a one-component with double action may represent a novel strategy to reduce undesiderable interactions between excipients and molecules in the formulation. Moreover, this may represent an interesting approach to simplify and speed up NPs preparation and to accelerate production scale up. Furthermore, here we provide new evidence on PEGylated PLGA NPs mucoadhesive properties, as indicated PEG content of 5% w/V allowed to obtain NPs with weak mucoadhesive properties as revealed by thermotropic analysis and the mucin particle method. Based on these results we support the use of PEG to confer a sufficiently hydrophilic and uncharged surface to minimize effectively mucin-NPs adhesive interactions, allowing particles to diffuse rapidly through human mucus and cross respiratory epithelium reaching trigeminal pathway. Considering that following IN delivery part of the drug could reach the

systemic circulation, PEG could be further useful to increase NPs stability in biological media, extending their circulating half-life because of reduced phagocytosis and clearance by reticuloendothelial cells (Couvreur et al., 2001).

Our first work, purely technological studies, allow us to outline and to tune an interesting new nano-formulation for nose-to-brain delivery. Taking into account these results, further perspectives will be aimed at studying the *in vivo* fate of PEGylated NPs after IN administration in order to achieve a full view of these systems similar among them but with specific surface differences.

In the second set of experiments we looked at the *in vivo* fate of PLGA NPs and PLGA NPs surface modified with Chitosan after IN administration in rats. These formulations, already well-known and consolidated by our research group, have been optimized, from the technological point of view and tested *in vivo* (Musumeci et al., 2014, Musumeci et al, 2013; Li Volti et al., 2012; Musumeci et al, 2006; Vicari et al., 2006; Musumeci et al., 2006). To the best of our knowledge, very few or no studies to date have looked into the distribution of polymeric NPs in subregions of the brain.

In particular, we contribute, with **Paper II**, to define that compounds encapsulated in NPs can be directly transferred from the nasal cavity to the brain after IN administration. Interestingly, our data demonstrated some regional localization differences in the uptake of NPs in the brain areas dependent on the NPs physicochemical properties and time after administration. Among all the brain areas studied, NPs showed a steady and intensive distribution in the hippocampus.

These findings raised the hypothesis that therapeutic agents loaded NPs may have a direct access to the CNS following IN administration. Our findings led us to hypothesize that different pathways, were involved in the transport of unmodified and

modified NPs. The rapid appearance of the fluorescent signal in rostral brain regions at early time points for PLGA NPs suggested the olfactory transport considering that the olfactory pathway may provide both intra-neuronal and extra-neuronal access into the brain, while the uptake of CS- PLGA NPs was different, since we found a weak fluorescent signal in all areas at early time points and a strong fluorescent intensity in caudal brain after 48h suggesting the involvement of the trigeminal nerve transport. Thus, maybe our contribution could encourage further investigation to enhance brain-targeted delivery in specific areas by attaching various ligands on the NPs surface.

Our findings were once again confirmed by further investigation (**Paper III**), in which the animal species, the dye encapsulated in the nanocarrier and the system used to detect the fluorescent NPs have changed, while the NPs and the delivery route remain unchanged. In particular, the biodistribution study carried out after IN administration of DiR-PLGA NPs in mice gave us further demonstration of a direct brain transport via nasal route. Interestingly, from this study findings have emerged that repeated IN administrations provided a significant increment of NPs-associated fluorescence in the brain while repeated IN administrations did not affect NPs accumulation in other organs and tissues. Indeed, NPs were quickly cleared from the thorax (mainly the lungs) and from the abdominal cavity. These findings let us answer our questions related to the possibility for NPs to reach the brain after IN administration and to identify the brain areas involved in their distribution and also to evaluate whether surface modification could affect NPs *in vivo* fate.

The promising nanosystem, PLGA NPs, was chosen to answer another important question: Can NPs improve the efficacy of a therapeutic agent?

Based on the results of Rhodamine-labeled NPs brain distribution, and considering that the hippocampus was particularly involved in their localization, PLGA NPs were loaded with OX, an antiepileptic drug, showing as NPs mediated nose-to-brain delivery can improve drug efficacy by reducing the symptoms appearance and duration of signs as well as the frequency of administration (**Paper IV**). Furthermore, after the NPs chronic administration up to 2 weeks, rats did not experience any evident side effects.

These data were confirmed by immunohistochemical analysis evaluating potential OX neuroprotection from brain damage induced by PTZ.

Our last question to be found with our last study focused our attention on nanotechnology-mediated nose-to-brain delivery for gene therapy (**Paper V**).

Emerging evidence indicates a potential role of siRNAs in the treatment of many diseases including cancers and infections. However, the partial negative charge and the susceptibility to degradation by nucleases have hampered its use in a naked form.

This is where nanotechnology-based systems may help, by protecting siRNA circulation and providing cell/tissue-targeting and intracellular siRNA delivery. A delivery vector is therefore required to protect the siRNA from enzymatic degradation, facilitate cellular uptake to the target cells and release the siRNA at the site of action. Here we provide new evidence on novel carrier systems, synthesized in our lab and effectively complexed with siRNA, able to effectively and safely transfect carcinoma cells and induce down-regulation of the model protein after IN administration in rats.

The silencing effect was more evident in some brain areas. Future work could focus on testing these systems in a specific *in vivo* animal model of disease or solid tumors.

These experiments improve our understanding of the possibility of a direct nose-to-brain delivery and of the mechanisms of NPs transport from the nose to the brain.

We cannot fully exclude the involvement of the systemic pathway after IN administration but we claim that the transport mediated by the olfactory and trigeminal nerves is predominant. This suggestion is due to our results, as we found an uneven biodistribution of Rhodamine through the brain regions with higher concentrations in the rostral or caudal areas depending on the nanocarriers considered following IN instillation, in comparison with the homogenous brain distribution pattern typical after IV injection, and the efficiently silencing effect of siRNA observed in specific brain areas (olfactory bulb, cerebellum, cerebral cortex). All this, strongly suggests the involvement of a direct transport of NPs from nose to brain.

With that said, the IN route induces lower blood exposure and consequently lower off-target effects.

IN administration and NPs systems may help especially for drug substrates of P-gp, which are unable to cross the BBB and when they are delivered through the IN route they are able to bypass the BBB.

Nose-to-brain delivery in combination with nanotechnology-based systems have an enormous potential that should not be underestimated, for brain targeting as well as in reducing the systemic exposure. Nano-sized carriers could really improve nose-to-brain delivery by their capability to increase the stability of the encapsulated compound against chemical and biological degradation. Furthermore, the use of drug delivery systems represents a convenient strategy to repurpose old drugs with known pharmacological activities reducing the time and cost needed for the development of new molecules. NPs formulation administered intranasally shows a great promise for use as a therapeutic modality in the treatment and prevention of neurodegenerative disorders and lastly but not least importantly IN administration also provides a painless

and convenient route for drug administration in children and older adults and could be used for self-administration by patients.

CHAPTER VIII

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AND

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CHAPTER IX

ANNEXES

ANNEXES

1. List of Pubblications and Scientific Contributions

1.1. Publications

Revisiting the role of sucrose in PLGA-PEG nanocarrier for potential intranasal delivery.
A. Bonaccorso, T. Musumeci, C. Carbone, L. Vicari, M. R. Lauro, G. Puglisi.
Pharmaceutical Development and Technology (accepted, in press).

Nose to brain delivery: localization and time-course of polymeric nanocarriers on different regions of brain rats.
A. Bonaccorso, T. Musumeci, M.F. Serapide, R. Pellitteri, I. F. Uchegbu, G. Puglisi.
Colloids and Surface B: Biointerfaces (revised).

Nose-to-brain delivery of DiR-loaded PLGA nanoparticles.
In preparation.

Controlling seizures by intranasal delivery of Oxcarbazepine-loaded nanoparticles in rats.
In preparation.

Nose to brain delivery and chitosan derivatives nanocomplexes for sirna delivery to the brain.
In preparation.

1.2. Conference proceedings

Oral communication

Treatment of brain diseases: nose to brain nanocarriers
A.Bonaccorso**,** Joint Annual PhD Students Retreat Donnalucata Resort, 5-7 July 2014, Scicli (RG);

Brain localization of polymeric nanoparticles after intranasal administration

A.Bonaccorso, Joint Annual PhD Students Retreat 31-May 2015-2-June 2015 Bordeaux Neurocentre Magendie, INSERM U862, Université Bordeaux;

International research project presentation

A.Bonaccorso, Euraxess road show, Catania,16 November 2015;

Presentation of research project

A.Bonaccorso, Ph.D. Days II edition "Per una ricerca di qualità"
24 November 2015 – Scuola Superiore di Catania;

Nanotechnology and intranasal drug delivery: a combined approach for brain targeting

A.Bonaccorso, **"**II corso permanente di aggiornamento per i dottorandi del settore tecnologico-farmaceutico", XVI Summer School, University of Calabria, Rende (CS), 5 September 2016.

Poster

Preparation and characterization of polymeric nanoparticles for potential administration of drug via nose-to-brain delivery.

A. Bonaccorso, T. Musumeci, G. Puglisi XIV Pharmaceutical Tecnology Summer School, XIV Summer School Arcavacata di Rende (CS) from 22 to 26 Semptember 2014.

Brain Target: bottom up strategies to design nanothecnological carriers for CNS disorders

T. Musumeci, A. Bonaccorso, L. Montenegro, C. Puglia, C. Carbone,
R. Pignatello, G. Puglisi.
DSF- PharmaDay, Department of Drug Science, Catania 20 February 2015.

Potential neuroprotection effect and increase of stability of curcumin loaded polymeric and solid lipid nanoparticles after intranasal administration.

T. Musumeci, R. Pellitteri, F. Serapide, C. Puglia, A. Offerta, A. Bonaccorso and G. Puglisi.
XXIII National Meeting on Medicinal Chemistry, Campus of the University of Salerno, Fisciano - Salerno, Italy, from September 6th to 9th, 2015.

Nose to brain delivery: localization and time-course of polymeric nanoparticles on different regions of brain rats.

T. Musumeci, M.F. Serapide, R. Pellitteri, V. Pepe, C. Carbone, R. Pignatello, A. Bonaccorso, G. Puglisi. 10th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Glasgow, United Kingdom, 4 to 7 April 2016.

Intranasal delivery of polymeric nanoparticles in the rat: an innovative brain targeting strategy for diseases of central nervous system.

M.F. Serapide,T. Musumeci, R. Pellitteri, A. Bonaccorso, G. Puglisi**.**
67th SIF National Congress, Catania, 21-23 September 2016.

Nose-to-brain delivery of polymeric nanoparticles

R. Dal Magro, T. Musumeci, A. Bonaccorso, E. Donzelli, E. Ballarini,
G. Puglisi, G. Sancini
67th SIF National Congress, Catania, 21-23 September 2016.

PLGA-PEG Nanoparticles loaded with antipsychotic agents: influence of drug molecules on the cryoprotective effect of sucrose

T. Musumeci, C. Carbone, A. Bonaccorso, G.M. Leggio, S. Salomone,
R. Pignatello, G. Puglisi.
4th Congress on Innovation in Drug Delivery Site-Specific Drug Delivery, Antibes-Juan-les-Pins, France; 25-28 September, 2016.