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Frontiers in Nanomedicine

Volume 2

Nanomedicine and Neurosciences: Advantages, Limitations and Safety Aspects

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Giovanni Tosi

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(Nanomedicine and Neurosciences: Advantages, Limitations and Safety Aspects)

Editor

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FOREWORD

Although neurosurgery is one of the youngest surgical specialties, it is also the one that has undergone the most dramatic progress in recent years. As neurosurgery interfaces with other surgical areas, in particular ear, nose, throat specialty (ENT), ophthalmology and orthopedic surgery, new subspecialties have aroused such as: otoneurosurgery, neuro-ophthalmology and neuro-orthopedic spinal surgery. More particularly, new surgical approaches have appeared thanks to the improvements made in the areas of medical devices, imaging and information technology.

Nonetheless, surgery of the nervous system and the spine still has to face therapeutic challenges, including the incurability of most cerebral tumors, low back pain and its socioeconomic impact, as well as the neurodisability associated with the evolution of a large number of afflictions of the nervous system. These challenges can only be addressed through a new technological revolution.

For instance, Huntington's disease (HD) is an incurable neurodegenerative genetic disorder manifesting in adulthood and causing motor, psychiatric and cognitive disturbances. It is caused by a mutation in the huntingtin gene (htt), which at first leads to the degeneration of striatal GABAergic neurons and then to other neuronal areas. This mutation (mhtt) is involved in repression of several neuronal genes, particularly brain-derived neurotrophic factor. The use of trophic factors, targeting particularly BDNF in a neuronal protection strategy, may be particularly relevant for the treatment of HD where genetic screening can identify individuals at risk, providing a unique opportunity to intervene early in the onset of striatal degeneration.

The "NBIC convergence" (convergence between Nanotechnology, Biotechnology, Information technology and Cognitive sciences) is a concept that appeared in 2002, in a report from the National Science Foundation. This concept appeared following a reflection on the potential impact of this convergence in the improvement of human capabilities, both at the individual and societal level. While this new concept, in particular its potential applications, has generated a philosophical and ethical debate, it has already been a source of progress in health technologies.

For the first time, this e-book aims to depict the state of the art using nanotechnologies as a promising tool for therapy and diagnosis of neurodegenerative diseases. It focuses on anatomy and pathology of the main related-diseases, and gives a clear overview of the last advances in the so-called nanomedicine as to target the blood brain barrier or to image the brain defects accurately. All main issues linked to the development of new nanomedicine platforms (liposomes, targeting molecules, nanoconjugates…) and their fate, *in vivo,* (biopharmaceutical performances, interaction with biological media, toxicity…) are clearly presented with a translational approach.

This e-book gives the reader a perfect overview of this very exciting field of medical research. It is intended to help scientists, technologists, and students who may use or need to use some aspects of nanomedicine in their work or who wish to be trained in this emerging and promising area of investigation.

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PREFACE

The era of nanomedicine is claimed to be effective now, in these years. But we experiment that is not true, in the field of medicine in particular. Obviously, there is a plethora of papers published in the major scientific and highly impacted journals, but it is not enough to claim clearly that medical application of nanotechnology is currently on the edge of technological approaches.

Considering the brain, there are several pathological changes affecting the Central Nervous System (CNS): **neurodegenerative** (Alzheimer's, Parkinson's, retinal degeneration), **neurological/neuropsychiatric** (epilepsy, amyotrophic lateral sclerosis, autism), **brain tumors** (gliomas, astrocytomas, etc.) and **rare neurometabolic disorders** (i.e. inherited Lysosomal Storage Diseases), all considered **major contributors to human death**. Neurological disease management deeply impacts on patients health, care providers activity and represents a substantial socio-economic burden. Due to the absence of targeted and cost-effective therapies and limited diagnostic tools, the costs to the national health systems are high.

For disease management, it is fundamental to achieve a **deeper understanding of basic neurobiology underlying each distinct disorder** and as an urgent unmet need, to develop **novel targeted therapeutic strategies**. *Nanomedicine* represents a powerful new approach providing novel carriers to deliver drugs to specific sites in the brain as well as to other organs (lung/liver/ breast/ tumor sites). Only a **joint multidisciplinary research coordination** effort can facilitate the full development of nanomedicine as valuable treatment and diagnosis strategy for these diseases.

This book provides for the first step in order to "fill in the blanks" with a part of aspects which should be considered in order to produce and propose a real and applicable *nanomedicine* for the cure of neurodegenerative disorders and neurological diseases.

With critical behavior and with deep knowledge, scientists of high experience and skills in their field of research or clinical settings analyzed different aspects of nanomedicine for brain delivery and targeting of drugs.

From the bases of neurodegenerative diseases as anatomy and pathology of brain disorders, this book opens to wide overview of the applications of nanosystem to brain disorders, addressed by means of application of nanomedicines to neuronopathic lysosomal storage disorders, or by the application of nanoparticles to target mitochondria in neurodegenerative diseases. Drug delivery to the brain by liposomal systems along with nanotopography sensing are also approached, together with the targeting of nanomedicine in mucopolysaccharidoses and brain compromission, along with the validation of drug nanoconjugates in vivo. Finally, safety aspects and benefit/risk focus is given by means of analysis of protein corona affecting the *in vivo* efficiency of polymeric NPs and neuroendocrine aspects of nanoparticles into neurodegeneration.

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CHAPTER 1

Nanomedicine and Neurodegenerative Diseases: An Introduction to Pathology and Drug Targets

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Abstract: Neurodegenerative diseases are debilitating conditions that result in progressive degeneration and death of neuronal cells. One of the hallmarks of neurodegenerative diseases is the formation of protein aggregates. Progressive accumulation of similar protein aggregates is recognized as a characteristic feature of many neurodegenerative diseases. Particularly in Parkinson's Disease (PD), aggregated forms of the protein α-synuclein (α-syn); and in Alzheimer's Disease (AD) and cerebral amyloid angiopathy (CAA), aggregated Aβ amyloid fibrils form the basis of parenchymal plaques and of perivascular amyloid deposits, respectively. In Amyotrophic Lateral Sclerosis (ALS), the RNA-binding protein TDP-43 is prone to aggregation. The focal aggregates at early disease stages later on result in the spreading of deposits into other brain areas and many neurodegenerative diseases display a characteristic spreading pattern. Here, we will summarize the anatomy and pathology of the predominant neurodegenerative diseases focusing on AD and PD and review their clinical manifestation to highlight the urge of novel therapeutic strategies. Additionally, given that development of treatments requires suitable animal models, the most commonly used model systems are introduced and their pathology compared to the human situation is mentioned briefly. Finally, possible drug targets in neurodegenerative diseases are discussed.

Keywords: Alzheimer's Disease, Amyotrophic Lateral Sclerosis, Animal models, Drug targets, Dementia, Lewy Bodies, Neurodegeneration, Parkinson's Disease, Synuclein, TDP-43 Tau pathology, β-Amyloid.

INTRODUCTION

The foundation for the definition of modern neurological disease entities was laid in the middle of the 19th century when Jean-Martin Charcot tried to relate - at this time mysterious - clinical phenotypes to neuro-anatomical findings. In *post*

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mortem studies, he demonstrated such a relation for Amyotrophic Lateral Sclerosis (ALS) and Multiple Sclerosis (MS). Subsequently, the increasing interest in therapeutic approaches, including disease modification and prevention, fueled the interest in longitudinally studies that formally assess disease pathology. To that end, the use of molecular markers for a specific pathology such as synuclein for Parkinson´s Disease (PD) and tau for Alzheimer´s Disease (AD) became a useful tool to describe the pre-symptomatic and symptomatic stages of a disorder. Findings from these studies led to the current understanding of the pathology of neurodegenerative diseases, which is characterized by an initiation- and propagation phase of the disease process.

Today, the term "Neurodegenerative disease" is used for a wide range of conditions primarily affecting neurons in the brain and spinal cord. Given the inability of neurons to perform cell division and to replace themselves, progressive neuronal cell death is an irrevocable and, over time, cumulative process. The most prominent examples of neurodegenerative diseases include Parkinson's, Alzheimer's, Huntington's Disease (HD) and Amyotrophic Lateral Sclerosis (ALS). Neurodegenerative diseases may be hereditary or sporadic conditions.

Ongoing neuronal loss ultimately leads to problems with movement (called ataxias), or mental functioning (called dementias). With approximately 60-70% of cases, AD represents the greatest burden within the group of dementias. Other neurodegenerative diseases are Prion Disease, Multiple Sclerosis, Spino-cerebellarataxia (SCA) or Spinal Muscular Atrophy (SMA). However, hundreds of different disorders fulfill the criteria for a neurodegenerative disease.

Currently, the life expectancies of the general populations in both developed and developing countries are increasing, which affect the prevalence of neurodegenerative disorders (Table. **1**). This creates an enormous socio-economic burden with a total cost of hundreds of billion Euro per year in Europe alone [1].

Table 1. Age and gender specific prevalence rates (%) of dementia and PD in Europe [2].

	Dementia		**Parkinson**	
Age group (years)	♂	♀	♂	♀
65-69	1.8	1.4	0.7	0.6
70-74	3.2	3.8	1	1
75-79	7	7.6	2.7	2.8
80-84	14.5	16.4	4.3	3.1
85-89	20.9	28.5	3.8	3.4

(Table 1) contd.....

	Dementia		Parkinson	
Age group (years)	♂	♀	♂	♀
>90	32.4	48.8	2.2	2.6

Thus, research in the field of neurodegenerative disorders and the translation of the findings in this area to novel treatment strategies are an urgent and important goal. Fortunately, in recent years, our understanding of the anatomy and pathology of neurodegenerative diseases have made good progress.

CLINICAL REPRESENTATIONS

Alzheimer's Disease

AD is a progressive neurodegenerative disorder, which is described as the most common form of dementia nowadays. It was first described in 1907 by the German psychiatrist and neuropathologist Dr. Alois Alzheimer after observing a 55 years old patient named Auguste Deter. In general, AD patients suffer from disturbances in cognitive function or information processing like reasoning, planning, language & perception; which lead to a significant decrease in the quality of life. Besides other factors, age is the main contributing factor (Table. **1**) where 30% of individuals aged more than 85, develop the disease. A new case of AD is diagnosed worldwide every 7 seconds [3] and it is estimated that at least 34 million people will be suffering from AD by 2025, in both industrialized and developing countries [4].

Core Features of AD

AD can be divided into two groups based on the onset of the disease- early onset AD and late onset AD. In early onset, the disease occurs before the age of 65 and in late onset, the disease occurs after 65. Most of the patients are usually late onset as early onset accounts for only around 5% of the total disease occurrence. However, studies show that early onset AD is associated with high mortality and morbidity whereas late onset is much more common with less morbidity and mortality [5]. The disease progression of early onset AD is often predictable and it is possible to express the stage numerically using scales like Global Deterioration Scale [6] or Clinical Dementia Rating Scale [7]. The symptoms usually start around the age of 70. Patients show impairment in memory, problem solving, planning, judgment, language and visual perception. Some also suffer from hallucination and delusion. Eventually, the condition worsens and the patients are unable to carry out normal day-to-day functions and become bed-ridden. They need extensive palliative care and often die of other medical conditions [8 - 10].

CHAPTER 2

Nanoparticles Targeting Mitochondria in Neurodegenerative Diseases: Toxicity and Challenge for Nanotherapeutics

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Abstract: In the past decades, the prevalence of neurodegenerative diseases (NDDs) has risen dramatically with the increasing age of human population. Neurodegeneration is a long-term and complex process resulting in the degeneration of neurons. So far, no causative therapy exists, urging the development of methods for the early diagnostics and efficient therapy. In this respect, nanoparticles (NPs) are considered a promising tool due to their efficient blood-brain barrier penetrance and specific interactions with the cellular components. They can localize to mitochondria, nucleus, and autophagosomes and also interact with the cytoskeletal structures as tubulin and Tau protein. Therefore, as mitochondria represent important target for NPs, the therapeutic potential of NPs together with their toxicity to mitochondria has become an emerging topic. In this review, we describe the current knowledge in targeting NPs into mitochondria in relation to Alzheimer's and Parkinson's disease. Furthermore, we propose a novel idea how to compensate the compromised mitochondrial functioning without the delivery of NPs into the mitochondrial matrix, specifically by the development of NPs targeting either cytoskeleton or the proteins of mitochondrial motility and fusion-fission machinery. As the latter face cytoplasm, this approach does not require targeting NPs into the mitochondrial matrix. At the same time, it could be a significant step to improve the therapy of NDDs, since the movement, fusion, and fission are necessary for mitochondria to exchange their membrane material, mitochondrial DNA, and to remove the damaged mitochondria.

Keywords: Alzheimer's disease, Cytoskeleton, Mitochondria, Mitochondrial dynamics, Mitochondrial fusion and fission, Nanoparticles, Neurodegeneration, Parkinson's disease, Tau protein, β-amyloid.

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INTRODUCTION AND OVERVIEW

The development and progression of neurodegenerative diseases (NDDs) is a long-term and complex process. Several factors, such as physiology, environment, and genetics are involved in the manifestation of the NDDs. So far, no causative and thus effective therapy exists, emerging the progress and research in understanding the pathomechanisms of the NDDs. From the NDDs, this review is focused on Alzheimer's (AD) and Parkinson's disease (PD), the two most prevalent NDDs due to their rapidly increasing incidence with the ageing of the world human population [1]. For AD as well as PD, there is the experimental and clinical evidence pointing out the significant contribution of mitochondria to the pathophysiology of both disorders. The importance of mitochondria in the context of NDDs is supported by the fact, that antioxidant therapy, although symptomatic, represents the most effective therapy for AD and PD treatment so far. Therefore, the involvement of mitochondria as the therapeutic target may be a great promise for the successful treatment of the NDDs in general. Herein, the use of nanoparticles (NPs) with their unique physico-chemical properties is a challenging issue. Their highly specific penetrance through biological membranes, focused delivery, and versatility to attach or encapsulate the active substances highlight NPs as a promising tool to target mitochondria and to use them, beyond the early diagnostics, especially for the therapy of the NDDs. In contrary, as any new invention, the toxicity of NPs is of critical note. Since the progress of NDDs is very slow, usually several decades, the long-term treatment is required. Thus, the low-dose long-term toxicity of NPs compared to their acute adverse effects has to be evaluated when using NPs for the treatment of NDDs.

Therefore, this review interconnects three topics: 1. the involvement of mitochondria in AD/PD, with a special focus on the mitochondrial dynamics, 2. the use of NPs as a tool in the treatment of AD/PD, and 3. the direct and indirect relation between NPs and mitochondria (the latter mainly through cytoskeleton and reactive oxygen species). Moreover, the therapeutic potential *vs.* toxicity of NPs is summarized (Fig. **1**) mainly focused on data obtained in experimental condition. At last, the aim of this chapter is to open the readers' mind, to give them inspiration for the future experiments, and to stimulate the paradigm shift in the area of the therapeutic use of nanoparticles in mitochondrial medicine in relation to the neurodegenerative diseases.

ALZHEIMER'S AND PARKINSON'S DISEASE

Progressive increase in the mean age of the human population is a positive end-point showing the progress in the healthcare system, on one side due to success in decreasing the birth deaths and improved care of elderly patients. However, this

progress is associated with the increased incidence of diseases associated with the old age – especially the number of patients with neurodegenerative disorders increases non-linearly, sometimes even exponentially [2]. Alzheimer's disease (AD), Parkinson's disease (PD), and related dementias have appeared to be one of the most critical public health problems in the aging population because they are major sources of disabilities, poor quality of life for the patients themselves and their families [3], and of caregiver strain as well [4, 5]. As published in the Delphi consensus study, there is an estimate of 24 million people with AD or dementia worldwide and the prognosis for the year 2040 is 81 million [6]. Regarding the PD, the World Health Organization's has reported estimation of 5 million patients in 2006. Patients suffering from these dementias have increased mortality [7, 8], where in 2006, around half a million of deaths in the world were directly related to AD and dementias, and roughly 100,000 were associated with PD (WHO, 2006). These numbers are proposed to increase significantly in the next years due to increase in the mean age of the world population [9].

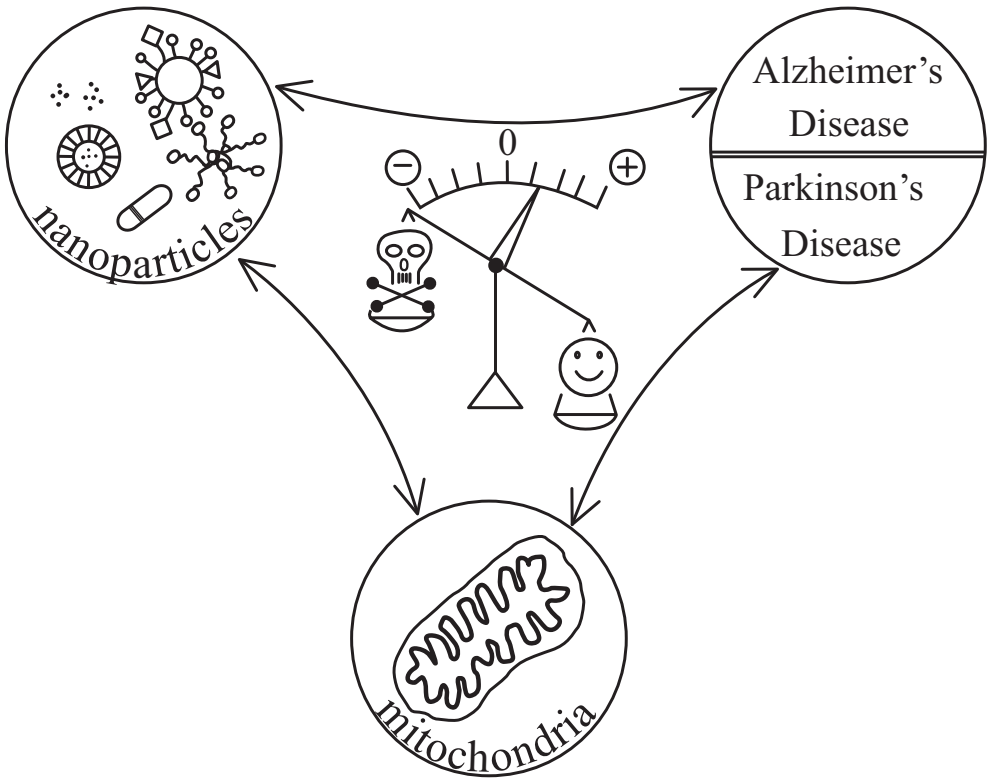

Fig. (1). The aims of the review – to present overlap in the fields of neurodegeneration, mitochondria and nanoparticles focusing on their potential therapeutic use.

CHAPTER 3

Neuronal Mechanisms for Nanotopography Sensing

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Abstract: Cell contact interaction with extracellular environment cooperates in coordinating several physio-pathological processes *in vivo*, and can be exploited to manipulate cell responses *in vitro*. Thanks to recent developments in micro/nano-engineering techniques, nano/micro-structured surfaces have been introduced capable of controlling neuronal cell adhesion, differentiation, migration, and neurite orientation by interfering with the cell adhesion machinery. In particular, this process is mediated by focal adhesion (FA) establishment and maturation. FAs cross-talk with the actin fibers and act as topographical sensors, by integrating signals from the extracellular environment. Here, we describe the mechanisms of nanotopography sensing in neuronal cells. In particular, experiments addressing the role of FAs, myosinII-dependent cell contractility, and actin dynamics in neuronal contact guidance along directional nanostructured surfaces are reviewed and discussed.

Keywords: Actin contractility, Contact guidance, Cytoskeleton, Focal adhesions, Mechano-transduction, Neuron, Nanostructured substrates, Neurite, Nanograting, Nanogroove.

INTRODUCTION

Nowadays it is accepted that cells respond to the morphology of the extracellular environment at the nanoscale level. The contact interaction of cells with extracellular physical features cooperates in regulating physiological (*e.g.* embryogenesis, cell migration) [1] and pathological processes [2] *in vivo*, and can be exploited to modulate cell responses *in vitro* [3 - 5]. In the central nervous system (CNS), the sensing of the extracellular environment combines with intracellular signaling patterns that is integrated by cells to establish the final neuronal polarity, differentiation, migration, neurite path-finding and the final

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Giovanni Tosi (Ed)

architecture of the functional network of neuronal connections [6 - 8]. The mammalian neuronal network is an example of highly polarized tissue, where cell development is driven by molecular stimuli, acting over long distance, and by physical signals that act locally through direct contact sensing [9, 10]. Neurons polarize and produce long cellular extensions (the neurites) whose development is governed by the formation and maturation of focal adhesions (FAs), the integrin-based cellular structures anchoring the cell to the external environment [6]. During neurite development, focal adhesions act as *topographical sensor*s and integrate both physical and chemical signals from the extracellular matrix [11]. The maturation of FAs is in fact finely tuned by multiple information regarding the extracellular matrix properties such as mechanical stiffness, density of adhesion points, their chemical identity and geometry, and surface topography [12]. Importantly, through the modulation of FA maturation, a specific extracellular configuration can regulate the cell fate and in particular neuronal polarization, migration [13] and function. For example, synaptic plasticity has been recently suggested to involve the surrounding extracellular matrix signaling [14,15]. These processes involve coordinated interactions between FAs and the cell cytoskeleton. In order to build a correct brain architecture, a coordinated rearrangement of the cytoskeleton in response to extracellular cues is essential, and focal adhesion kinase (FAK) was recognized as a key neuronal enzyme [16]. For sake of example, the activation of the FA effector FAK is dispensable for glial-independent migration of interneurons but is required for the normal interaction of pyramidal neurons with radial glial fibers during cortical migration [13]. FAK is also required by both attractive and repulsive stimuli to control cytoskeletal dynamics and axon outgrowth and disassembly, working as a versatile molecular integrator that can switch to different functions depending on its activation site [17].

All cells grow and live while embedded in a dense and complex environment, the extracellular matrix (ECM), which contains an array of structural and directional cues. In particular, beyond chemical recognition, three independent ECM parameters play a major role in governing cell behaviour: topography, stiffness, and density of adhesion points. Topographical features in the micron and submicron range act as physical boundaries providing a local constraint to the formation and maturation of FAs. Cells apply force to the developing adhesions through acto-myosin contractility and the mechanical response of the matrix controls the further maturation of the adhesion points in a molecularly regulated feedback loop. Thanks to recent developments in micro/nano-engineering tools, the processes that control cell and, in particular, neuronal guidance and polarization can now be investigated *in vitro* using nano/micro-structured surfaces [18 - 21]. Nano/micro-textured substrates were demonstrated to be capable of tuning neuronal and glial cell adhesion, differentiation, polarization, migration,

neurite orientation and even stem-cell fate [3, 22 - 25]. In particular, non-conventional lithographic technologies such as nanoimprint lithography (Fig. **1a**), electrospinning, or soft replica moulding to name but a few, allow the production of biocompatible substrates with customizable topography in the critical ranges affecting cellular functions. The application of these methods yielded patterned surfaces with lateral resolution ranging from few microns (*e.g.*, photolithography) down to tens of nanometers (*e.g.*, electron beam lithography [26]) and showed good versatility and reliability coupled with the use of polymeric materials [27 - 29]. The combination of these techniques with optically transparent polymers such as tissue culture polystyrene (TCPS), polyethylene terephthalate (PET) and cyclic olefin copolymer (COC) yielded patterned substrates where contact guidance could be observed by means of high resolution microscopy in living cells [28, 30, 31].

Here we focus on reviewing the influence of topography on the responses of neuronal cells. In order to control the assembly and maturation of FAs, and thus to induce specific cellular guidance, biomimetic scaffolds can provide modulation of the topographical parameters within the physiological ranges that are resolved by cells. The physical parameters in the substrate topography, which were reported to modulate contact guidance and formation of FAs in mammalian cells are the size, aspect ratio, and lateral spacing of the topographical features together with the isotropy and degree of disorder of the pattern.

In this framework, governing neuronal cell adhesion, migration, and axonal outgrowth are critical elements for regenerative medicine applications and for developing artificial neuronal interfaces, but at the same time these substrates open new experimental perspectives for the study of the molecular mechanisms at the base of neuronal environmental sensing. The molecular/cellular processes regulating synaptic plasticity, and learning are in fact an adaptation of the mechanisms used by all cells to regulate cell motility and shaping [32] as they involve the same complex machinery (*i.e.* actin fiber regulation and cell-cell/-ECM interaction signalling) where also the activation of FAs is emerging as pivotal [33]. Therefore, new knowledge about the sensing mechanisms of neuronal cells might impact also our understanding of several CNS disorders and providing new insight into the mechanisms leading to several neurological and neuropsychiatric disorders associated with connectivity and cognitive impairments.

Focal Adhesions and Cytoskeleton during Neuronal Cell-nanograting Interaction

In the last years nanogratings (NGs), anisotropic topographies composed by

CHAPTER 4

Drug Delivery to the Brain by Liposomal Carrier Systems

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Abstract: Endothelial cells of brain microvessels limit the entry into the brain for xenobiotics and many drugs, which otherwise may be therapeutically active in the central nervous system. The ABC transporters, P-glycoprotein and Breast cancer resistance protein, which are predominantly located in the luminal surface of capillary endothelial cells, are key players for this barrier function. Thus, particular efforts have been made to overcome the blood-brain barrier or to circumvent these efflux pumps. The various options for drug transport into the brain include encapsulation of active compounds into delivery systems, *e.g.* liposomes, which are able to by-pass the export pumps and to convey their payload across the endothelial barrier. The applied systems target receptors at the luminal surface of the blood-brain barrier by using antibody-coupled immunoliposomes, liposomes conjugated to receptor-targeting vectors such as insulin, transferrin and apolipoproteins or cationized albumin-coupled liposomes.

Keywords: Albumin, ApoE, Blood-brain barrier, Immunoliposomes, Insulin, P-glycoprotein, Transferrin.

INTRODUCTION

Delivery of drugs to the central nervous system (CNS) remains to be a major challenge in modern pharmacotherapy. In 2007, William Pardridge gave an excellent summary on drug distribution to the CNS [1]. He stated that out of >7000 drugs in the Comprehensive Medicinal Chemistry database only 5% are used for CNS treatment, and that these compounds are limited to depression, schizophrenia and insomnia [2]. Most drugs do not reach the brain because they are not able to cross the blood-brain barrier (BBB), which is formed by endothelial cells of brain microvessels. In principle, drugs may reach the brain by several paracellular and transcellular routes across the BBB (Fig.**1**), from which

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the paracellular pathway is neglectable due to the very tight junctions between adjacent cells of brain capillaries. In addition, a key problem is the expression of export proteins in the luminal membranes of the endothelial cells, mainly P-glycoprotein (P-gp, ABCB1), Breast cancer resistance protein (Bcrp, ABCG2) and several Multidrug resistance-related proteins (Mrps, ABCCs) recognizing a huge variety of different substrates including most drugs and drug candidates. One possibility to overcome this obstacle is the use of colloidal carriers, which are able to by-pass these export proteins and to be transferred across the barrier by cytotic transport processes. Such carriers may include nanocrystals, micelles, polymeric nanoparticles, solid lipid nanoparticles and liposomes, respectively. The ultimate goal of these nanocarriers would be an exclusive delivery of drugs to the CNS. This idea of an unfailing "magic bullet" was originally developed by Paul Ehrlich at the beginning of the 20th century and it is more acute in medicine and pharmacy than ever. However, it is difficult to get a drug to the brain without coming into contact with other parts of the body. All hitherto existing efforts clearly show the main problems of drug targeting. Drug delivery systems must feature a satisfactory loading capacity and signal molecules or vectors, that recognize their targets with sufficient selectivity, have to be attached to the surface of the nanocarriers. Finally, they have to be inert and biocompatible, implying no own pharmacological effects by the inserted materials, no immune response of the body and biodegradability. Amongst the available nanocarriers liposomes fulfil these requirements quite well and are, therefore, suitable for CNS drug delivery. In the present article we describe features of such liposomes.

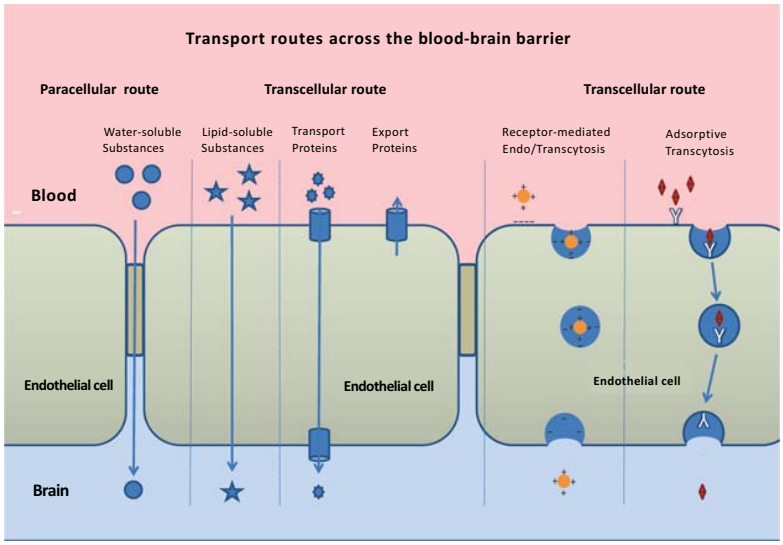

Fig. (1). Para- and transcellular as well as carrier-, receptor and adsorption-mediated transport pathways across the blood-brain barrier (modified from Abbott *et al*. [39]).

LIPOSOMES

Generally, liposomes are self-assembling phospholipid vesicles with an aqueous inner space, surrounded by a bilayer of naturally occurring phospholipids. Intravenously (i.v.) administered they may bind unspecifically to blood components, the opsonins, and are subsequently trapped by the reticuloendothelial system (RES) of liver, spleen, lung or bone marrow. A significant improvement allowing to overcome this phenomenon is the hydrophilisation of the liposomal surface by attachment of sterically hindering polyethylene glycol (PEG) chains (stealth liposomes). Circumvention of the RES can be accomplished by coupling signal molecules to the ends of PEG residues, thus avoiding internalization by the RES and improving the targeting effect. Over the past 2 decades interesting developments within molecular biology have offered new dimensions for the identification of ligand-receptor interactions and for the manufacture of appropriate signal molecules. *E.g.* "immunoliposomes", have successfully been tested in animal studies, in which selective antibodies targeting epitopes of receptors at the surface of brain microvessels have been coupled to PEG residues. These receptors include the insulin receptor, which transports insulin of peripheral origin into the brain [3, 4], insulin-like growth factor I and II receptors (IGFIR, IGFIIR) [1, 5], LDL receptor, leptin receptor (OBR) [6], or the receptor of advanced glycation endproducts (RAGE) [7]. Further receptors at the BBB are the low-density lipoprotein-related receptors 1 (LRP1) and 2 (LRP2, megalin) [8] and the transferrin receptor (TFR), which is expressed at the luminal as well as at the abluminal membrane of endothelial cells in the BBB, thus acting in a bidirectional way [9, 10].

TRANSFERRIN RECEPTOR

The transferrin receptor (TFR) moves apotransferrin fast in the brain-to-blood direction and holotransferrin from blood-to-brain. It is one of the most promising receptors for effective brain-targeting as it is highly expressed at the BBB. Here, some interesting examples are mentioned to demonstrate its capabilities: One of the first studies focussing this receptor used immunoliposomes for the delivery of the antineoplastic agent daunomycin to the rat brain [11]. Thiolated antibodies (OX26) were coupled to maleimide-grafted 85nm liposomes sterically stabilized with polyethylene glycol (PEG). Whereas no brain uptake of PEG-conjugated liposomes carrying [^{3}H]daunomycin was observed, coupling of thirty OX26 antibodies per liposome resulted in optimal brain delivery, which showed saturation at higher antibody densities. Brain targeting was not seen in immuno-liposomes conjugated with a mouse IgG2a isotype control. Further on, coinjection of free OX26 saturated the plasma clearance of the immunoliposomes. The study impressively demonstrated that these PEG-conjugated immunoliposomes might

CHAPTER 5

Neuronopathic LSDs: Quest for Treatments Drives Research in Nanomedicine and Nanotechnology

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Abstract: Lysosomal storage diseases (LSDs) are due to mutations in genes coding for high molecular weight lysosomal enzymes, which result in a deficiency or complete loss of enzyme activity and the consequent storage of undegraded substrate within lysosomes. Therapeutic approaches capable of modifying the natural history of the disease are available today and many have already entered into clinical practice. Among these, enzyme replacement therapy (ERT) represents an approved key treatment for a number of LSDs. Unfortunately, none of the used therapeutic replacement enzymes have, so far, proved to be effectively able to reach the central nervous system (CNS) in significant amounts and arrest neurodegeneration. Thus, currently, only the peripheral disease can be treated with ERT while storage product continues to accumulate in the CNS, resulting in severe neurodegeneration and premature death in childhood for all neurologically affected patients. In recent years, scientific advances in nanotechnology have led to development of revolutionary approaches potentially capable to provide a solution to the still unmet problem of increasing drug delivery across the Blood Brain Barrier. In particular, the growing interest in the medical applications of nanotechnology has contributed to the advent of a new field of applied science named nanomedicine that offers promising strategies to overcome several of the current impediments and disadvantages of ERT. The combination of existing nanotechnology with already available enzymes can, in fact, significantly improve the enzyme delivery opening a promising new era in the treatment of LSDs. This chapter aims to review the most recent advancement in nanomedicine and nanotechnology presenting novel therapeutic approaches designed to address neuronopathic LSDs.

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Keywords: Blood-brain barrier, Enzyme replacement, Lysosomal storage diseases, Neurodegeneration, Nanomedicine, Nanotechnology.

INTRODUCTION

Lysosomal Storage Diseases (LSDs) are a heterogeneous group of more than 50 inherited metabolic disorders characterized by the absence or deficiency of a functional lysosomal enzyme or lysosomal component implicated in the degradation and recycling of macromolecules, or due to errors in enzyme trafficking/targeting or defective function of non-enzymatic lysosomal proteins [1]. Any of these defects prevents the complete breakdown and recycling of target macromolecules that consequently accumulate inside the lysosome. Such accumulation of undegraded compounds alters lysosomal function resulting in a progressive and systemic disease process commonly affecting multiple organs and tissues including the central nervous system (CNS). These conditions, each year, affect the lives of numerous children worldwide with an overall prevalence of about 1 every 5000 live birth affected by LSDs, but these data are probably underestimated because LSDs heterogeneous phenotypes make the diagnosis complicated [2]. In fact, for reasons still not well understood, age of onset and clinical manifestations may vary widely among patients affected by a given LSD, and significant phenotypic heterogeneity between family members carrying identical mutations has been reported. Commonly LSDs clinical phenotypes range from classical severe forms to very attenuated ones with limited disability. Main classic symptomatology includes organomegaly (mainly hepatosplenomegaly), connective-tissue and ocular pathology, musculoskeletal abnormalities, coarsening of hair and facial features and, in the neuronopathic forms, CNS pathology [3]. Typically, in the absence of a family history of the disease, pregnancy develops in an uneventful natural manner, and the infant appears normal at delivery and develops normally during the first year of life. Nevertheless, progressive lysosomal accumulation of stored undegraded product starts very early in life and usually begins to affect normal neurological development by the first - second year of life. The fundamental neuro-developmental steps of childhood are not attained as expected and mental retardation becomes recognized by both parents and medical staff. It has been estimated that more than 70% of LSD affected children suffer from different grades of CNS involvement with various grades of neurodegeneration and CNS cell death depending on their disease phenotype [4]. Generally affected children manifest a progressive deterioration of movement, skills, speech and cognition. Communicating hydrocephalus and progressive profound mental retardation are the major CNS features and are frequently responsible for the demise of these children in early childhood with devastating consequences on their immediate environment and relatively high costs for society [1].

The science of the last decade has provided more tools, discoveries and scientific insights to develop novel LSDs treatments but the translation of ideas into drugs that are available is a greater challenge as the drug development system was not designed for these complex and heterogeneous diseases, most of which have never before been studied. For long time LSDs have not been considered a public health priority by the pharmaceutical industry, since, because of their low-prevalence, the market was usually seen as unprofitable. Before 1987, no treatments were in fact available and therapeutic management essentially consisted of simple symptomatic care of disease manifestations, with no possibility for cure. The introduction across the globe of special Orphan Drug legislations, providing incentives for pharmaceutical companies to develop and market needed medicinal products to treat rare diseases, marked important milestones for LSDs patients. Since 1983, year in which the first Orphan Drug Act was signed, these Regulations have been, and continue to be, an important force in driving treatment innovation for rare diseases mostly stimulating the research toward the clinical development of drugs for rare diseases, including LSDs [5]. In addition, recent advances in molecular biology and biochemistry have allowed a very thorough knowledge of the basic genetic mechanisms responsible of LSDs pathology, further contributing a rise in bio-company attention to LSDs [6]. Rare disease research has particularly exploded in recent decades with the development of several new therapeutic strategies capable of modifying a disease's natural history and improving a patient's quality of life [7]. A number of different approaches for treating the LSDs already exist and are commercially available. They include: (i) Substrate reduction therapies (SRT) consisting of the administration of a drug that inhibits an early stage in the degradation pathway and reduces the production of the accumulating substrate and (ii) Small molecules, named chaperones, which play an essential role in the regulation of protein conformation states. Chaperones bind to the active site of the defective and misfolded enzyme and induce its proper conformational folding, stabilizing it and preventing its degradation and restoring enzymic activity, thus ensuring the proper intracellular trafficking and delivery of the functional enzyme to the lysosomal compartment and last but not least, (iii) enzyme replacement therapy (ERT), consisting in the replacement of the defective enzyme by the regular intravenous infusions of the functional enzyme [7, 8]. ERT is considered a key treatment which has already proved to be safe and effective for peripheral manifestations in patients with Gaucher disease (GD), Fabry disease, mucopolysaccharidosis (MPS) types I, II, and VI, and Pompe disease (PD) where it reduces the lysosomal substrate load positively modifying the natural course of the disease as confirmed by the many extensive clinical trials (see www.ClinicalTrials.gov). However, there are still several obstacles that have to be overcome for the achievement of successful ERT since benefits are only evident

CHAPTER 6

Targeting Brain Disease in Mucopolysaccharidoses

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Abstract: Mucopolysaccharidoses (MPSs) are a group of inherited disorders due to the deficit of the lysosomal enzymes involved in the degradation of the mucopolysaccharides, which thus accumulate within different organs, taking to a heavy progressive malfunctioning. The disorders involve most of the organ-systems and in the patients affected by MPS I, II, III and VII, also the neurological compartment may be severely affected. Many therapeutic strategies have been proposed along the years, and, following the identification of the genes underlying each disorder, in the last decade some MPSs have taken advantage on the availability of the recombinant enzymes, systemically administered to the patients. Such treatment, however, has hardly shown any effects on the CNS disease, given the inability of the enzymes to efficiently cross the blood-brain barrier. Therefore, the efforts of the last years have been focused on developing new therapeutic strategies targeting this aspect. This chapter summarizes the most relevant proposed, discussing their advantages, limitations and potential applications. Treatment of the brain disease in neuronopathic MPSs, conjugated with an early diagnosis, would represent a milestone in the improvement of patients' and families' life condition.

Keywords: Brain therapy, Blood-brain barrier, BBB, BBB crossing, Brain, Enzyme Replacement Therapy, ERT, Mucopolysaccharidosis, Neurological disease.

INTRODUCTION

Mucopolysaccharidoses (MPSs) are a group of inherited metabolic diseases belonging to the wider group of the Lysosomal Storage Disorders (LSDs), of which they represent about 30% of the patients [1]. Each MPS is due to the deficit

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of one of the eleven lysosomal hydrolases, normally degrading mucopolysaccharides or glycosaminoglycans (GAGs), this determining a pathological accumulation of such molecules inside cell lysosomes as well as in the extracellular matrix (Table **1**).

Table 1. Mucopolysaccharidoses classification.

Type	Eponym	MIM ID	Gene	Enzyme Name	EC number	Stored GAG
I	Hurler syndrome	#607014	IDUA	Alpha-L-iduronidase	3.2.1.76	DS, HS
	Hurler/Scheie syndrome	#607015				
	Scheie syndrome	#607016				
II	Hunter syndrome	#309900	IDS	Iduronate 2-sulfatase	3.1.6.13	DS, HS
IIIA	Sanfilippo A syndrome	#252900	SGSH	N-sulphoglucosamine sulphohydrolase	3.10.1.1	HS
IIIB	Sanfilippo B syndrome	#252920	NAGLU	Alpha-N-acetylglucosaminidase	3.2.1.50	HS
IIIC	Sanfilippo C syndrome	#252930	HGSNAT	Heparan-alpha-glucosaminide N-acetyltransferase	2.3.1.78	HS
IIID	Sanfilippo D syndrome	#252940	GNS	N-acetylglucosamine-6-sulfatase	3.1.6.14	HS
IVA	Morquio A syndrome	#253000	GALNS	N-acetylgalactosamine-6-sulfatase	3.1.6.4	C6S, KS
IVB	Morquio B syndrome	#253010	GLB1	Beta-galactosidase	3.2.1.23	KS
VI	Maroteaux-Lamy syndrome	#253200	ARSB	Arylsulfatase B	3.1.6.12	DS
VII	Sly syndrome	#253220	GUSB	Beta-glucuronidase	3.2.1.31	C4S, C6S, DS, HS
IX	Hyaluronidase deficiency	#601492	HYAL1	Hyaluronidase-1	3.2.1.35	HYAL

C4S=chondroitin-4-sulfate; C6S=chondroitin-6-sulfate; DS=dermatan sulfate; GAG=glycosaminoglycan; HS=heparan sulfate; HYAL=hyaluronan KS= keratan sulfate.

Being lysosomal hydrolases housekeeping enzymes, accumulation of GAGs affects most of the organ-systems, although each MPS is characterized by specific clinical manifestations, variously affecting liver, spleen, heart, bones, joints, eyes, ears and other organs [2, 3]. Many MPS patients also present an important, progressive neurological deficit. In particular, brain disease affects at various degree of severity MPSs type I, II, III and VII. Nevertheless, a general brain

involvement including brain and spinal cord compression may be observed in all MPSs [4].

MPS treatment has been at first attempted by procedures of bone marrow transplantation and, more recently, of hematopoietic stem cell transplantation, mainly applied to Mucopolysaccharidosis type I. Following the identification and cloning of the genes underlying each disease, in the last 10-15 years many efforts have been directed to the production of the recombinant functional forms of some enzymes. Starting 2004, protocols of Enzyme Replacement Therapy (ERT) have been settled for MPS I, II, VI and, more recently, for MPS IVA [5]. A trial has recently reached phase III for MPSIIIA [6] and preliminary encouraging data derive from a pilot study for the treatment of MPS VII [7]. Some efficacy has been demonstrated for the systemic compartment involved in these disorders, although with alternative success [8]. However, the present formulations and therapeutic concentration of the recombinant lysosomal hydrolases have shown to be unable to target the brain involvement, due to their inability to efficiently cross the blood-brain barrier (BBB). Therefore, most of the efforts of the last years have been dedicated to the design of new drug formulations or to the set-up of safe and efficient delivery systems, hopefully able to render the drugs available to the neurological compartment.

The present chapter reviews several of these approaches, underlying their features, perspectives and limitations of their potential applicability to the treatment of these diseases.

LYSOSOMES AND LYSOSOMAL STORAGE DISORDERS

Lysosomes are intracellular organelles deputized to several functions within the cell compartment. They have been long considered mainly the site of cellular degradation of materials deriving from the outside as well as from the inside of the cell. Along the years these functions have been progressively widen and it is now quite clear of how many other functions these organelles are entitled. They exert a control on cellular homeostasis, they are implied in the repair of plasmatic membrane and also in cell signalling and, together with mitochondria, in the energy metabolism of the cell. For their degrading functions, lysosomes contain about 50 hydrolases able to “digest” complex macromolecules as proteins, lipids and sugars, as well as “multiple-molecule” complexes, as small organisms endocytosed by the cell, or cellular debris.

All these functions help to maintain a healthy cellular environment and a correct equilibrium between anabolic and catabolic reactions of biological macro-molecules. Lysosomal hydrolases are coded by housekeeping genes, therefore their deficit functionally involves most tissues and organs. In a few cases, non-

CHAPTER 7

Functional Validation of Drug Nanoconjugates *in vivo*

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Abstract: Preclinical development of nanotechnology formulated-drugs shares many features with the development of other pharmaceutical products. However, there are some relevant differences. Nanoparticulated therapeutic systems have challenges related to their production, physicochemical characterization, stability and sterilization, but offer special advantages regarding drug solubilization, bioavailability and biodistribution. A good design of the nanoconjugate, should take into account these *pros* and *cons* in the specific setting of the target disease. Moreover, researchers should also bear these in mind when planning *in vitro* and *in vivo* proof-of-concept assays. In this chapter we will focus in assays required to test the efficacy of a therapeutic nanoconjugate and how appropriate animal models and imaging technologies help to speed up preclinical development. In addition, we will also describe how basic *in vivo* pharmacokinetic and biodistribution assays aid researchers to optimize the design of a highly active and non-toxic nanoconjugate.

Keywords: Animal models, *In vivo* preclinical validation, Nanomedicine, Nanoconjugate, Optical bioluminescence and fluorescence imaging, Proof-of-concept, Toxicology, Whole-body biodistribution.

INTRODUCTION

In the last two decades reformulation of drugs by means of nanotechnology has changed the way drug development is foreseen. Nanomedicine holds now the promise to revolutionize medical treatments with more potent, less toxic, and smart therapeutics that could home into disease areas like a magic bullet [1]. Indeed, the number of nanoformulated drugs entering clinical trials is growing exponentially and there are reports indicating that 80% of the pharma market will be related to nanotechnology by 2020.

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Nowadays, reformulation of drugs by nanothecnology has open a new door in the treatment of many diseases, especially those neglected by the conventional therapeutic approaches. Many neurological diseases fall into this category because large and hydrophilic drugs are not able to cross the blood brain barrier (BBB) and reach efficiently their target cells in the central nervous system (CNS). Indeed the market of nanotechnology in the area of CNS products is valued for 2016 in almost $30 billions [2].

As with any other drug, nanomedicines also need to prove their efficacy and safety *in vivo*. Once the proof-of-concept is defined and activity of a nanoconjugate is demonstrated, preclinical safety studies help to select the best candidate for development and furthermore, clinical studies. In this chapter we will describe the key steps of this pipeline, from design and manufacturing of the nanoconjugate to its characterization and biological testing.

Finding the Right Nanoconjugate for the Right Disease

Both the indication and the therapeutic agent define the type and characteristics of the nanoconjugate to be used. Intensive work is required to translate such design into a reproducible scale-up and manufacturing process to achieve a consistent product, sterile and endotoxin-free. Reproducibility of the production is extremely important, because safety and efficacy of a given nanoformulated drug can be influenced by small variations along the synthetic, purification or storage procedures. Most *in vitro* proof-of-concept assays are performed during the optimization of the production procedure, which should be well defined when moving to *in vivo* assays. Indeed, many nanoconjugate systems fail *in vivo* after performing excellently in cell culture assays due to uncontrolled deviations on the production and characterization that ultimately change their biodistribution and safety profile [3].

Regarding the physicochemical characterization of nanoconjugates, size/size distribution, shape, charge, composition, purity, and stability are key parameters that should be consistent and reproducible among batches. Whenever possible, characterization must be performed under biologically relevant conditions (*i.e.* human plasma instead of PBS or water), since size, charge and composition of the nanoconjugate may widely vary depending on the dispersion media. Moreover, in the case of drug delivery systems it becomes very relevant to characterize the maximum loading capacity of the system and its kinetic release even before performing any *in vitro* experiment. Many drug conjugates, especially those where the drug is covalently linked to the carrier, are limited by the amount of drug they can incorporate and by the solubility of the nanoconjugate. Such systems might perform well *in vitro*, where achieving a high local drug

concentration is feasible, but might fail when tested in animal models because not enough drug concentration is achieved at the target site.

Overall, drugs with a small plasma half-life are usually encapsulated in nanosystems that protect them from fast metabolization and excretion. These nanosystems can be decorated with polyethylene glycol (PEG) or other molecules than reduce opsonization and phagocytosis by the reticuloendothelial system. When nanoconjugates are not efficiently scavenged by macrophages, the resulting increase in blood circulation time and hence bioavailability is expected to extend the duration of the controlled drug delivery or to improve the prospects for nanoparticles to reach target sites by extravasation [4]. This becomes very relevant for targeting solid tumors and inflammation areas, where extended circulation time is combined with a vascular enhanced permeability and retention (EPR) [5, 6]. Accordingly, a leaky vasculature together with a defective lymphatic drainage passively increases the retention of nanoconjugates within the tumor and inflammatory tissues. Nanoconjugates with or without "stealth" modifications that relay in the EPR effect, are considered passive, non-targeted systems. Many of the nanomedicines already marketed are of this type of passive, first generation nanomedicines, including different liposomal formulations, polymers, micelles and nanoparticles, among others [7].

Nowadays however, nanomedicines are evolving towards actively targeted systems. This is particularly relevant for those drugs intended to reach the central nervous system which have no ability to cross the BBB by themselves. In these cases, therapeutic nanoconjugates must use active systems in the form of specific components or mechanisms that will help the drug cross the BBB. Lysosomal storage diseases (LSD) with neurological affectation are the perfect example of how nanotechnology can help to improve current therapies. These diseases are caused by defective lysosomal enzymes or transporters that can be replaced by the exogenous addition of the active proteins. The therapy is known as enzyme replacement therapy (ERT) and it has been successfully applied to six different lysosomal storage diseases. However, classical ERT strategies are suboptimal in attenuating the strong neurological deterioration associated to more than half of LSDs, because enzymes are not able to cross the BBB. Since transient disruptions of BBB by hyperosmolar solutions, solvents, adjuvants, ultrasound, and surgical interventions (intracerebroventricular or intracerebral delivery) have proven to be too invasive and non-efficient, other approaches have been explored. Enzymes have been targeted to certain cell receptors expressed on brain endothelial cells involved in transcytosis. Examples include the use of human insulin receptor [8] and the HIV-1 trans-activator protein transduction domain (TAT) [9]. Further, the aminoacidic sequence known as cRGDfK binding αvβ3 integrins has also been capable of facilitating the cross of the BBB [10, 11]. Recently, we also found that

CHAPTER 8

How does "Protein Corona" Affect the *In vivo* Efficiency of Polymeric Nanoparticles? State of Art

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Abstract: Nanomedicine is increasingly considered as one of the most promising ways to overcome the limits of traditional medicine and conventional pharmaceutical formulations. In particular, polymeric nanoparticles (NPs) represent one of the most important tools in the nanomedicine field due to their potential in a wide range of biomedical applications such as imaging, drug targeting and drug delivery. However, their application is strongly hampered by limited knowledge and control of their interactions with complex biological systems. In biological environments, NPs are enshrouded by a layer of biomolecules, predominantly proteins, which tend to associate with NPs, forming a new surface named 'protein corona' (PC). Thus, the resulting nano-structure is a new entity, defined as PC-NP complex, featured by new characteristics, different from the original features of the bare NPs. In this chapter, starting from the definition of PC, we critically discuss the physico-chemical properties of polymeric NPs (*e.g.*, size, shape, composition, surface functional groups, surface charge, hydrophilicity/hydrophobicity) and the environmental biological parameters (blood concentration, plasma gradient, temperature) affecting PC formation and composition. We further discuss how the new "entity" generated by the interactions between NPs and proteins *in vivo* mediates the ability of all the nanosystems to circulate, biodistribute and selectively release the drugs to the target site. We conclude by highlighting the gaps in the knowledge of the PC in relation to polymeric NPs and by discussing the main issues to be addressed and investigated in order to speed up the translatability of NPs into clinical protocols.

Keywords: *In vivo* outcome, Protein corona (PC), Polymeric nanoparticles (NPs), PC-NP complex, Protein –NPs interaction.

INTRODUCTION

Recent years witnessed a progressive growing interest in developing innovative formulations for drug delivery to solve the limits of conventional pharmaceutical

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formulations. Innovation in pharmaceutical technology means to stabilize and selective deliver drugs to the site of action without affecting healthy organs, and achieve drug dose maintenance in the organism without the need of repeated administrations [1]. These needs have led to the development of nanomedicine, defined as the science of studying nanoscale-sized structures for diagnostics, therapeutics and specific drug-delivery [2].

One of the primary challenges of nanomedicine is to deliver a drug to the target site, avoiding side effects to non-targeted organs [3]. During the last several decades, numerous lipidic and polymeric nanosized drug delivery systems were proposed. Examples of lipidic carriers are liposomes and solid lipid nanoparticles, while polymeric carriers are mainly represented by nanoparticles (NPs), dendrimers and micelles [4].

These nano-systems can be directed to a specific target site by means of different strategies, *i.e.* passive targeting and active targeting. Passive targeting exploits differences in patho-physiological features of diseased tissues, enabling delivery of drugs to the target, as diseased tissues are often altered in terms of facilitated accumulation or permability aspects. As an example, within tumoral tissues, nano-sized systems can escape from nonspecific trapping by the reticuloendothelial system and accumulate in target tissues after circulating in the blood by passive targeting exploiting the enhanced permeability and retention (EPR) effect (high interstitial pressure, enhanced vascular permeability, and the lack of functional lymphatic drainage). On the other hand, active targeting is complicated regarding the design of surface engineered nanocarriers; frequently, these systems are stabilized by polyethylene glycol (PEG) moieties and conjugated with ligands, specifically able to recognize structures or specific environments on or inside the target. This kind of targeting also includes carriers sensitive to physical stimuli such as temperature, pH or magnetism [5].

In this context, NPs were extensively studied in the last couple of decades, due to their potential for a wide range of biomedical applications. These systems represent a versatile tool in drug delivery, able to load a large variety of drugs with different chemico-physical features.

Additionally, chemical moieties present on the surface of NPs can be suitable for functionalization with different ligands directed to a specific target [6 - 10]. This surface modification approach allowed to reach significant results with pre-clinical application considering both polymeric and inorganic NPs, which highlighted their role in medicine fields [11 - 14]. These evidences, even if considered “promising” *in vivo* proof-of concept and results, were considered as “enough for now” over a long time [15].

Nowadays, aiming to speed up the translatability of nanomedicine and NPs into clinical protocols, another concept and relatively un-explored field are turning on around NPs: understanding their fate *in vivo*.

The destiny of NPs after their administration and their interaction with biological fluids is an interesting but complex field of research. The first observation was that, as happens with any foreign materials, NPs are immediately covered by proteins from the blood stream, leading to the formation of what is called "protein corona" (PC) [16].

Several studies pointed out that the PC plays an important role in the NPs behavior *in vivo* and may impact on biodistribution, drug targeting, intracellular uptake and toxicity of NPs [17]. Thus, the characterization of NPs is not sufficient anymore, but the relative PC must be also characterized and possibly controlled, in order to completely predict the real fate and efficiency of these drug delivery systems *in vivo*.

In literature, the studies on PC mainly involve inorganic NPs; moreover, in this review, we tried to combine and to critically comment the outputs relating to the behavior of polymeric NPs. By understanding the PC, we focalized on the parameter affecting the formation of the new biochemical entity, namely NP-PC and then on the evaluation of the complex interaction NP-PC/body.

Protein Corona: Composition and Structure

Nowadays it is almost clear that any foreign material that enters in contact with a biological fluid interacts with its components, particularly its "resident" proteins. This event happens also to NPs when injected into the bloodstream; their surface is immediately covered by circulating proteins [18], leading to the formation of a complex and variable structure, called PC [16, 19, 20]. There is no "universal" corona for all the nanomaterials: the PC composition strongly depends on the synthetic identity of the NPs. In addition, the relative densities of the adsorbed proteins generally do not necessarily correlate with their relative abundances in plasma [20]. Walkey and Chan identified a subset of plasma proteins detected on at least one nanomaterial surface, and called it "adsorbome". According to their results, in general, the plasma PC consists of 2-6 proteins adsorbed with high abundance and many other adsorbed with low abundance. In particular, they pointed out that the most abundant identified protein generally represents the 29% of the total adsorbed proteins, while the top 3 most abundant proteins represent 56% of the total amount [21].

The structure of PC consists of two components, known as *hard* and *soft* corona (Fig. **1**) .

CHAPTER 9

Safety of Nanomedicine: Neuroendocrine Disrupting Potential of Nanoparticles and Neurodegeneration

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Abstract: The development of nanomaterials (NMs) for applications in biomedicine inclusive of drug delivery as well as medical imaging is currently undergoing an enormous expansion. NMs may have many different forms and characteristics, depending on their size, chemical composition, manufacturing method, and surface modification. The use of NMs in the field of neurodegenerative diseases diagnosis and treatment implies the ability of NMs to cross the blood-brain barrier (BBB) and enter the central nervous system (CNS) in dependence on their physico-chemical properties, composition, and functionalization. The same properties that make the NMs beneficial for their applications may also affect their interactions with biological systems and have unintended consequences on human health. Several *in vivo* and *in vitro* studies have demonstrated that intentional exposure to NMs with potential use for diagnostic and therapeutic purposes might induce neurotoxic effects resulting in neuro-degeneration in different CNS regions. Recent evidence has indicated that neuro-endocrine disrupting effects by the action of NMs in dopaminergic, serotoninergic, and gonadotropic systems might be relevant to neuropathogenesis and neurodegeneration. In line with developmental origin of adult diseases, it is forewarning the evidence that pre- and post-natal exposure to different risk factors including NMs may lead to phenotypic heterogeneity and susceptibility to neurodegenerative diseases in later stages of the life. In the light of the above mentioned events, relevant test models are required to assess: i) the role of NMs in the development and progression of neurodegenerative disease; ii) the effects of NMs on neurodevelopment upon *in utero* exposure of foetuses or neonatal exposure of pups; or iii) the neuroendocrine disrupting effects during critical period being crucial for the development of neurodegenerative diseases. Early identification of potential negative features of NMs using interdisciplinary research approaches (biological, toxicological, clinical, engineering) could minimize the risk of newly designed/developed nanomedicines.

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NANOTOXICOLOGY AND SAFETY OF NANOMATERIALS

Nanomaterials (NMs)/**nanoparticles** (NPs) cover a heterogeneous group of materials, including inorganic metal and metal oxide NMs, polymeric particulate materials and carbon-based NMs in a wide range of shapes. NMs possess unique physico-chemical properties, such as ultra small size (1-100 nm), large surface area to mass ratio, and high reactivity, which considerably distinguish from the bulk microscale material of the same composition. A wide range of NMs is already accessible on the market, and NMs for future applications like, novel robotic devices, targeted drug delivery systems, molecule-by-molecule design, and self-assembly structures are in the course of development. According to the European Commission (EC), the global quantity of NMs may achieve around 11.5 million tones with a market value of circa 20 bn € per year [1].

Nanotoxicology is a newly-formed discipline which focuses on the understanding of the properties of engineered NMs and their interactions with biological systems emphasized to elucidate the relationship between the physico-chemical properties of NMs and induction of toxic biological responses [2, 3].

Several leading scientists [4] have suggested five grand challenges that need to be achieved in line with safety and sustainability of the developed **nanotechnologies** (NTs). These require to develop: 1) instruments to monitor NM exposure in water and air; 2) validation of methods for the evaluation of the toxicity of NMs; 3) models predicting the impact of NMs on the human health and environment; 4) robust systems for evaluation of NMs impact on health and environment over entire life cycle; and 5) strategic programs intent on relevant risk-focused research. These challenges have been chosen to initiate strategic research aimed at the safety of NT.

Many questions should be opened before NPs would be widely implemented in the marketplace. These are concerning the medicine and environment and say: will NPs induce nano-specific qualitatively distinct and novel toxic effects; how will be measured and predicted nano-specific effects; what will be the relationship between the shape, size, and surface chemistry of NPs on the one hand and their *in vivo* behavior on the other hand; how will be the NMs degraded or metabolized, will be the NMs and/or their degradation products effectively excreted from the body?

At the European level, the discussion about NMs at legislative and scientific level has been ongoing for several years. To date, the current regulatory guidelines are

summarized in Table **1**.

Table 1. Legislative activities in the European Union on regulation of nanomaterials.

Date	Regulatory subject	Action	Conclusions	Ref.
May 2004	EC	Communication "Towards a European strategy for nanotechnology"	- proposed actions to promote a strong role of Europe in nanoscience and nanotechnology - the need to address potential risks for health and environment	a
June 2005	EC	Action plan "Nanosciences and nanotechnologies" for 2005-2009		
2008	EC	First Regulatory Review of EU legislation with respect to nanomaterials	"Current legislation covers in principle the potential health, safety and environmental risks in relation to NMs. The protection of health, safety and environment needs mostly to be enhanced by improving implementation of current legislation."	
2009	EP	EP resolution of April 2009 on regulatory aspects of nanomaterials	1. Call for a regulatory and policy framework that explicitly addresses NMs. 2. Call on the Commission to review all relevant legislation. 3. Call for an inventory and product labeling. 4. Call on the Commission to evaluate the need to review REACH concerning: a) simplified registration for NMs manufactured or imported below 1 tonne; b) consideration of all NMs as new substances; c) a chemical safety report with exposure assessment for all registered NMs; d) notification requirements for all NMs placed on the market on their own, in preparations or in articles	b
October 2012	EC	Second Regulatory Review on Nanomaterials	- the REACH registration and proof of safety use for NMs should be based on a case by case approach, and each type of NM should be clearly described	c
February 2013	EC	REACH Review	- revision of annexes	d

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Since November 2005, Dr. Giovanni Tosi is researcher in the scientific field of Pharmaceutical Technology at the University of Modena and Reggio Emilia. Since 2015, he is associate professor in the same field.

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His works on Nanoparticles for CNS delivery and targeting were honored with national prices and awards. His work on Nanoparticles for CNS drug delivery was awarded as one of the Hot Topic (150 selected research over 19.000 proposals) at the World Neuroscience Meeting (Society for Neuroscience) 2011 and 2012.

He is author or co-author of more than 90 publications in international journals, he gave over 150 presentations (invited speaker, oral presentations and posters) in international and national congresses. He is or has been PI of a number of national and international research programs, especially focused on nanomedicine application to neurodegenerative and neurometabolic disorders.