

Chapter 2

Improved Targeting of Cancers with Nanotherapeutics

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Abstract

Targeted cancer nanotherapeutics offers numerous opportunities for the selective uptake of toxic chemotherapies within tumors and cancer cells. The unique properties of nanoparticles, such as their small size, large surface-to-volume ratios, and the ability to achieve multivalency of targeting ligands on their surface, provide superior advantages for nanoparticle-based drug delivery to a variety of cancers. This review highlights various key concepts in the design of targeted nanotherapeutics for cancer therapy, and discusses physicochemical parameters affecting nanoparticle targeting, along with recent developments for cancer-targeted nanomedicines.

Key words Targeting, Antibodies, Ligands, EPR, Cancer, Oncology, Nanoparticles, Nanotherapeutics, Nanomedicine, Multivalency, Drug delivery, Translation

1 Introduction

Cancer chemotherapy is fundamentally limited by dose-limiting toxicity. This has long been true of nonspecific cytotoxic agents that are still the most widely used anticancer therapies, which in addition suffer from a low therapeutic index. The last 20 years has seen the development of molecularly targeted agents, which have an improved toxicity profile, especially for acute events. However, these newer agents are often given continuously over longer time periods and chronic toxicity remains a key limiting factor [1, 2].

With our improved understanding of cancer biology and pathways, targeted agents and cancer immunotherapy approaches have gained considerable interest and investment, resulting in positive outcomes, and a rise in the use of these treatments. Targeted agents primarily include antibody drugs that are capable of specifically blocking proliferative cancer pathways, and immunotherapy approaches being aimed at priming the patient's own immune system to attack and destroy cancers [3, 4]. Surgery and radiotherapy are also still routinely used in the arsenal of anticancer treatments—along (or in combination) with chemotherapies,

targeted therapies, immunotherapy or newer treatments such as adoptive cellular therapy [5]. Cancer nanomedicines are intended to add to this arsenal of therapeutics, by packaging and specifically delivering existing chemotherapies to where they are needed the most: within cancer cells.

Nanoparticles (NPs) enable the encapsulation of poorly soluble drugs, protection of a variety of therapeutic payloads from blood components, increase systemic circulation times and improve bio-distribution, leading to minimized systemic toxicities [6]. These properties are ideal for oncology applications where systemic toxicity is a major issue. Doxil, which is the first anticancer nanomedicine to enter the clinic, is liposome-encapsulated doxorubicin, which was shown to drastically increase the systemic circulation half-life of doxorubicin (Dox) from 0.8 h to 2 days, in addition to reducing cardiotoxicity [7]. Albumin associated paclitaxel (nab-PTX) has also led to higher tolerated doses [8].

Once injected into systemic circulation, the localization of NPs within the body can be influenced via “passive” or “active” targeting strategies. The term passive targeting describes the accumulation of NPs bearing no affinity ligands at disease sites, where the degree of accumulation depends on their inherent physicochemical properties (size, shape, charge, flexibility, etc.). If suboptimal, these properties may also impede the effective concentration of NPs at active sites due to increased sequestration by the mononuclear phagocytic system (MPS), limiting their systemic concentration and potential to extravasate into target tissues [9]. Active targeting is a term used to describe the mode of action of NPs with surface-bound affinity ligands having specificity to diseased tissue and/or cells. Actively targeted particles rely on the principle of passive targeting discussed above, but targeting ligands have an additional effect, aiding accumulation in the tumor or uptake of NPs into cancer cells via endocytosis, or both.

The development of actively targeted NPs was facilitated by the maturation of antibody technologies, together with techniques for bioconjugation of targeting moieties including antibodies, antibody fragments, peptides, aptamers (Apts), sugars, and small molecule ligands to the surface of NPs [10–13]. Although more than 30 years has passed since the first targeted NPs were developed, only a handful have reached clinical translation, and none have yet been approved [14–17]. The lack of effective translation of targeted NPs could be attributed to the following limitations; (1) our insufficient understanding of events at the nano–bio interface both *in vitro* and *in vivo*; (2) our inadequate knowledge of the fate of NPs at the body, organ, and cellular levels; (3) difficulty in achieving reproducible and controlled synthesis of targeted NPs at larger scales; (4) lack of technologies enabling screening of a large number of targeted NP candidates under biologically relevant conditions that could be reliably correlated to clinical performance; (5) potential

lack of or low occurrence of the so-called enhanced permeation and retention effect (EPR) in patient tumors, and; (6) inter- and intra-patient heterogeneity in receptor expression levels. In this review we discuss effective targeting of tumors via passive and active mechanisms, and highlight the various tumor biology and NP physicochemical properties that affect targeted nanodrug delivery for oncology applications.

2 Passive Targeting

The growth of a solid tumor requires development of a blood supply and lymphatic drainage. However, the development of these systems is pathological and this has profound implications for delivery of nanomedicines. Characteristics of the tumor vasculature such as excessive branching, chaotic structures, enlarged gaps between endothelial cell lining of vessel walls and associated breakdown of tight junctions, and a disrupted basement membrane, facilitate the extravasation of particulate materials from vessels into tumor tissues [18, 19]. An impaired lymphatic drainage system further entraps macromolecular particles and delays their clearance. Starting in the 1980s, the observation of increased accumulation of macromolecules and colloids such as the polymer-drug conjugate poly(styrene-co-maleic acid)-neocarzinostatin (SMANCS) in tumor tissues led to the term “enhanced permeability and retention” (EPR) effect [20]. Together with grafting of polyethylene glycol (PEGylation) to the surface of NPs (to enhance systemic circulation), this has become the most widely exploited concept in nano-oncology applications [20–24]. The EPR effect has been observed with a wide range of macromolecular agents such as: proteins, including immunoglobulin G (IgG); drug-polymer conjugates; micelles; liposomes; polymeric NPs and many other types of NPs [25–28].

The distribution of NPs within tumors begins with margination toward the vascular wall, followed by extravasation from the blood vessels, diffusion throughout the extravascular tissue and interaction with extracellular and intracellular targets [6]. The degree of vascular permeability in patients is far more heterogeneous than in pre-clinical models. Variable tumor microenvironments can affect the cutoff size for NP accumulation in tumors, restricting their effective penetration range, and accounting for the lack of observable EPR effects in certain tumor types [29, 30]. The negative pressure gradient present within the tumor interstitium can substantially limit the convection of NPs from the intravascular to the extravascular space within tumors, regardless of the presence of leaky vasculature [29, 31, 32].

The EPR effect is more highly multifaceted than first thought. Only recently are we beginning to understand the contribution of other tumor microenvironment (TME) related parameters such as

blood circulation in tumors, extravasation to the perivascular tumor microenvironment, tumor-associated macrophages (TAMs), fibroblasts, collagen, penetration distance within tumor tissue, tumor cell internalization and intracellular trafficking (and other tumor matrix components). Although the EPR effect has been the main principle governing the use of nanomedicines for tumor therapy, a number of important limitations need to be considered. The main observations of EPR have been studied in small animal subcutaneous or orthotopic xenografts, and genetically engineered mouse models [33]. These settings are drastically different from tumors in humans where the EPR effect may not be present or manifested in a similar manner. Other than the observation of marginal progression-free survival in the case of ovarian cancer patients (receiving Doxil as a second line therapy or platinum-sensitive cohorts), passively targeted NPs up to now have not led to substantial improvement in patient survival rates, which may suggest patient populations with lowered tumor susceptibility to NPs and/or lack of EPR effect [6, 34–36]. More importantly, it appears that the EPR effect can also vary substantially between patients, with variability arising even within the same patient or tumor type [6]. To circumvent these problems, in the same manner that molecularly targeted treatments are currently offered to patients following genetic profiling of their tumors, more personalized approaches could be envisioned for the future where “nanomedicine-responsive” patient populations can also be identified. Research on companion diagnostics is helping to address this complex problem. In preclinical models, iodine loaded liposomes have been used to predict animal cohorts that were responsive to nanotherapeutics [37]. In another recent preclinical study magnetic resonance imaging (MRI) was used to predict treatment response and drug accumulation using co-administered 30 nm magnetic iron oxide NPs and paclitaxel encapsulated polymeric NPs in tumor-bearing mice [38]. In the clinic, a study was initiated to ascertain the safety of co-administering ferumoxytol as a tumor imaging agent, in addition to irinotecan-loaded liposomes (clinicaltrials.gov, NCT01770353). These approaches have yielded valuable insights into the in vivo kinetics of NP biodistribution and demonstrated how clinically relevant imaging modalities and agents can be utilized to select patients with high EPR and to predict therapeutic nanomedicine efficacy using companion diagnostics.

3 Active Targeting

Active targeting describes the use of affinity ligands to direct NP binding to antigens that are overexpressed in diseased tissue. Actively targeted particles rely on the principle of passive targeting

Table 1
Examples of targeting ligands and targeted NPs

Ligand Type	Nanoplatfrom	Target	Indication (Ref)
<i>Antibodies and fragments</i>			
• F(ab') ₂	Liposome	Non-muscle myosin heavy chain type A	Gastric cancer [63]
• F(ab')	Liposome	HER2	Breast cancer [144]
• scFv	Liposome	HER2	Breast cancer [58]
<i>Proteins</i>			
• Transferrin	Polymeric NPs	Tf receptor	Cancer [59]
• Ankyrin repeat protein	siRNA complexes	EpCAM	Cancer [145]
• Affibodies	Polymeric NPs	HER2	Breast cancer [146]
<i>Peptides</i>			
• CGNKRTRGC (LyP-1)	Protein NPs	gC1qR (p32)	Cancer [147]
• F3 peptide	Iron oxide NPs	Nucleolin	Cancer (imaging) [148]
• iRGD	Iron oxide NPs	αvβ3/5	Cancer (imaging) [149]
• iRGD	Polymeric NPs	αvβ3/5	Cancer [150]
• KLWVLPKGGGC	Polymeric NPs	Collagen IV	Inflammation [151]
• KLWVLPK	Polymeric NPs	Collagen IV	Vascular wall [152]
• SSPIQGSWTWENGK-WTWRGIIRLEQ	Iron oxide	Fibronectin	Cancer (Imaging) [153]
• SSPIQGSWTWENGK-WTWRGIIRLEQ	Liposomes	Fibronectin	Cancer
<i>Nucleic acid ligands</i>			
• A10 aptamer	Polymeric NPs	PSMA	Prostate cancer [154]
• A9 CGA aptamer	Gold NPs	PSMA	Prostate cancer [155]
<i>Small molecule ligands</i>			
• Folic acid	Liposomes	FA receptor	Cancer [56]
• Folic acid	Polymeric NPs	FA receptor	Cancer [156]
• TPP	Polymeric NPs	Mitochondria	Various [157]
• ACUPA	Polymeric NPs	PSMA	Cancer [158]

HER2: human epidermal growth factor receptor 2. Tf: transferrin. EpCAM: epithelial cell adhesion molecule. PSMA: prostate specific membrane antigen. FA folic acid, TPP triphenyl phosphonium, ACUPA 2-[3-[5-amino-1-carboxypentyl]-ureido]-pentanedioic acid

discussed above, but the addition of ligands can aid accumulation in the tumor and uptake of NPs into cancer cells via endocytosis.

A wide variety of targeting ligands are used to create targeted NPs (Table 1). These ligands are often directed toward overexpressed receptors or antigens on proliferating cancer cells. Important parameters that should be taken into account when choosing a targeting ligand include binding affinity, ligand size, immunogenicity, and cost of manufacturing. The ideal ligand should also facilitate deeper tumor penetration.

The first antibody to be approved for clinical use was muromonab-CD3 (an immunosuppressive agent) in 1986 [39]. Since then numerous antibody platforms have been developed

including murine, chimeric, humanized and human monoclonal antibodies (mAbs) [40]. For example, rituximab (Rituxan) is a chimeric mAb, which binds to CD20, and was approved for the treatment of non-Hodgkin's lymphoma in 1997 [41]. Trastuzumab (Herceptin) which was approved for the treatment of breast cancer in 1998 is a humanized mAb that binds to the HER2/neu antigen [42]. Natural or fully synthetic antigen-binding fragments (Fab and Fab' ~ 50 kDa), variable fragments (Fv ~ 15 kDa), and single chain variable fragments (scFv ~ 30 kDa) of antibodies have been engineered and tested [43]. Antibody fragments are engineered to control properties such as affinity (K_D usually lower than 1 nM) or internalization capability. The advantage of antigen-binding fragments is that they lack the Fc-antibody region, which is the most immunogenic component of antibodies.

Several other classes of binding ligands, including antibody mimetics, peptides, nucleic acid ligands, and small molecules, have been developed against a number of target antigens [43]. These types of ligands can also be conjugated to radioisotopes or drug molecules to create more effective targeted imaging and therapeutic modalities [44–47]. Similar ligands can be conjugated to the surface of NPs in order to achieve antigen-specific active targeting [43, 48]. In contrast to antibody–drug conjugates, which typically carry 1–8 drug molecules, targeted NPs are capable of carrying up to 10^5 drug molecules, allowing for a higher amount of drug delivery per bio-recognition or binding event.

For effective cancer therapy, drug encapsulating NPs should ideally be delivered within cancer cells, and therefore therapies that act on intracellular active sites are most effectively delivered with targeted NPs since this facilitates uptake of NPs via either clathrin-dependent endocytosis, caveolin-assisted, cell adhesion molecule directed, or lipid raft associated mechanisms [49]. In the case of hydrophobic small molecule drugs that can easily permeate through the endosomal lipid bilayer, NP entrapment within endosomes following endocytosis can still lead to effective intracellular drug concentrations as the drug molecules are released from the carrier over time. In contrast, for the effective delivery of biological macromolecules such as nucleic acids (DNA, siRNA, miRNA), charged/and or hydrophilic small molecule drugs (that are impermeable to the endosomal membrane), endosomal escape is an important prerequisite [50]. This is crucial since intracellular payload release should occur prior to fusion of endosomes with lysosomes where biological payloads can be degraded as a result of low pH levels [51]. Studies to identify mechanisms that lead to endosomal escape based on pH buffering and osmotic swelling, which cause endosome bursting or endosomal membrane destabilization for the purposes of effective subcellular drug delivery are helping to further understand this effect [52–55].

Ligand mediated cell internalization can result in therapeutic benefits as compared to equivalent non-targeted NPs [56–59]. For example, accumulation of siRNA-loaded NPs at tumor sites is largely a function of effective EPR via passive targeting; however, cellular internalization and effective gene silencing are largely a function of targeting ligands, which facilitate intracellular uptake. Therefore, the colloidal properties of NPs determine their biodistribution, whereas the targeting ligand serves to facilitate and enhance cellular uptake at specific sites [60]. For effective tumor targeting and margination and extravasation of NPs for cancer therapy deeper tumor penetration and retention is important. Targeting strategies that focus on the TME can also be utilized to improve penetration. For example tumor-specific penetrating peptides such as iRGD with a R/KXXR/K C-terminal peptide motif have been used to stimulate neuropilin-1-mediated vascular permeability [61].

For targeting of nanomedicines in oncology applications parameters such as ligand binding affinity (K_D) and receptor expression levels throughout the treatment period also need to be considered.

3.1 Clinical Stage Actively Targeted Nanoparticles

Over the last four decades since the first actively targeted NPs were reported [62], only a handful have progressed to clinical trials (Table 2). MCC-465, the first targeted NP to enter trials, consists of liposome encapsulated Dox with surface bound PEG for immune shielding and dimers of F(ab') fragments for targeting [15]. The F(ab')₂ used is a fragment of the tumor specific human mAb (GAH), which has shown affinity to >90 % of human stomach cancer cells [15]. MCC-465 exhibited significant antitumor response against GAH-positive xenografts leading to 80 % reduction in tumor mass [63]. Phase I trials with MCC-465 were carried out in order to determine the maximum tolerated dose and further dosing regimens. MCC-465 does not appear to have progressed through clinical development after phase I completion.

Anti-EGFR (epidermal growth factor receptor) immunoliposomes loaded with doxorubicin (anti-EGFR ILs-dox) have been tested in the clinic on patients with advanced solid tumors overexpressing EGFR no longer amenable to standard treatment and a maximal tolerated dose was defined (50 mg/m²) from the phase I trial [64]. MM-302 is a HER-2-targeted PEGylated liposome that encapsulates doxorubicin for delivery to HER-2-overexpressing tumor cells, it is currently undergoing phase II and III trials for breast cancer and HER-2 positive breast cancer respectively [65]. MM-302 has been recently shown to improve the antitumor activity of oxaliplatin in HER-2 positive breast cancer, when administered after cyclophosphamide priming [66]. The overexpression of Tfr, EGFR, and HER-2 occurs in a range of cancer types, making these cancer ligands attractive targeting strategies for drug delivery [67].

Table 2
Actively targeted nanoparticles in the clinic

Name	Nanopatform	Targeting	API	Dose (Recommended)	Size (nm)	ζ potential (mV)	Ref
MCC-465	Liposomes	Metastatic gastric cancer, (Ab fragment)	Doxorubicin	6.5–45.5 mg/m ² (32.5 mg/m ²)	143	-	[15]
anti-EGFR-ILs- dox	Liposomes (Doxil [®] / Caelyx [®])	Solid tumors, EGFR (Ab, cetuximab)	Doxorubicin	5–60 mg/m ² (50 mg/m ²)	100–120	-	[64]
MM-302	Liposomes	HER2+ breast cancer, Her2 (Ab)	Doxorubicin	8–50 mg/m ²	80–100	-	[159] [65]
MBP-426	Liposomes	Gastric, esophageal, gastroesophageal Adenocarcinoma, Tf-Receptor (Tf)	Oxaliplatin	6–400 mg/m ² (226 mg/m ²)	-	-	[66, 160]
SGT-53	Liposomes	Solid tumor, recurrent glioblastoma, metastatic pancreatic cancer, Tf-Receptor (Ab scFv)	p53 plasmid DNA	0.6–3.6 mg (2.4 mg)	114	28.2	[161]
SGT-94	Liposomes	Solid tumor Tf-Receptor (Ab scFv)	RB94 plasmid DNA	-	207	6.6	[162]
2B3-101	Liposomes	Brain metastases, glutathione transporter (glutathione)	Dox	-	95	-	[163]
BIND-014	Polymeric NPs	Non-small-cell lung, metastatic castration-resistant prostate cancer, others, PSMA (small molecule)	Docetaxel	15–75 mg/m ² (60 mg/m ²)	100	–6	[158]
CALAA-01	Polymeric NPs	Tf-Receptor (Tf)	siRNA M2 subunit of ribonucleotide reductase	18–30 mg/m ²	70	15	[164]

Table adapted from refs. [6] and [75]. API: active pharmaceutical ingredient

SGT-53 is a transferrin receptor (TfR)-targeted liposome, which binds to TfRs on the surface of cancer cells using single-chain antibody fragments (TfRscFv). Its payload is a plasmid encoding wild type tumor suppressor P53 [68]. Preclinical studies showed that SGT-53 could sensitize tumors to the effects of radiation and chemotherapy [68]. It is currently undergoing phase I and II clinical trials in combination with Dox for treatment of solid tumors and metastatic breast cancer. SGT-94 is a similar formulation to SGT-53. In SGT-94 the P53 plasmid was replaced with an RB94 plasmid encoding a fragment of the wild-type retinoblastoma tumor suppressor protein. SGT-94 is undergoing Phase I trials in patients with RB negative tumor biopsies.

MBP-426 is another TfR-targeted liposome that encapsulates oxaliplatin [14]. In a phase I study, patients with advanced or metastatic solid tumors refractory to conventional therapy received MBP-426 as 2–4 h infusions every 3 weeks and it was well tolerated [14]. 2B3-101 is a targeted liposomal formulation of Dox using glutathione as a ligand. By targeting the glutathione receptor it is able to traverse the blood–brain and blood-CSF barriers. Phase II trials have taken place for glioma and for metastases of breast cancer to the brain or the leptomeninges.

BIND-014 is the first targeted, controlled-release polymeric NP for cancer chemotherapy to reach clinical development. It is composed of PLA-PEG diblock polymer, that targets prostate-specific membrane antigen (PSMA) and encapsulates a docetaxel (Dtxl) payload [69]. PSMA is a transmembrane protein that is overexpressed on the surface of prostate cancer cells and tumor-associated neovasculature of virtually all solid tumors [70, 71]. BIND-014 is currently in phase II trials for second-line therapy for patients with non-small-cell lung, metastatic castration-resistant prostate cancers, and squamous cell carcinoma of the head and neck [72].

CALAA-01 was the first targeted polymeric NP to reach clinical use for siRNA delivery in 2008 [73]. The CALAA-01 NP consists of siRNA that reduces the expression of the M2 subunit of ribonucleotide reductase (R2), cyclodextrin containing polymer (CDP) for siRNA condensation, adamantine-PEG (AD-PEG) for steric stabilization, and adamantine-PEG conjugated to human Tf (AD-PEG-Tf) to target the TfR which is overexpressed on the surface of many cancer cells [74, 75]. CALAA-01 currently has no further active trials after the completion of the phase I trial.

4 Optimal Biophysicochemical Parameters for Targeted Nanoparticles

Properties such as size, shape, surface charge, surface chemistry, hydrophobicity, roughness, rigidity, and composition are termed the physicochemical properties of NPs and can influence the uptake and/or targeting of NPs to tumors and cancer cells (Fig. 1) [76].

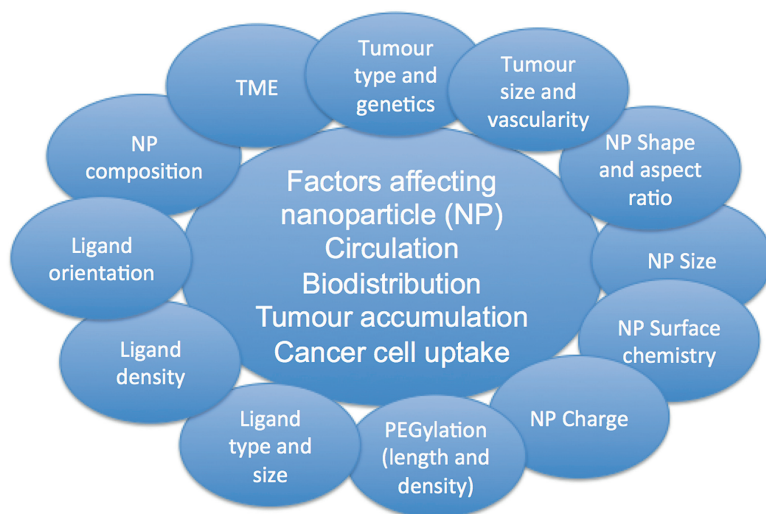


Fig. 1 Physicochemical properties of ligands and nanoparticles and the TME can affect their in vivo performance

Tumor targeted NPs must be able to effectively circulate, marginate, and successfully bind to vascular targets or extravasate into tumor interstitium and finally become internalized in cancer cells. The combination of these events requires that the NPs have optimal size, shape and surface properties. Each of these aspects merits investigation when developing optimal tumor targeted NPs. In this section, we discuss these properties, which generally affect both passively and actively targeted NPs.

NP biophysicochemical properties can significantly affect their cell uptake, cell cytotoxicity, pharmacokinetics (PK) and biodistribution (BD) in vivo. There is a strong interplay between each of these properties. Computational techniques ranging across scales from molecular dynamics through to simulation of fluid flow and tumor growth can help gain insight in to the optimal combination [77, 78]. However, the complexity of the bio–nano interface still demands a comprehensive experimental approach.

4.1 Influence of Nanoparticle Size

Nanoparticle size crucially effects three phases of biodistribution: circulation and clearance; diffusion into and through tumor tissue; and cellular internalization by tumor cells. NP sizes are most often given as hydrodynamic sizes, measured by dynamic light scattering. Other techniques, such as atomic force microscopy or electron microscopy are also sometimes used, and will generally measure smaller sizes for the same particles.

Based on many studies, 10–150 nm is a generally accepted size range for the development of NPs for in vivo applications with most favorable in vivo circulation and biodistribution, and tumor uptake patterns [79]. These upper and lower sizes are mostly determined

because of interactions with the immune system and kidney filtration cutoffs, respectively. In general the larger the NP, the lower the curvature will be, which can lead to increased interactions of opsonins and more rapid *in vivo* clearance [80]. Larger NPs are more likely to be filtered by the sinusoids in the spleen and cleared by the MPS cells, which include the Kupffer cells of the liver [81]. Furthermore, NPs smaller than approximately 5.5 nm have been shown to be rapidly cleared by glomerular filtration in the kidneys [82].

For tumor accumulation, the upper limit for extravasation into solid tumors appears to be ~400 nm and it is generally observed that NPs <200 nm in size can accumulate effectively within tumor tissue, with the 70–200 nm range considered optimal for tumor passive targeting [83]. In relation to diffusion within the tumor, intercapillary distances must be considered, since it is important for NPs to reach tumor cells that are located further away from these microvessels. Tumor matrix components such as collagen fibers, extracellular proteins, increased interstitial fluid pressure, heterogeneous blood flow, and impaired lymphatics towards the tumor core present challenges for the effective passive diffusion of NPs within tumors [84, 85]. Work has shown that NPs of about ~10–20 nm are ideal for maximum tumor penetration [86].

Cellular internalization of NPs is highly dependent on the size of the NPs, and in general, particles in the 40–50 nm range exhibit maximal uptake *in vitro* [87]. For example, Herceptin (HER) gold NPs (2–100 nm) were synthesized and their size-dependent binding and uptake was investigated in ErbB2 receptor expressing cells [88]. Interestingly, studies have shown that NP uptake as a function of size can also be tumor cell type-dependent, and just a 10 nm deviation from an investigated optimal size, results in a significant decrease in NP uptake [88]. Firstly it was shown that the number of HER antibody binding sites on the NPs was dependent on the NP surface area and increased with particle radius. Surface antibody density also increased linearly with NP radius—demonstrating that these multivalent antibody-conjugated NPs can allow for a high degree of ErbB2 cross-linking and can be tuned by varying NP size. Therefore, NP shape and size can change ligand presentation, affecting NP binding avidity.

As noted above, animal models understate the extent of variability of the EPR found in patient tumors. Yet even examples from the preclinical literature clearly show that optimal size is dependent on the model chosen.

The size dependent accumulation of fluorescently labeled PLA-PEG polymeric NPs using two different tumor xenograft models, HT20 colon and A2780 ovarian carcinoma, which result in different tumor structures, growth rates, and microenvironments was investigated [89]. The biodistribution and accumulation of near-infrared dye-loaded PLA-PEG NPs was tracked using *in vivo*

fluorescence imaging technique. NPs with sizes 111 and 141 nm accumulated efficiently in tumors and a larger 166 nm NP was shown to undergo rapid clearance in the liver. The pattern of accumulation was variable in both tumors, although fluorescence was mostly observed from the tumor core region in the case of HT29 tumors, which was not observed in the A2780 tumors. NP accumulation in the necrotic HT29 tumor core was shown to be size-independent, but size dependent in A2780 tumors, which were more vascularized. In general the larger NPs led to lower tumor accumulation.

Tumor permeability and degree of vascularization are key concepts for the assessment of NP biodistribution in tumors. In hyperpermeable murine colon adenocarcinoma polymeric micelles of sizes between 30 and 100 nm were found to be similar in their accumulation, tumor extravasation, and treatment efficacy [90]. However, in hypopermeable pancreatic tumors, only 30 nm micelles were shown to be effective [90]. Considering the diversity of nanomaterials and cell types used to investigate targeted NPs for cancer therapy, it is important that an optimal NP size be determined experimentally for a given NP type and cell type.

4.2 Influence of Nanoparticle Shape

The interaction between NP shape and hemodynamic forces (fluid dynamics of blood flow) affects circulation through the blood. The interaction between shape and cellular internalization affects both the rate of clearance by the MPS and the rate of uptake by target cells.

In relation to circulation of NPs in vivo, spherical NPs tend to be less susceptible to transport across streamlines by hemodynamic forces in the bloodstream. Certain studies, on the other hand, have shown that non-spherical particles with longitudinal lengths reaching cellular diameters and discoidal shapes can exhibit longer circulation times than spherical particles [91, 92]. Discoidal particles have highly oscillatory trajectories that can increase their contact with vessel walls. Oblate-shaped NPs are susceptible to torques resulting in tumbling and rotation, leading to an increase in the lateral drift of NPs towards blood vessel walls in microvessels [93]. Furthermore, oblate-shaped particles are less susceptible to uptake by macrophages leading to increased blood circulation [91, 94, 95]. Vascular endothelium or cancer cell targeting seems to also favor oblong and more elongated NPs since these shapes offer greater avidity towards targets due to an increased multivalency, maximizing NP surface contact with targets [96]. In particular for vascular targeting, geometrically enhanced targeting can avoid hemodynamic forces that can lead to NP detachment from the endothelium [97].

After systemic injection of a NP, before it reaches the cancer cells within tumors, it must first evade phagocytosis by macrophages, and marginate across the vascular wall against blood

hemodynamic forces. This activity can be influenced by the tumbling rate of the NP which is affected by its buoyancy, drag, van der Waals interactions, electrostatic double layer interactions, and steric repulsive behavior [98]. The movement of spherical NPs and their margination is size dependent [99]. This is because transport of larger spherical NPs is dominated by advection while smaller NPs are more prone to diffusion, which facilitates their extravasation across blood vessels. For examples, 65 nm liposomes were more than three times likely to marginate than 130 nm liposomes [100].

Nanoparticle shape is also an important factor in the rate of cellular internalization [87], with shapes that can accommodate cellular membrane wrapping processes most effective at cellular uptake. Amongst NPs of either rod or sphere design, the spherical shaped NPs were taken up by cells more readily [101].

A wide variety of non-spherical NPs with different sizes and geometries have been manufactured using different methods [98]. A top-down fabrication method termed Particle Replication in Non-wetting Templates (PRINT) utilizes lithography techniques to create polymeric NPs of a wide variety of geometries, shapes and aspect ratios, and a study using cylindrical PLGA NPs created using this technique showed an increase in tumor levels of docetaxel with decreased liver and spleen uptake [102, 103]. However, typical processes used to fabricate therapeutic NPs (bottom-up fabrication, self-assembly) renders most nanomedicines spherical. In addition to further studies of the in vivo effects of shape, new methods of synthesis for non-spherical NPs suitable for industrial scale-up is therefore an important area for future research.

4.3 Influence of Nanoparticle Surface Charge

The degree of nonspecific binding of proteins to NPs in the blood is related to NP surface charge, because highly positively charged NPs are more rapidly cleared from circulation by cells of the MPS since they are more prone to opsonization [79]. Although cationic NPs promote cellular binding (due to interactions with the negatively charged phospholipids, proteins, and glycans on cell surfaces) and can be ideal for in vitro applications of drug delivery to cancer cells [104], in vivo this charge should be masked until the NPs are within the tumor. Negatively charged NPs can also exhibit selective cellular uptake compared to NPs with neutral surfaces [101]. Either the uptake of positively charged NPs could be an energy-dependent process involving the proteins dynamin and F-actin, or positively charged NPs can by-pass endocytic pathways and enter cells by creating holes in the cellular bilayer [104, 105].

In vitro studies have shown that amino terminated surfaces induce the highest levels of complement activation [106], and NPs with neutral methoxy surface groups were most immunocompatible. Post systemic administration, NPs with zeta (ζ) potential <15 mV exhibited minimal macrophage uptake and led to longer

circulation times and overall tumor retention [107]. When near neutral polymeric micelles (ζ potential 1.3 mV) and anionic (ζ potential -10.6 mV) were compared, the more negatively charged NPs resulted in lower liver and spleen accumulation [108]. Cationically charged NPs have a high nonspecific uptake in a variety of cells, and can also facilitate endosomal release through the so-called “proton sponge effect” [109].

In summary, the charge of NPs, which is related to their surface chemistry influences the degree of opsonization, and circulation time of the NP, and interaction with macrophages within organs of the MPS. Positively charged NPs are more prone to uptake by macrophages in the lungs, liver, and spleen. Neutral and slightly negatively charged NPs exhibit prolonged circulation lifetimes and lowered uptake by the MPS.

4.4 Influence of NP Rigidity

Particle rigidity can highly influence both circulation and cellular uptake in vivo. This has long been studied in the case of larger structures, such as red blood cells. Synthetically engineered microparticles with both rigid and deformable properties were developed and their biodistribution evaluated in vivo [110]. The more deformable synthetic blood cells were eliminated 30 times more slowly than their rigid counterparts from blood, with the more rigid microparticles accumulating in the lungs 2 h post-injection.

More recently, attention has been paid to the rigidity of NPs although this is still a preliminary area of investigation [111, 112]. Rigidity affects circulation in capillaries. Healthy red blood cells tend to accumulate in the center of the capillary, leaving a cell-free sheath zone of several microns. In normal physiology this zone is also occupied by white blood cells and platelets that interact with the capillary wall. As referenced above, NPs tend to marginate toward the cell wall on the basis of size. Whether the correlation between stiffness and margination found for red blood cells extends down to the NP size regime is an open question.

In relation to cellular uptake there is in vitro evidence that macrophages take up harder particles more efficiently, by phagocytosis, and softer particles less efficiently by macropinocytosis [112]. Although evidence is more scant, the same in vitro preference for harder particles is found in endothelial cells. Cancer cell lines show much greater variation, which is not surprising in view of their heterogeneity [112]. It may be that tuning particle rigidity is another parameter for personalizing tumor treatment.

A key study which controlled for rigidity while holding other properties constant and investigated the NP size range was Zhang et al. who synthesized 120 nm poly(carboxybetaine) particles [113]. They used variable cross-linking of their zwitterionic monomers to achieve bulk moduli ranging from 180 to 1350 kPa. They showed that softest, more deformable NPs had a circulation half life

of 20 h, more than twice that of the most rigid particles at 9 h. The softest particles also showed less than half as much spleen accumulation, probably aided by a capacity to squeeze through splenic filtration. Another study found similar behavior with PEG based hydrogel particles, although at the upper end (greater than 200 nm) of the size range relevant to NP drug delivery [114].

Considering the highly flexible and deformable biconcave discoid shape of red blood cells, it is not surprising that the elastic modulus of NPs can also play an important role in their pharmacokinetics and biodistribution, with more flexible NPs navigating and traversing vessels and gaps more easily in vivo. Investigating targeted NPs with variable degrees of structural rigidity and flexibility could be of high interest for effective tumor targeting strategies.

4.5 Influence of Nanoparticle PEGylation

The large surface area-to-volume ratios of NPs can attract a “corona” type binding of blood proteins to their extremely curved surfaces [109], with up to hundreds of proteins adsorbing onto their surfaces [115]. Hydrophobic surfaces tend to lead to higher levels of protein adsorption, and IgG proteins (opsonins) have high affinities for hydrophobic surfaces [116].

In the case of small drug molecules plasma protein binding by abundant proteins such as albumin and alpha-1-acid glycoprotein can lead to increased bioavailability through the reduction of first pass hepatic extraction. However for NPs, this can lead to their enhanced blood clearance by the MPS [9]. Binding of plasma proteins onto the surface of NPs (opsonization) is extremely rapid once the NPs are injected into the blood stream. The ability to graft hydrophilic neutral polymers of polyethylene glycol (PEG) onto the surface of liposomes and polymeric NPs goes hand-in-hand with EPR discoveries and led to increased blood circulation times and improvements in tumor accumulation [12, 23, 75, 117]. The grafting of PEG molecules onto the surface of NPs is termed PEGylation and is currently the most utilized technique for improving the pharmacokinetics, plasma half-life, biodistribution, and elimination of NPs in vivo [118]. Indeed, uncoated NPs have been observed to be rapidly cleared by the MPS [116], and the density and thickness of the PEG layer can also influence opsonization and biodistribution of injected NPs. Surface-grafted PEG NPs exhibit reduced uptake by liver cells [119].

PEG configurations on the surfaces of NPs can present as extended brush-like structures, coiled mushroom or mushroom/brush intermediates. Brush-like PEG surfaces were shown to be more optimal to sterically suppress the approach and binding of opsonins such as the C3b protein [116]. PEGylation as a strategy for improving NP-based drug delivery has been recently discussed in an excellent review elsewhere [120].

5 Emerging Considerations for Improving Targeted Cancer Nanomedicines

The effective homing of targeted NPs to the required site of action faces numerous biological barriers, including the immune system and the TME. Opsonization driven sequestration, compromised vasculature, erratic blood flow and high interstitial fluid pressures all need to be overcome. In previous sections we discuss the variety of parameters that can be tailored and optimized in order to improve nanomedicine targeting to tumors and cancer cells. Further considerations that are more recently gaining momentum and interest are the realization of the importance of TME components and stimuli-responsive design elements for more controlled targeting and drug delivery to cancer cells—and are discussed in the following sections.

5.1 The Tumor Microenvironment

The tumor microenvironment (TME) includes subpopulations of genetically diverse cancer cells, genetically normal cells, vascular/endothelial cells, blood cells (e.g., erythrocytes, leukocytes, and thrombocytes) together with the tumor interstitial medium—with all these components having a role in cancer progression [121]. TAMs, cancer-associated fibroblasts and endothelial cells have been shown to play a role in tumor progression. It is anticipated that reprogramming of the TME via non-cancerous cells can lead to tumor regression. For example targeting stromal cells has gained interest recently [122, 123]. Stromal cells play a major role in tumor growth and maintenance—and their reprogramming can be a potential next-generation cancer treatment approach [124]. Another striking example is the use of immune checkpoint blockade inhibitors to reprogram immunosuppressive TMEs. Nanotechnology can offer solutions for the effective targeting of each of these components, and pathways. In particular the combination delivery of multiple chemotherapeutics can be achieved using targeted NPs. Nano-enabled synergistic combination drug delivery to cancer cells can more effectively kill these cells and minimize resistance [125].

5.2 Stimuli-Responsive Nanomedicines

Cancer produces an inevitable shift in homeostatic chemical equilibrium, such as amplified or triggered enzymatic activity, a change towards acidic pH, reductive or oxidative states, or an increase in reactive oxygen species [126]. These differential biochemical signatures can be exploited for the development of more precise therapies through NPs which sense these differences (“endogenous stimuli”) to trigger drug release. In addition to internally triggered drug release, externally controlled physical parameters (“exogenous stimuli”) such as local induction of thermal, electrical, ultrasound, or magnetic energy can also be used to trigger release [127]. Interest is growing in adding biologically responsive elements to

NP design, to achieve more controlled output behaviors and therapeutic outcomes. Release can be triggered by causing structural changes such as NP building block degradation, shedding of surface layers (e.g., PEG) and charge switching (neutral to positive), which can ultimately allow for better uptake into cancer cells. As with targeting generally, the goal of stimuli-responsive drug delivery systems is to further minimize systemic toxicities and unfavorable drug-plasma interactions to allow more efficient dosing and treatment of disease.

A range of endogenous stimuli such as changes in pH, redox state and ionic content within tissues and cells can be utilized for the development of chemically triggered drug release from polymeric NPs. Solid tumors have acidic pH environments (pH 6.2–6.9) [128], that can be used to trigger chemical changes in the NP structure leading to increased drug release. Subcellular compartments also offer various low pH environments post uptake of NPs (endosome, lysosome, cytosol, etc.), which can cause either surface layer shedding or conformational changes in NPs, leading to site-specific and increased drug release.

5.3 Subcellular Targeting

Following cancer cell internalization through different endocytosis pathways, NPs need to release their therapeutic payload, which can further diffuse through the cellular compartments to reach the biological target. Cell cytosolic internalization is not always sufficient for drugs to reach their targets via diffusion alone [129–135]. The cytoplasm contains a cytoskeletal network and numerous dispersed organelles, with many dissolved macromolecules ranging in concentration between 50 and 400 g/L [136, 137]. In the case of drugs that are recognized by efflux pumps (e.g., P-glycoprotein) NPs that can be internalized by endocytosis and thus release their active drugs within specific subcellular organelles can be a way to reduce multidrug resistance in cancer cells [138, 139].

Folic acid, low-density lipoprotein, cholera toxin B, mannose-6-phosphate, Tf, riboflavin, the tripeptide RGD, ICAM-1 antibody, and nicotinic acid are suitable endocytic targeting ligands that can be useful for subcellular localization of NPs [49]. Cellular internalization with these ligands can occur via clathrin-dependent receptor mediated endocytosis, caveolin-assisted endocytosis, lipid raft-associated endocytosis, or cell adhesion molecule (CAM)-directed cellular uptake [49, 140, 141].

Encapsulation of Dox into liposomes bearing Tfs on the distal end of PEG chains was shown to increase Dox uptake into glioma cells (that overexpress TfRs) [142]. The dynamic subcellular fate of polymeric micelles formed from (1,2-diaminocyclohexane) platinum(II) (DACHPt/m), the parent complex of oxaliplatin, was investigated in tumor tissues [143]. Potent antitumor activity was shown and the micelles were able to overcome Pt resistance both in vitro and in vivo. The extravasation of DACHP/m NP was

observed from blood vessels into tumors in addition to polymer dissociation within the cells. The polymeric NPs selectively dissociated in the late endosomes and facilitated Pt drug delivery to the nucleus relative to free oxaliplatin, by circumventing the cytoplasmic detoxification systems of metallothionein and methionine synthase, suggesting that NP intracellular targeting via compartmentalization is an effective strategy for drug delivery. It would be of interest to understand the exact mechanisms of subcellular transport of NPs in order to improve their design and targeting functions.

6 Conclusions and Outlook

Improving and fine-tuning our understanding of tumor heterogeneity and discovering EPR biomarkers can help identify “nanomedicine-responsive” patients and further improve their clinical outcomes. Our understanding of NP transport to tumors and factors involved in their biodistribution and uptake within the TME is constantly expanding and will result in safer and more efficacious nanotherapeutics. Investigating the challenges of controllable, reproducible and scalable NP synthesis, as well as large-scale NP screening and evaluation, will facilitate their more rapid clinical translation.

Oncology is one area where nanomedicine products are set to make the most impact, where cell and tissue targeting approaches can be used to efficiently deliver cytotoxic and molecularly targeted drugs to cancer cells. Ultimately physicochemical parameters need to be investigated for successful design of targeted NPs, which include optimization of NP biophysicochemical properties and the demonstration of the efficacy of targeted NPs in a clinical setting on their impact on patient treatment. Indeed, the value of tailoring these parameters with the purpose of minimizing toxicity, unfavorable interactions with the immune system, rapid renal clearance, and minimal accumulation in organs such as the liver and spleen is beginning to be more systematically recognized and more routinely investigated. Other than identification of optimal ligands and ligand targets suitable for highly selective NP targeting, other important practical challenges in the development of targeted therapeutic NPs should also be considered including: (1) the use of biocompatible, biodegradable/bioeliminable materials; (2) the use of simple, robust, and reproducible bioconjugation chemistries for the attachment of precursors and targeting ligands; (3) facile NP assemblies that avoid multistep NP preparation and purification steps; (4) optimization of NP biophysicochemical properties to achieve, optimal drug load/release, long circulation half-life, suitable biodistribution, differential target tissue accumulation, efficacious target tissue drug concentration and drug exposure kinetics;

(5) validation of NP stability and predictable shelf life; and (6) development or adaptation of scalable processes and units of operations amenable to the manufacturing of large quantities of targeted NPs for clinical development and commercialization. The field is steadily progressing and we will see targeted nanomedicines en route to becoming valuable therapeutics in oncology with greater impact in the near future.

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