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Frontiers in Nanomedicine
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FOREWORD

The urgent need to reduce the side effects of drug administration requires to increase the concentration of drug to the targeted body part of interest. The introduction of nanotechnology, which involves creation and utilization of materials, devices or systems on the nanometer scale, allowed to design and develop nanocarriers able to deliver the drug to a target organ, minimizing the drug's effects on the healthy tissues, thus reducing its side effects. The application of nanotechnology to medicine, designated as nanomedicine has greatly accelerated not only the treatment of many diseases, but also the diagnosis and the imaging. Target-specific drug therapy and methods for early diagnosis of pathologies are the priority research areas where nanotechnology would play a vital role.

However, even with excellent new materials, some questions have to be solved: how to improve a nanocarrier for real applications? What is the state of the art of the research into the drug delivery systems? This e-Book is written with these questions in mind. How nanomaterials are employed in the disease treatment and in diagnostic purposes.

This e-Book edited by Dr. M. Luisa Bondì, Dr. Chiara Botto and Dr. Erika Amore is of interest for not only specialists in pharmaceutical technology, but also graduate students and researchers in materials for drug delivery, biomolecular recognition and medical imaging since "*Frontiers in Nanomedicine"* links academic knowledge to both research know-how and the avant-garde industrial application of advanced materials for drug delivery systems of industrial application.

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PREFACE

The problems related to the side effects of conventional drug administration led to a growing interest towards the development of systems able to release drugs selectively into the target site and at controlled rate. Basically, a good targeted drug delivery system should localize and prolong the drug action, with the inherent advantage of reducing the administered drug dose, decreasing consequently side effects.

In the field of drug delivery systems, colloidal carriers, and in particular nano-scaled carriers, had a great impact, being made of biocompatible materials and suitable for any route of administration and different pathologies, difficult to treat with conventional therapies.

The application of nanostructured materials to medicine has greatly accelerated not only the treatment of many diseases, but also the diagnosis. In fact, the development of nanotechnology-based imaging devices improved the specificity and sensitivity of diagnostic imaging by allowing the non-invasive and quantitative detection of specific biomolecules in humans, enabling the characterization of biological processes at the cellular and/or molecular level.

Chapters written by experts will make the reader acquainted with a variety of topics ranging from nanoparticles for drug, gene delivery and for bio-imaging purposes.

In particular, Chapter 1 discusses the use of nanoparticles in biomolecular recognition and imaging applications, describing nucleic acids and biomarker detection and focusing on the systems already being translated into clinical settings.

Chapter 2 and Chapter 3 focus their attention on the use of nanostructured systems for the treatment of liver diseases. In Chapter 2, the physiology and anatomy of the liver, the epidemiology, natural history and current clinical treatments of liver diseases are summarized, followed by a description of the most common nanoparticle types employed in the treatment of liver diseases, as well as a description of preclinical and clinical evidence for the treatment of liver diseases by nanotechnology approaches. Chapter 3 discusses the available strategies to realize targeted drug delivery to hepatocytes by galactose-decorated nanostructured systems based on polymers, *i.e.,* in viral hepatitis and in liver cancer, when hepatocyte is the key target cell for therapeutic interventions.

Breast cancer is the subject of Chapter 4, presenting an overview of different nanoparticulate drug delivery systems developed for breast cancer treatment either under preclinical or clinical evaluation and also discussing different ways to obtain targeted delivery.

Targeted drug delivery systems are particularly required for brain-located pathologies, since the blood-brain barrier (BBB) seriously impedes any treatment approach. Recent results demonstrating the ability of nanoparticles to traverse the BBB provide potential alternate means for targeted drug delivery to the central nervous system and novel therapeutic and/or early diagnosis applications.

Chapter 5 describes the most recent attempts to develop nanoparticulate systems for diagnosis and/or therapy of Alzheimer's Disease. All types of nanoparticles which have been employed up-to-date to target Alzheimer's Disease are described and also a brief description of BBB physiology and methodologies used for studying transport of drugs across the BBB are mentioned.

Gene therapy is an important strategy for providing treatment for diseases that have been left uncured for decades. Therapeutic nucleic acids comprise different types of DNA and RNA, that need an effective and safe drug delivery system to cross the cell plasma and arrive to the nucleus.

In this regard, synthetic nano-vectors are emerging as safer alternatives to viral vectors as carriers for nucleic acid delivery. Compared to the viral vectors, the synthetic ones are more biocompatible, less cytotoxic and can be designed and synthesized to overcome biological barriers and target specific areas of interest. Moreover, they can protect nucleic acids from enzymatic degradation in the harsh biological environment. Chapter 6 highlights recent progress about synthetic nano-vectors for delivery of nucleic acids, discussing some of the common nucleic acids and the mechanisms currently used in synthetic nano-vectors for their delivery.

This e-Book provides to scientists a short but reasonably comprehensive overview of the most recent research strategies for the design of drug delivery systems based on advanced materials for nanomedicine applications. It explains the use of nanotechnology in medicine to improve the diagnosis of disease and the role of nanoparticles as targeted drug delivery systems for application in disease therapy and diagnostics. The goal of the authors is to offer to readers an up-to-date understanding of these materials with either a clinical or research interest in this field.

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CHAPTER 1

Nanoparticles for Diagnostics and Imaging

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Abstract: Nanoparticles possess unique optical and physic-chemical properties that may potentiate applications in biomedicine, in particular in diagnostics, therapy and imaging. Advances on biomolecular diagnostics strategies have greatly focused on single molecule detection and characterization of DNA, RNA or proteins through improved nanoparticle-based platforms. Nanoparticles improve analytical capability when compared to traditional techniques with high resolution and medium-high throughput. Also, particular interest has been directed at SNP detection, gene expression profiles and biomarker characterization through colorimetric, spectrometric or electrochemical strategies.

Molecular imaging has also benefited from the introduction of nanoparticles in standard techniques towards non-invasive imaging procedures that can be used to highlight regions of interest, allowing the characterization of biological processes at the cellular and/or molecular level. Several imaging modalities are associated with low sensitivity, an issue that can be tackled by the use of probes, *e.g.* contrast agents for X-ray and magnetic resonance imaging, radiolabelled molecules for nuclear medicine. Furthermore, nanoparticles can be used as vehicles that deliver specifically these contrast agents, leading to overcome the limitations of conventional modalities.

This chapter will discuss the use of nanoparticles in biomolecular recognition and imaging applications, focusing those already being translated into clinical settings. Current knowledge will be addressed as well as its evolution towards the future of nanoparticle-based biomedical applications.

Keywords: Cancer diagnostics, contrast enhancement, cross-linking assay, DNA detection, gold nanoparticles, gold nanoprobes, liposomes, magnetic nanoparticles, metal enhanced fluorescence, metal nanoparticles, molecular resonance imaging, nanomedicine, nanoparticles, optical imaging, photoacoustic imaging, Positron

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Emission Tomography, quantum dots, RNA detection, silver nanoparticles, theranostics.

INTRODUCTION

Nanotechnology has brought forth new materials suitable for application in biomedicine that greatly boost current methodologies for clinic diagnostics, including gene expression profiling, biomarker quantification and imaging [1, 2]. Such strategies have focused on i) the development of nanoscale devices and platforms that can be used for single molecule characterization (nucleic acids or proteins) at an increased rate when compared to conventional systems; and ii) the construction of novel contrast agents to improve existing diagnostics *via* tissue/organ imaging [3]. Most platforms specifically designed for protein detection often include standard assembly concepts using antigens and antibodies for molecular recognition (*e.g.* sandwich immunoassay), coupled to distinct detection strategies, such as spectroscopy [4] or electrochemistry [5]. Similarly, systems designed towards nucleic acid sensing, whether for screening of nucleotide sequences or single base mismatch discrimination (*e.g.* single nucleotide polymorphism, SNP), are usually based on defective hybridization stringency due to mismatch, causing a conformational shift in the duplex, which is then detected [6]. Identification of specific relevant molecules by using nucleic acid probes or aptamers can also be performed and detection is often carried by means of an intercalating agent or dye [7]. Currently employed bioassays for detection of known biomarkers or nucleotide sequences, have progressively been integrated into nanoparticle-based systems, increasing sensitivity and lowering costs [8, 9].

Imaging methodologies for *in vivo* diagnostics have also greatly benefited from the development of nanotechnology, particularly the incorporation of nanoparticles (NPs) or NP-conjugates into imaging techniques as novel contrast agents [10, 11]. NPs possess elevated surface area and relative ease-of-derivatization, which present advantageous for increased interaction and site-specific delivery, which in turn may amplify the signal and boost the diagnostic sensitivity.

Due to the high variability in nanoparticle-based applications, we have organized this chapter around in i) nucleic acid detection using NP-based systems focused around 3 types of nanoparticles: gold, silver and magnetic; platforms for diagnostics *via* biomarker detection and quantification divided by nanomaterial; iii) NPs for imaging organized by technique; all these discussions will show a

particular focus on those already being translated into clinical settings or targeting clinically relevant targets.

NANOPARTICLES FOR DIAGNOSTIC APPLICATIONS

Molecular diagnostics requires highly-paralleled and miniaturized assays capable of incorporating the vast available information on disease biomarkers [12], either abnormal profiles resulting from disease onset or nucleic acid characterization with increased potential for prognosis of disease. Over the past couple of decades, noble metal NPs, due to their optical and physic-chemical properties, have been used for development of biosensing tools capable of specific identification of nucleic acid sequences associated to relevant phenotypes or capable of characterizing specific protein profiles of disease, often as substitutes of fluorescence or chemiluminescence based detection [13, 14].

Nanoparticles for Nucleic Acid Sensing

One of the greatest potential that makes NPs a practical biological tool is the ease of synthesis and functionalization with DNA/RNA molecules, proteins and other biomolecules. Due to their nano-size scale they present high surface-area-to-volume ratio, with great capability of interaction in the same scale of target biological molecules. Diagnostics strategies using nanoparticles have been reported for the detection of nucleic acids, proteins, pH variations or small analytes *via* colorimetric, fluorescence, mass spectrometry, electrochemical and scattering approaches. Most of the reported systems are still in pre-clinic with few commercially available products being transposed to clinic.

Gold and Silver NPs

Due to their optical, chemical and electrical properties, noble metal nanoparticles have been extensively used as tags for nucleic acids probes, since they may be easily functionalized with oligonucleotides through a thiol bond [15]. Several methods for detection of DNA hybridization are described in the literature, taking advantage of NPs plasticity of detection, *i.e.* optical absorption, fluorescence, Raman scattering, atomic or magnetic force and electrical conductivity. The biggest contributes of NPs for nanodiagnostics are associated with the detection of biomarkers of genetic diseases, SNP genotyping and detection of pathogens' nucleic acids (bacteria and virus). Most of the methods describe a detection limit in the fmol/L scale, however there are some reports that claim an amol/L limit of DNA. For in depth reviews of this matter, please refer to [1, 16] and references therein.

CHAPTER 2

Emerging Use of Nanotechnology for the Treatment of Liver Diseases

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Abstract: Liver diseases, including viral hepatitis, resulting from hepatitis B or C virus infection, liver cirrhosis and hepatocellular carcinoma pose great global health challenges due to the limited curative treatment options. The application of nanoparticles has emerged as a rapidly evolving area for safely delivering therapeutic agents (*i.e.* drugs and nucleic acid) to treat a range of diseases, including those of the liver. In this chapter, we give an overview of various nanotechnology approaches which can be employed when treating liver diseases, focusing in particular on liver infections, fibrosis and cancer.

Keywords: Drug delivery, HBV, HCC, HCV, liver cirrhosis, liver diseases, liver fibrosis, nanoparticles.

INTRODUCTION

Liver diseases, such as those secondary to hepatitis B and C viral infections, hepatic fibrosis or hepatocellular carcinoma (HCC) are among the main causes of morbidity and mortality globally [1-7]. Despite improvements in the diagnosis and management of liver diseases, they continue to constitute a significant problem for health, as adequate therapies are lacking [8-10]. Clearly, there is an urgent need for new therapies and/or alternative pharmacological interventions for these diseases.

Current therapies have a number of disadvantages, the major ones being that organ selectivity is not sufficient, so they are consequently toxic to disease-free

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tissues, and drug bioaccessibility to the diseased target areas is limited, which means that high doses of drugs are required. Emerging therapies based on nanotechnologies have begun to offer alternatives to traditional therapy which are both promising and innovative. Nanotechnology, a field in rapid growth, focuses on developing, manipulating and applying materials in the size range of 10-500 nanometers (nm). To do so it can either scale up from single atom groups or refine or reduce bulkier materials to produce nanoparticles (NPs). A therapeutic nanoparticle is generally defined as a nanostructure incorporating a therapeutic drug, peptides, proteins or nucleic acids loaded in carriers with at least one length in the nanometer range. NPs can be engineered as nanoplatforms allowing effective and targeted drug delivery, and as imaging labels, being able to overcome a number of biological, biophysical, and biomedical barriers. The possibility to incorporate drugs and genes into NPs, which may also be functionalized, *i.e.* ligands specifically binding to targeted cells or tissues are conjugated or coated (Fig. **1**), is opening up a new frontier for the selective delivery of drugs and genes to the disease site.

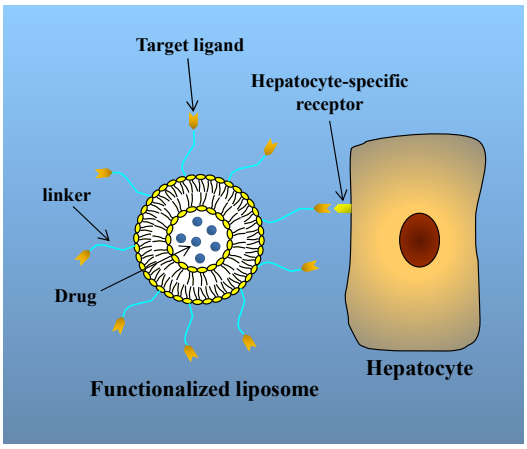

Figure 1: Functionalized nanoparticles are loaded with drugs to actively target the disease site by using cell-specific homing devices.

In general, the advantages in the use of nanoparticle systems for agent administration are manifold: i) they increase stability of the therapeutic agent, due to protection against inactivation of the active agent, especially nucleic acid, until it reaches the site of action; ii) prevent the fast uptake and subsequent buildup of reticuloendothelial system (RES) by macrophages; iii) feasibly incorporate hydrophilic and hydrophobic agents; iv) optimize pharmacological effectiveness (ability to increase the bioavailability of drugs which are transported through anatomical barriers); v) reduce toxicity and side effects of the drugs; vi) reduce

drug blood fluctuations (lower risk of ineffective or toxic concentration); vii) offer a potential broad spectrum of administration routes (external, ophthalmic, oral and parenteral); viii) allow controlled (sustained) drug release, and ix) enable active targeting due to the possibility of obtaining a greater affinity of the nanoparticle system (functionalized nanoparticle) for certain tissues. All these properties could solve the common problem of non-compliance to prescribed therapy. However, following systemic administration, plasma proteins opsonize conventional NPs, which are then rapidly recognized as foreign bodies and quickly captured by the RES. NPs mainly accumulate in the liver and spleen [11, 12] owing to their rich blood supply and the abundant tissue-residing phagocytic cells, the so-called Kupffer cells in the liver. However, liver targeting of nanoparticles may be favorable when treating liver diseases like tumors or hepatitis. Nanoparticle uptake and distribution are size-dependent. In general, NPs with a mean diameter > 400 nm are quickly captured by the RES and therefore cannot circulate in the bloodstream for long, whereas in NPs with a diameter < 200 nm blood circulation time is prolonged and the RES uptake rate is relatively low [13]. Nevertheless, covalent bonding at the surface of the nanocarrier of certain biologically inert hydrophilic polymers, including polyethylene glycol (PEG), has been shown to prolong bloodstream circulation time by limiting RES uptake and to reduce immunogenicity and antigenicity. In addition, these polymers shield the nanoparticle surface and thereby reduce opsonization by blood proteins [14, 15]. Such NPs are commonly called “stealth” NPs. Long circulating nanoparticles can thereafter slowly accumulate in pathological sites, *i.e.* solid tumor tissues with leaky vasculature *via* the well-known *enhanced permeability and retention effect* (EPR) [16, 17] (Fig. **2**).

Moreover, as lymphatic drainage is greatly reduced or absent in tumor tissues, the tumor interstitium retains NPs for a lengthy period. These processes are called “passive targeting”, a phenomenon which occurs in all types of tumor [16, 17]. Matsumura and Maeda first reported the EPR effect in 1986 [18]; it is a phenomenon present only in solid tumors, linked to differences in anatomy and pathophysiology compared to normal tissues: large gaps exist between endothelial cells in the blood vessels of the tumor, which facilitates macromolecule transport into the diseased tissues.

The field of medicine dealing with the application of nanotechnology to address medical problems such as prevention, diagnosis, treatment and monitoring of various pathologies, including cancer, is referred to as nanomedicine [19-21]. In recent years, nanomedicine-based approaches have been explored for liver disease

CHAPTER 3

Galactose-Decorated Polymeric Carriers for Hepatocyte-Selective Drug Targeting

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Abstract: In this paper, the current available strategies to realize galactose-decorated nanostructured polymeric systems are summarized. These carriers are designed in order to obtain targeted drug delivery to hepatocytes *via* galactose (GAL) moieties, *i.e.* for the treatment of viral hepatitis or liver cancer that are the greater causes of global disability and mortality. Usually, the main followed strategy to obtain galactosylated polymeric carriers is to use galactosylated copolymers. The chemical modifications of preformed polymers with sugar-containing reagents is followed for obtaining lactosaminated human albumin, galactosylated phospholipid-polyaminoacid and polylactide (PLA)-polyaminoacid conjugates obtained from α,β-poly(N-2-hydroxyethyl)-D,L-aspartamide (PHEA) or lactosaminated carboxymethyl chitosan (CMC). Galactosylated polymers are also obtained *via* the polymerization of GAL-bearing monomers, that is for obtaining galactosylated polycarbonates. Finally, the surface galactosylation of preformed polymeric carriers is an alternative strategy that can be used to obtain a GAL-decorated system, that is for obtaining dendrimers based on polyamidoamine (PAMAM)-GAL conjugates.

Keywords: Asialoglycoprotein receptor (ASGP-R), carboxymethyl chitosan (CMC), galactose (GAL), hepatocytes, lactosaminated albumin, liver targeting, poly(ε-caprolactone) (PCL), polyamidoamine (PAMAM) dendrimers, polycarbonates, polylactide (PLA), xyloglucan, α,β-poly(N-2-hydroxyethyl)-D,L-aspartamide (PHEA).

INTRODUCTION

Liver diseases are the greater causes of global disability and mortality [1]. Many potent drugs are often not or little effective *in vivo* or cause adverse effects; thus liver targeting of drugs represents a new promising therapeutic opportunity compared to the only satisfactory curative options such as liver resection and

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transplantation and considering that drugs could accumulate rapidly in the liver but not in the proper hepatic cell-type [2].

Targeted drug and gene delivery in particular to hepatocytes could be promoted by the development of galactosylated polymeric or lipid carriers directed to the asialoglycoprotein receptor (ASGP-R), which was identified as the most useful target protein for hepatocytes-selective carriers. In fact, although hepatocytes represent the most part of resident hepatic cells, other cell-types like Kupffer cells (KCs) could highly uptake drugs or other therapeutics leading to complete degradation of such materials [3]. The ASGP-R is abundantly present on the plasma membrane of hepatocytes [4-7] and thanks also to its hepatocyte specificity, it has been used to deliver therapeutics such as proteins, antivirals [8] and anticancer drugs [9, 10] into hepatocytes. Consequently, the delivery of the drug-loaded carrier specifically to hepatocytes could allow for the optimization of the drug pharmacological effects and also reduces side effects on other hepatic cells due to non-specific cellular uptake. The scope of this paper is to summarize the available strategies to realize galactose-decorated nanostructured systems based on polymers for targeted drug delivery to hepatocytes *via* galactose (GAL) moieties, *i.e.* in viral hepatitis and in liver cancer, when hepatocyte is the key target cell for therapeutic treatments [11].

Active Targeting To Hepatocytes

In the human liver, the various physiological functions are performed through the specific activities of various cell types, such as the non-parenchymal sinusoidal endothelial cells (SECs), KCs, hepatic stellate cells (HSCs) and the predominant parenchymal hepatocytes. These cells express surface receptors for several carbohydrate molecules. In particular, hepatocytes specifically interact with GAL or with molecules having residues of GAL thanks to presence of ASGP-R, while KCs and endothelial cells have mainly receptors for mannose [12, 13]. KCs also express fucose receptors, so that they could can internalize fucosylated drug carriers [14]. Hepatocytes are functional cells implicated in the metabolic and secretory activities of the liver. Hepatocytes play also a critical role in liver diseases like viral hepatitis A, B or C, some genetic diseases like Wilson's disease, steatohepatitis induced by alcohol (ASH), α1 antitrypsin deficiency, hemochromatosis and several other metabolic disorders. Drug uptake by hepatocytes is generally allowed thanks to the first pass effect and due to the presence of many endocytotic receptors and transporters on hepatocyte plasma membrane that permit internalization of drugs. Despite that, in order to enhance the therapeutic effects or to minimise side effects of drugs, many strategies for

selective targeting of drugs to hepatocytes have been explored. Small colloidal drug carriers (<150 nm) can diffuse out of the hepatic capillaries through the fenestrations and interact with hepatocytes that can take up them through mechanisms such as pinocytosis and receptor-mediated endocytosis. Specific internalization in hepatocytes through targeting of their receptors can also be achieved. Targeting to hepatocytes through the ASGP-R is the most followed strategy to enhance endocytotic uptake mediated by clathrin of nanostructured systems by hepatocytes. The success of this approach originates by the innate binding affinity between the ASGP-R and a broad range of molecules exposing GAL and N-acetyl-galactosamine (NAc) residues, such as asialofetuin (AF), sterylglucoside and lactose (LAC), for targeting to hepatocytes [15].

Recently, Alnylam Pharmaceuticals designed two novel GalNAc-siRNA conjugates to achieve targeted delivery of RNAi therapeutics to hepatocytes. In particular, conjugation of siRNA to NAc ligand result in higher liver exposure *in vivo* and lead to a significant increased potency and durability of effect in pre-clinical studies [16, 17].

Galactosylated Polymeric Carriers

Polymers today represent an indispensable component to design either conventional or innovative drug delivery systems (DDS). Polymeric drug carriers such as nanoparticles, microspheres, dendrimers and micelles, have already well showed their successful applications in modified and targeted drug delivery [18-22]. For their *in vivo* fate, size, morphology, matrix composition and surface charge, play a basic role, and all these characteristics can be modulated in order to obtain appropriate compatibility, modified release profile and biological activity. To enhance the internalization specifically in the needed pathological cells, a successful strategy could be the decoration of polymeric carrier surface with recognition moieties that impart an affinity for cellular receptors or components that are present on the target cells and/or are up-regulated by these specific cells [23, 24]. The active targeting allows that nanocarriers are retained in the microvessels of the target tissue within a few hours, moving from the blood into the target tissue by endothelial transcytosis. Galactosylation of these carriers is a exploited strategy in the field of drug delivery because targeting *via* galactosylated carriers induces highly specific interactions of GAL ligands with endogenous lectin receptors such as ASGP-R [13]. Molecular recognition of GAL ligand on the polymeric carrier is strongly influenced by the oligosaccharide chain. Thus, the sugar units necessary for molecular recognition by ASGP-R should be incorporated in the carrier in such a way to be available for binding

CHAPTER 4

Nanomedicine for Delivery of Drugs in Breast Cancer: Recent Advances and Prospects

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Abstract: Breast cancer is one of the most invasive cancers, with a high mortality rate; almost all treatment options are invasive, with significant side effects, and not always curative. Nanomedicine, the application of nanotechnology to medicine, is an interesting approach to improving the delivery of anticancer drugs, thus increasing their therapeutic index and specificity, while reducing side effects. This chapter describe the most widely studied nanoparticulate drug delivery systems, also discussing nanocarriers in clinics and recent advances in targeted drug delivery approaches for breast cancer treatment.

Keywords: Active targeting, anticancer drugs, breast cancer, cancer therapy, clinical trial, clinical use, dendrimers, drug delivery, EPR effect, inorganic nanoparticles, lipid nanoparticles, liposomes, multidrug resistance, nanomedicine, passive targeting, polymeric micelles, polymeric nanoparticles.

INTRODUCTION

"Cancer is the second cause of death in the world after cardiovascular diseases. Breast cancer is one of the four common cancers that account for over 50 percent of total cancer case [1]. Moreover breast cancer is the most commonly diagnosed cancers and the leading cause of cancer death in women in the world [2]". In cancer treatment, chemotherapy is used either as neoadjuvant therapy, to decrease tumor size before surgery and/or radiation, or as an adjuvant therapy after surgery. In neo-adjuvant or pre-operative treatment, anti tumor drugs are given to patients with a primary tumor, but without clinical evidence of distant metastases. Treatments composed of chemotherapy, or hormonal therapy, with radiotherapy can also be included in this category. The aim of this approach is to reduce tumor bulk, the risk of tumor seeding, and to facilitate surgery or radiotherapy of the primary tumor. A combined treatment of an antracycline/cyclophosphamide/taxane regimen is the

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recommended neodjuvant chemotherapy for breast cancer bearing patients [3]. The aim of adjuvant treatment is to eliminate tumor cells, that could be still present after surgical treatment of the primary tumor. Adjuvant treatment is generally given to women with a high risk of micrometastases and its efficacy is statistical. It has been demonstrated statistically that adjuvant treatment can be efficacious for the treatment of subclinical, micrometastatic disease in patients with breast and other cancers. Therefore classical chemotherapy remains the keystone of cancer therapy and deals with the administration of drugs that interfere with cell's cycle, inhibit its division or eventually destroy them. However, the anticancer agents used still present some drawbacks, that can reduce or limit their efficacy. Most anticancer drugs have reduced specificity, because they do not discriminate between tumour and normal cells, leading to the onset of toxicity and to adverse effects that limit treatment efficacy [4]. These drugs have a narrow therapeutic window and are normally administered around the maximum tolerated dose [5]. Moreover, acquired drug resistance may further reduce the efficacy of chemotherapy and of other adjuvant treatments. The heterogeneity of breast cancer also restricts the efficacy of cancer treatment. In fact, it is a complex disease that include a variety of biological types and is characterized by a dysregulation of pathways related to cell differentiation, cell cycle control, apoptosis, angiogenesis and development of metastasis. The prognosticative symptoms for breast tumor are: nodal condition, tumor size, grade of malignancy, age, and hormone receptor state. Breast cancer is heterogeneous and can be classified in three main groups: i) hormone receptor positive, ii) HER2 (human epidermal growth factor receptor, 2) positive, and iii) the triple-negative disease (with negative expression of estrogen receptor, progesterone receptor and HER2 overexpression/amplification). Hormone receptor positive cancer is expressed in about 60% of all breast cancers and has a better prognosis [6]. This disease is normally treated with hormonal therapy, with drugs that either block the receptors or block hormones production or activity. The selective estrogen receptor modulators such as tamoxifen are indicated for both the treatment and prevention of estrogen-responsive breast cancer [7]. Sex steroids and their agonists/antagonists are employed in hormone replacement therapy, and also in the treatment of breast cancer [8]. Anti-HER2 agents combined with chemotherapy or hormonal therapy are the standard treatment for tumors overexpressing HER2. In particular trastuzumab and lapatinib are the approved targeted agents in breast cancer [9-11]. Trastuzumab is a monoclonal antibody directed to the extracellular domain of HER2. It is reported that trastuzumab is effective in patients with HER2-positive metastatic breast cancer either alone [12] or in combination with anticancer drugs [13]. Recently it has been demonstrated to be efficacious as adjuvant in the treatment of early HER2-positive breast cancer [14]. Lapatinib is an orally-active drug and acts as a potent and

reversible inhibitor of the ATP binding site at the tyrosine kinase domains of both HER2 and epidermal growth factor receptor (EGFR). "It is thus a dual tyrosine kinase inhibitor that is able to inhibit EGFR and HER2 with high specificity. This is relatively unique among the small-molecule tyrosine kinase inhibitors; this property is potentially advantageous as lapatinib is able to inhibit a greater number of cell signaling pathways that result from homo- or heterodimerisation of EGFR and/or HER2 with themselves or other HER family members" [15]. At present, treatment possibilities for the triple-negative breast cancer are very restricted and effective treatments for patients with metastatic breast cancer are also reduced; in these cases chemotherapy still remains the treatment of choice. In the last years, in order to improve the efficacy and to reduce the side effects of anticancer drugs, carrier mediated drug delivery systems have been proposed as a new class of drugs thanks to their ability to deliver antitumoral agents directly to the target site [16, 17]. In fact the main aims of nanoparticles as drug delivery systems are to target specific tissues or cells, to deliver drugs in a controlled manner, and/ or to lower the necessary dose in order to decrease the toxicity of the corresponding treatment. The majority of these systems have been defined nanovectors. "Nanovectors offer the promise of providing breakthrough solutions to the problems of optimizing efficacy of therapeutic agents while simultaneously diminishing the deleterious side-effects that commonly accompany the use of both single chemotherapeutic agents as well as multimodality therapeutic regimens. In general, nanovectors are comprised of at least three constituents, which include a core material, a therapeutic and/or imaging "payload", and a biological surface modification, which aids in both appropriate biodistribution and selective localization of the nanovector and its cytotoxic and/or imaging agent" [18]. Thus, nanovectors can be highly useful either as drug delivery systems (to enhance the pharmacological activity of conventional anticancer drugs), or in the field of diagnostic imaging in the preparation of new and potent diagnostic device able to identify cancer in the early stage [19]. This chapter present an overview of different nanoparticulate drug delivery systems developed for breast cancer treatment either under preclinical or clinical evaluation.

NANOTECHNOLOGY AND NANOMEDICINE

Nanotechnology is an emergent part of science, through which tools and devices in the 1-100 nm size range can be designed [20] in which particular properties, such as optical, magnetic, electronic and structural characteristics not present in macromolecules, offer new utilization [21]. In the last years, nanotechnology has been widely used in different industrial fields such as: electronic storage systems, biotechnology, food, chemical sensor, energy, magnetic separation and

CHAPTER 5

Applications of Nanoparticles for Alzheimer's Disease Diagnosis and/or Treatment

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Abstract: This chapter is dedicated to the applications of nanoparticles (NPs) for the diagnosis and treatment (or therapy) of Alzheimer's disease (AD). Today, the Blood Brain Barrier (BBB) is considered to be one of the main challenges for treatment and diagnosis of AD. In this chapter, after a brief introduction about the disease and its related pathologies, the BBB physiology and the methodologies used for investigation of the transport of drugs across the BBB are described, together with the main physicochemical characteristic prerequisites of NPs that may be proposed as therapeutic and/or diagnostic systems for brain-located pathologies. In the second part, all the types of NPs (delivering drugs and/or imaging agents) which have been explored up-to-date for diagnosis or therapy of AD are presented, together with the advantages and disadvantages of each type. Finally, current accomplishments and future challenges are summarized.

Keywords: Alzheimer's disease, Aβ-plaques, BBB, brain, liposome, nanoparticle, neurodegenerative, neurofibrillary-tangles, targeting, theragnosis.

INTRODUCTION

One of the most challenging diseases in therapeutics today is Alzheimer's disease (AD), which is the most common form of dementia over the age of 65 and its occurrence is constantly increasing, as a consequence of population aging [1]. For this reason, interventions for early diagnosis and treatment of AD are urgently required, since the disease has a heavy social and economic impact, which will increase exponentially and highly affect health care systems in the coming years. The BBB seriously impedes diagnosis or treatment approaches, since the location of AD pathological features is in the central nervous system (CNS). Nanoparticles

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(NPs) have the ability to transcytose across the BBB providing a potential for delivery of APIs (or imaging agents) to the CNS, for diagnosis and/or therapy of AD [2]. Different NP-types are currently being evaluated as potential systems to target the pathological features of AD, providing the basis for development of diagnostic or therapeutic systems. For these types of applications NPs need to have the ability to be transported across the BBB and target specific AD-related pathologies, in order to be successful as therapeutic or diagnostic systems, (with the exception of NPs that may utilize the "sink theory" approach [3, 4], which will be described below). A brief introduction of AD pathologies and BBB physiology will be initially presented, for better understandment of the physiological parameters of the disease. The currently used methodologies for screening potential formulations that can pass the BBB and target AD-related pathologies will be also described, before the presentation of the most current examples of NPs which are being investigated as systems to provide diagnosis or therapy of AD.

CURRENT METHODOLOGIES TO TARGET AD

AD is a neurodegenerative disease which today affects 24 million people or more, worldwide. AD causes cognitive dysfunction and it progressively leads to learning and memory impairment and behavior/cognition changes, while there is no currently available therapy, but only symptomatic treatment [2, 5]. AD diagnosis is based on detection of behavioral changes (as confusion, mood swings or irritability). The basic AD-related pathologies are two: The amyloid, plaques (which are formed by β-amyloid (Aβ) peptides) and the neurofibrillary tangles. The most important Aβ peptides consist of 39-42 aminoacids, and are produced due to the abnormal cleavage of the Amyloid-Precursor-Protein (APP) [6]. These amyloid deposits are insoluble and accumulate in the brain, leading to destructive effects on neurons, and increasing the oxidative stress. Metal ions (mostly copper (Cu) and zinc (Zn)) further promote the deposition of extracellular plaques [7]. The second major pathological finding in AD is that of neurofibrillary tangles (NTFs) which are hyperphosphorylated filaments of a (microtubule-associated) protein, tau. Neurofibrillary tangles are linked to growth and development of healthy cells, but their accumulation in cells leads to cell death, due to disturbance of the normal cytoskeleton. AD thus leads to neuronal loss. In addition to the two basic pathologies, other important clinical findings related to AD are: (i) chromosomal mutations, (ii) deposition of amyloids in blood vessels, (iii) oxidative stress and vascular degeneration [8, 9]. The two main pathologies of AD are described in more detail below; several recent review articles are available for those interested in more details on AD pathophysiology [10-12].

The methodologies for diagnosis and treatment of AD which are currently under development, target the two pathologies mentioned above: tau neurofilaments and amyloid plaques. Below, various approaches are described, together with a number of potential ligands which are evaluated as homing devices, to target NPs to the two basic AD-pathologies.

Tau-Neurofilament Targeting Strategies

As explained above, one category of primary markers of AD are the Neurofibrillary Tangles (NFTs) which are aggregates of the tau protein (also present in other diseases, known as Tauopathies). These aggregates are formed after hyper-phosphorylation of the microtubule-associated protein, tau, which causes aggregation when the phosphorylation equilibrium in the neurons is disturbed. Hyperphosphorylation is required, for induction of tau aggregation, and subsequent toxicity. The tau protein level in AD brains is 8-fold higher compared to the levels in healthy brains, mainly due to NFT presence [13]; while the reduction of tau concentration has been proposed as a neuroprotective strategy against tauopathies [14]. Tau oligomers can be targeted (directly or indirectly) by different strategies such as: a) tau phosphorylation prevention: Since the phosphorylation of tau is controlled by enzymes (kinases and phosphatases), tau kinase-inhibitors can be used for AD treatment. Indeed, protein phosphatase (PP)-2A may result in dephosphorylation of tau; b) tau misfolding prevention: Activation of molecular chaperones for tau misfolding might be prevented and this could potentially reduce NFTs. Heat shock proteins were demonstrated to activate chaperones that prevent misfolding and subsequently promote tau binding with microtubules; c) tau immunotherapy: One immunotherapeutic approaches to treat AD, is the selective reduction of the pathological forms of tau.

Ligands for Tau Targeting

Different ligand types which were found to have ability to target tau proteins are seen in Table **1**. Certain small molecules are capable to prevent tau protein aggregation and in some cases to even dissolve aggregates, as it has been demonstrated in recent studies. Such small molecule ligands include aminothienopyridazines, thiazolyl-hydrazides, thiacarbocyanine dyes, polyphenols, anthraquinones, N-phenylamines, rhodanines (thioxothiazolidinones), quinoxalines, phenothiazines, phenylthiazolyl-hydrazide, benzothiazoles and others [15]. In several cases their activity was linked to their structure. A few examples follow: i) The importance of structural properties of the rhodanine core for inhibition of tau aggregation was investigated by synthesis of appropriate small molecule ligands. The activities (IC50/DC50) of the compounds were found to be: rhodanine > thiohydantoin >> oxazolidinedione = thioxooxazolidinone >> hydantoin. From this

CHAPTER 6

Non-Viral Nano-Vectors for Nucleic Acid Delivery

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Abstract: The development of therapeutic nucleic acids has led to new strategies for treating various diseases. Non-viral, synthetic nano-vectors in gene therapy have attracted increasing attention due to their low immunogenicity and low toxicity compared to viral counterparts. Due to the molecular structure of nucleic acids, they are very prone to degradation in pH sensitive biological environments. Therefore, synthetic nano-vehicles for therapeutic delivery, known as 'nano-vectors', need to be cleverly designed and engineered to protect and deliver appropriate therapeutic nucleic acids to the targeted sites for action. In this chapter, a brief overview of various types of therapeutic nucleic acids is first provided, followed by analysis of the synthetic nano-materials under development as delivery systems to carry nucleic acids. The nucleic acid-encapsulated nano-vectors discussed here open a window for a new generation of nanomedicine.

Keywords: Cancer, chitosan, co-delivery, delivery, dendrimer, gene therapy, gold, inorganic, iron oxide, liposomes, magnetic, nanocarriers, nanoparticles, nano-vectors, nucleic acid, pDNA, polymer, rheumatoid arthritis, silica, siRNA.

INTRODUCTION

Genetic engineering involves the modification/manipulation of an organism's genome. In general, living organisms, whether plants or animals, that have been revised through genetic engineering are defined as genetically modified organisms (widely known as GMOs). Genetic modification is an important strategy for providing treatments for diseases that have been left uncured for decades. An early example is the delivery of plasmid DNA (pDNA) for treatment [1]. It has been recognized since 1970 [2, 3] that there are a range of medical conditions that are potentially amenable to treatment *via* direct modification or repair of the genes within a patient's cells. For example, rather than regulate the concentration of some biologically important molecules within the body by externally administering some

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substances, it could be far better to re-program the body's own cellular machinery to produce (or suppress) the molecule of interest.

Initially, nucleic acids such as antisense oligodeoxyribonucleotides (ODN) and pDNA were employed in gene therapy. In particular, pDNA has been widely used by biotechnologists and biomedical scientists for delivery to express therapeutic transgenes [4]. Antisense ODN binds with the complementary strand on a messenger RNA (mRNA), and results in the degradation of ODN/mRNA complex or by blocking splicing without degradation [5], while pDNA will allow the expression of therapeutic transgenes. The latest development of nucleic acid therapeutics is the discovery of RNA interference (RNAi). The discovery of RNAi in the late 1990s and microRNA (miRNA) lin-4 in *C. elegans* in 1993 [6, 7] eventually led to the emergence of specific gene silencing using synthetic small interfering RNAs (siRNA) [8-12].

Viral and calcium phosphate vectors were the earliest strategies for mimicking the body's cellular interactions in order to achieve delivery of nucleic acid. However, the advancement of nanotechnology and biotechnology has more recently enabled the use of synthetic carriers for delivery of nucleic acids into various types of mammalian cells. This has value both for the purpose of pure research and for commercial or medical applications. In addition, viral vectors tend to be more immunogenic compared to appropriate benign synthetic nano-vectors, they may display irregular cytotoxicity and/or limitations in targeting specific cell types, they have a low DNA carrying capacity and, finally, they may not infect non-dividing cells [13].

The use of synthetic nano-vectors as a carrier for nucleic acid delivery is a vast and emerging field. These types of vectors have attracted increasing attention compared to their viral vector counterparts [14, 15] due to their benign characteristics, low cytotoxicity and biocompatibility of the proposed synthetic non-viral delivery systems [16]. Synthetic delivery systems can be designed and synthesized to overcome biological barriers and target specific areas of interest using chemical synthesis [17-20]. Nucleic acids can be sequestered and protected from enzymatic degradation in the harsh biological environment. In particular, polymeric nanocarriers have been widely studied for nucleic acid delivery. Chemical synthesis and controlled/living radical polymerization (CLRP) have assisted polymer chemists in engineering well-defined macromolecules to deliver payloads to the desired region of the diseased cells [21]. For example, negatively-charged nucleic acids are not readily bound to the surface of cells, which are themselves negatively charged due to having an abundance of sulfated

proteoglycans. However, the negative charge of nucleic acids can be neutralized if bound to cationic polymers (the conjugate being known as a polyplex) and then they can attach to the negatively-charged cell surfaces [22]. This creates attractive interactions rather than repulsive interactions between the polyplexes and disease cells. Other alternatives include DNA encapsulation using a biodegradable polymer and DNA adsorption which combines electrostatic interaction and encapsulation. DNA encapsulation has remarkable advantages due to polymer biodegradability and the possibility of controlling DNA release. On the other hand, the DNA adsorption technique has been demonstrated to have many advantages too, including those of improving DNA bioavailability and augmenting load efficiency [23].

Inorganic particles for nucleic acid delivery are also being investigated as synthetic nano-vectors. Common inorganic vectors include Spherical Nucleic Acid (SNA) developed using a gold core [24, 25], and iron oxide nanoparticles, Plank *et al.* pioneered the combined used of iron oxide-based magnetic nanoparticles (MNP) with surface functionalized organic polyethylenimine (PEI) under the influence of magnetic field (magnetofection) for improved transfection efficiency [26, 27]. Magnetic force is being applied to drive vectors towards target cells leading to rapid and highly effective nucleic acid delivery. However, these delivery systems still need to be carefully investigated using further *in vitro*, *ex vivo* and *in vivo* studies.

The present chapter will highlight recent progress in respect of synthetic nano-vectors for delivery of nucleic acids. Synthetic nano-vectors have been sub-divided into organic and inorganic derived materials. Examples provided in the two sections are vectors most commonly investigated as delivery systems. Due to ease of condensing negatively charged nucleic acids with cationic polymers, some very common cationic polymers and their derivatives are reported. However, in most cases, cationic polymers can only encapsulate nucleic acids. They lack specificity and targeting properties and are sometimes toxic to cells, therefore, cationic polymers are most often functionalised with biomimetic molecules to enhance their uptake and release in specific targeted cell types. This aspect is discussed in the subsequent sections. Another leading area of research in nucleic acids delivery is the use of inorganic materials such as gold which can offer optical and plasmonic properties, and iron oxide having its magnetic attribute. Owing to the intrinsic capabilities of inorganic materials, they are typically functionalised with organic materials to improve the delivery efficiency of nucleic acids.

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Maria Luisa Bondì

Dr. Maria Luisa Bondì was born in Altavilla Milicia (Palermo) on the March 7, 1964. She graduated in Pharmacy in 1987 at the University of Palermo presenting an experimental thesis on synthetic work concerning synthesis of derivatives 1,2,4-thriazines with potential biological activity.

From September 1990 to March 1992 she worked as fellow (CNR borsa di studio di formazione Mezzogiorno) at the "Department of Organic Chemistry" of the University of Palermo carrying out research on isolation, purification, semisynthesis and characterization of natural products from plants (Compositae, Labiatae families) at aim to obtain molecules with antifeedant activity.

She has got great experience on chemistry of organic natural compounds, chromatography, purification of natural compounds and their chemical transformation.

She was selected as researcher (art. 23 CNR) at the ICTPN-CNR research team developing several products with biological activity. In December 2000 she was confirmed as permanent researcher at the same institute. Actually Dr. Maria Luisa Bondì works at Institute for Studies of Nanostructured Materials (ISMN) of Italian National Council Research of Palermo. She is author of about 80 scientific publications on international journals and of over 120 presentations at national and international congresses.

Since 2005 she has been Contract Professor of Course "Veicolazione e direzionamento di farmaci" at the Science Faculty, Chemistry Department, Univ. of Palermo.

The field of prevailing interest is the study of the preparation and characterization of lipid and polimeric nanoparticles for drug delivery and targeting into the tumors, Central Nervous System, lung and posterior region of eye. She has authored several publications on internationally reputed journals and author of two international patents licensed to pharmaceutical industries. She is scientific referee for internationally respected journals and member of Editorial Board of international journals such as “Recent Patents on Nanomedicine”, Journal of Biotherapy

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Erika Amore

Chiara Botto was born in Palermo on the 9th September, 1989. In July 2012 she graduated with highest honors in Pharmacy from the University of Palermo, presenting an experimental thesis on the delivery of nanostructured lipid carriers containing antitumoral drugs for the treatment of hepatocellular carcinoma. Since 2013 she is a PhD student in “Technology of biologically active substances” at the University of Palermo, and works in collaboration with the Institute for the Study of Nanostructured Materials (ISMN) of the National Council of Research (CNR) of Palermo. Her activity is mainly focused on the preparation and characterization of nanoparticles for targeted drug and gene delivery, in particular for ocular, pulmonary and hepatic applications. During the second year of PhD she worked at the Laboratory for General Biochemistry and Physical Pharmacy of the University of Gent (Belgium), studying the colloidal stability of nanomedicines in biological fluids by fluorescence microscopy techniques and their effects on living cells.

Chiara Botto

Erika Amore was born in Augusta on the 3th Dicember, 1988. In March 2013 she graduated with highest honors in Pharmacy from the University of Palermo, presenting an experimental thesis on the preparation and characterization of nanostructured lipid carriers for the treatment of liver tumors. She continued to carry out research by assigning a collaboration contract and after a fellowship at the Institute for the Study of Nanostructured Materials (ISMN) of the National Council of Research (CNR) of Palermo. In particular she worked on the preparation and characterization of nanostructured lipid systems for drugs delivery to the liver and to the eyes.

Since January 2015 she is a PhD student in “Technology of biologically active substances” at the University of Palermo, and works in collaboration with the Institute for the Study of Nanostructured Materials (ISMN) of the National Council of Research (CNR) of Palermo. Her activity is mainly focused on the preparation and characterization of lipid microparticles for drug delivery to the lungs.