The Ligand Nanoparticle Conjugation Approach for Targeted Cancer Therapy

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Abstract: Cancer therapy often requires frequent and high drug dosing. Yet, despite the significant progress in cancer research and the wide versatility of potent available drugs, treatment efficacy is still hurdled and often failed by the lack of pharmaco-selectivity to diseased cells, indiscriminate drug toxicities and poor patient compliance. Thus, innovative pharmaceutical solutions are needed to effectively deliver the cytotoxic drugs specifically to the tumor site while minimizing systemic exposure to frequent and high drug doses. Polymeric nanocarriers, particularly nanoparticles, have been extensively studied for improved oncological use. Such nanocarriers hold great potential in cancer treatment as they can be biocompatible, adapted to specific needs, tolerated and deliver high drug payloads while targeting tumors. Active targeting, as opposed to passive targeting, should add value to selective and site specific treatment. Active targeting of nanosized drug delivery systems is firmly rooted in the Magic Bullet Concept as was envisioned by Paul Ehrlich over 100 years ago. This targeting strategy is based on the molecular recognition of tumor biomarkers which are over-expressed on cancer cells, via specific vector molecules conjugated to the surface of the drug carrier. These vector molecules dictate the carrier's biodistribution and its biological affinity to the desired site of action. Many recent publications have shown encouraging results suggesting that targeting nanocarriers represent a highly-promising strategy for improved cancer treatment. This chapter will focus mainly on polymeric nanoparticles as the main drug carriers to be conjugated to various ligands able to deliver the drug to the specific desired pathological tissue.

Keywords: Cancer, ligands, monoclonal antibodies, nanoparticles, targeted delivery.

I. INTRODUCTION

Cancer is the second leading cause of death worldwide. About 1,529,560 new cancer cases were expected to be diagnosed in 2010. In the US, cancer-related death is exceeded by heart diseases only, and accounts for nearly 1 of every 4 deaths. The National Institute of Health estimated the overall costs of cancer in 2010 at $263.8 billion [1]. Thus, cancer imposes an unbearable economical burden on health authorities and on society. As a result, efforts are continuously being invested in an attempt to ameliorate treatment and alleviate patient suffering.

In recent years, with the mapping and profiling of specific tumor biomarkers characterizing cancer cells and the understanding of signal cascades involved in the pathogenesis of tumors, targeted therapy has become the mainstay of cancer research. A targeted treatment approach is aimed at designing intelligent new anticancer therapeutics meant to specifically target some unique aspect of tumor biology. The targeted therapy approach is a broad and colorful niche where multiple fields of research often intersect.

Notably, nanomedicine, the application of interdisciplinary fields of nanotechnology in medicine, has gained a major role in the targeted therapy efforts. In the past three decades, nanotechnology and nanomedicine have evolved as multidisciplinary fields of research with the potential of providing breakthrough solutions in cancer diagnosis and treatment. The use of nanoscaled drug delivery systems (DDSs) is becoming a widely investigated approach for the improved delivery and treatment of cancer diseases. The commercial availability of two nanosized delivery systems, liposomal doxorubicin (Doxil®) and albumin-based nanoparticles (NPs) of taxol (Abraxane®) has reigned the interest in this exciting technology of drug delivery. Furthermore, nanoparticulate drug delivery systems offer major advantages such as the ability to deliver insoluble lipophilic drugs, increase drug stability by preventing its early degradation, provide sustained drug release and promote favorable drug bioavailability, biocompatibility and pharmacokinetic profile. NPs also possess the potential ability to bypass multi drug-resistance mechanisms (MDR), by various approaches [2-7]. The first approach resides merely in the encapsulation and hiding of the potent substrate drugs within the polymer matrix which is not recognized by the transporters, thereby avoiding the efflux of the chemical active ingredient by the molecular pumps [3, 8, 9]. Currently, there are additional proposed mechanisms for the bypassing of MDR by different kinds of NPs, including direct interference with P-gp expression and function, P-gp inhibition and ATP depletion by the carrier [2, 7]. Other approaches are based on targeting moieties that enhance the internalization of the nanoparticles within the resistant cells through specific receptor mediated binding and endocytosis [5].

Such delivery systems may be designed to carry high payloads of a wide range of molecules and elements, including small molecules, either hydrophilic (such as doxorubicin [10]) or hydrophobic (like taxanes [11]), peptides (insulin [12, 13]), siRNA (small interference RNA) [14, 15], optical-imaging dyes [16, 17], and even radionucleotides [18-21]. While micron sized particles may also possess the ability to deliver lipophilic drugs and other therapeutic molecules, they cannot be administered intravenously. Thus, the nano scale size of NPs, especially if they are pegylated, exhibits a marked added value since they are suitable for intravenous delivery. There are other advantages which include their lower recognition and clearance by hepatic macrophages, their favorable prolonged circulation time, and enhanced cellular and tissue penetration properties [22, 23]. Furthermore, particle size plays an important role in the biofate and efficacy of the colloidal carrier in cancer therapy. The penetration of NPs to the tumor tissue is highly affected by their size, and smaller particles will likely exhibit higher rates of extravasation into permeable tumor tissues through the abnormal loose neovascularure, which is one of the characteristics of growing solid tumors [22].

Particular focus has been directed towards the active targeting of nanosized delivery systems for cancer treatment. This arises primarily from the shortfall of conventional treatments, but also from the evolving understanding of cancer biology, and the discovery of new molecular targets involved in carcinogenesis, as well as the advent and clinical use of monoclonal antibodies (MAbs) and other macromolecules used for targeting specific markers. The concept of active targeting of nanoscaled delivery systems based on the surface accessibility and the high level of expression of specific
cancer antigens, has gained major importance, as it is now widely recognized that successful selective targeting to cancer cells will not only result in highly efficient treatments and markedly reduced systemic toxicities, but will also serve as a unique platform for the design of advanced multifunctional NPs. The clinical applicability of these nanocarriers in cancer diagnosis and treatment is versatile, ranging from specific tumor imaging to targeting conventional chemotherapy, as well as targeting immunotherapy, gene therapy, radiotherapy, and chemoradiation. Particularly, the combined use of NPs as a therapeutic, imaging and diagnostic tool, referred to as theranostics, is highly promising in the emerging field of personalized medicine because it will allow both early-stage detection of an individual patient’s cancer, and will hopefully provide clinicians with a rapid and sensitive tool for real-time, non-invasive monitoring of the theragnostic NPs and of the tumor’s response to treatment[24]. Thus, these targeted nanoscaled delivery systems hold great potential to provide a revolutionary improvement in current cancer treatment.

The present review highlights the key principles of targeting cancer treatment by the ligand-nanoparticle approach, and summarizes cumulative and recent data with emphasis on ligand-conjugated polymeric NPs. Various targeted epitopes and their versatile ligands are reviewed. Furthermore, some recent advances and insights in the field are mentioned.

NANOCARRIERS- MORPHOLOGICAL DESCRIPTION AND DEFINITION

Nanotechnology, nanomedicine, and NPs are terms which are often mentioned interchangeably to describe the multidisciplinary use of various submicron colloidal systems in the diagnosis, imaging and treatment of various diseases, particularly cancer. These colloidal carriers may be of diverse composition, shape and physicochemical characteristics which dictate their in vivo behavior and thus their actual translation into clinical use. Such nanoscaled constructs include mainly liposomes, nanoemulsions, polymeric micelles, dendrimers and other stealth organic and inorganic NPs (The various carriers are illustrated in Fig. 1).

NP dimensions may range from 1-1000 nm [25]. It is currently accepted that the diameter of NPs for cancer therapy should be in the range of 10-100 nm, so they may easily penetrate the leaky tumor vasculature and accumulate within the tumor tissue [26].

Liposomes, first discovered by Dr. Alec Bangham in 1961[27], are closed vesicles encapsulating water by a lipid bi-layer membrane usually composed of phospholipids. Their hydrophilic core can be used for the entrapment and delivery of water soluble drugs. These vesicles can be uni or multi lamellar, with the potential of carrying not only water-soluble drugs but also lipophilic molecules entrapped within the lipid bi-layer.

Nanoemulsions or sub-micron emulsions usually depict oil droplets dispersed in water (o/w emulsions), and are usually suitable for the intravenous, oral or ocular delivery of lipophilic compounds.

Polymeric micelles are nanosized vectors that form spontaneously in aqueous solutions of amphiphilic block copolymers. At a specific concentration, termed the CMC (critical micelle concentration), amphiphilic molecules with the appropriate geometry, orient in such a way that the hydrophobic segments are isolated from the aqueous surrounding, achieving a state of minimum energy that leads to the formation of core-shelled colloidal structures termed micelles.

Dendrimers are a class of polymeric materials. First discovered in the early 1980’s by Donald Tomalia and colleagues [28], these hyper-branched polymeric molecules were called dendrimers, originating from the Greek word dendron, meaning a tree. These are multi-branched, monodispersed macromolecules, with nearly perfect 3-D geometrical architecture. As the chains growing from the core molecule become longer and more branched, dendrimers adopt a globular structure. Dendrimers become densely packed as they extend out to the periphery, forming a closed membrane-like structure. Their sizes range between 1.9 nm and 4.4 nm, the smallest nanocarriers so far developed. Dendrimer -drug interactions or drug loading in dendrimers may be achieved by various approaches: simple encapsulation in the interior of dendrimers (as illustrated in Fig. 1), electrostatic interactions and covalent conjugations to the surface of the dendrimers. The empty internal cavities interact with poorly soluble drugs through hydrophobic interactions [29, 30]. These cavities may also possess nitrogen or oxygen atoms which can interact with the drug molecules through hydrogen bonds [31, 32]. The presence of functional groups in high density on the surface of dendrimers (such as amine or carboxyl groups) may be exploited to bind drug molecules with relevant functional groups through electrostatic or covalent interactions [29, 30, 33-35].

The general term nanoparticles (NPs), describes a wide range of sub-micron colloidal systems including organic polymeric NPs, composed of synthetic or natural polymers or proteins (i.e, albumin), solid lipid nanoparticles comprising physiological lipids, as well as inorganic NPs such as semiconductor NPs, iron oxide NPs, quantum dots and gold NPs.

Polymeric NPs usually consist of a general core-shell structure and are also subdivided into various categories according to their basic chemical composition (biodegradable as opposed to non-biodegradable), their core shell composition and their morphology; these include nanocapsules (NCs) and nanospheres (NSs). According to literature definitions, nanocapsules are hollow spherically-shaped vesicular particles, where the drug is confined to a hollow core, usually composed of oil droplets, which is surrounded by a polymeric shell or membrane. On the other hand, nanospheres are solid colloidal matrix systems, ideally uniform in their core-shell polymer partition, where a drug is dispersed or dissolved in the polymer matrix [36] (Fig. 1).

Polymeric NPs have a very similar design as a polymeric backbone, usually formed from one or more biodegradable monomers of simple organic biocompatible molecules. Various synthetic and natural polymers are currently being investigated for the design and potential applications of NPs including, poly ethylene glycol (PEG), poly lactide (PLA), poly glycolide (PGA), poly acrylates, poly alkyl cyano acrylates (PCA) [37, 38], polycarpolactone [39, 40], polyethilenimine [41], and their derivaties, PEG, PLA and poly(D,L-lactide co-glycolide) (PLGA) and their copolymers PEG-PLA, PEG-PGA, PLGA and PEG-PLGA are the most widely investigated synthetic polymers for drug and gene delivery [42-46].

In addition, protein based platforms have emerged as attractive drug delivery carriers due to their biodegradability, biocompatibility and low toxicity. These platforms comprise naturally self-assembled protein subunits of the same protein or a combination of proteins making up a complete system [47]. Various proteins have been reported and characterized for such DDSs, including the ferritin/apoferritin protein cage, plant-derived viral capsids, the small heat-shock protein (sHsp) cage, albumin, soy, collagen, and gelatin. These platforms may be designed in different types and shapes, including various protein cages, microspheres, NPs, hydrogels, films and more [47].

NPs may be prepared by two general approaches:

1. In situ polymerization of monomers, for example by suspension or dispersion polycondensation methods [48].

2. Dispersion of pre-formed polymers is the commonly-applied approach for NP preparation. Typically, the hydrophobic polymer and drug are first dissolved in an appropriate organic solvent and then mixed into an aqueous phase which generally consists of a surfactant solution. When water-insoluble solvents are employed (acetone, DMF or dimethylsulfoxide), upon diffusion of the solvent into the surrounding water phase...
The polymer will precipitate, and drug-loaded NPs stabilized by surfactant molecules will form. This process is termed interfacial deposition, nanoprecipitation or nanodeposition. In contrast, non-water-miscible solvents, such as dichloromethane and chloroform, will result in an oil-in-water (o/w) emulsion upon mixing with water, and the solidified particles will form during solvent evaporation [42, 49, 50].

Drugs can be incorporated or loaded into NPs by various approaches: Molecular dispersion (solubilization) in the polymer matrix; entrapment or encapsulation in a hollow oily core; chemical attachment through covalent conjugation to the polymer backbone; or adsorption onto the surface of NPs. Covalent conjugation of therapeutic molecules is usually attained using functional groups of carboxyl, hydroxyl, thiols, amines, phosphonate and carbonyls.
where the resulting bonds are usually esters, carbonates, carboxylates, dithiols, amides, phosphates and oximes, which are then hydrolyzed or enzymatically cleaved in vivo [51].

Polymeric NPs, especially those composed of PLA and PLGA, have emerged as promising carriers for targeted delivery of a wide variety of drugs. Major advantages of these NPs include their bio-compatibility, biodegradability, ease of formulation, and tunable sustained release properties. The nanodelivery systems currently approved by the FDA for cancer treatment are liposomes (Doxil®) and albumin NPs (Abraxane®). It should be emphasized that although the biodegradable polymers, PLGA and PLA are worldwide approved for subcutaneous and intramuscular use, there is not yet an approved intravenous (i.v.) nanodelivery system based on such polymers in the market.

In the present chapter, we will mainly address targeted NPs of various biodegradable polymeric compositions (natural and synthetic) as they are widely investigated nanodelivery systems for clinical and diagnostic use. This chapter will review recent advances and developments in the design and applications of targeted-polymeric NPs in cancer treatment. NPs targeting brain tumors and NP delivery across the blood brain barrier (BBB) will not be discussed. Likewise, targeted liposomes, nanoemulsions, polymeric micelles, dendrimers and inorganic NPs will not be addressed in the present review. For additional information on these topics, the reader is referred to Refs [44, 52-69]. Furthermore, direct antibody/ligand-drug molecular conjugates (ADCs) or drug-polymer-antibody/ligand molecular conjugates will not be addressed either.

III. THE NECESSITY FOR AND THE UNDERLYING PRINCIPLES OF DRUG TARGETING

In recent years, the advances in molecular biology and in the understanding of the different signaling pathways involved in tumor development and progression have led to the discovery of potential drug targets and to the design of novel drug entities for cancer treatment. However, despite the versatility of available potent drug molecules, their clinical use and positive outcome are far from accomplishing the desired clinical endpoint.

In vitro, many agents demonstrate potent anticancer activity, by the induction of cancer-cell death through numerous complex pathways, such as apoptosis or prevention of further cell division and proliferation. However, when transferred from a cancer-cell culture to a whole-organism system, their simultaneous effect on normal healthy tissue is practically inevitable and limits their effective use in a clinical setting. Treatment efficacy is thus often burdened by the indiscriminate drug distribution and the lack of drug specificity to the pathological tissue. If rephrased in clinical terms, conventional chemotherapeutics are characterized by a narrow therapeutic index and systemic toxicities which enforce limited dose settings and eventually lead to poor drug concentrations at the tumor site and to suboptimal tumor response. Chemotherapeutic side effects may range from unpleasant hair loss, sensory neuropathy, diarrhea, nausea and vomiting to life threatening myelosupression, neutropenia, nephrotoxicity and cardiotoxicity. Consequently, despite the wide variety of available drug molecules, their use is often discontinued far before attaining the therapeutic endpoint. Ideally, enhancing drug accumulation at the tumor site while lowering systemic exposure should result in a more efficient and patient friendly treatment. Thus, innovative pharmaceutical solutions that will enable efficient treatment and adequate patient response are constantly being sought.

Targeted delivery of anticancer agents is a rapidly evolving and highly promising field of research in anticancer therapy. In recent years, scientists from various disciplines have concentrated enormous efforts in the design and investigation of targeted nanocarriers, as a means to potentially enhance treatment efficiency and circumvent non-specific toxicities of traditional chemotherapy, by altering biodistribution profiles of anticancer agents. Indeed, targeted drug delivery may potentially increase the fraction of the systemically administered dose reaching the disease site resulting in a high local drug concentration at the tumor site, while minimizing collateral damage to adjacent healthy cells.

III.a. Nanoparticle Biofate

Most advanced cases of cancer present a disseminated disease. Therefore, systemic intravenous injection of NPs for the delivery of therapeutic compounds is still one of the preferred routes of administration. As a result, a prerequisite for the design of a successful nanoparticulate delivery system is the prolonged circulation and moderate degradation rate that will provide enough time for the nanocarrier to reach the target tissue. The most prominent factors affecting NP circulation time and biodegradation rate are related to the NP properties as a whole assembly. They are: (a) the nature of the polymer. Such important parameters include, particle size distribution, NP surface chemistry, steric hindrance, surface hydrophobicity/hydrophilicity, surface charge, as well as the basic monomer unit composition and the molecular weight of the polymer [22]. Among these parameters, particle size may markedly affect NP biodistribution. Small particles below 10 nm are cleared by renal excretion, while larger particles are cleared by the mononuclear phagocyte system (MPS) and by hepatic filtration [22].

Upon intravenous injection, most of these NPs are recognized as foreign bodies and cleared by phagocytic cells, mainly cells of the mononuclear phagocyte system and the polymorphonuclear leukocytes. Macrophages located in the reticuloendothelial system (RES) of which the Kupffer cells in the liver comprise 85-95% of the total intravascular phagocytic capacity, play a crucial role in the phagocytosis of injected particles. This rapid phagocytosis is generally hypothesized to be mediated by an opsonization process, i.e. the adsorption of certain blood components (opsonins) to the surface of NPs, apparently by hydrophobic interactions. Opsonins include components of the innate immune system, such as IgG, IgA and complement cascade components. The likelihood of opsonization and liver uptake augments with the increase in particle size. NPs around 100 nm exhibit an improved circulation half life [22] compared to larger NPs.

The idea of NPs coating or grafting with the hydrophilic polymer chain moiety poly(ethylene glycol) (PEG) was proposed by Gref et al, as a means to circumvent the rapid opsonization and phagocytic clearance of NPs, hence prolonging their circulation in the blood [70]. Today, it is a well-documented fact that pegylated NPs, especially where the PEG chain is chemically attached, have longer residence time in the blood than non-pegylated NPs. The stealth properties provided by these PEG chains arise from a combination of mechanisms, including steric hindrance, flexibility and hydrophilicity of the particle’s cell surface [71]. It should be kept in mind however, that pegylation does not completely prevent or eliminate opsonization and phagocytosis, but when appropriately designed, it significantly delays and reduces these inevitable processes.

III.b. Passive Targeting Mediated by the EPR Effect

Therapeutic drug concentrations in the tumor tissue appear to be a key parameter in cancer treatment success. It is therefore of great importance to assure NP delivery to the tumor site. The enrichment of the drug-loaded NPs in tumor tissue might occur by passive or active-targeting mechanisms. Passive targeting results from the Enhanced Permeability and Retention (EPR) effect originally described by Maeda [72, 73]. Growing tumors are characterized by the formation of new blood vessels (neovascularization). This rapid angiogenesis results in defective and chaotic vasculature characterized by hyperpermeability and leakiness, allowing NPs and macromolecules to diffuse into the tumor tissue. Naturally, smaller particles will more readily penetrate into the tumor site and to suboptimal tumor response. Chemotherapeutic side effects may range from unpleasant hair loss, sensory neuropathy, diarrhea, nausea and vomiting to life threatening myelosupression, neutropenia, nephrotoxicity and cardiotoxicity. Consequently, despite the wide variety of available drug molecules, their use is often discontinued far before attaining the therapeutic endpoint. Ideally, enhancing drug accumulation at the tumor site while lowering systemic exposure should result in a more efficient and patient friendly treatment. Thus, innovative pharmaceutical solutions that will enable efficient treatment and adequate patient response are constantly being sought.

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tissue, on the other hand, is attributed to the lack of lymphatic drainage which also characterizes the tumor environment. However, it is known that this EPR effect is not sufficient for efficient accumulation of low-molecular-weight drugs at the target site.

III.c. Active Targeting Using Recognition Moieties

Even though NPs with appropriate stealth properties possess the inherent ability to passively accumulate in the tumor tissue through the EPR effect, this accumulation is mainly at the interstitium and within tumor associated macrophages (TAM), and does not provide specific targeting and sufficient intracellular delivery into cancer cells [74]. Thus, specific intracellular uptake of accumulated NPs depends on the presence of a targeting vector molecule, a ligand that enables receptor-mediated endocytosis [74].

The rationale that lies in the basis of active targeting is that the various types of cells that compose the tumor mass carry molecular markers that serve as tumor signatures distinguishing them from normal cells. Such markers are either not expressed in normal cells or are expressed at much lower levels than in tumor cells. These findings are the targeting basis for possible implementation of the modulation of the bio distribution and preferential accumulation of nanocarriers and their drug cargo in the diseased cells. Thus, a rational targeted approach would require the identification of unique molecular targets by the unravelling of the versatile antigenic landscapes of various tumors.

Cancer biomarkers include an altered presentation of a variety of molecules and components of the cell, ranging from mutant genes, RNAs, to proteins, lipids and even small metabolite molecules [75]. Molecular profiling of tumors using cDNA microarrays, tissue microarrays and immunohistochemical validations, may discover cancer biomarkers that might provide valuable information on cancer behavior and characteristics such as, the stage, grade, clinical course, prognosis and even response to treatment [76].

Other than serving as diagnostic and prognostic tools, cancer biomarkers may serve for the individualized design of cancer treatment, either by modulating the expression of the biomarker and knocking it down specifically, or by exploiting these unique patterns of expression for the design of targeted drug delivery systems.

Having discovered the potential of tumor receptors to serve as an attractive homing modality, the search for new ligand molecules to target these biomarkers has become in recent years an eminent objective with high priority for scientists. The pursuit for the isolation of novel targeting molecules, including antibodies, peptides and aptamers, is a growing field of research.

There are currently several widely-accepted methodologies for the screening of potential tumor markers and ligands. Traditionally, antibody-based screens have been used for the detection of tumor markers. In recent years, in vivo phage-displayed library technology has emerged as a powerful tool for the isolation and generation of peptides and antibodies that target specific tumor markers with high affinity and specificity [77]. It is a validated method that relies on the binding of ligands to accessible targets on the cell surface rather than on the overall expression levels of an epitope [78, 79].

III.d. Key Principles of Active Targeting

In order to be able to actively and specifically target a DDS to a solid tumor, a pre-requisite is the presence of a target molecule, a tumor specific epitope, expressed at the membrane surface of the tumor cell. Targeting efficiency is dependent on an ensemble of parameters, some of which will be presented herein.

On a molecular level, the interaction between the targeting moiety and the targeted epitope is highly affected by the binding affinity and selectivity of the targeting unit and by the capacity of the targeted receptors [79].

First, the number of cell-surface receptors and their availability dictate the number of targeting molecules that will eventually bind specifically to the tumor. Once the surface receptor is saturated by the carrier, the effect of specific targeting will be screened by non-specific background leading to reduced tumor-homing efficiency [79, 80].

Binding affinity of the targeting ligand is another important factor affecting tumor-homing efficiency; low affinity of a ligand to its receptor might seriously limit and hamper targeting efficiency. However, in certain cases, such a disadvantage may be compensated for by the multivalent binding character of targeted NPs [79]. Thus, NPs binding to cell-surface receptors may be tuned by both the binding affinity of the targeting ligand and by NP surface density (i.e. the number of conjugated units decorating the surface). This multivalency principle of NPs enables the expanding of the repertoire of available ligands that may be used as targeting moieties, such as low-affinity peptides [26, 81-83]. However, it should be taken into consideration that the pursuit of improved targeting ability by increasing NP ligand density and multivalency might increase the probability of NPs to be recognized and cleared by the MPS [79] ultimately leading to poor targeting efficiency. High density of ligands at the surface of pegylated NPs might shield the PEG chains and annul their protective effect against RES organs [84]. This dilemma has been addressed by Gu et al. [84], who have investigated the effect of an aptamer ligand’s surface density on the in vivo biodistribution, tumor localization and immune evasion of the targeted nanocarrier in a tumor-bearing mouse model. The authors developed a series of targeted NPs with increasing ligand densities that inversely affected the amount of PEG exposure on the NP surface. They eventually identified the narrow range of aptamer density when the NPs were maximally aimed and maximally stealth, resulting in the most efficient prostate-cancer-cell uptake in vitro and in vivo [84].

The ability of the attached ligand to reach the target receptor may also be influenced by the PEG chain length and coverage densities at the NP surface. This interference has been discussed by Wang and Thanou, with emphasis on liposomes [75], concluding that the optimal surface design and the fine tuning of the interplay between stealth properties and targeting efficiency are not an easy task.

An important feature that should characterize ligands chosen for tumor targeting is their innate ability to induce receptor-mediated endocytosis. The rate of internalization is affected by the nature of interaction between the targeting ligand and the targeted surface epitope. This process enhances the internalization of the drug carrier into the desired cells and eventually affects the uptake of the NPs into the tumor tissue, rather than its accumulation within the tumor interstitium. The importance of cell-mediated endocytosis and internalization of the targeting ligand in treatment efficacy was clearly demonstrated both by Kirpotin et al. [74] and Bartlett et al. [85]. The authors reported that although both non-targeted and targeted nanocarriers exhibited similar biodistribution and tumor localization, targeted liposomes and NPs were correlated with superior antitumor activity relative to the respective non-targeted carrier. Thus, the primary advantage of targeted NPs was associated with processes involved in cellular uptake in tumor cells rather than overall tumor localization. Optimization of internalization may therefore be a key factor for the successful development of effective NP-based targeted therapeutics [85]. Finally, it should be ensured that the ligand–nanoparticle conjugate will exhibit sufficient blood circulation time to reach the desired target. Indeed, regarding ligand-molecule conjugates (mostly, ADCs), intended for drug delivery, it is generally assumed that the targeting ligand should possess a relatively long half-life to provide the drug sufficient time to reach the target at therapeutic levels [79, 86]. However, as a rule this is true for direct ligand-molecule conjugates but does not apply necessarily to the biofate of nanoparticulate carriers. As previously mentioned, NP circulation time and elimination rate is dictated by RES uptake which in turn is influenced mainly by the NP
size and stealth properties. Moreover, Debotton et al. [87], recently reported that despite a superior tumor response elicited by MAb-targeted NPs as compared to non-targeted NPs, the pharmacokinetic behavior of the targeted NPs was similar to that of non-targeted NPs. This is again consistent with the early findings of Kirpotin, that ligand attachment does not increase tumor accumulation, but enhances specific intracellular internalization [74]. Thus, at least in this particular case, it was suggested that upon conjugation of the MAb to the NPs, the intrinsic pharmacokinetic properties of the MAb were altered and they acquired the behavior of the nanocarrier [87].

**III.e. The Potential Advantages of Drug Targeting with Polymeric Nanoparticles**

Several unique features of polymeric NPs render them highly appealing DDSs and distinguish them from other anticancer modalities:

Polymeric NPs may serve as efficient vehicles for the solubilization and delivery of otherwise insoluble drugs such as paclitaxel and docetaxel [11]. In addition, they may provide an increased stability to encapsulated drugs, preventing their early degradation and elimination, thus increasing their bioavailability.

Another property of polymeric NPs is their ability to serve as sophisticated drug reservoirs able to exhibit pharmacokinetics which can be tailored according to the specific drug, disease and clinical needs. When properly pegylated, NPs exhibit a long circulation time and provide prolonged and sustained drug levels. Their tunable biodegradability and controlled drug release properties can be exploited to design targeted delivery systems, where important pharmacokinetic (PK) parameters, such as half life, area under the curve (AUC), maximal concentration (Cmax) and mean residence time (MRT) may be modulated. Controlled drug exposure is particularly important in cancer treatment, as systemic and local drug levels at the tumor site play a critical role both in systemic toxicities and tumor response.

When compared to ADCs, ligand-drug conjugates and drug polymer immunoconjugates, targeted polymeric NPs possess several additional advantages:

- In contrast to polymeric NPs, the release profile and pharmacokinetics of direct ADCs and drug polymer immunoconjugates are highly dependent on the cleavage of the conjugated functional group, which could turn out to be a rate limiting step in drug release [88] or result in an uncontrolled drug pharmacokinetic behavior. Moreover, the environmental conditions required for cleavage of the conjugates may work against the stability of the unshielded drug [89], thus reducing its effective concentration.

- Unlike the abovementioned conjugates, drug-loaded NPs offer the possibility of delivering a large payload of drug in an encapsulated protective form while avoiding coupling reactions and the subsequent production of a new chemical entity (NCE) [90]. Moreover, while a single immunoconjugate unit usually carries several conjugated drug molecules, one single NP may be loaded with several thousands up to few hundred thousands of encapsulated drug molecules (either solubilized or dispersed). Similarly, the large surface area of a single NP available for conjugation of targeting moieties allows a higher density of conjugated ligand molecules than the one provided by direct immunoconjugates, resulting probably in improved targeting efficiency of ligands with moderate affinity to specific targets.

**III.f. Basic Components of a Targeted Nanocarrier Platform**

Delivery systems used for active tumor targeting most often comprise five basic components: **a.** A polymeric carrier scaffold **b.** A shielding stealth corona of a hydrophilic polymer designed to reduce opsonization and prolong circulation time (PEG chains). **c.** A targeting ligand that binds to a specific over-expressed epitope at the disease site. **d.** A linker molecule or functional group connecting the carrier to the ligand. A drug and/or an imaging probe incorporated within the scaffold, either encapsulated or chemically bound.

Targeting ligands may be covalently or non-covalently attached to the surface of NPs by various approaches and functional groups (Fig. 2). MAbs are among the most widely used ligands to be conjugated to NPs and the methods described herein were mainly reported with MAbs. Nonetheless, some of the covalent methods mentioned could potentially be applied to other ligands as well, provided the presence of functional groups amenable to conjugation.

**Non-covalent Ligand Conjugation Approaches:**

The most widely investigated non covalent approaches include

1. Adsorption of the ligand/Ab to the surface of the NPs.
2. Biotin-Avidin complexes (These are illustrated in Fig. 2).

Adsorption is not an ideal conjugation method, as competitive displacement of the ligand/Ab by blood components could occur upon intravenous injection of the NPs and infinite dilution in the blood [91]. Biotin-avidin complexes exhibit a very strong non-covalent natural bond, however, as avidin is derived from bacterial streptavidin or from the egg white, its potential immunogenicity limits its use in vivo [92]. Thus, covalent binding is currently the preferred approach for antibody Conjugation.

**Covalent Ligand Conjugation Approaches:**

This can be achieved by various methods. We will only mention the two most commonly described linkage processes (Fig. 2):

1. Amide linkage – Activation of the end groups of carboxyl terminated PLA and PLGA by a carbodiimide (such as EDC-1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide Hydrochloride) will result in an active ester intermediate that can be coupled to the amine functional groups of an antibody by carbodiimide chemistry [93, 94].
2. Thioether linkage – The reaction between thiol functional groups and maleimide groups is highly efficient and leads to stable thioether bonds. Such a linkage may be formed between maleimide-bearing NPs and thiolated antibodies or other thiol-bearing ligands [87, 95, 96]. Alternatively, thiol-surface-activated NPs may also react with maleimide-activated antibodies [97, 98].

**TARGETING STRATEGY APPROACHES**

**IV.a. Definition and Type of Potential Targeting Ligands.**

Many approaches and tools are currently available for active targeting to tumor cells. Traditionally, monoclonal antibodies have been used to target cell-surface epitopes. However, the extensive screening of peptide and aptamer libraries has greatly expanded the repertoire of ligand tools available for targeted nanocarrier delivery [99]. All ligands that can be bound to the surface of NPs may possibly serve as targeting moieties, including antibody fragments, small peptides (EGF, RGD), aptamers (PSMA aptamer, VEGF aptamer), oligosaccharides and even small molecules (Folate) provided they can specifically recognize an over expressed target on the cell surface.

**1. Monoclonal Antibodies (MAbs)**

Monoclonal antibodies are macromolecules widely used as targeting ligands because of their immediate and variable availability and their high affinity and specificity to molecular targets. These targeting ligands usually possess a molecular weight of about 150kDa and exhibit high binding affinities (in the µM-nM concentration range). One of the drawbacks in the use of MAbs as therapeutic or targeting agents is the concern of their immunogenicity. The use of antibody fragments such as Fab’ and single-chain vari-
able fragments (ScFv), affibodies and peptides might overcome partly the immunogenicity issue.

2. Single-chain Variable Fragment (scFv)

ScFvs are fusion proteins of the variable regions of the heavy and light chains of an antibody (V<sub>H</sub> and V<sub>L</sub>) connected with a short linker peptide of 10-25 amino acids. The molecular weight of an scFv is around 27kDa.

3. Affibodies

An affibody is a small, stable 58-amino acid Z-domain scaffold, derived from the IgG binding domain of staphylococcal protein A. Its binding pocket is composed of 13 amino acids, and it is able to bind to a variety of targets, depending on the randomization of the amino acids. As opposed to IgGs, its small size (~6-15kDa) enables tumor tissue and cell penetration. Affibodies possess a high receptor affinity that mimics the active portion of the Fab' region of the corresponding antibody. Their short half life makes them good candidates as tumor imaging probes but not ideal tools for targeting direct drug conjugates, where long circulation times are required [86].

5. Aptamers

Aptamers are an emerging class of targeting ligands which, like antibodies, may also serve as biological drugs in the treatment of various diseases. The advent of monoclonal antibodies over the past decade and the use of peptide hormones, growth factors and cytokines have been continuously providing a spectrum of protein-based ligands needed for a selective targeting of tumor-associated antigens and cancer biomarkers. However, issues concerning the size, cost and immunogenicity of such protein-based ligands have led to the search for alternative ligand families.

Aptamers, are short single-stranded synthetic nucleic-acid oligomers, DNA or RNA oligonucleotides (ssDNA, ssRNA), that can form complex three-dimensional structures with the ability to bind to the internalized surface markers and target molecules with high affinity and specificity [100]. Advantages of aptamers include availability and ease of chemical synthesis, small molecular weight and lack of immunogenicity. Numerous publications have reported the conjugation of aptamers to polymeric NPs as targeting ligands [100, 101].
6. Miscellaneous Ligands

Endogenous ligands, such as folic acid, epidermal growth factor (EGF) and transferrin, are highly attractive to target their respective receptors, as they have low immunogenicity and should ensure binding affinities to the targeted receptor.

To conclude, the choice of the ligand type is usually based on numerous considerations including availability and ease of production, variety, affinity, applied conjugation approaches, immunogenicity and cost.

IV.b. Common ligand Targets for Targeting Tumors

Pathologically up-regulated cancer epitopes exploited for targeted drug delivery include solid tumor markers as well as hematologic cancer markers and angiogenesis related markers. A non-exhaustive list of such epitopes includes HER2, epidermal growth factor receptor (EGFR), transferrin receptor (TfR), ferritin receptor, folic acid receptor (FR), PSMA, intercellular adhesion molecule 1 (ICAM-1), epithelial cell adhesion molecule (EpCAM), carcinoma embryonic antigen (CEA), vasoactive intestinal peptide, CA15-3 antigen, MUC1 protein, hyaluronan, CD20, CD33, integrins, and many more. It is beyond the scope of this chapter to discuss all potential nanocarriers able to target the above mentioned surface antigens. Hence, we will mainly review targeted delivery systems for the most commonly described antigenic targets, as well as papers of special interest and recent developments.

IV.c. Targeting Tumor Cells

1. Targeting the Tyrosine Kinase Receptors

The tyrosine kinase receptors play key roles in signaling pathways of fundamental cellular processes, such as proliferation, metabolism, differentiation, survival etc. Although tyrosine kinase receptor's activity in normal cells is tightly regulated, their abnormal activation in transformed cells is implicated in the development and progression of many human cancers [102]. For example, HER2 and EGFR, both tyrosine kinase receptors belonging to the ErbB/HER receptor family, are highly expressed in numerous types of cancer. This receptor family includes four related members: the EGFR (ErbB1/EGFR/HER1), ErbB2 (HER2/neu), ErbB3 (HER3) and ErbB4 (HER4). These receptors are trans-membrane glycoproteins formed by an extra-cellular ligand growth factor binding ectodomain, a trans-membrane region and an intracellular catalytic tyrosine kinase domain with a tyrosine-containing cytoplasmic tail [102]. When specific ligands (growth factors) bind to the extracellular domain of the tyrosine kinase receptor, the receptor dimerizes, thus activating the intracellular kinase domain which in turn leads to the triggering of the downstream signaling cascade.

HER2 has no identified ligand, but it is a preferred partner to form heterodimer with other HER members resulting in subsequent activation of intracellular signal transduction cascades, including PI3K and MAPK pathways which can lead to carcinogenesis [86]. HER2 gene amplification and protein over-expression lead to malignant transformation and are directly associated with poor clinical outcome [86]. HER2 may be found in high levels of expression in breast, cervix, colon, endometrial, gastric, esophageal, ovarian, lung, prostate and pancreatic cancer [86]. EGFR's (ErbB1) expression ,on the other hand, is highly up-regulated in bladder, breast, head and neck, kidney, non-small cell lung, prostate, colon, and pancreatic cancers [103-105]. Hence, these tyrosine kinase receptors and their ligands have become common targets for therapeutic interventions with the use of both monoclonal antibodies targeting their extracellular binding domain and small molecules inhibiting the intracellular kinase domain. The MAbs directed against the extracellular portion of the receptor may be used as therapeutic entities per se, or merely as potential targeting vectors for nanosized drug delivery systems.

1.1. HER2-Targeting NPs

Among numerous tumor bio-markers present on cell surface, the HER2 membrane receptor is one of the most appealing and promising targets for immunotherapy. The over-expression of HER2 antigens (c-erbB-2, neu) in 20–30% of breast and ovarian cancers is correlated with a high occurrence of metastasis and angiogenesis processes, as well as with a poor prognosis [106]. Thus, many therapeutic modalities have been developed in the attempt to target and modulate HER2 expression and dimerization.

A clinically-used approach is the targeting of the extracellular domains (ECD) by monoclonal antibodies, and the subsequent prevention of the dimerization of HER2. Trastuzumab (Herceptin®), a humanized MAb designed to specifically antagonize HER2 function, was approved in 1998 for metastatic breast cancer over-expressing HER2 antigens. Trastuzumab prevents the dimerization of HER2 receptors. Another MAb, pertuzumab, binds to a different sub-domain of the HER2 ECD and blocks the dimerization of HER2 with other HER family members [86].

Other modalities to modulate HER2, include inhibition of phosphorylation of the intracellular tyrosine kinase domain with small molecules and silencing of HER2 by oligonucleotides (asODN and siRNA) [86].

On account of its high level of expression in many cancer diseases and its surface availability on cancer cells, HER2 has naturally become an attractive target for the specific targeted delivery of chemotherapeutics. Many works, both early and recent, have used the Herceptin MAb or its fragments and anti-HER2 affibodies as targeting molecules in various cancer models with different conjugation techniques, and in a wide range of nanocarriers. These include immunoliposomes [107, 108], immunomulsions [52] and inorganic NPs [69].

With regard to polymeric NPs, early works by Steinhauser et al. [109] and Anhorn et al. [110] reported the formulation of HER2-targeting human serum albumin NPs by their covalent attachment to thiolated trastuzumab. The binding procedure optimization, the specific transport of the drug-loaded NPs, their release, and their biological activity were demonstrated in HER2 over-expressing breast cancer cells.

In vitro, targeting and enhanced internalization in breast cancer cells was also reported by Sun et al. for trastuzumab-conjugated poly(D,L-lactide-co-glycolide)/montmorillonite nanoparticles (PLGA/MMT NPs) [111]. A similar conjugation methodology of trastuzumab was adopted by Debotton et al. [87] using a maleimide-bearing cross-linker molecule that was anchored at the interface of PEG-PLA NPs. The superior efficacy of trastuzumab-guided drug immunonanoparticles (INPs) over non-targeted NPs was again demonstrated in an orthotopic metastatic prostate cancer model over-expressing HER2 receptor [87].

Another conjugation approach was undertaken by Nobs et al. [97] and Cirstoiu-Hapca et al. [98] using thiol-functionalized PLA NPs where the MAb is covalently bound by a bi-functional cross linker, m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (sulfo-MBS). These trastuzumab INPs exhibited successful targeting and internalization in vitro in human ovarian cancer cells. Recent works by the same group also reported the formulation and in vitro characterization of paclitaxel-loaded INPs. In vivo, even though no difference in overall tumor accumulation was observed between HER2 targeting and non-targeting NPs, trastuzumab INPs demonstrated superior antitumor efficacy and prolonged survival in a disseminated xenograft model of ovarian cancer [112, 113].

In another work by Gao et al. [114], PE38KDEL immunotoxin-loaded anti-HER2 PLGA NPs exhibited higher efficacy in inhibition of progression of a breast tumor xenograft, with reduced toxicity and immunogenicity as compared to PE38KDEL conjugated...
directly to anti-HER Fab. This clearly demonstrates the possible advantage of the use of polymeric INPs for the encapsulation of therapeutic entities as opposed to direct drug–Ab conjugates.

Other authors have used different moieties such as affibodies and peptides for the targeting of anti-HER2 NPs. For example, Alexis et al. [115] described the preparation of paclitaxel-loaded NPs and the conjugation procedure of an anti-HER2 affibody to poly-(D,L-Lactic acid)-poly(ethylene glycol)-maleimide (PLA-PEG-MAL) copolymer.

1.2. EGFR-Targeting NPs

Like the HER2 receptor, EGFR (ErbB1, HER-1), is a transmembrane receptor tyrosine kinase that belongs to the HER family. EGFR’s dysregulated expression in tumors is a very well-known phenomenon and its over-expression has been reported in many human solid tumors, including, breast, prostate, ovarian, bladder, NSCLC (non-small lung cancer) and glioblastoma [104, 105]. As such, various ligands including EGF, anti-EGFR antibodies (Cetuximab, Erbitux™), antibody fragments and EGFR specific peptides are often exploited as escort molecules decorating the surface of various nanocarriers, for the purposes of EGFR-specific tumor imaging and therapy [116-118].

Acharya et al. have developed a poly(lactide-co-glycolide)-based targeted DDS, carrying high payloads of rapamycin and conjugated to an anti-EGFR antibody [94]. Confocal microscopy demonstrated the preferable cellular uptake of coumarin-6-loaded EGFR targeting INPs in MCF 7 breast cancer cells, as compared to non-targeting NPs. This preferable internalization resulted in an enhanced cytotoxic effect of the encapsulated drug, as determined by the MTT assay and cell cycle analysis [94].

Yu et al. recently reported the formulation of nanocapsules loaded with indocyanine green (ICG) within salt-cross-linked polyallylamine aggregates [119]. ICG was used as a model of a photothermal agent. These nanocapsules were further coated with an anti-EGFR Ab. The authors suggested that the observed thermal toxicity and targeting ability of these nanocapsules (NCs) in cancer cell lines demonstrates the possibility of using the targeting nanocarrier platform for the design of NCs with antitumor photothermal capabilities [119].

In the works of Tseng et al., the epidermal growth factor (EGF) rather than anti-EGFR antibodies served as the targeting moiety of gelatin-based nanoparticles (GPs) [120, 121]. GPs were modified with NeutrAvidin™-biotinylated EGF to form EGF-receptor-seeking NPs (GP-Av-bEGF). Biodistribution studies were carried out in A549 lung-cancer-xenografted mice following aerosol administration of the targeted NPs. GP-Av-bEGF targeted NPs accumulated in the cancerous lung tissue far more than non-targeted NPs. Targeting specificity to cancer cells was clearly demonstrated, as GP-Av-bEGF’s accumulation in vivo in cancerous lungs was 3.6 folds higher than in healthy lungs [120]. These results were further confirmed with targeted cisplatin-incorporating GPs (GP-Pt-bEGF): these targeted GPs exhibited higher in vitro anticancer efficacy in A549 lung cancer cells, and higher cisplatin concentrations in cancerous lungs following aerosol administration, as compared to non-targeted GPs [121]. When discussing targeted drug delivery by NPs, it is usually mentioned in the context of systemically administered therapy. Nonetheless, it seems that local administration could also profit from a targeted DDS, as it is now recognized that one of the major contributions of targeting moieties to therapeutic efficacy is mediated by a higher internalization into the cells and intracellular drug delivery.

The targeting ability of EGFR MAb-modified poly(lactic acid-co-L-lysine) (PLA-PLL-EGFR Mab) NPs was also demonstrated in vitro and in vivo in an SMMC-7721 xenograft hepatocellular carcinoma model [122].

Another work examined the possible use of EGFR-targeting NPs as a safe and effective gene delivery vector for pancreatic cancer where EGFR is highly over-expressed [123]. Hence, an EGFR-specific peptide was conjugated to gelatin-based engineered nanocarrier systems (GENS) using a PEG spacer. Plasmid DNA encoding for enhanced green fluorescent protein (EGFPN1) was encapsulated in the control and peptide-modified GENS. Remarkably, the transgene expression efficiencies were significantly enhanced in Panc-1 human pancreatic adenocarcinoma cells upon plasmid DNA administration via EGFR-targeting GENS, as compared to the control non-targeting NPs [123]. This work exemplifies the potential of targeted NPs to serve as efficient specific transfection vectors intended for targeted gene therapy.

2. Tranferrin Receptor-Targeting NPs

Transferrin receptor (TfR) is a dimeric trans-membrane glycoprotein of 180kDa, that has long been known to be upregulated in many types of malignancies [124]. Transferrin (Tf), the 80 kDa glycoprotein, is formed upon the binding of the circulating apotransferrin to ferric ion. It then binds to the TfR, internalizes into the cell, fuses with the endosome and finally releases the iron ion.

Tf and TfR are extensively studied as tumor-targeting vectors. Over the past years, a great deal of work has been performed and published regarding the targeting potential of nanocarriers to TfR. The receptor-mediated transport system existing for the endogenous peptide transferrin is often exploited to enable DDSs to better cross the BBB in vivo [46, 96, 125]. However, the targeting of BBB will not be addressed in the present chapter. We will only mention a few recent works describing TfR targeting in various cancer models.

A unique transferrin receptor targeting drug delivery system was recently developed by Krishna et al. [126] for the delivery of doxorubicin to a hepatocellular carcinoma (HCC) model: the authors used the apotransferrin protein as a sole scaffold and drug carrier for the preparation of NPs. Both apotransferrin-doxorubicin conjugate-based NPs (conj-nano) and doxorubicin-encapsulating apotransferrin NPs (direct-nano) were prepared and compared. The specificity of both nanocarriers in cellular interactions and uptake was shown to occur through TfR-mediated endocytosis as confirmed by competitive inhibition of the receptor using an anti-TfR antibody. The drug encapsulating NPs however, proved to be more efficient than the drug conjugating NPs in terms of cellular uptake, drug release kinetics and in hepatic localization following intraperitoneal and i.V. injections, with lower accumulation in the heart, kidneys and spleen. Finally, drug-encapsulating apotransferrin NPs induced significant tumor regression in an ascitic HCC rat model with negligible toxicity. The authors suggested this targeted nanocarrier as a promising tool for HCC treatment with the advantage of the nano vehicle’s low intrinsic burden, as apotransferrin is secreted from the cell and recycled [126].

Another work by Zheng et al. describes the use of the transferrin protein as the targeting moiety of lipoid-coated PLGA NPs for the specific delivery of an aromatase inhibitor (7a-APTADD) to breast cancer cells [127]. A slightly different approach was used to conjugate the NPs with the targeting ligand; a post insertion method was adopted to incorporate Tf in the form of micelles (Tf-DOP micelles) to the surface of lipoid-coated PLGA NPs. The resulting targeted NPs exhibited specific uptake by SKBR-3 breast cancer cells and superior aromatase inhibition and cytotoxicity in vitro than that of non-targeted NPs [127].

Interestingly, Sundaram et al. reported that transferrin-surface functionalized PLGA NPs are able to enhance the trans-nasal delivery of plasmin as compared to non-functionalized NPs, and that the use of NPs for the encapsulation of plasmin retains their transfection efficiency following trans-nasal transport. The authors suggest that trans-nasal delivery of such Tf-functionalized NPs could enhance gene therapy at remote target cancer cells [128].
A most recent and exciting work published by Davis et al. [15] reports the results of the first in-human phase I clinical evaluation of a transferrin-targeted siRNA delivery system. The reported nanocarrier consists of a linear cyclodextrin-based polymer with poly(ethylene glycol), where the targeting moiety is the human transferrin protein and the delivered siRNA is designed to reduce the expression of a specific anticancer target, RRM2. The targeting ability and intracellular tumor localization of the Tf-siRNA NPs following systemic delivery is clearly demonstrated by a tumor biopsy taken from the treated melanoma patients. Moreover, the specific gene inhibition mechanism by the targeted siRNA delivery is also reported to be successful, as both the miRNA and the protein levels of RRM2 were reduced as compared to initial pretreatment levels [15].

3. Folic Acid Receptor-Targeting NPs

Folate receptor (FR) is another common targeted epitope in drug delivery research. It is a glycosylphosphatidylinositol-anchored glycoprotein (38–40 kDa). Notably, folate receptor (FR) is highly expressed in several types of solid tumors such as ovarian, uterine, lung, breast, and head and neck cancers [129]. The use of its correspondence, the vitamin folic acid (Folate, FA), as a highly efficient targeting moiety is already long acclaimed due to its small size, high binding affinity for folic acid receptor (FR) (Kd = 10^{-10} M), lack of immunogenicity, high stability, ready availability and low cost [130-132]. Moreover, normal tissues lack FR expression, avoiding any possible deleterious effects on normal cells. Folic acid is reported to be taken up by FRs by a hypothesized process known as potocytosis [133].

Recently, an asODN designed to reduce the production of P-gp was encapsulated in folate-conjugated hydroxylpropyl-chitosan NPs (FA-HPCS- asODNs) [134]. The targeted delivery of the asODN was reported to successfully reverse multidrug resistance as compared to non-targeted administration of an asODN solution or NPs. This effect was clearly demonstrated by the down regulation of the MDR1 gene, the reduced expression of P-gp and most importantly by the enhanced antitumor efficacy of doxorubicin solution, all exhibited both in vitro and in vivo [134]. Another recent work describes the in vivo antitumor efficacy of folate-mediated poly(3-hydroxybutyrate-co-3-hydroxyoctanoate) NPs carrying doxorubicin, in a HeLa xenograft mouse model [135].

On a slightly different note, an interesting green approach was adopted by Narayanan et al. [136], to use folate-conjugated PLGA NPs for the targeted delivery of a non-conventional therapeutic, the polyphenolic phytodrug, grape-seed extract (GSE). The specific uptake and the improved apoptotic index and anti-proliferative effect of GSE by its targeted nanodelivery (FA-NanoGSE) was demonstrated in vitro in various cancer cell lines [136].

A comprehensive pharmaceutical approach was undertaken by Santra and colleagues [137] who recently introduced a multifunctional folate-targeted theranostic nanocarrier, for the concomitant delivery of membrane impermeable proteins and optical imaging probes to tumor cells. The designed nanoparticulate system is based on a novel water-soluble hyperbranched polyhydroxyl polymer (HBPH) possessing a dual encapsulation ability of a hydrophilic, apoptosis inducing cytochrome C protein, and an amphiphilic Near Infra Red fluorescent dye appropriate for in vivo imaging. The authors systematically demonstrated the receptor mediated specific uptake of the cytochrome C loaded folate-HBHP-NPs and the subsequent apoptosis induction in cells over-expressing FR (A549), as opposed to folate receptor negative cells (MCF 7). These in vitro findings further corroborate the generally acclaimed idea that folate-receptor targeting nanocarriers could efficiently target cancerous cells while sparing normal cells. This novel organic biocompatible targeted DDS is suggested by the authors as a potentially applicable multifunctional theranostic tool [137].

4. Prostate Specific Membrane Antigen (PSMA)-Targeting NPs

PSMA is a membrane protein over-expressed on the surface of prostate cancer cells and tumor neovasculature, internalized by clathrin-coated pits [138, 139]. The PSMA expression on malignant cells is not only upregulated but also displayed in a different way; while cancer cells display a full-length surface protein, normal cells display an alternatively spliced cytosolic form [140].

The main methodology reported for PSMA targeted drug delivery has been based on the employment of aptamer-conjugated nanocarriers. However, several works have also employed anti-PSMA antibodies for the targeted delivery of magnetic iron oxide NPs and dendrimers [141, 142].

Farokhzad and colleagues conjugated a PSMA-specific RNA aptamer, A10, to docetaxel-loaded NPs formulated with biocompatible and biodegradable poly(D,L-lactic-co-glycolic acid)-block-poly(ethylene glycol) (PLGA-b-PEG) copolymer. One intratumoral injection of the biconjugated construct, (Dtxl-NP-Apt) to nude mice harboring a LNCaP xenograft elicited significant tumor regression compared to non-conjugated Dtxl-NPs, with no apparent immunogenicity [101]. More recently, the same aptamer–NP conjugates were loaded with cisplatin [143]. Targeted NPs incubated with PSMA-positive LNCaP prostate cancer cells were internalized by endocytosis, resulting in higher cytotoxicity represented by the significantly lower IC_{50} value, as compared to the non-targeted NPs and to the free drug. This was not the case when incubated with PSMA negative PC3 cell line[143]. In addition, the in vivo specific binding of these NPs to PSMA-targeted prostate cancer cells was also demonstrated [84, 144].

Another PSMA-targeting nanoparticulate system was reported by Tong et al. Here, paclitaxel-PLA nanoconjugates were prepared through a unique drug-initiated controlled ring-opening polymerization of lactide followed by nanoprecipitation and conjugation to the PSMA aptamer. The resulting product exhibited efficient in vitro targeting to PSMA-positive LNCaP cells [145].

Chandran and colleagues [146] introduced a urea-based small-molecule peptidomimetic inhibitor of PSMA as the targeting moiety of docetaxel-encapsulating NPs. This targeting moiety also proved to be an efficient vector for the specific targeted binding and uptake of NPs in PSMA over-expressing cells.

5. Targeting Additional Markers

Except the abovementioned surface epitopes which are so often exploited for the targeted delivery of nanocarriers, other tumor antigens are also reported to serve as potential anchors for delivered NPs.

For example, Debotton et al. have demonstrated the in vitro targeting ability of anti-H ferritin INPs in lung and pancreatic cancer cells [147]. Total ferritin is known to be increased and shifted toward acidic (H-rich) ferritin in the serum of patients with various malignancies such as colon, breast and pancreatic cancer. The authors reported the conjugation of AMB8LK, an anti-H Ferritin MAb, to paclitaxel-palmitate-loaded PEG-PLA NPs. Notably, a quantitative evaluation of the molecular-binding affinity of anti-H ferritin INPs by SPR (Surface Plasmon Resonance) demonstrated that the binding affinity of the AMB8LK Ab was not affected by its thiolation and subsequent conjugation to NPs, as the association/dissociation constant and affinity kinetics were similar to those observed for the native antibody [147]. Furthermore, significantly increased uptake of INPs as opposed to plain NPs was demonstrated in both human lung and pancreatic cancer cell lines over-expressing H ferritin. A549 and CAPAN, respectively. Finally, a higher anti-proliferative effect was observed in A549 cells upon treatment with drug-loaded INPs as opposed to plain NPs, indicating the potential application of such a DDS in the treatment of lung cancer.
The epithelial cell adhesion molecule (EpCAM) is a membrane protein that was originally identified as a tumor-associated antigen [148], attributable to its high expression on rapidly proliferating tumors of epithelial origin. Expression of this panepithelial marker EpCAM is known to be upregulated in a variety of carcinomas, including those of the lung and colon. Moreover, it has been associated with metastasis formation. Recent evidence is suggestive of EpCAM to serve as a cancer stem cell marker [149]. Upon binding to specific ligands, the EpCAM receptor is internalized within the cell. Owing to these features, it is a potentially efficient targeting moiety to cancer cells [150]. Indeed, Das and Sahoo most recently reported the conjugation of an anti-EpCAM antibody to nutlin-3a-loaded PLGA NPs [151]. Enhanced uptake of the EpCAM-nutlin-3a-NPs as compared to the free drug and the non-targeted drug NPs was demonstrated in both HCT116 and A549 colon and lung cancer cell lines, respectively. As a result, a superior anti-proliferative effect was observed with the EpCAM NPs, as demonstrated by reduced cell viability, cell cycle arrest and apoptosis. Thus, the overall expression and reactivation of the p53 tumor suppressor gene by nutlin-3a was improved by the targeted approach [151].

Over-expression of ICAM-1 (Intercellular adhesion molecule 1) has also been found in many carcinomas, such as breast, colon, non-small cell lung, renal-cell, pancreas, and gastric carcinomas, as well as in the tumor microenvironments and in other inflammatory conditions [152]. Park et al. reported the use of the I domain of lymphocyte function-associated antigen-1 (LFA-1) as a ligand for the targeted delivery of urethane acrylate nonionomer (UAN) NPs to ICAM-1 over-expressing ovarian cancer cells [152]. UAN NPs encapsulating the water insoluble drug celastrol were functionalized with LFA-1 I domain by a novel high affinity non-covalent method. Using additional LFA-1 I domain mutants with variable binding affinities, the authors demonstrated that the cellular uptake in HeLa cells is dependent on the binding affinity of the ligand. Furthermore, using various cancer cell lines, the authors demonstrated a correlation between the level of UAN binding and ICAM-1 expression on the cells. As a result, the cytotoxicity of celastrol was also affinity dependent and dictated by the chosen targeting ligand [152].

Gly-Ile-Arg-Leu-Arg-Gly (GIRLRG) peptide, isolated by phage display biopanning, was recently reported by Passarella and colleagues [153] as a peptide that selectively recognizes tumors responding to ionizing radiation (XRT) and targets the radiation-induced cell surface receptor, GRP78. Paclitaxel polyester NPs were prepared from poly(valerolactoneoxyvalerolactone-allylvalerolactone-oxepanedione), and were conjugated to GIRLGRG. GIRLGRG-guided NPs yielded increased paclitaxel concentration and apoptosis in irradiated breast carcinomas for up to 3 weeks. Furthermore, a single dose of GIRLGRG-paclitaxel NPs delayed the in vivo tumor tripling time by 55 days in a human breast cancer xenograft, and by 12 days in a syngeneic mouse glioma model. In contrast, these effects of the targeted DDS were not observed in unirradiated tumors, indicating the specific targeting ability to GRP78 [153].

Carcinoembryonic antigen (CEA) is over-expressed in 90% of pancreatic cancers [154] but was originally identified by Gold and Freedman in colon cancer [155]. It was also reported to be present in other malignancies. Hu et al. reported the maleimide-thiol coupling of half antibodies targeted against CEA (hAb) to hybrid PLGA–phospholipids NPs [156]. The hAb-targeted NPs exhibited specific targeting to BxPC-3 human pancreatic cancer cell lines, as opposed to CEA negative XPA-3 cells and to non-targeted NPs. In addition, paclitaxel-loaded hAb-NPs exerted superior cytotoxicity in BxPC-3 cancer cells compared to the non-targeted counterparts [156].

Another recent work describes the use of a lung cancer-targeting peptide (LCP) as a targeting ligand [157]. LCP binds to αvβ3 which is up-regulated in many human non-small cell lung cancers (NSCLC) [158]. LCP was conjugated to a multifunctional polymeric micelle system encapsulating superparamagnetic iron oxide and doxorubicin for both magnetic resonance imaging and therapeutic delivery [157].

An intriguing anticancer approach involves a targeted induction of a pro-inflammatory response at the tumor microenvironment, resulting in a targeted antitumor immune response. This was exemplified by Dominguez and Lustgarten who reported the design of a bi-functional INP, where the anti-neu Ab served as a targeting moiety and the anti-CD40 Ab served as the immunomodulator [159]. The authors reported the ability of the bi functional anti-neu/anti-CD40 INPs to induce an efficient antitumor immune response, eliminating RNEU+ tumors. Interestingly, no effect was observed with the anti-neu INPs or with anti-CD40 INPs, suggesting that the delivery mechanism of the anti-CD40s Ab determines its therapeutic outcome [159]. Notably, the authors reported that animals previously treated with bi-functional INPs did not develop tumors upon re-challenge with tumor cell implantation, suggesting the development of a cellular memory response by the bi-functional INPs vaccine [159].

To summarize, the abovementioned works are only examples of the diversity of available cancer cell markers that are continually being discovered and targeted by nanocarriers.

IV.d. Targeting Tumor Vasculature

1. The Underlying Principles of Targeting Tumor Vasculature

Angiogenesis, the development of new blood vessels, is considered a vital process in controlling tumor growth and metastasis. Folkman demonstrated that all growing tumors are angiogenic [160] and that the expansion of a malignant mass beyond the initial microscopic size is dependent on the recruitment of its own vascular supply. Thus, the development of highly malignant tumors occurs through what is known as the angiogenic switch, driven by angiogenic imbalance favoring the pro-angiogenic state [161]. This imbalance is mainly characterized by increased expression of positive angiogenic regulators (such as vascular endothelial growth factor, VEGF) and decreased expression of endogenous angiogenesis inhibitors (thrombospondin-1, endostatin, and angiostatin) by tumor cells and by stroma fibroblasts [161]. The abnormal features of the chaotic tumor vasculature (leakage, hemorrhage and tortuosity), even though in part, are responsible for the EPR effect of NPs, might result in a markedly decreased delivery of low molecular weight drugs into the tumor tissue and render tumor cells resistant to certain drugs [162]. Indeed, it was demonstrated that VEGF causes impaired delivery of therapeutic agents to tumor cells [163]. Thus, the positive outcome of angiogenesis inhibition on cancer treatment could be related not only to its direct influence on tumor growth and metastasis, but also to indirect effects arising from normalization of the tumor vasculature, which could result in improved drug diffusion and penetration within the tumor.

As opposed to conventional chemotherapeutics aimed at disseminated and distant tumor cells, the site of action of antiangiogenic therapy, endothelial cells, is readily accessible upon intravenous administration. However, the majority of the pharmaceutical considerations that were raised with regard to targeted NPs of cytotoxic drugs also apply for the delivery of the various antiangiogenic inhibitors (small molecules, proteins and antibodies) [164]. Thus, advantages of such a delivery system include potentially improved stability, solubility, pharmacokinetics and most importantly site-specific action with reduced non-specific distribution and toxicities.

2. Targeting Prevalent Epitopes on Tumor Vasculature

In recent years, multiple reported tumor vasculature markers have been described in the context of drug targeting [79]. Amongst them, the αvβ3 integrin is an angiogenic marker that is commonly targeted by NPs. αvβ3 is a receptor for extracellular matrix ligands...
such as vitronectin, fibronectin, fibrinogen, and laminin. While most tissues and cell types are characterized by low levels or absence of αvβ3 integrin expression, it is over-expressed on activated endothelial and smooth muscle cells, especially in tumor blood vessels [165]. It is also known to be over-expressed on some tumor cells, rendering it a dual target receptor, even more appealing and potentially more effective in cancer treatment [79].

The Arg-Gly-Asp (RGD) peptide motif is known to selectively bind to αvβ3 and αvβ5 integrins [166]. RGD peptides and other RGD peptidomimetics have been conjugated to NPs for the targeted delivery of drugs, genes (siRNA) and even radiotherapy both to tumor vasculature and to tumor tissues over-expressing integrins [167-171].

Indeed, as opposed to non-targeted NPs, RGD-grafted paclitaxel-loaded NPs exhibited enhanced in vitro cellular uptake in TNF-α activated endothelial cells, and higher targeting ability in a syngeneic transplantable liver tumor xenograft, resulting in superior tumor growth inhibition and prolonged animal survival [172]. Doxorubicin-loaded human serum albumin NPs were also conjugated to an anti-integrin antibody, (DI17E6), and were also shown to efficiently target integrin-positive melanoma cells [168]. This antibody-mediated targeting resulted in significantly increased cellular uptake and in vitro cytotoxicity in melanoma cells as compared to non-targeted drug NPs and drug solution [168].

Further evidence for successful in vitro and in vivo tumor and vasculature targeting by cRGD-conjugated PLGA NPs were also reported by Toti et al. [95]. A two to three fold enhanced uptake of cRGD-conjugated NPs was observed in various tumor cells (4T1, NCI-ADR/RES, MCF-7) as well as in human umbilical endothelial cells (HUVEC). The specificity of the internalization in a receptor dependent mechanism was also validated in 4T1 tumor cells. Furthermore, a dose response experiment showed that upon saturation of cRGD receptor at high doses of cRGD-NPs, the added value of targeted versus non-targeted NPs is reduced. Interestingly, in vivo studies exhibited enhanced tumor targeting represented by an increased total accumulation of the targeted NPs at the tumor tissue. This is in disagreement with the works of Kirpotin, Bartlett and others [74, 85, 87] who have demonstrated that the targeting mainly affects the NP specific cellular internalization rather than the total accumulation at the tumor site. However, it could be that the discrepancy in various reports as to the biodistribution pattern of targeted NPs is related, among other possible factors, to the differences in the surface properties of the various employed nanocarriers. In fact, a faster tumor accumulation was also reported for folate-targeted liposomes as opposed to the non-targeted controls [173]. This could also be attributed to the recognition of folate-targeted liposomes by tumor-associated macrophages (TAM) [174] which were found to over-express the folic receptor. Thus, multiple factors could have contributed to the accumulation pattern of the reported cRGD-NPs.

RGD-conjugated chitosan NPs were also shown to serve as efficient targeting vectors for siRNA delivery and gene silencing in relevant orthotopic human ovarian cancer models [175].

A slightly different double-targeting approach was undertaken by Sundaram and colleagues, who investigated the combination of tumor cell targeting with tumor vasculature targeting, using drug and gene targeted constructs, respectively [176]. A ligand-cytotoxic drug conjugate (deslorelin-docetaxel conjugates targeted to the LHRH receptor on tumor cells) and RGD-conjugated NPs carrying an anti-VEGF intraperitoneal pilsmard, were concomitantly used to treat lung cancer xenografted mice. Upon weekly intravenous injections, the combination of the two resulted in a significantly greater inhibition of tumor growth than that observed with the individual targeted therapies [176]. This work further strengthens the long-acclaimed hypothesis, that efficient cancer treatment should be based on combinational therapy, with at least two to three synergetic drugs. In fact, combinational therapy is implemented in the clinic, where a cancer patient usually receives a cocktail of drugs rather than monotherapy. This idea was also executed by non-targeted NPs co-encapsulating two anticancer drugs [177, 178]. Such an approach is based on the rationale of simultaneously attacking different cellular and tumor targets, thus achieving pharmacological synergism, and most importantly, avoiding and inhibiting possible salvage or escape mechanisms for cancer cells, eventually reducing drug resistance. This synergism can be further confirmed by the improved overall survival established by the combination of chemotherapy with the anti-angiogenic antibody, Avastin®, in NSCLC patients [179] and by the design of novel tyrosine kinase inhibitors with dual EGFR and VEGFR blockade abilities [180].

The abovementioned reports exemplify the plausibility of using anti-integrin ligands for the targeted delivery of therapeutics to various tumor cells and related tumor vasculature. Nevertheless, there are other angiogenesis cell-surface markers that could be exploited for this purpose. For instance, KDR/Flik-1, also named VEGFR-2, is one of four VEGF binding receptors, and a primary mediator of tumor angiogenesis. As such, it was chosen by Yu et al. as the targeted aperture for the enhanced delivery of paclitaxel-loaded NPs to tumor vasculature [181]. Indeed, the conjugation of a KDR-binding peptide, K237, to NPs (K237-Ptx-NPs) established a favorable internalization into HUVEC cells, leading to their reduced proliferation, migration and tube formation. These results were reinforced in vivo in a breast cancer xenograft model; confocal microscopy of tumor tissue sections revealed an accurate targeting of the tumor neovasculature following intravenous administration of fluorescent K237-Ptx-NP to tumor bearing mice. This in turn induced significantly higher apoptosis of endothelial cells and subsequent tumor necrosis [181].

Other tumor angiogenesis cell-surface markers that could potentially be targeted by ligand conjugated NPs include nucleolin, annexin, plectin and more [79]. In addition, ICAM-1, is constitutively expressed at endothelial cells, and it can be further upregulated by pathologic factors such as endotoxins, cytokines, oxidants and other inflammatory signals [182]. Such inflammatory processes may take place at the tumor microenvironment, thus promoting angiogenesis and tumor growth [183]. Indeed, ICAM-1 has been used as the targeting moiety for various NPs to endothelial cells, even in non-cancerous conditions [184, 185].

IV.e. Targeting Tumorous Lymph Nodes

The lymphatic system is an important route of tumor metastases dissemination and many cancers preferentially spread through the lymphatics [186]. Several mechanisms may give rise to lymph node metastases from a primary tumor: cancer invasion into intratumoral lymph vessels, invasion into pre-existing lymphatics at the tumor periphery or growth of new lymphatics induced by tumor cells. Lymphatic vessels are reported to be relatively abundant in the periphery of tumors [187, 188] although poor lymphatic drainage occurs within the tumor [188]. Moreover, the number of lymphatics in the surroundings of a tumor, (and possibly their size), and the expression of lymphangiogenic growth factors could be important determinants in the ability of a tumor to metastasize [186, 188, 189]. However, target-specific NPs for drug delivery to lymphatic metastases are not common due to the lack of specific markers in the tumor lymphatics. A proof of concept of such a delivery system was reported recently by Luo and colleagues [190], using a unique lymphatic targeting moiety, LyP-1. LyP-1.a cyclic 9-amino acid peptide, binds to the lymphatic vessels in certain tumors but not to the lymphatics of normal tissues [189, 191]. LyP-1-conjugated PEG-PLGA NP (LyP-1-NPs) exhibited an eight fold increased uptake in metastasis lymph nodes as compared to non-targeted NPs, demonstrating the potential ability to deliver drugs specifically to lymphatic metastasis [190].
IV.f. Tumor-penetrating Peptides

A recent advancement in the field of molecular biology which seems to possess an enormous impact on drug delivery in cancer treatment is the discovery of a tissue cell penetration system described by Ruoslahti and colleagues [192]. The possibility to design peptides with both tumor-homing properties and a peptidic tissue penetration-enhancing motif, designated as R/KXXXR/K, should promote both the specific recognition of the tumor cells and also improve tumor penetration ability of the currently available targeting ligands. As previously explained, in many (if not most) reported cases of targeted DDSs, the overall tumor accumulation, when compared to the EPR of non-targeted NPs, is not affected by the active targeting. The resultant benefit of targeting arises mainly from the endocytic intracellular delivery at the tumor cell level, even when both angiogenesis targeting and tumor targeting are combined. However, the added value of a tumor penetration system would be an increase in total tumor accumulation of NPs, or as explained by Ruoslahti et al.,[79] increased volumetric concentration of the targeted NPs at the tumor site. Indeed, this was clearly demonstrated in two recent reports with tumor-penetrating-peptide-targeted NPs:

Karmali et al. [193] reported the targeted extravascular delivery of LyP-1 conjugated abraxane® NPs in MDA-MB-435 human cancer xenografts. The aforementioned LyP-1 peptide, which binds to tumor lymphatics, was shown to possess extravasation properties. Thus, the targeted NPs were found to co-localize with extravascular tumor islands that express the respective p32 receptor, resulting in significant tumor inhibition. Untargeted abraxane on the other hand, was detected in the form of a faint meshwork in the tumor interstitium [193].

Sugahara et al. reported similar extravascular parenchymal accumulation in tumor tissue when NPs were functionalized with iRGD [194].

The current accepted mechanism of action of these peptides is mediated through the R/KXXXR/K peptidic motif present at the C terminal end of the ligand (CEndR) in a cryptic form. For example, iRGD contains both the integrin binding RGD sequence and the tumor penetrating CEndR motif. Upon binding of the iRGD to the integrin, the cryptic CEndR of the tumor penetrating peptide is proteolyzed, exposing the C-terminal which in turn binds to a neuropilin-1 (NRP-1) receptor [194]. The latter mediates extravasation, tissue penetration and cell uptake of the bound peptide and any cargo that it carries. Hence, this penetration system merges the two unique features of both tumor-homing ligands and of cell penetration peptides such as the non-specific HIV-related Tat protein, resulting in tumor-specific extravasation. However, it is hypothesized that there are still other unraveled direct and indirect mechanisms for tumor-penetrating peptides, and specifically for the iRGD peptide, which might also contribute to the observed pharmacological effects and positive outcome [195].

Currently, this approach may be conceived as an extension of the ammunition of targeting ligands, especially when dual neovasculature and tumor cell targeting ligands like RGD are employed [195].

IV.g. Targeting Non-solid Hematologic Malignancies

Various nanocarriers such as liposomes and other lipid NPs are also reported to be a potential targeted treatment of various hematologic malignancies, using ligands that target specific markers expressed on various cells of the immune system [196-198]. Polymeric NPs were also described as plausible targeting systems in hematologic malignancies. For example CD8-targeting NPs were suggested as drug delivery systems for lymphoblastic leukemia treatment [199]. Additionally, LFA-1 (Lymphocyte function associated antigen-1)-targeting NPs may be applicable for various leukemic diseases [200].

V. THE CELL SURFACE - OUR FINAL DESTINATION? The Intracellular Trafficking and Bio fate of Targeted Nanocarriers: Aiming the Bullet at the Right Intracellular Destination

We often tend to determine a nanocarrier's success by one main endpoint - shrinkage of the tumor mass in animal models. This judgment of success or failure based on this macroscopic endpoint sometimes disregards other important mechanistic factors of the DDS. In the previous section we revealed new exciting vectors with the potential to further enhance the accumulation of the NPs at the target site. So, it seems we have unveiled the optimal ways to arrive to the long-awaited destination, the tumor cell surface. However, a long underestimated question relates to the bio fate of the specifically-delivered nanocarrier and its drug cargo once it reaches the harboring point at the tumor endothel or even at the tumor cells. As previously explained, ligand-conjugated NPs enhance intracellular delivery by receptor-mediated endocytosis. However, endocytosis is only the starting point of internalization and a potential portal for a much more complex cytosolic path, dictating the carrier's intracellular trafficking, compartmentalization and degradation. Hence, in order to develop an efficient cytosolic delivery system, there is a clear need to understand the molecular mechanisms and the various aspects of the intracellular trafficking of NPs and their nanocarrier and its cargo between sub-cellular compartments such as the endoplasmic reticulum, golgi apparatus, endosomes (late and early), lysosomes and the plasma membrane. For example, particles entering the cell through an endocytic pathway will be trapped in the endosome, and to some extent will be degraded in the lysosome. Thus, the intracellular track through which the delivered nanocarrier passes will dictate its degradation, its recycling and the effective amount of therapeutic cargo that will eventually reach the desired target inside the cell.

Generally, if we undertake a simplistic approach, the parameters that influence the NP cell interactions and subsequent intracellular bio fate can be divided into two main categories: DDS-related factors and cell-related factors. DDS-related factors include the size, the surface charge, the targeting ligand (for example a MAb with innate ability for endocytosis mediated through a specific receptor or a cell-penetrating peptide), the polymer type and the polymer’s molecular weight. While the size and surface charge might influence the cell-surface interactions and specific cellular pathways of uptake [201], the polymer type and weight might influence the rate of the NP biodegradation in the cell. The choice of pH-responsive polymers will also influence both the rate and the site of NP degradation and their cargo release. For example, HBPH NP stability in pH=7.4, prevents the payload’s constitutive release to the aqueous milieu [137]. Thus, traceable cargo is released only in the presence of esterases and acidic pH, which are found in endocytic vesicles, upon receptor-mediated cellular uptake [137]. Plausibly, such NPs are suggested as suitable for the controlled release of therapeutics at the acidified microenvironment of carcinomas that arise from the Warburg effect and enhanced glycolysis [137]. Thus, cell-related factors that might influence the intracellular degradation of NPs include endocytic vesicles, pH changes and the presence and levels of esterases. These issues have been discussed and reviewed in detail by Vair and Labhasetwar [43] and Harush-Frenkel et al. [202].

Furthermore, a thorough knowledge of a trafficking pathway can be used to maximize the efficient intracellular delivery via a specific carrier protein. Hence, effective intracellular drug delivery requires a comprehensive understanding of the relevant cellular physiological and pathological processes. For example, an early work of Muro et al. tracked down in detail the intracellular trafficking of anti-ICAM-1 Ab-conjugated NPs in endothelial cells and investigated some factors which might affect it [184]. Nevertheless, with regards to targeted NPs, even if the exact trafficking mechanism of the targeting ligand is well established, one cannot assume that the ligand will preserve its intracellular trafficking behavior.
upon nanoconjugation. This question has been recently raised by Bhattacharyya et al. [203] who have investigated the influence of nanoconjugation on the mechanism and pattern of cetuximab MAb-induced EGFR endocytosis in various primary and metastatic pancreatic cancer cells. The authors reported that the conjugation of cetuximab Ab to gold NPs promoted faster endocytosis of EGFR as compared to the native Ab, and altered the endocytic patterning of the receptor in the cells. Furthermore, conjugation of the antibody to gold NPs altered the intracellular trafficking of EGFR to the specific organelles and influenced the mechanism of endocytosis. This shows that the nanoconjugation process in not merely an innocent chemical bond between the Ab and the NPs, and even if the binding affinity of the Ab to its receptor is preserved upon conjugation, subsequent intracellular processes may be altered at the molecular level.

The targeted cells' specific phenotype is another key factor that is poorly addressed as a contributor to ligand and NP intracellular biofate. Indeed, it was demonstrated by Bhattacharyya et al. [203] that the targeted cell type, of primary or metastatic origin, significantly influenced endocytosis patterns and intracellular trafficking, induced by either a free antibody or by Ab-conjugated NPs [203]. This was also exemplified by Barua and colleagues [204] who demonstrated that unconjugated anionic quantum dots exhibit dramatically different intracellular localization profiles in three closely related human prostate cancer cells, PC3, PC3-flu and PC3-PSMA [204].

The recent insights into the intracellular trafficking of internalized targeted constructs reveal many uncovered research fields and unanswered questions. The vivid understanding of these mechanisms will help to design clever DDSs which will be specifically delivered not only to the desired cell, but to the specific desired cell organelle [205]. Surely, a comprehensive targeting approach of DDS should take into consideration all the above mentioned factors for the successful translation of biomedical nanotechnology into clinical practice.

VI. CONCERNS AND HURDLES

Specific drug targeting has evolved as the most desirable goal in cancer treatment. The platform provided by nanosized targeted delivery systems offers opportunities and advantages for the design of efficient individualized treatments and multifunctional theragnostic carriers. The numerous publications reviewed in the present chapter present encouraging results as to the versatility, feasibility and in vivo efficacy of this approach in various tumor models. The successful clinical translation of these carriers could result in an optimal tool of high therapeutic significance in oncology following:

1. Individual molecular screening of the tumor to isolate the specific epitope that should be targeted.
2. Individualized therapy using tailor made delivery system with the relevant targeting ligand based on the molecular screening.
3. Sensitive and specific monitoring of tumor response with targeted diagnostic imaging NPs.

In the past few years, with the understanding of cancer's mutli factorial and complex nature, the concept of active targeting has expanded and is now extending to the multiple components and aspects of the disease. Cancer is a pathology implicating various interconnected signaling pathways and it induces changes in various cells promoting tumor growth and comprised in the tumor mass. Indeed, tumor mass components include parenchymal cells (for example epithelial cells) as well as angiogenic endothelial cells, various stromal cells composing the connective tissue of the tumor (pericytes, fibroblasts) and lymphatics. Thus, a comprehensive approach for targeted cancer therapy would be a multi pronged process, taking into consideration the complexity of the tumor mass and the microenvironment surrounding it. In light of these insights, an ideal treatment should combine a delivery system targeting concomitantly tumor cells, vasculature, lymphatics and metastases (Fig. 3).

Such a multi-targeted construct may be tackled by various approaches:

- A cocktail of various targeted NPs where each kind targets a different tumor component.
- A single targeted NP conjugated to a single ligand able to recognize various tumor components (tumor cells, angiogenic endothelials and tumor lymphatics). For example, anti-avrF3 integrin NPs might exhibit such an effect on specific tumors.
- A single targeted NP conjugated to various ligands where each ligand is aimed at a different target, thus enabling the same NP to bind to multiple targets.

The payload of the targeted NPs may be a drug, a gene-silencing sequence or a radioisotope, or even a combined payload. Thus, each of the abovementioned approaches offers unlimited possibilities and combinations of NPs and therapeutic payloads.

The choice of the most appropriate approach will be based on both pharmaceutical and physiological considerations, starting from the feasibility of designing and up-scaling such a DDS up to the clinics.

Having the path laid by a plethora of preclinical evidence demonstrating the tremendous potential concealed in targeted nanocarriers, it seems that we have come to the dawn of a new era in cancer treatment. And yet, despite nearly three decades of extensive research, the number of targeted nanocarriers that have actually reached clinical trials and essentially the market is limited. The translation of these targeted NPs from laboratory practice to clinical settings could face various scientific obstacles and regulatory difficulties. These issues have also been raised by Davis et al.[26].

First, the formulation complexity of these targeted DDSs, could produce logistic difficulties anywhere from laboratory to industrial settings: issues such as reproducibility and long-term stability of the product, which are usually not fully addressed in the preclinical setting, might complicate the manufacturing procedure and significantly increase the cost of this already expensive nanotechnology.

Despite the potential therapeutic benefits of NPs, a great deal of concern has been raised regarding their safety and possible toxicities. Many works have addressed these issues in an attempt to better elucidate the main contributors to possible toxicities. However, the long-term implications of these nanomaterials on human health are not known, and will surely require further investigation. Other uncertainties concern the targeting moiety. Even though the targeted epitope should ideally not be expressed on normal cells, most cancer markers have some basal level of expression on normal tissues as well. In some cases, the expression on normal cells could be influenced and induced by other co-existing conditions, such as inflammation. Thus, it is unknown whether the basis for the selectivity of the target will be maintained in real clinical settings, where co-morbidities exist. Hence, induced non-tumor expression of the targeted epitope could result in exacerbated toxicities.

Furthermore, it should be kept in mind that efficacy data of targeted therapeutics in animal models, usually murine or human xenografts or orthotopic, cannot always be projected to clinical settings, as these models do not accurately represent the actual cancer pathophysiology in humans. Additionally, the genetic and phenotypic patient heterogeneity which is not present in carefully selected animals, might highly influence treatment success. Thus, even when positive preclinical evidence is achieved, the outcome in clinical trials is unpredictable.

CONCLUSION

With the extensive ongoing research of targeted drug delivery nanocarriers, we have elucidated some of the key factors for efficient targeting. However, the optimal conditions for a clinical suc-
cess in humans remain somewhat elusive, as there are still many unraveled issues. Primarily, the intracellular trafficking of targeted DDS is not fully understood. Even though many recent insights and publications have been made regarding the intracellular behavior of internalized NPs, one cannot deduce any universal or generalized conclusions for all targeted NPs and surface epitopes. Most importantly, the intracellular behavior is also affected by the phenotype of the targeted cell. In a clinical setting, this might have a huge impact on the treatment outcomes, as cancer patients might exhibit various disease phenotypes. A better understanding of the intracellular trafficking and final biofate of the targeted NPs might turn out to be of great influence on treatment outcomes. Such knowledge might significantly contribute to a clever design of organelle-specific NPs. Indeed, targeted cancer treatment is a highly challenging and extremely complicated task. However, the most recently trialed CALAA-01 (targeted siRNA NPs) proves that up-scaling and clinical translation of this complex nanotechnology can be accomplished, and brings hope for additional successes.

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