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Cancer Nanotechnology: The impact of passive and active targeting in the era of modern cancer biology☆

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Abstract

Cancer nanotherapeutics are progressing at a steady rate; research and development in the field has experienced an exponential growth since early 2000's. The path to the commercialization of oncology drugs is long and carries significant risk; however, there is considerable excitement that nanoparticle technologies may contribute to the success of cancer drug development. The pace at which pharmaceutical companies have formed partnerships to use proprietary nanoparticle technologies has considerably accelerated. It is now recognized that by enhancing the efficacy and/or tolerability of new drug candidates, nanotechnology can meaningfully contribute to create differentiated products and improve clinical outcome. This review describes the lessons learned since the commercialization of the first-generation nanomedicines including DOXIL® and Abraxane®. It explores our current understanding of targeted and non-targeted nanoparticles that are under various stages of development, including BIND-014 and MM-398. It highlights the opportunities and challenges faced by nanomedicines in contemporary oncology, where personalized medicine is increasingly the mainstay of cancer therapy. We revisit the fundamental concepts of *enhanced permeability* and *retention* effect (EPR) and explore the mechanisms proposed to enhance preferential "retention" in the tumor, whether using active targeting of nanoparticles, binding of drugs to their tumoral targets or the presence of tumor associated macrophages. The overall objective of this review is to enhance our understanding in the design and development of therapeutic nanoparticles for treatment of cancers.

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Keywords

enhanced permeation and retention effect; active targeting; nanoparticles; nanomedicine; personalized medicine; tumor microenvironment; drug delivery; patient enrichment; vessel normalization; imaging

1. Introduction

More than 40 years ago, the foundations were laid down for nanotechnologies to deliver therapeutic and diagnostic agents in a safer and more efficient manner [1]. Achieving this vision became more realistic in recent years, with increasing numbers of nanotherapeutics and nanodiagnostics being commercialized or having reached clinical stage. In addition other important bench-to-bedside milestones are being achieved. In 2010, the first clinical evidence of gene silencing was obtained by systemically-administered targeted nanoparticles (NPs) delivering siRNA therapeutics [2]. Other clinical evidences of RNA interference have been obtained since then [3]. In parallel, clinical investigation of the first actively-targeted polymeric NPs, BIND-014, for the delivery of a small molecule drug was reported [4]. Although only a relatively small number of nanosized drug delivery carriers have been approved for human use so far, it is now accepted that nanotechnologies will likely constitute a growing share of the oncologist's therapeutic arsenal over the next decades to come [5-7]. There are many nanoparticle technologies under development and a great majority are still without animal proof of concept. However, what is exciting is the momentum in this field and of the 73,000 articles on "nanoparticles" reported in Pubmed as of May 2013, more than half were published since 2010, emphasizing that research efforts are growing exponentially.

Nanoparticles offer the possibility to encapsulate poorly soluble drugs [8, 9], protect therapeutic molecules [10], and modify their blood circulation and tissue distribution profiles [11, 12]. These properties are attractive in oncology in order to encapsulate cytotoxics exhibiting wide-ranging toxicities and physicochemical properties. For instance, liposome-encapsulated doxorubicin (DOX) decreases cardiac toxicity of the cytotoxic drug [13, 14], and albumin-stabilized paclitaxel (nab-PTX) allows higher tolerated doses in patients [15]. Lately, drugs that modulate cancer signaling pathways (*i.e.*, molecularly targeted therapies) have shifted the paradigm of cancer treatment in patients exhibiting specific genetic mutations [16]. Like their cytotoxic counterparts these targeted drugs have toxicities and suboptimal tumor distributions that motivate their encapsulation in therapeutic NPs [17]. Furthermore, the robustness and redundancy of the signaling networks and the cross-talk between molecular pathways often promote resistance in cancers treated with molecularly targeted therapies [18, 19]. Many of the kinase inhibitors are sensitive to plasma drug concentration to maintain on-target activity and molecular pathways can reactivate as plasma concentrations decline [20]. Using NPs to precisely control the tumor levels of protein kinase inhibitors could theoretically circumvent that problem and result in improved efficacy.

Nanotechnologies are also appealing because they can facilitate the combination regimens which are commonly practiced in cancer therapy. Having a single NP encapsulating multiple

active pharmaceutical ingredients (API) could potentially offer synergistic effects to promote the efficacy of therapies, while limiting the risk of resistance. When multiple drugs are administered separately, each API acts according to its own distinct pharmacology. Because drugs differ in their pharmacokinetics, biodistribution and duration of effect, there is no certitude that all cells will synchronously receive optimal levels of each therapeutic entity. Conversely, when drugs are combined in a single NP carrier, cells are always exposed to synergistic amounts of API. The timely co-delivery to cancer cells of multiple agents inhibiting distinct, essential pathways could provide improved anticancer effects. This synergy has been demonstrated for combinations of small molecular weight drugs *in vitro* [21], combinations of a cisplatin prodrug and siRNA *in vivo* [22] or the combination of siRNA targeting 2 different genes in humans [3], highlighting the potential of encapsulating multiple API in a single carrier. Nevertheless, the determination of optimal therapeutic combinations using NPs is challenging. In opposition to conventional anticancer regimens where the dose of each single drug can be adjusted individually in patients (*i.e.*, based on their response or susceptibility to toxicities), the ratios of the different APIs encapsulated in a NP need to be optimized *a priori*, during the development phase. The large diversity of regimens possible makes the selection of ideal treatment combinations difficult. Furthermore, temporal exposure to the different components of the combination might also be important. Anticancer treatments undeniably impact transcriptional response in the tumor microenvironment and cancer cells, sometimes with important impact on subsequent response to chemotherapy (for example through p53 gene down-regulation or WNT16B expression) [23, 24]. Combination regimens must therefore be designed so that the chronology of exposure to one agent does not affect the efficacy of the second drug. This might be particularly true for antiangiogenic agents where the shutdown of oxygen supplies has been shown to significantly reduce susceptibility of cancer cells to other chemotherapeutics [23]. Although it is feasible to independently control the release of APIs from a NP, the sequence of exposure must be specifically considered to maximise synergism [25].

Currently, despite the broad interest surrounding nanomedicines, the development and clinical translation of NPs remain laborious. Among the clinically-validated nanomedicines, only nab-PTX has become officially part of the first-line treatment of cancer, but the picture is changing rapidly. Very recently, a combination of gemcitabine with nab-PTX proved to significantly improve survival in pancreatic cancer patients from 6.7 months to 8.5 months (25% increase) [26]. These findings led, during the writing of this article, to the approval of nab-PTX as first-line treatment of advanced pancreatic cancer (in combination with gemcitabine). These NPs are now established as leading treatments for 2 types of cancer (pancreatic and locally-advanced or metastatic non-small cell lung cancer (NSCLC) (in combination with carboplatin). In all likelihood, the true potential of nanomedicines to really improve clinical outcome will continue to emerge as we begin to understand which patients can benefit further from treatment with anticancer therapeutic NPs. In the era of personalized medicine and as our understanding of genomics, tumor biology and nanotechnology progresses, sophisticated strategies may be able to address interpatient variability when initiating a treatment and ultimately improve therapeutic response and cancer survival.

Molecularly targeted therapies have paved the way for the personalized treatment of patients based on the genetic profile of their tumors and it is now acknowledged that all patients do not respond equally to therapies [16]. Given that inter- and intratumoral variability can affect the architecture of the neovasculature and the tumor microenvironment, it becomes apparent that the passive targeting of NPs to tumors may be more complex than originally assumed [27]. The development of future therapeutics based solely on passive targeting strategies might not capitalize on the full potential benefit of therapeutic NPs. More than ever, increasing the response rates by screening patients for optimal response to nanomedicines based on specific tumor characteristics or the presence of certain biomarkers seems attractive, if not yet fully achievable. While patients who are naturally more responsive to NPs might be more exposed to efficacious treatments, regimens increasing the susceptibility of the tumor to the nanomedicine could also be envisioned for others [28].

The parameters governing effective passive transport and retention of NPs in tumors hold true for actively-targeted NPs which are decorated with ligands. Although this approach has been proposed for quite some time [29-32], the first actively-targeted NPs have relatively recently made their way to human clinical trials [33]. This strategy exploits the affinity between surface ligands and antigens on the surface of cancer cells to facilitate the delivery of the NPs. Actively-targeted NPs therefore display an increased degree of complexity. To potentially benefit from the active targeting strategy, it is imperative that the specific antigen be present on the targeted cells and accessible to bind the NPs. It is also important that antigen localisation and expression remains adequate throughout the treatment. In this context, identification of predisposed patients goes beyond relatively simple genetic profiling.

Understanding the biological processes involved in the distribution and retention of nanomaterials inside the tumors is therefore essential to the development of personalized nanomedicine approaches. The concepts apply to therapeutic nanomaterials in general, whether drug carriers or nanoparticulate therapy mediators responding to external stimuli to exert therapeutic effects (*e.g.*, light [34], magnetic field [35, 36]}, ultrasounds [37, 38]). In this review, we examine the fundamentals behind passive and active targeting of nanomedicines to tumors and cancer cells. We will discuss how the morphology of the tumor vasculature, the components of the extracellular matrix or the presence of immune cells in the tumor microenvironments can affect the distribution of NPs. With the perspective of developing new therapeutic NPs, we will also examine how the physicochemical parameters of the nanomaterials will affect their localization, retention, cell binding, internalization, efficacy and toxicity. We will highlight the opportunities that can be exploited and the pitfalls to be avoided by assessing the most recent developments in the fields of cancer biology and cancer nanotechnology. This work encompasses the current corpus of knowledge obtained from preclinical studies in animal models through results obtained in humans.

2. Passive targeting: Nearly 30 years of the EPR effect…

The observation that certain macromolecules accumulate preferentially to tumors was witnessed more than 30 years ago [39]. The tumor accumulation of therapeutic

macromolecules was first reported for poly(**S**tyrene-*co*-**M**aleic **A**cid)-**N**eo**C**arzino**S**tatin (SMANCS), a 16 kDa polymer conjugate that non-covalently binds albumin in the circulation to reach a molecular weight of around 80 kDa [40, 41]. The distribution of SMANCS to the tumor vicinity was observed in early preclinical development and led Matsumura and Maeda to further investigate the phenomenon [39]. Using labeled albumin and other proteins in addition to the polymer conjugate, they showed that proteins larger than 30 kDa (*i.e.*, SMANCS, murine (67 kDa) and bovine albumin (69 kDa) and IgGs (160 kDa)) could preferentially distribute to the tumor interstitium and remain there for prolonged periods of time (up to 144 h) [39]. This preferential distribution to the tumors was ascribed to the presence of fenestrations in the imperfect tumor blood vessels and to the poor lymphatic drainage in the tissue. The combination of these two phenomena was coined as the enhanced *permeation* and *retention* effect. Since then, the EPR effect has become the *leitmotiv* of many scientists for the efficient delivery of anticancer drugs to tumors, whether using polymer conjugates, liposomes or NPs.

Nonetheless, the EPR effect is much more complex than initially defined and the phenomenon has somehow become a blanket term encompassing dozens of complex biological processes (*e.g.*, angiogenesis, vascular permeability, hemodynamic regulation, heterogeneities in tumor genetic profile, heterogeneities in the tumor microenvironment and lymphangiogenesis). These factors vary among patients and their tumor types. Likewise, the distribution and accumulation of NPs in tumors is also multifaceted and is affected by the biological and physicochemical properties of each material. For these reasons, and with the advent of personalized medicine, the designation of EPR as a simple self-explaining phenomenon might be becoming outdated.

2.2. The fundamentals of EPR

When a solid tumor reaches a given size, the normal vasculature present in its vicinity is not sufficient to provide all the oxygen supply required for its further proliferation. As cells start to die, they secrete growth factors that trigger the budding of new blood vessels from the surrounding capillaries [42]. This process, known as angiogenesis, promotes the rapid development of new, irregular blood vessels that present a discontinuous epithelium and lack the basal membrane of normal vascular structures [43, 44]. The resulting fenestrations in the capillaries can reach sizes ranging from 200 to 2,000 nm, depending on the tumor type, its environment and its localisation [45]. When blood components reach the abnormal, discontinuous vascular bed, the fenestrations offer little resistance to extravasation to the tumor interstitium. This denotes the *enhanced permeation* portion of the EPR effect.

In normal tissues, the extracellular fluid is constantly drained to the lymphatic vessels at a mean flow velocity around 0.1-2 μm/s [46]. This allows the continuous draining and renewal of interstitial fluid and the recycling of extravasated solutes and colloids back to the circulation. In tumors, the lymphatic function is defective, resulting in minimal uptake of the interstitial fluid [47]; the colloids cannot rely on convective forces to return to circulation. While molecules smaller than 4 nm can diffuse back to the blood circulation and be reabsorbed [48-50], the diffusion of macromolecules or NPs is hindered by their larger hydrodynamic radii. Therefore, NPs that have reached the perivascular space are not cleared

efficiently and accumulate in the tumor interstitium. This aspect represents the *enhanced retention* component of the EPR effect.

Since the early works of Matsumura and Maeda in the mid-1980s, the EPR effect has been comprehensively documented using various tumor types and animal models. The parameters which affect the distribution of macromolecules and NPs to the tumor are better understood, and we are slowly unravelling the subtleties of the EPR effect [44, 51]. Importantly, it is now recognized that lymphatic drainage is not homogenous throughout the cancerous mass. Vessels in the bulk of the tumor experience higher mechanical stress, and the functional loss in the intratumoral regions is therefore more important than in the margin [47]. In fact, residual lymphatic activity and *de novo* lymphangiogenesis are believed to be in part responsible for the progress and dissemination of metastases [52]. The heterogeneity of lymphatic function within the tumor is therefore a factor that should be considered when addressing tumor NP accumulation.

2.3. Factors affecting the EPR effect

In a solid tumor, the distribution of molecules to the tumor is governed by at least three distinctive but interrelated phenomena: the extravasation of colloids from the blood vessels, their further diffusion through the extravascular tissue and their interaction with intracellular and/or extraceullar targets within the tumor environment (Figure 1). The first two aspects are the result of diffusive and convective forces and can be influenced concurrently by the tumor biology and the characteristics of the diffusing species. The third parameter is more complex and less understood. It represents the interactions of the colloids with the tumor whether through adsorption phenomena, cellular uptake or degradation and metabolism. These aspects can all affect the equilibrium of accumulation inside the tumor; they depend on the nature of the material, its affinity for all components of the tissue and the tumor composition (*e.g.*, nature of the extracellular matrix, the type of cells present). Because we still lack the full understanding to completely assess all these interactions, they are represented as a “black box” that will be further detailed below.

2.3.1. Extravasation—Extravasation of colloids from the circulation is influenced by many parameters ranging from their concentration in the blood, the relative permeability of the vascular wall to macromolecules and NPs as well as the nature of the extravascular environment. In order to reflect the relative involvement of each factor on the escape of colloids from the blood, the phenomenon can be described by simple and general mathematical relations. Although these equations are difficult to use experimentally because of the great number of variables involved, they can be useful to represent and understand the consequences of each biological and physicochemical characteristic independently.

The total flux of material toward the tumor is described by adding the contributions of diffusive (in red) and convective forces (in blue) as well as possibly a number of unknown phenomena (black box):

$$J_{Total} = PA(C_v - C_i) + L_p A[(P_v - P_i) - \sigma(\pi_v - \pi_i)](1 - \sigma_F)C_v + \_BLACK\ BOX\_ \quad (1)$$

The diffusive component ($PA(C_v\text{-}C_i)$) originates from the Brownian motion of colloids resulting in a positive net flux towards the interstitium because a gradient exists between the vascular (C_v) and interstitial concentrations (C_i) [46]. It is given by a modification of Fick's first law to account for the permeability of the vascular wall (P) and the area of the blood vessel (A). The permeability incorporates both the diffusion coefficient of the colloid (D) and how the vascular barrier restricts its passage. This hindrance effect depends on the physicochemical properties of the colloid as much as on the properties of the vessel wall [44].

The outflow of fluids from the vessel also creates a convective force pushing the colloids toward the tumor. In that case the flux of fluid ($L_pA[(P_v\text{-}P_i)\text{-}\sigma(\pi_v - \pi_i))]$) is described by the Starling Law where L_p is the filtration coefficient of the fluid through the vessel wall, P_v and P_i are the vascular and interstitial hydrostatic pressures, π_v and π_i are the vascular and interstitial oncotic pressures and σ is the capillary osmotic reflexion coefficient [46]. This later parameter reflects the permeability of the capillary to proteins and how effective the oncotic pressure gradient is to pull the fluid back in the vascular space. The flux of fluid is adjusted for the imperfect permeation and drag of the colloid by the fluid (σ_F) and the colloid concentration in the vascular compartment (C_v).

Finally, despite the recent progresses in cancer biology and nanotechnology, our understanding of the tumor microenvironment and how colloids extravasate and reach the tumor cells is far from complete. The "black box" in equation 1 highlights this opportunity for further exploration. When measuring the tumor uptake of a compound, the distinction between extravasated material in the vicinity of the vessels and the material which is taken up by tumor endothelial cells in the neovasculature is difficult. Some researchers describe enhanced interactions with endothelial cells, for example by cationic charges (see section1.3.2), as an increase in the effective P of the vessel [53, 54]; others consider these interactions to be the product of absorption and endocytosis by the endothelium [55, 56]. The black box accounts, among other things, for that uncertainty on the effective concentration in the vasculature available for extravasation (*i.e.*, if endothelial cells are internalizing the colloids relatively fast, the actual concentration left to permeate through the vessel is lower than the concentration supplied by the systemic circulation, C_v).

Similarly, authors have shown that tumor accumulation of NPs increased with higher levels of phagocytic cells in the tumor interstitium [57]. The interactions of NPs with macrophages and dendritic cells can therefore affect the concentrations of NPs effectively diffusing in the tumor microenvironment (C_i). Finally, it is also important to decouple the accumulation of the NPs by EPR effect from the accumulation of their encapsulated payload. The tumor distribution of therapeutic NPs is usually measured by tracking their loaded cargo, whether it is a small molecular weight drug, a therapeutic macromolecule, a fluorescent dye or a radiolabelled tracer. Those entities may have very distinct properties when they are encapsulated in NPs compared to when they are released; the kinetics of their release as well as their possible interactions with the tumor micro-environment must therefore be accounted for.

The black box is introduced to address all parameters which factor in the retention of the NPs in the tumor whether they are the product of absorptive or intracellular trafficking forces or the result of degradation, metabolism and drug release. It also acknowledges that many aspects of tumor accumulation are still not fully understood.

2.3.2. Diffusion and convection in the interstitium—Once the colloids are extravasated to the tumor, their surrounding environment is composed of interstitial fluid (with a composition similar to plasma), cancer and stromal cells and the extracellular matrix (ECM). Their movements in the interstitium are also guided by convective, diffusive forces and other phenomena and can be described by:

$$\frac{\partial C_i}{\partial t} = D_{eff} \nabla^2 C_i + \varphi_i \underline{v} \nabla C_i - R_i \quad (2)$$

Here, the changes in interstitial concentration over time ($\delta C_i/\delta t$) result from the diffusive component ($D_{eff}\nabla^2 C_i$), the convective component ($\varphi_i \underline{v} \nabla C_i$) and the effects of the tumor microenvironment on the colloid's transport (R_i). The diffusive factor is governed by the effective diffusion coefficient (D_{eff}) and the change in concentration in all directions (*i.e.*, the Laplace operator of the interstitial concentration, $\nabla^2 C_i$). The convective portion results from direction and intensity of the convective motion (*i.e.*, the fluid velocity vector ($\underline{v}$)), the spatial concentration gradient (∇C_i) and a coefficient (ϕ_i) to account for the fact that the colloid velocity in the porous ECM might be different from the velocity of the fluid (*e.g.*, drag, adsorption or exclusion effects). This difference can be explained by interactions with the ECM retaining the colloid (ϕ_i<l) or by size-exclusion effects hastening it through larger pores (ϕ_i>l). Finally, the factor R_i also highlights the possible degradation, metabolism or binding of the colloids to ECM, their capture by extravasated components of the mononuclear phagocyte system (MPS) as well as their uptake by tumor cells. The negative sign acknowledges that higher interactions with the tumor result in decreased movement.

2.4. Tumor biology

The tumor environment is a complex milieu where normal anatomy and physiology principles are defied. The untamed growth of cancer cells and the secretion of angiogenic factors both contribute to the highly disorganized vasculature and congested extravascular environments. Paradoxically, these structural imperfections are both the cause of the EPR effect and the principal source of uneven tumor accumulation and retention of nanomaterials.

2.4.1. Tumor vasculature—The release of angiogenic factors from cancer cells dictates the blood vessel architecture and morphology of the vascular wall [42]. The neovasculature sprouting from capillaries is disordered, discontinuous and highly fenestrated [28]. The degree of leakiness of the endothelium and the enhanced vascular permeability to macromolecules and nanomaterials depend on many factors like the cancer type, its stage and in xenograft models, the implantation site of the tumor [44, 45, 58]. In equation 1, the vascular biology affects both the diffusion (*via* the permeability, P) and the convection through the vascular wall (*via* the filtration coefficient (Lp)).

While the fenestrations offer escape routes for the colloids, the discontinuities and irregularities in the architecture of the vessels also affect the blood flow and the hydrostatic pressure in the vessels [28, 44]. The mass of hyperproliferative cancer cells also exert mechanical pressure on the different vessels further impeding perfusion [47]. The reduced pressure might be associated with a decrease in the convective forces responsible for the extravasation of blood components and nanomaterial ($\downarrow P_v$).

2.4.2. Tumor extravascular environment—The tumor extravascular environment is a congested entanglement of collagen fibres and glycosaminoglycans (GAG), with inhomogeneous distributions of solutes, proteins and cellular debris. In opposition to healthy tissues were interstitial flow is regulated to efficiently disseminate the fluid throughout the cell population [46, 49], the disorganized structures in tumors strongly hinder the diffusion and fluid convection. The abnormal traffic of fluid influences local interstitial hydrodynamic (P_i) and oncotic pressures (π_i), two parameters which impact the convection of NPs through the vascular wall [59].

Once NPs have extravasated, the nature of the ECM further regulates the diffusive and convective movement of NPs in the tumor. Various studies *in vivo* and *ex vivo* have shown that the effective diffusion coefficient (D_{eff}) in the tumor interstitium is below that measured for colloids in simple solutions [60, 61]. The GAGs (*e.g.*, hyaluronic acid, heparan sulfate), covalently-attached to proteins (*e.g.*, collagen, laminin), affect the viscosity of the environment and the tortuosity of the diffusive paths. GAGs chains organized in blotches of low and high viscosities which translate into a two-phase transfer process where colloids of different sizes show high and low mobilities, respectively (Fig. 2, closed and open symbols) [61]. Although these distinct mobilities can probably be explained by differences in the diffusion of colloids (D_{eff}, in equation 2), the presence of some steric exclusion effects (ϕ_i) caused by the inhomogenous environment cannot be completely ruled out.

The collagen content and its degree of organisation in the ECM also correlates with the resistance exerted on the interstitial transport ($\downarrow D_{eff}$) [62]. The disruption of the collagen network by collagenase can break up the protein entanglement and restore some mobility to slow diffusing species [61, 62]. Similarly, intratumoral injections of collagenase were shown to rearrange the ECM in a manner that allowed enhanced mobility of 150-nm viral vectors in the tumor [63], further consolidating the influence of diffusion on the two-phase transport (in opposition to steric effects).

On the other hand, the effects of GAG-disrupting enzymes are less clear. In xenograft models of various tumors, relations between the GAG content and the transport restriction could not be established [62]. In some instances, hyaluronidases were reported to decrease the diffusion of macromolecules by collapsing the hydrated protein structure and increasing the overall viscosity ($\downarrow D_{eff}$ and ϕ_i) [61]. In other cases, heparinases that cleave the matrix heparan sulfate moieties can restore mobility of positively-charged macromolecules [60], in this case most probably by decreasing the adsorptive interactions of the colloids with the ECM ($\downarrow R_i$ in equation 2) .

Finally, cells from the MPS have been found to extravasate to the tumor interstitium. The affinity of these macrophages for the colloid and the resulting phagocytic activity can further impede the NPs movement toward the cancer cells while increasing their retention in the tumor interstitium [27]. Recently, Zamboni and colleagues showed that xenografts of ovarian cancer showing increased amount of CD11c-positive cells (a marker specific for dendritic cells) had increased liposomal accumulation compared to melanoma cells with lower dendritic cell expression [57]. Although it is unclear if the increased tumor accumulation seen in this study has a positive or negative influence on the NPs' therapeutic efficacy, it certainly implies that the MPS plays a role in their retention in tumor (decreasing movement in equation 2 by $\uparrow R_i$). In humans, the age of patients and their intrinsic MPS activity was also correlated with the clearance of liposomes from the blood [64]; older patients or patients with hepatic metastases were shown to experience higher blood exposure to the NPs. Interestingly, older patients also had less hematologic toxicity compared to patients below 60 years old [65], further suggesting that interactions between NPs and the MPS affect the pharmacodynamics of nanomedicines. Nevertheless, this Phase 1 clinical trial did not focus on how the different MPS functions in the tumor correlate with the efficacy of the NPs. To answer this question, a new clinical trial was recently initiated to use iron oxide NPs as an MRI contrast agent in combination with therapeutic liposomes (clinicaltrial.gov, NCT01770353). This pilot study will be discussed in more details in section 2.6.

2.4.3. Improving EPR by changing tumor biology—As our knowledge about the EPR effect grows, pharmacological approaches are proposed to optimize the tumor microenvironment for enhanced distribution of macromolecules and nanomaterials. The injection of enzymes that remodel the ECM to augment the intratumoral mobility of colloids ($\uparrow D_{eff}$) has been introduced before [63]. In a similar fashion, other methods remodelling the perivascular environment have been exploited using photo-immunotherapy [66] or small molecular weight drugs [67]. These approaches showed increased tumor distribution and therapeutic efficacy of oncolytic viruses [63, 67] and/or NPs [66, 67] in preclinical models.

Another approach relies on improving the transvascular convective movement by increasing the perfusing pressure (P_v). To that end, the cyclic or continuous administration of hypertensive molecules, like angiotensin II, was studied [59]. This approach resulted into significantly increased extravasation only when the antibodies had sufficient affinity to bind to the tumor and avoid being translocated back to the circulation upon pressure normalization ($\uparrow R_i$). The administration of enalapril, an angiotensin-converting enzyme (ACE) inhibitor, was also proposed to augment tumor accumulation of antibodies [68]. In this case, the ACE inhibitor was used to block the degradation of bradikinin, a potent physiological vasodilatory peptide that increases the vascular permeability to macromolecules ($\uparrow P$ and L_p). The combination of enalapril with angiotensin II was effective to counter the hypotensive effect of the ACE inhibitor and further improve EPR. Other vasodilatory pathways ($\uparrow P$ and L_p), like nitric oxid [69], prostaglandins [70] or carbon monoxide [71] were also found to improve tumor accumulation of NPs.

The normalization of the blood vasculature was also proposed as an alternative to improve the EPR effect [28]. This approach is believed to rectify the blood flow in the tumor ($\uparrow P_v$)

and consequently normalize the interstitial fluid exchanges (↓ P_i and πi). In a murine model of orthotopic allograft breast adenocarcinoma, the blocking of the vascular endothelial growth factor receptor-2 (VEGF-R2) improved penetration of small (~12-nm) albumin-based drug complexes while not affecting the diffusion of larger (> 60 nm) nanoparticles [72]. It is believed that the restoration of the transvascular pressure gradient also impacted the size of the endothelial fenestrations and resulted in decreased permeability for the larger particles (↓ P and L_p).

The impact of the modulation of the vessel morphology strongly depends on the initial tumor architecture. The administration of various small molecular weight tyrosine kinase inhibitors affecting the vascular endothelial growth factor (VEGF) activation pathways had different effects in murine xenograft models of colon and pancreatic cancers [73]. In the former, VEGF-inhibition lead to decreased permeability by reducing the endothelial density in the tumor (↓ A and possibly P and L_p), while in the latter, it decreased the coverage of the vessels by pericyte and improved permeability (↑ P and L_p). The administration of transforming growth factor-β inhibitors (TGF-β inhibitors) in a model of hypovascular, poorly-permeable pancreatic cancer, was also found to increase the permeability of blood vessels to large nanoparticles (↑ P and L_p) [73-75].

Together, these results in preclinical models highlight certain therapeutic interventions that could possibly modulate the tumor biology and regulate the EPR effect. However, further efforts are needed to fully understand how to efficiently exploit these different strategies in a clinical setting.

2.5. The physicochemical parameters

The physicochemical parameters of the colloids affect their extravasation by influencing, among other things, their diffusivity (D_{eff}) , their permeability through the vascular wall (P and L_p), their drag coefficient in the fluid (ρ_F and ϕ_i) and their interactions in the tumor with the cells and the ECM (R_i). Furthermore, the physicochemical characteristics of exogenous materials used for therapeutic or diagnostic applications also impact on how the host's defense mechanisms clear them from the blood circulation [11, 12]. In fact, because individual physicochemical parameters can affect the overall blood circulation kinetics, the extravasation processes and the intratumoral diffusion, directly measuring the influence of each specific characteristic on the EPR is difficult.

Nevertheless, the total blood exposure to the NP is believed to be a key factor influencing its distribution to the tumor in the EPR effect [76]. The diffusive and convective elements forces are both influenced by the concentration of colloid in the bloodstream (C_v). Maintaining high blood concentrations is also very important to ensure unidirectional diffusion towards the tumor and prevent the efflux from the tumor when $C_i > C_v$ [77]. Therefore, longer circulation times in the blood result in higher amounts extravasated to the tumor interstitium. The tumor deposition of soluble polymers like poly(ethylene glycol) (PEG) and N-(2-hydroxypropyl)methacrylamide (HPMA) augments proportionally to blood exposure when the molecular weight is increased above the glomerular filtration threshold and the polymer cannot be eliminated by the kidney (Fig. 3) [50, 78]. The correlation

between the tumor accumulation and the blood circulation kinetics has since then been generalized to other polymers [79], liposomes [80, 81] and nanoparticles [82].

2.5.1. Size—The size of the nanomaterial influences the kinetics and extent of tumor accumulation. The material needs to be smaller than the cut-off of the fenestrations in the neovasculature, but size is also factored in the various parameters affecting extravasation to the tumor and diffusion in the interstitium (P, L_p, D_{eff} and, possibly to a lesser extent, ρ_F and ϕ_i). In mice xenograft models, when the kinetics of intratumoral accumulation were studied over 30 min, smaller macromolecules (3.3 and 10 kDa, R_H : 2 and 3 nm) were shown to accumulate faster and diffuse deeper in the tumor than larger molecules (70 and 2,000 kDa, R_H: 7 and 25 nm) [77]. However, this accumulation was transitory as smaller molecules would rapidly diffuse back in the vascular compartment [77]. By focusing on a short accumulation period during which the blood concentrations of each polymer were relatively constant, the authors of this study could somehow alleviate the influence of the distinct circulation times on their findings. However, in general, measuring the direct impact of size on the EPR is hampered by the effect that size usually has on the circulation kinetics of NPs and polymers (see Fig. 3).

A recent article addressed this issue by comparing the tumor deposition of different-sized particles which ostensibly showed the same circulation profiles [74]. This study found that particles (sizes: 30, 50, 70 and 100 nm) distributed comparably when the tumors were hyperpermeable (murine colon adenocarcinoma), but that only NPs smaller than 70 nm could accumulate efficiently in poorly permeable tumors (human pancreatic adenocarcinoma). In that case, the degree of tumor accumulation of these platinum-loaded particles correlated with tumor shrinking efficiency. This study highlights the dual effect of the tumor biology and the size of nanomaterial on the endothelial permeability (P and L_p) and nanomaterial reflexion coefficient (ρ_F). Given the relatively small difference in the hydrodynamic radii of the NPs, it suggests that the neovasculature wall in this pancreatic cancer model acts as an all-or-nothing barrier with a 30 to 70 nm threshold (P and $L_p = 0$ above the threshold). This cut-off could be raised above 70 nm when the tumors were treated with TGF-β inhibitor (see section 1.3.3). Preferential tumor accumulation of smaller particles (*i.e.*, < 50 nm) was confirmed by others, however differences in the blood circulation profiles of the compared NPs could not rule out other interfering parameters [83].

Insightful results were also obtained by Wong *et al.* who developed 90-nm NPs that disintegrated into 10-nm quantum dots when degraded by tumor proteinases [84]. The evaluation of their novel nanomaterial design emphasized the importance of size on the interstitial diffusivity of particles (D_{eff}). The quantum dots loaded in cleavable particles showed improved spatial distribution and enhanced penetration depth in the tumor compared to quantum dots loaded in similarly-sized, non-cleavable silica NPs.

2.5.2. Charge—Like other physicochemical parameters, the charge of macromolecules [85] and nanomaterials [11, 12] can influence the EPR effect by changing the systemic circulation times and the intratumoral processes. Once again, it is usually difficult to address both phenomenon independently [54]. The presence of surface charge can alter the opsonisation profile of the material, its recognition by cells in the organs of the MPS and its

overall plasma circulation profile (↓ C_v) [11, 12, 86-92]. Negative surface charges can either increase, decrease or have no impact on the blood clearance of NPs [92-97], but positive charges are generally recognized as having a negative effect on the plasma exposure to the nanomaterial [90,91, 98].

In tumor-bearing animals, despite the reduced blood circulation times, non-PEGylated, positively-charged liposomes containing the lipid 1,2-diacyl-trimethylammonium propane (DOTAP) display higher ratios of concentration in tumor *vs.* surrounding tissue compared to their negative or neutral counterpart [55, 56, 98]. The preferential distribution to the tumor is attributed to localization of the vesicles to the epithelium of the tumor neovasculature, with very little extravasation or very shallow interstitial diffusion. The positive charges possibly favour interactions of the NPs with the tumor blood vessels and eliminate their predisposition to diffuse deeper in the tumor while preventing their redistribution in the systemic circulation. This phenomenon has been utilized for therapeutic purposes by targeting the tumor vessel endothelium with antiangiogenic drugs in preclinical models [55, 99] and, very recently, in humans [100, 101].

Sterically-stabilized colloids with positive charges have also demonstrated enhanced tumor accumulation [102, 103] while others seem less efficacious [90, 91]. Parameters like the degree of ionisation, the relative blood circulation times of the control NPs [102] or the architecture of the construct (*i.e.*, charge on the core surface [102, 103] *vs.* charge on the corona [90, 91]) might explain these conflicting findings. Recent work has highlighted that the architecture of charge presentation by zwitterionic material influences the type of non-specific interactions with endothelial cells [104].

Besides the limited extravasation of NPs due to interactions with the tumor endothelium, charges can also bind to the ECM and limit diffusion in the interstitium (↑ R_i) [60]. *Ex vivo* studies conducted on ECM isolated from mice sarcoma showed that charges (positive or negative) had deleterious effects on the movement of NPs through the matrix. In fact, the presence of charge above a certain threshold (> 30 % DOTAP or >60 % 1,2-dioleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (DOPG), *i.e.*, ζ-potential ~ +10 mV and -30 mV, respectively) abolished the diffusion of the NPs [60]. This is in accordance with the results obtained from the intratumoral injection of neutral and charged NPs, which showed that charged colloids interacted with the tumor longer than their neutral counterparts [105, 106]. *Ex vivo*, the mobility of the particles can be restored by masking the surface charges with high amounts of salt (*e.g.*, adding 1 M KCl) or, in the case of the positively-charged material, small amounts of polyanionic proteins (*e.g.*, 0.5 mM Heparin), suggesting that this effect might be the result of ionic interactions between the NPs and the ECM [60].

2.5.3. Shape—The shape of NPs has been shown to influence blood exposure by modulating interactions with the MPS [12, 107-109]. Furthermore, single-wall carbon nanotubes with high aspect ratio (*i.e.*, from 100:1 to 500:1) were shown to be cleared efficiently by the kidneys despite dimensions 10-20 times above the usual glomerular filtration threshold (*i.e.*, 100-500 nm), suggesting that elongated shapes could provide benefits to the filtration process through porous structures [110]. These factors prompted

investigation into the ability of nanomaterials with different aspect ratios to accumulate in the tumors.

Recently, the tumor distribution kinetics of nanorods with a length of 44 nm (aspect ratio: 10) were compared to those of 35-nm nanospheres showing the same hydrodynamic radius [111]. Despite similar blood circulation profiles, the nanorods were shown to extravasate to the interstitium 4 times faster and to diffuse deeper in the tumor, interacting with a 1.7-fold larger volume. Similarly, regardless of their increased splenic clearance and shorter blood circulation times, elongated viral nanofilaments derived from plant viruses showed increased tumor homing and accumulation compared to other spherical viral constructs [112]. All together, these findings suggest that elongated architecture might present beneficial EPR properties (possibly by influencing P, L_p and ϕ_i) .

2.6. The EPR effect in humans

Although the EPR effect has been observed and studied in many rodent models using subcutaneous and orthotopic xenografts, as well as genetically-engineered mouse models, its prevalence in human primary and metastatic tumors require further investigations [113, 114]. This is particularly important since only a few first-generation anticancer nanomedicines materially improve overall survival in patient cohorts, conceivably because subpopulations with higher susceptibility to NPs might be masked by non-responsive patients [27]. Provided that such subpopulations exist, careful patient selection based on the degree of EPR could potentially further enhance the effectiveness of nanomedicines in clinical practice [27].

Many reasons can account for the paucity of data for the EPR effect in human, the most important being that obtaining meaningful biodistribution data in human is challenging. In clinical studies, surrogates to the analytical methods employed in animals (*i.e.*, tissue extraction and digestion) must be employed [115]. During the initial evaluation of PEGylated liposomes as drug carriers for DOX and other drugs, some studies assessed the distribution of liposomes and their payload in patients' tumors [116-121]. These studies measured the drug concentration in malignant exudates [116], tumor biopsies [117, 118] or using radionuclide-based imaging of modified liposomes (^{99m}Tc or ^{111}In) [119-121].

These studies usually report advantageous drug tumor deposition of the liposomes compared to the free DOX: the DOX levels achieved in the tumor 3 to 7 days after the administration are usually 4 -16-fold higher with the liposomes [116, 117]. This difference might be due to preferential accumulation of the liposomes in the tumor by EPR or to fact that liposomes maintain higher DOX concentrations in the blood, supplying the tumor for a longer time (free DOX $t_{1/2}$: 10.4 h, liposomal DOX $t_{1/2}$: 45.9 h).

In opposition to the animal studies where EPR of PEGylated liposomes can be evidenced by high tumor-to-blood ratios [122, 123], figure 4 shows that, in humans, the only cancers which show preferential tumor accumulation for liposomes (*i.e.*, ratio > 1) are sarcomas (with the exception of 1 patient with bone metastases for which plasma and biopsy samples were not analyzed at the same laboratory) [118]. Evidently, the direct comparison of these studies is imperfect because the data is scarce, the methodologies employed vary and the

studies do not provide information on the antitumor response [64, 124]. Also, it is still unknown how the data obtained with liposomes translates to other systems; it is probable that factors like drug release from the NPs and cellular uptake also affect the drug concentrations measured in the tumor. Nevertheless, these observations support the idea that diverse cancer types might show distinct predisposition to the EPR effect.

It is also important to note that systemic chemotherapy is usually reserved for patients with metastatic cancers, while preclinical models usually focus on primary tumors. Since primary and metastatic lesions may exhibit different behavior [113, 114], it is still not clear if the deposition of NPs in metastases correlates to that of primary tumors [125]. To further complicate the picture, patients enrolled in early clinical phases are rarely naïve to therapeutic agents, and most have received multiple treatments that may have altered the complex tumor microenvironment.

Finally, another reason why the assessment of the EPR effect in humans is not simple is that the optimal parameters to ensure maximum distribution and retention in humans can be different to those required in animal models. Both clearance mechanisms and tumor biology differ between animals and man [126, 127]. Animal models, especially xenograft models, used to study NPs at the preclinical stage, seem to offer limited predictive value of the clinical outcome in humans [128]. Furthermore, while the screening of NPs with different physicochemical properties in mice is relatively easy, the parallel evaluation of NPs candidates in humans remains largely impractical. Phase 0 clinical trials allow the concomitant study of multiple drug candidates (including NPs) at sub-therapeutic doses in a small number of patients to achieve a proof of principle [129]. However, this approach is potentially complex and would require the manufacturing of clinical supplies and suitable preclinical toxicology studies for each NP candidate. Furthermore, assessment of tumor accumulation would also necessitate the development of robust endpoint assays, with adequate sensitivity regardless of the sub-therapeutic doses used and sufficient reproducibility considering the low number of patients. Hence, while possible, the parallel evaluation of multiple formulations in humans remains a considerable challenge.

In the context of personalized medicine, it therefore becomes very important to identify what are the characteristics that predispose cancers to the accumulation of available NPs. In animals, iodine-loaded liposomes as EPR imaging modalities were able to determine *a priori* which mice would benefit from the injection of therapeutic nanomaterials [130]. A similar strategy in humans may provide useful information by correlating EPR effect with biomarkers and response to therapeutic NP. A pilot clinical study was recently initiated to ascertain the safety of the concomitant administration of an imaging agent with irinotecan-loaded liposomes (clinicaltrial.gov, NCT01770353). In this study, ferumoxytol, a clinically validated iron-oxide NP approved for the treatment of iron-deficiency, is being administered prior to the administration of MM-398, a liposomal irinotecan. Subsequently, intratumoral concentration of irinotecan and SN-38 (the active metabolite of irinotecan) will be measured. Given that ferumoxytol is efficiently phagocytosed and has been used to image tumor-associated macrophages (TAM) in preclinical models of solid tumors [131], this study might reveal insightful information about the possible predictive value of iron-oxide NP accumulation on the efficacy of MM-398. Similar iron-oxide NPs have been efficiently

used in humans for the early detection of lymphatic metastases [132] and to measure increased vascular permeability in the context of inflammation [133].

2.7. Future perspective on passive targeting

The last 30 years have shown that EPR plays an important role in the delivery of macromolecules and nanomaterials to tumors. Nevertheless, a full understanding of the degree of interpatient variability and the importance of tumor heterogeneity in the EPR effect in humans has yet to be established [27]. As we unravel which parameters are the most important to improve the distribution of chemotherapeutics to tumor cells, the individual patients and the cancer types benefiting the most from carrier-mediated drug delivery might be identified.

Furthermore, it is now appreciated that the physicochemical properties of the nanomaterial are as important as the tumor biology. Meaningful exploitation of the EPR effect in humans will require precise understanding and control of the physicochemical properties of nanomaterials [134] and, possibly artificial fine tuning of the tumor microenvironment [28]. In the era of modern cancer biology, assessing the tumor microenvironment in individual patients and predicting their susceptibility to the EPR effect may eventually become the mainstay of therapy when choosing between therapeutic regimens. Although it is hard to currently predict which markers better correlate with NPs efficacy in humans, relationships will most likely delineate as tumor genotyping and bioassays continue to progress. Ideally, a broad biomarker correlating the susceptibility of tumors to nanomedicines in general (*i. e.*, levels of tumor-associated macrophages; levels of ECM proteins/GAG; tumor infiltration of specific proteins/cells; endothelial expression of surface biomarkers) would certainly facilitate the screening of patients to optimise therapy.

In parallel, the EPR phenomenon affects the distribution of the drug-carrier to the tumor without necessarily increasing the ability of the drug to reach its pharmacological target. The optimisation of NPs' efficacy therefore also involves optimal drug release rates whether through controlled diffusional release [135], covalent conjugation of the drug to a polymer backbone [136, 137] or environment-triggered release [138, 139]. Without specific affinity of the nanomaterial for the cancer cells, the chemotherapeutic payloads will have to reach their pharmacological targets by their own means or risk diffusing back into the vasculature [77]. In some instances, drugs exhibit sufficient affinity for their pharmacological target to remain trapped in the tumor for prolonged periods of time. For example, the high affinity of docetaxel for the microtubules translates into very low efflux from the tumor; consequently, the elimination half-life of the drug from tumors is approximately 15-20 times higher than its elimination from the blood and normal tissue [140]. The behavior of drugs and their affinity for the intratumoral environment needs to be taken into account individually when designing passively-targeted NPs and the optimal drug release profiles should be optimised on a case-by-case basis. In this regard, tools that can assess the interactions between drugs and their substrates in tumors will be of particular interest [141].

Similarly, most macromolecular drugs (including nucleic acids and some proteins) cannot readily permeate through the cell membrane and reach their pharmacological target. For these classes of API the modification of the NPs with targeting ligands may be more

appropriate. Active targeting increases the affinity of the NPs for tumor cells, increasing its tumor residence times and allowing the drug-loaded NPs to efficiently enter the cells through receptor mediated endocytosis. The principles and future development of active targeting using affinity ligands on the surface of NPs will be presented in the next section.

3. Active targeting: Toward magic bullet?

Active targeting, also called ligand-mediated targeting, involves utilizing affinity ligands on the surface of NPs for specific retention and uptake by the disease cells targeted. To that end, ligands are selected to target surface molecules or receptors overexpressed in diseased organs, tissues, cells or subcellular domains [6, 33, 142-144]. Actively-targeted material need to be in the proximity of their target to benefit from this increased affinity. Therefore, the approach is aimed towards increasing interactions between NPs and cell and enhancing internalization of drugs without altering the overall biodistribution [6, 145, 146].

The design of actively-targeted NP drug carriers is complex because the NP architecture, the ligand conjugation chemistry and the types of ligand available all contribute to the efficacy of the system. Other factors like the administration route or the non-specific binding of proteins during the NP's journey through the bloodstream have been shown to affect the targeting ability of NPs. [12, 147]. Physicochemical properties like the ligand density [148], the size of the NPs [149] or the choice of the targeting ligand [150] might also possibly affect the efficacy of the active targeting strategy *in vitro* and, most importantly *in vivo*. The following section will highlight the strategies, benefits and drawbacks of combining targeting ligands with NP drug delivery systems in the targeting of solid tumors.

3.1. The fundamentals of active targeting

The main mechanism behind active targeting is the recognition of the ligand by its target substrate. Representative ligands include antibodies, proteins, peptides, nucleic acids, sugars, and small molecules such as vitamins [151]. Target molecules can be proteins, sugars or lipids present in diseased organs or on the surface of cells [152, 153]. The interactions of ligand-functionalized NP systems with their target antigen are enhanced by the multivalent nature of the NP architecture: multiple copies of the ligand increase the avidity of the NP for its target [154]. The targeting specificity and the delivering capacity are two important aspects to evaluate the efficiency of an active targeting system. The specificity is determined by the biodistribution of the ligand-functionalized NP and by how the conjugated ligand and the NP system interact with off-target molecules and cells; it is defined by the ligand and NP properties. The delivering capacity is directly related to the NP material and structure [33, 155]. Currently, actively targeted NPs are envisioned as a promising complementary strategy to EPR to further augment the efficiency of cancer nanomedicines.

Actively-targeted NPs require being in the vicinity of their target antigen to recognize and interact with it. That intrinsic characteristic is considered a major challenge to the development of actively-targeted NPs [156]. Here again, the systemic clearance of the NPs affects the amounts available in the bloodstream supplying the tumor. Because tumor blood flow is small compared to those observed in the organs of the MPS [12], the increase in the

NPs' affinity for the targeted tumor-antigens cannot always compensate for the natural clearance processes. Actively-targeted NPs need to be designed to have extended blood circulation times. Similarly, because molecular targets are usually situated in the extravascular space of the tumor, NPs rely on the EPR effect to reach their targets [113, 114]. Together, these factors explain why active targeting strategies cannot radically change the biodistribution profiles of nanomaterials [157-159] and why the blood circulation times of ligand-decorated NPs need to be optimized to achieve optimal targeting [4, 33, 160].

Active targeting has been efficiently exploited to increase the NP internalization by the target cells and improve the efficacy of their payloads [4, 158, 159, 161]. Evidently, NP intracellular trafficking is complex [162] and receptor-mediated internalization can qualitatively affect the vesicular transport [163, 164]. Actively targeted NPs that increase therapeutic efficacy will circumvent these issues by being capable of sufficient endosomal escape or by encapsulating drugs that are impervious to the endosomal/lysosomal environment. Anti-HER2 targeting moieties on the surface of liposomes strongly increase the uptake of the NPs in HER2-expressing cancer cells [158]. In opposition, non-targeted liposomes or targeted liposomes injected to mice bearing non-HER2-expressing tumors were shown to accumulate in the perivascular and stromal space in higher proportions [158]. In those cases, the liposomes were highly captured by the macrophages and showed reduced interactions with the cancer cells. Similarly, the intracellular delivery of nucleic acids can also take advantage of active targeting. For example, Bartlett *et al.* showed that the targeting of the transferrin-receptor is essential to the silencing of a luciferase beacon in a neuroblastoma xenograft [159].

Recently *in vitro* works on targeting concomitantly multiple surface receptors with a single NP were conducted [165, 166]. The presence of both ligands (ligands targeted to folic acid combined to either EGFR antibodies [165] or glucose [166]) seemed to quantitatively improve cellular internalization. *In vivo* evaluation of non-sterically stabilized polystyrene nanoparticles functionalized with antibodies (against transferrin and intercellular adhesion molecule-1) seemed to show that multiplexed ligand could affect biodistribution [167]. However, the nanoparticles in this study being so remote from potential therapeutic NPs (in terms of size and hydrophobicity, for example), it remains difficult to predict if such approach will really prove beneficial to target cancer cells.

3.2. Ligand Conjugation/Attachment Strategies

Since NP avidity is directly related to the ligand density, the introduction of ligands on the surface of the NP is one of the key steps of designing actively-targeted systems. In most cases, covalent attachment of the ligand is the preferred strategy, but physical adsorption using affinity complexes can also be used effectively [168].

Organic and inorganic materials having different physicochemical properties, the type of particle will determine the difficulty of the ligand conjugation step [169]. For example, the surface functionalization of gold surfaces can be carried by directly reacting thiols with the surface [170], but other inorganic materials need the introduction of functional groups (e.g. NH_2 and OH) to increase their reactivity [171-173]. Organic polymers require different

strategies where side chains or terminal reactive functions are reacted before or after NP synthesis.

In all cases, the stability of the ligand-NP bond will dictate how the particle retains the targeting moiety on its surface. In that regard, other design considerations must also be taken into account. For example, polymer matrices that erode homogenously in bulk [174, 175] might be preferable to surface-eroding polymers [176] for which the tethered ligand can shed as the polymer degrades. Similarly, ligands that are non-covalently inserted in a lipid bilayer might require bulkier hydrophobic anchors to remain stable *in vivo* [138, 177]. The following section will discuss the ligand conjugation/adsorption strategies in detail, with a specific emphasis on commonly utilized biodegradable polymers [178].

3.2.1. Pre-conjugation vs. post-formulation strategies—The conjugation of the ligand to the NP material is relatively straightforward when it is done before the assembly of the NP formulation. Pre-conjugation can be achieved with small molecules [150], peptides [179] and aptamers [142]. It is less adapted to native proteins with complex secondary structures as the conjugation step usually involves exposure to organic solvents. The pre-conjugation enables a subsequent one-step formulation procedure that reduces the risks of side reactions, forms covalent bonds between the ligands and NPs, and allows greater control over NP properties and drug release. Furthermore, this strategy allows the introduction of multiple types of ligands to NPs and facilitates the purification steps after NP formulation.

The alternative to this pre-formulation strategy is post-formulation conjugation where the ligands are reacted with formulated NPs directly to form covalent bonds. This strategy works for all types of ligands: antibody, protein, peptide, aptamer and small molecules, and might be preferable when the stability of the ligand in organic solvents is an issue, when the size of the ligand is too large or when the presence of the ligand changes the physicochemical properties of the copolymers. For example, some proteins lose their functions in organic solvents and they are too large to participate in the NP polymer self-assembly process [180]. In other instances, hydrophobic molecules tethered at the end of flexible copolymer chains modify their solubility and the ligands end up embedded in the hydrophobic core during the self-assembly procedure [150]. In those cases, it can usually be beneficial to conjugate these ligands after NP formation, directly onto the NP surface.

3.2.2. Synthetic strategies for conjugation—For the chemical conjugation strategies, bifunctional linkers are used to couple the ligand with polymers or NPs *via* a series of chemical coupling reactions. The formation of a peptidic bond between the ligand and the NP surface is usually done by activating carboxylic groups (using, for example, N-hydroxysuccinimide (NHS) and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)) and reacting it with nucleophilic groups (*i.e.*, amine) on the ligand. Although this approach is straightforward and can be carried in both aqueous and organic environments, the selectivity of the conjugation depends on the number of reactive amine functions on the ligand and the final orientation of the ligand can be compromised if more than one reactive group exists on the ligand.

Similarly, another coupling strategy couples a maleimide group on NP surfaces or polymers with a thiol group of ligand (*i.e.*, cysteines in proteins or peptides) to form a stable thioether bond. In proteins or peptides, disulfide bonds can be reduced to free thiols to allow the reaction, provided that the reduction does not alter the three-dimensional structure and affinity of the ligand for its substrate. Similarly, free thiols can be introduced by first reacting 2-iminothiolane to primary amines, but this approach introduces a positive charge on the NP-ligand construct [181]. Other chemical efforts have also been reported for introducing thiols to molecules [182].

Recently, a bioconjugation method called “click chemistry” was developed which is a single step reaction that involves heteroatom bonds with or without catalysts [183]. For example, alkyne groups on peptides or small molecules will readily form a bond with the azide group on the NP surface or polymer backbone without side reactions. This method is very selective and provides very good yields. Copper-catalyzed click-chemistry is limited by the strenuous purification required to remove the toxic catalyst and by the fact that copper can have deleterious effects on the ligand themselves. For these reason, non copper-catalyzed click-chemistry has been developed using cycloadditions such as cyclooctyne, tri(benzyltriazolylmethyl)amine, and sulfonated bathophenathroline [183]. Finally, another limitation is that alkyne and cyclooctine groups are artificial chemical moieties and must be introduced synthetically to the ligand structure. When the ligand is a peptide, this can be easily done by introducing a reactive aminoacid at the C- or N-terminus. This strategy is less viable for small molecules where the new, relatively bulky functional groups can compromise affinity or for larger, more complex proteins produced through recombinant or bioengineering strategies.

3.2.3. Non-covalent approaches—Streptavidin-biotin interaction may be helpful to overcome limitations of synthetic bioconjugation [168]. Nanoparticles coated with avidins have complementary pairings with biotinylated ligands. Biotin is commonly used due to its small molecule size allowing it to not inhibit or change the functions of the ligands [184]. This strategy is more versatile in comparison to bioconjugation method and has been applied to antibodies, peptides and aptamers [185]. It is particularly useful to establish proof of concepts or to screen different ligands without interference from the conjugation chemistry or to study fundamental ligand decoration parameters [185, 186]. The weakness of this method is that it may cause immunogenicity due to the presence of the exogenous protein on the surface and it is not usually suitable for human use [187]. Also, because avidin can bind biotin in a multivalent fashion, cross-linking between the NP constituents can also occur.

Alternatives to the avidin-biotin strategy use other high affinity complexes like Vancomycin/D-Ala-D-Ala [188] or adamantane-cyclodextrin coupling [189]. For example, the later strategy allows the conjugation of ligands to hydrophilic polymers (like PEG) using very mild conditions and the subsequent tethering of the PEGylated-ligand on the surface of the nanoparticle *via* non-covalent interactions [190].

3.3. Influence of the architecture of actively-targeted NPs

The conjugation of ligands on the surface of NPs changes their properties [33, 142]. While they lose the rotational and translational freedom bestowed to free molecules, the new targeted entity achieves improved avidity because of the increased valency [149, 154, 191]. Similarly, the properties of the NP like size, geometry, surface properties (charge and hydrophobicity), and composition (NP material) also affect the behavior of the targeted constructs (Figure 5). In some cases, NPs have shown benefits that go beyond the simple delivery of drug. For example, strands of nucleic acids immobilized on the surface of nanomaterial are more resistant to nuclease degradation [192, 193]. To fully understand the properties of actively-targeted NPs, it is critical to determine how the physicochemical properties of the NPs affect the interactions with the targets.

3.3.1. The ligand density—Because increased valency allows cooperative effects, the density of the targeting molecules on the surface of NPs impacts their affinity for the substrate. Thermodynamically, the binding of a ligand to its substrate facilitates the subsequent binding of its neighbors [191, 194]. Biologically, the multiple interactions of the NP with the cell membrane force the clustering and local concentration of receptors. This triggers the wrapping of the membrane and leads to internalization [195]. Together, these incidences impede the detachment of the NP from the cell surface and result in increased avidity.

This allows the use of multiple relatively low affinity ligands to efficiently bind targets with high avidity [196]. *In vitro*, this increasing ligand density usually results into improved cellular uptake [148, 197]. However, this increase in affinity is not always linear. In some cases, the cooperative effect of the ligand can saturate and further increases in ligand density can have deleterious effects on cell binding [197, 198]. This effect can be explained by improper orientation of the ligand, steric hindrance of neighboring molecules or competitive behaviors for the binding of the receptor. Similar negatively cooperative systems have been observed with folic acid-targeted micelles where the ligands are arranged in patchy clusters [199]. In these NPs, the display architecture of the targeting ligands influenced the extent of receptor-mediated tumor uptake in cancer cells both *in vitro* and *in vivo*. In other cases, the use of high densities of hydrophobic ligands can increase the macrophage uptake of the NPs without providing significant advantages in terms of receptor-mediated internalization [150].

Similar effects are seen *in vivo* where higher densities do not however always result in improved efficacy. The alteration of the NP surface to incorporate the ligands can modify the blood circulation and biodistribution profiles of the material. Using aptamers to target polymeric NPs to prostate cancer cells, Gu *et al* showed that increasing the ligand density above 5 % caused increased clearance of the NPs by the liver and the spleen and impeded the distribution to the tumor [148]. In fact, the increased clearance of targeted NPs by the MPS is critical and has been responsible for the demise of most actively targeted systems [200].

Finally, it has been observed that tumor selective antibodies could suffer from a binding-site barrier preventing their in-depth diffusion in the tumor [201-203]. This phenomenon is observed when high affinity molecules rapidly bind perivascular cells upon their

extravasation to the tumor (↑ R_i in equation 2) [204-207]. The diffusion of drugs with high affinity for tumor cells is limited by the binding-site barrier [208]. This effect has been recently detected with 25-nm NPs targeted to the epidermal growth factor which showed limited tumor penetration compared to their non-targeted counterpart [209]. Interestingly in that case, the binding-site barrier effect was not observed with larger NPs (*i.e.*, 60-nm particles), presumably because of differences in the rates of tumor penetration and/or cell internalization. Therefore, it seems probable that adequately tuning the ligand density on the NP surface could mitigate the binding-site barrier effect and translate into adequate retention time and maximal cellular uptake throughout the tumor.

3.3.2. The NP size and shape—Size and shape of the nanomaterial must be taken into consideration early in the design of targeted NPs. For spherical particles, smaller sizes represent higher curvatures which can be problematic for post-synthesis ligand functionalization. For example, when focusing on the adsorption of relatively rigid protein A on the surface of gold NPs, very small NPs (5 nm) can result in poor or uneven ligand surface densities [210]. In this study, this effect was not observed with larger NPs (*i. e.*, 15 nm).

In addition, the tethering of high molecular weight ligands *(e.g.*, antibodies, proteins, aptamers) on the surface of NPs increase their hydrodynamic radius beyond that of the unfunctionalized material [149]. This increase in size must always be considered in light of the possible size restrictions involved in tumor accumulation (*see* section 2.5.1).

Besides the abovementioned effect, size can also affect cellular uptake. Using gold and silver NPs targeted with anti-HER2 antibodies, Jiang and colleagues showed *in vitro* that optimum cellular uptake in breast adenocarcinoma cells was obtained with a very narrow size range, i.e., 25-50 nm (R_H with antibodies: 45 to 80 nm) [149]. Although, the avidity of the particle increased with size between 2 to 70 nm (R_H with antibodies: 13 to 100 nm), the authors explain that maximum uptake is a compromise between high avidity and optimal cell membrane wrapping around the NP. As these experiments were conducted in culture, these size considerations only took into account interactions with the surface of the cells and not convective or diffusive aspects of intratumoral transport. Additionally, it is not clear how these conclusions can be expanded to other systems, as NP-cell interactions highly-depend on the physicochemical properties of the material [211, 212].

In vivo, Lee and colleagues reported that the size of actively targeted NPs (25 *vs*. 60 nm) could affect the intracellular deposition of the nanomaterial [209]. In that case, although the intratumoral distribution of smaller NPs was decreased due to shorter blood circulation times, the cytoplasmic and nuclear distribution of the 25-nm NPs was superior to that of the 60-nm colloids.

Besides the effect on circulation properties and tumor accumulation aforementioned [82, 109], the shape of NP seems to influences the cell uptake kinetics and internalization pathways by modulating the interactions between the nanomaterial and the cell surface [211, 213]. The internalization of non-spherical targeted NPs has recently been studied [214, 215]. Barua *et al*. showed that, when compared to 200-nm diameter spheres, HER2-targeted 370 ×

125 nm nanorods improved specific uptake by 1.6 fold while reducing the non-specific uptake [215].

3.3.3. Surface and ligand charge—From a synthetic perspective, the charge of the unfunctionalized NP and that of the ligand can affect the conjugation yield and the spatial display of the ligand on the surface [216]. Repulsive or attractive forces between the surface of the NPs and the ligand can interfere with the conjugation [217, 218] or affect the final ligand structure and conformation. A chemical spacer with reasonable length, such as PEG, can be helpful to reduce the effect, but might simultaneously complicate synthesis and increase the final particle size [197].

As discussed in section 2.5.2., the final surface charge will affect the efficacy of the targeted NPs. Due to the interaction between cationic NPs and negatively charged cell membranes, positively-charged NP show increased cellular binding and uptake, in a non-specific manner [219]. As most ligands are charged molecules, the NP surface charge is determined by the combinations of ligand densities, materials, and NP formulation strategies. Although recent work was recently carried to address how charge density affects interactions of actively targeted NPs with cells [220] and how optimization of the ligand densities and NP charge can affect cellular uptake [221], it remains unclear what parameters offer the best tumor targeting *in vivo*.

3.3.4. Surface hydrophobicity—Besides surface charge, hydrophobicity can also affect the architecture of the ligand display [148]. This can have serious effects since most polymeric NPs have hydrophobic cores (e.g., polyesters, polyamides) [222]. Valencia *et al*. showed that during the self-assembly of polymeric hydrophobic particles, folic acid, a model hydrophobic ligand, could remain trapped in the particle core without being properly displayed on the surface [150]. This resulted in a non-linear rise of NP affinity with increased feed-ratios of ligands in the formulation. Since this effect was not observed for a more hydrophilic ligand (the RGD peptide), that study highlighted the necessity of thorough physicochemical characterization of NPs after synthesis.

The final surface hydrophobicity of the NPs can also affect non-specific interactions with cells. On the one hand, actively targeted NPs without steric stabilization seem to lose their substrate-binding capacity when proteins adsorb on their surface [223]. Using silica NPs functionalized with transferrin, Salvati and colleagues showed that NPs lost their selectivity for cells expressing the transferrin-receptor upon plasma incubation. Although raising an important issue, these *in vitro* works are preliminary: these particles were not optimized for prolonged blood circulation times, and probably would have faced many other issues *in vivo*.

On the other hand, while PEG surface-functionalization can delay adsorption of opsonins and plasma proteins, the use of long or dense PEG chains can also prevent ligands from reaching their targets. This phenomenon has been demonstrated *in vitro* [197, 224] and *in vivo* where the efficacy is dependent on both circulation times and ligand-substrate interactions [225]. Since minimal PEG coverage seems required to maximize circulation times and tumor distribution, NPs that lose their PEG protection in the vicinity of cancer cells might provide an opportunity worth investigating [139].

3.4. Targeting Ligands

Choosing the right type of ligand is critical to the efficiency of actively-targeted NPs. The first targeted systems centered mainly on the use of antibodies as targeting moieties because of their high specificity and wide availability [200]. Since, then other proteins, peptides, nucleic acid-based ligands and small molecules have all been described (Table 2). In the following section, the current understanding of the benefits and limitations of each system will be highlighted. Various issues of ligands that may affect ligand directed NP active targeting will also be discussed, including the ligand MW/size, ligand surface property, and ligand density on NP surfaces.

3.4.1. Antibodies and their fragments—An antibody (Ab, also called immunoglobulin (Ig)) is a large Y-shaped glycoprotein that can recognize the specific parts of a foreign target (an antigen). The dimeric functional region (hypervariable region (HVR), also called $F(ab')_2$ fragment) at the tip of the antibody can have very large numbers of slightly different structures (binding sites), offering the possibility to recognize a variety of antigens. Oppositely, the Fc fragment, at the base of the Y-shape, is much less variable and is responsible for the recognition of the protein by the MPS and the immune system.

Interest in using Ab as targeting moieties stems from the important role the play in modern therapeutics. Since the 1980s, antibodies have been the most widely investigated targeting ligands in the clinic and more than 30 types of monoclonal antibodies (mAbs) have been approved for clinical use, including rituximab, trastuzumab, cetuximab and bevacizumab [30-33]. The conjugation of Abs on the NP surfaces aspires to combine the Ab's specificity and affinity with the unique properties of the NPs themselves. The first reported examples of targeted NPs are liposomes decorated with mAbs [30-32]. Although these systems showed improved cellular uptake *in vitro*, antibody-targeted NP delivery systems still face many limitations and challenges, which greatly limit their pertinence *in vivo.*

First, antibodies are large proteins with a molecular weight of 150 kDa and a hydrodynamic radius of 15-20 nm. This large size impedes the effective surface conjugation and causes notable increases in the diameter of the NPs [159, 244]. To circumvent this problem, smaller fragments of Abs were proposed as targeting moieties, but the monomers and dimers of the Fab recognition patterns still represent bulky molecules with sizes around 50 and 100 kDa, respectively [6, 200].

Second, the physiological role of antibodies is to clear antigens from the circulation by facilitating their recognition by immune cells and the MPS. The conjugation of Abs on a nanosized carrier therefore results in very effective clearance from the blood [245, 246]. Removing the Fc fragment (*i.e.*, using only Fab fragments) resulted in slight improvements of blood exposure, but still compromised circulation times compared to undecorated NPs [247]. Others have proposed different conjugation strategies using the Abs carbohydrates to proper position them on the NPs and minimize the display of the Fc segment on the surface of the particle [248], but the results of this novel preparation procedure had mitigated effects on the circulation times [249].

A third limitation of using Ab-decorated NPs is the relative sensitivity of these proteins to environmental challenges (temperature, salt concentration and enzyme) and their low resistance to organic solvents. This creates technical challenges for the reproducible scale-up of NP preparation, affects the cost/efficiency ratio of the preparation, and restricts their stability and shelve-lives.

Despite, these challenges, a few Ab-targeted NPs have made it to the clinical stages; MCC-465 and SGT-53 are two examples of antibody fragment-targeted NPs (Table 3). MCC-465 showed positive results in preclinical studies with adequate biodistribution and highly efficient delivery of doxorubicin to stomach cancer cells [250]. Another example of an NP with a single chain antibody fragment is SGT-53, which targets transferrin-receptors (Tf-R) on tumor cell surfaces by targeted delivery of the p53 suppressor protein [251]. With evident tumor growth inhibition in multiple cancers including head and neck, prostate, and breast, this platform has great potential for future clinical trials [252]. Finally, a group of scientists at the University Hospital of Basel recently published their results of the clinical investigation of PEGylated DOX liposomes (Doxil®/Caelyx®) incorporating a F(ab') fragment of cetuximab (Erbitux®, anti-EGFR mAb) in 29 patients with solid tumors [253]. Their liposomes, manufactured according to good manufacturing practices at the hospital's pharmacy, showed promising activity including 1 complete response in a patient with head and neck cancer.

3.4.2. Other proteins—The three-dimensional shape of proteins provides affinity for specific substrates, and therefore non-antibody proteins can be used as targeting moieties. Numerous naturally-occurring proteins have endogenous targets that can be exploited for therapeutic applications. For example, transferrin (Tf) is a 80-kDa glycoprotein which is one of the most abundantly studied targeting ligands [189, 254, 255]. Physiologically, Tf is responsible for the transport and regulation of iron concentration in biological environments. On the surface of cells, it binds the internalizing transferrin-receptor (Tf-R) with high affinity. Because Tf-R is upregulated on the surface of cancer cells, NPs decorated with Tf have attracted much attention for the delivery of anticancer therapeutics [256]. The presence of Tf-ligands was shown to be essential for the intracellular delivery and gene silencing efficacy of siRNA nanocomplexes [159, 189]. The strategy of using Tf to target Tf-R is currently under clinical investigation for various NPs [143].

Synthetic proteins can also be exploited as targeting ligands. For example, affibodies [230] or ankyrin repeat proteins [229] were developed to decorate NPs. This approach possesses the advantage of using high affinity, artificial ligands which do not have to compete against highly abundant, naturally-occurring proteins. Peptide aptamers are also synthetic fusion proteins where short variable peptide domains are confined by a constant protein scaffold [257]. This unique double-constrained structure over a loop of 10-12 amino acids offers binding affinities similar to those of antibodies. The protein scaffold may be any soluble compact protein, such as bacterial Thioredoxin-A [258]. Peptide aptamer selection can be made from combinatorial peptide libraries constructed by phage display and other surface display technologies such as mRNA, ribosome, bacterial and yeast displays [259]

Protein ligands share some limitations with antibodies: their bulky nature results in significant increases in size and their patterning on the surface of NPs can trigger immune responses. In fact, the conjugation of proteins on polymeric substrates can affect their metabolism and elimination pathways [260, 261]. Also, because the amino acid sequences are generally more complex, the conjugation procedures are less straightforward. The many reactive functional groups in their structure (*i.e.*, amine groups or cysteines) complicate their optimal arrangement on the NP surface and can lead to cross-linking between NPs. Also, because their tertiary structure is usually essential to their affinity for their antigen, proper precautions must be used to ascertain that the conjugation processes does not abolish their ligand properties.

3.4.3. Peptides—Peptides are linear or cyclic sequences of amino acids. They are typically differentiated from their larger counterparts (*i.e.*, proteins) by having sequences limited to less than about 50 residues. These shorter chains lead to smaller molecular sizes and simpler three-dimensional structures which result in improved stability and resistance to the environment as well as easier synthesis and conjugation. Their smaller sizes also allow the use of pre-formulation conjugation techniques for the preparation of NP systems. These advantages, combined with improved screening techniques to isolate ligand-substrate combinations have contributed to the increased role of peptides as targeting moieties in the past decade.

The most widely investigated peptide ligand is RGD (arginine-glycine-aspartic acid) peptide family, which can strongly and specifically bind to αvβ3 integrin receptors [33, 142]. Many peptide containing RGD sequences have been developed to target both cancer and angiogenic endothelial cells expressing the αvβ3 receptors. One example of RGD is the cyclic peptide cyclo(-RGDfV-) (Cilengitide) used as an anti-angiogenic agent [262, 263]. Despite the widespread applications, the nonspecific targeting of RGD restricts its drug delivery applications because αvβ3 integrin is also widely expressed on normal or inflamed tissues. Some of the intrinsic properties of RGD may also affect its targeting efficiency, such as molecular geometry. Colombo *et al*. investigated the structure-activity relationship of linear and cyclic RGD peptides. In their hands, the cyclic ligand showed more than 10-fold higher anti-tumor efficiency than its linear counterpart [264]. In parallel, other new generations of RGD analogue ligands with improved targeting potential are underway [33].

For reasons explained earlier, vasculature targeting is an interesting complement to the EPR effect. Endothelial cell-penetrating peptides can facilitate the transport of NPs across the cell membrane and into specific cell organelles. Some of these penetrating peptides contain a specific C-terminal C-end Rule (CendR) sequence [233], (R/K)XX(R/K), which is responsible for the penetration activity. These penetrating peptides (e.g. LyP-1 [265], iRGD [233], and F3 [266] peptide sequences) can interact with neurophilin-1 to facilitate the cell and tissue penetration of NPs [267]. Roth *et al*. showed that the conjugation of the cyclic peptide LyP-1 (Sequence: CGNKRTRGC) on NPs can penetrate through blood vessels [267]. Sugahara *et al*. also reported that iRGD peptide (peptide sequence: CRGDKGPDC) could enhance the efficiency of drug delivery [233]. Luo *et al*. reported that the LyP-1-functionalized NPs target the lymphatic tumor cells with high binding affinity and increase cellular uptake of the NP by 8-folds compared to non-targeted NPs [268]. In addition, Lyp-1

is found to be internalized by its targeted tumor cells indicating its cell-penetrating abilities [233, 269]. Other cell penetrating peptides for targeted NPs include Cys-Arg-Glu-Lys-Ala (CREKA) [270], Asn-Gly-Arg (NGR) [271], and Ile-Thr-Asp-Gly-Glu-Ala-Thr-Asp-Ser-Gly (LABL) [272].

Finally, peptides can elicit agonist or antagonist pharmacologic activity on their substrate. This activity can alter the fate of NPs decorated with such peptides [273]. In this study, the targeting of quantum dots to G protein-coupled receptors using peptide agonists led to the internalization of the nanomaterial. On the other hand, the use of a small molecule antagonist as a ligand commanded the specific binding of the NPs without cellular uptake. Presumably, this concept could be further exploited to more closely control the fate of targeted NPs.

3.4.4. Nucleic acid based ligands—Nucleic acid-based aptamers are another class of ligands with completely different structures from peptide-based aptamers. Nucleic acid aptamers (Apts) are single-stranded (usually short) oligonucleotides, such as DNA, RNA or modified nucleic acids (xeno-nucleic acids or XNA) [259, 274]. Thanks to their unique conformational structures that originate from intramolecular Watson-Crick interactions, Apts show high affinity and specificity. Candidates are screened from large oligonucleotide libraries with random sequences by taking advantage of the nucleic acid sequences. Binders are selected and specifically amplified at the detriment of non-binders using the polymerase chain reaction. Apts with strong binding characteristics for various targets, including small molecules [275] and proteins [276] have been isolated. Although the relative ease of isolating high affinity ligands against a variety of substrates remains the largest advantage of Apts, their reproducible synthesis and the simplicity of their chemical derivation are also among the other advantages of using Apts as ligands for targeted NPs [259].

Although Apts can exist naturally in riboswitches [277-279], most of the Apts used for the design and synthesis of targeted NPs are artificially-engineered. However, although the diversity of possible sequences offers virtually unlimited binding options, the key element remains in the isolation of successful binders from non-binding candidates. Since the 1990s, the isolation of ligands has gone from simple chromatographic separation [280-282], to cell-based screening methods [283, 284] and *in vivo* selection [285]. These processes evolved with the objective of finding candidates for increasingly complex substrates and specific applications. When screening for ligands intended to design actively-targeted NPs, the internalization of the target remains an important benchmark. Recently, a method was developed to exclude non-internalizing ligands from the enrichment procedure [284].

In vitro feasibility of Apt-decorated NPs to target the prostate-specific membrane antigen (PSMA) cancer marker was reported in 2004 [286]. Since then, the proof of concept was achieved *in vivo* for NPs incorporating multiple types of drug [160, 161, 241, 287, 288]. Overall, the targeting of PSMA showed improved efficacy in decreasing tumor growth and increasing survival. For example, Bagalkot *et al.* reported that the physical conjugate of Apt-Dox could result in almost 2-folds increase in survival rates in comparison to non-targeted NPs [287]. Similarly, intratumor injections of PSMA-targeted NPs were shown to delay tumor growth for more than 3.5 months [161].

Despite their added promises at the preclinical stage, the clinical development of Apt-decorated NPs has been impeded by certain limitations. First, because nucleic acids in biological environments are easily degraded by nucleases, the stability of the ligand on the surface of the NPs is a concern. Although many strategies have been employed to prevent the degradation and excretion of free therapeutic Apts in the bloodstream [289], not much work has been conducted to specifically address degradation of Apts on the surface of NPs. By analogy, the conjugation of the Apt on the surface of NPs should provide steric protection similar to that conferred by PEG, sugars and cholesterol modifications [289]. Similarly, the use of nucleic acids modified with fluoro, amino, or methoxy groups [259] or artificially-locked nucleic acids (LNA) [289] should also delay degradation by nucleases.

Another concern for the surface functionalization of NPs with Apts is the effect that these ligands have on the circulation kinetics of the nanomaterial. Given the phosphodiester backbone of the nucleic acid chain, Apts have negative charges. The presence of charges on the surface of nanomaterial can affect their circulation times [12]. Gu *et al*. showed that Apt densities above 5 % resulted in decreased distribution to the tumor and increased capture by the organs of the MPS 24 h after intravenous injection [148].

Besides these fundamental considerations, other technical aspects of using Apts to decorate NPs are also important. Like other macromolecular ligands, Apts can increase the hydrodynamic radius of NPs. In fact, despite the fact their size is relatively small (around 10-20 kDa), the stiff chain structure of the nucleic acid chains makes their size (length) similar to that of proteins (*i.e.*, from 3 to 10 nm) [259, 290] Furthermore, although the production costs have fallen recently due to technological developments and the expiration of patents, the selection and large-scale production of Apts on solid phase supports remain expensive procedures[192]. The production costs might be partially mitigated by secondary selection techniques which delete or shorten the non-critical regions while keeping the functional binding domains [259].

3.4.5. Small molecules—Small molecular weight compounds have properties which strongly contrast from the targeting ligands presented above: small sizes, low production costs, and improved stability. These advantages translate into simple pre-formulation conjugation strategies and simple, tunable NP synthesis. Hrkach *et al*. recently reported the preclinical developments leading to the clinical evaluation of PSMA-targeted NPs in an article that highlights many of the benefits of using small molecules as targeting agents [4].

In fact, the main challenge with small molecules is the identification of new affinity ligands for the substrates of interest. In general, the screening of small molecules is difficult to multiplex because selection and signal intensifying mechanisms like bacteriophage-mediated replication or PCR amplification do not exist. Notwithstanding a few examples of DNA-encoded libraries that facilitate the screening of binders using nucleic acid footprints [291, 292], the identification of small molecular weight ligands involve serendipity or tedious high throughput screening procedures. For that reason, clinically-relevant ligand/substrate pairs are scarce and most of the examples of actively-targeted NPs rely on using widely known ligands.

Among the synthetic and natural small molecule ligands reported, a common example is folic acid (*i.e.*, folates or vitamin B9). Folates have been widely utilized because of their very high affinity (K_D = ~10^{-9} M) and specificity for folate receptors (FR) which are frequently over-expressed on the surface of a variety of human tumors, including ovarian, brain, breast, colon, renal and lung cancers [293, 294]. For example, folate-targeted NPs co-encapsulating paclitaxel and yittrium-90 showed improved survival in a xenograft model of ovarian cancer [242] and folate-targeted fluorescent dyes were investigated in humans to improve surgical debulking of tumors using intraoperative tumor-specific visualization [295]. On the downside, FR expression seems to be patient-dependent and must be assessed individually for each type of cancer [33]. Recently, folate-targeted imaging modalities were proposed to identify FR-positive patients [296]. In a personalized medicine setting, such an agent would help screen *a priori* the patients that could benefit from folate-targeted therapeutic NPs. More importantly, as opposed to methods based on the *ex vivo* quantification of FR in lysed patient biopsies [297], *in vivo* imaging modalities might enable the quantification of FR on the surface of cancer cells, actually available for interactions with folate-decorated NPs. This is particularly valuable as it has been observed that as much as 50-75 % of the FR pool can be localized in the endosomal membranes [294, 298]. Finally, another limitation of folate-mediated cancer nanomedicines is that FR is constitutively-expressed in healthy tissues and normal epithelia of many organs, potentially limiting the selectivity of the ligand for diseased cells.

Another example of small molecule targeting ligands is triphenylphosphonium (TPP) and its derivatives for mitochondria targeting [243, 299]. TPP is a cationic, relatively large and hydrophobic molecule that can penetrate easily through the cell membrane. An investigation indicated that the positively charged TPP could accumulate several hundred folds within mitochondria [299]. An *in vitro* study recently established the mitochondria targeting potential of TPP-decorated NPs [243]. These studies showed that NPs could efficiently be internalized and escape the endosomal compartment when their size was around 100 nm and their zeta-potential was higher than +22 mV. Although this positive charge, conferred in part by TPP, seems important for *in vitro* performance, it might possibly undermine its blood exposure *in vivo.* This factor will have to be considered in the future developments of this targeting strategy.

Carbohydrate moieties [300], including mannose [301], glucose [166], galactose [302], and their derivatives, have also been widely utilized as targeting ligands. Carbohydrates target lectins, ubiquitous cellular membrane proteins that bind carbohydrates [303, 304]. In fact, one of the first actively-targeted anticancer nanomedicines evaluated in humans was a DOX-conjugate, which incorporated a galactosamine targeting moieties to target primary and metastatic liver cancers [305]. Although this product failed in phase II due to lack of efficacy [306], it was observed in humans that the ligand conferred some level of liver targeting to this polymer-drug conjugate [305].

In order to break away from common small molecule ligands, Weissleder and colleagues screened 146 different small molecules to act as targeting ligands [196]. Using highly reactive small molecules and magnetic particles to facilitate large-scale synthesis and purification, they screened their multivalent particles for cellular uptake in 5 different cell

lines. The multivalency of 2 low affinity ligands on the surface of the NPs resulted in specific and efficient tumor targeting *in vivo*, after intravenous administration.

Finally, one of the most successful stories of actively-targeted NPs to date is based on the use of ACUPA, a small molecule targeted to PSMA [4, 142]. ACUPA was initially identified as part of a prostate cancer specific imaging agent [307]. The decoration of the surface of docetaxel-loaded NPs with ~200 molecules of ACUPA resulted in optimal targeting of PSMA-positive prostate cancers without interfering with blood circulation kinetics [4]. The PSMA-specific, actively-targeted NPs, BIND-014 has now promisingly completed its phase I evaluation in humans and is moving toward further clinical investigation.

3.5. Active targeting in humans

Although no actively-targeted NPs are currently commercially-available, at least 5 targeted liposomes and 2 targeted polymeric nanoparticle therapeutics have made it to clinical development stages. Among the NPs studies MBP-426 [308], MCC-465 [309], SGT53 [310], MM-302 [311, 312], BIND-014 [4], CALAA-01 [2], cetuximad-decorated Doxil®/Caelyx® liposomes [253] and a retroviral vector [313-315] have results of Phase I/II clinical trials available (Table 3). More details about these NPs and others ligand-functionalized therapeutics currently under clinical evaluation are examined in details elsewhere, in a very good review article published during the preparation of this manuscript [316]. Figure 6 also presents some examples of promising technologies currently studied in human. The main therapeutic targets of these NPs are the Tf-R [2, 143], the epidermal growth factor receptor [253, 317], PSMA [4], the surface of gastric cancer cells [309] and the human epidermal growth factor receptor 2 (HER-2) [312]. In the next few years, as the evaluation of these NPs in patients continues, our understanding of the parameters governing active targeting will hopefully improve. Nevertheless, a few important considerations regarding the use of actively-targeted NPs in humans must be highlighted.

3.5.1. The choice of the target—The design of actively-targeted NPs for clinical use involves scientific and economic considerations alike. Evidently, all important tumor biology and NP design aspects discussed earlier must be accounted for in order to develop the most efficient product possible. However, because of the large amount of investment involved in the development of therapeutic products, the probability of success, the prospective therapeutic impact and many other economic factors must be evaluated before choosing the target of interest [143].

The development of targeted drug delivery platforms is often considered to be more costly than the development of non-targeted NPs [143]. This belief likely stems from the increased complexities associated with the preparation and manufacturing of industrial-scale, clinical-grade NPs which display targeting ligands. That being said, the financial burden of scaling up and manufacturing must be considered in the development of any type therapeutic; while it represents a significant proportion of the expenses associated with early development, the costs associated with large-scale clinical trials are certainly much more prohibitive. Once the engineering obstacles are surpassed and NPs show clinical efficacy, targeted NP platforms

present unique designs which possibly confer long-term commercial advantages [318]. All things considered, the major challenge in the development of targeted and untargeted NPs alike remains to show sufficient efficacy in Phase III clinical trials to warrant commercialization. If the presence of the targeting ligand confers better clinical efficacy, the risk and expenses related to more complex chemistry, manufacturing and control (CMC) procedures might potentially be compensated when the NPs reach commercialization.

Additionally, the population that would eventually benefit from the treatment must be considered carefully. Given the wide range of cancer types in which the Tf-R is upregulated [319], it is not fortuitous that many NPs currently under study target this specific surface protein [33]. The prospect of having one versatile targeted platform that could simultaneously treat multiple cancers is very appealing to clinicians and investors alike. The shortcoming of this approach however, might emanate from the use of proteins as the targeting ligands; large and complex molecules can CMC issues that might hinder or limit the large-scale production and commercialization of the NPs. Furthermore, the targeting of ubiquitously-expressed proteins increases the risks of non-specific distribution and off-target toxicities. Poor tolerability and dose-limiting side-effects restrain the therapeutic regimens that can be utilized in patients. Such limitations have been the demise of many drug delivery systems (both targeted and non-targeted), when, doses could not be titrated high enough to achieve therapeutic efficacy [320-322]. Even for commercially-approved nanomedicine, patients in clinical trials experienced unique toxicities associated with the encapsulated form of the drug (*e.g.*, hand-foot syndrome or complement activation-related pseudoallergic reactions with PEGylated liposomal DOX [323, 324]). Since the clinical experience with targeted NPs remains limited, the appearance in clinical trials of non-expected toxicities due to the targeting ligand can never be completely ruled-out. As such, improved tolerability of anticancer therapeutics should therefore remain one of the main objectives throughout development.

In contrast, using molecular targets which are specific to a small population raises the hallmarks of required performance. When the number of prospective patients is small, a drug needs to be paradigm-shifting and become an unavoidable part of this population's therapeutic regimen to achieve good return on investment. The relative commercial successes of some small molecules targeted to very specific cancer metabolic pathways exemplify this phenomenon (*e.g.*, crizotinib and the ALK mutation in 3-5% of NSCLC patients). On the other hand, a drug that provides only marginal benefits might be overlooked and diluted by other comparable treatments. Such problems were encountered by SMANCS, a treatment mainly intended for hepatocellular carcinoma [70], and by others [305] for which the interest dwindled as clinical hurdles were exposed.

A good compromise is using targets like PSMA which are not constitutively present in healthy tissues, but expressed in the neovasculature of multiple types of cancer. Despite its name which suggests specificity for prostate cancer, PSMA is found in the vasculature of almost all types of solid tumors [325]. From an economic perspective, the versatility of the target therefore opens the door to multiple therapeutic indications and potentially higher return on investment. This is particularly interesting in the context of developing a high risk drug delivery platform. It also explains why, despite the fact that almost all of the preclinical

data leading to PSMA-targeted NPs was obtained in prostate cancer models, the initial clinical investigation enrolled patients suffering from all types of cancer [4]. The results of the Phase 1 clinical trial in 28 patients showed one complete response (cervical cancer), 3 partial responses (prostate, non-small cell lung carcinoma (NSCLC) and ampullary cancer) and 5 stable diseases (pancreatic, colorectal, gall bladder, tonsillar and anal cancers), demonstrating that the targeted therapy could be beneficial in a variety of different solid tumors. These promising results offer the opportunity to continue further clinical investigations by stratifying patients in multiple types of cancer. In the case of BIND-014, phase 2 clinical trials will be shortly initiated, simultaneously in 3 independent studies, for the 1st line treatment of chemotherapy-naïve, castrate-resistant prostate cancer as well as for 2nd line treatment of docetaxel-naïve NSCLC and bladder cancer.

3.5.2. Assessing the impact of active targeting—Measuring the impact of active targeting in humans is not simple. In preclinical models, each individual component can be evaluated distinctively and in an iterative manner by testing different NPs. In humans, assessing the efficacy of a treatment requires time and resources; the parallel evaluation of matching targeted and non-targeted NPs remains therefore unlikely.

One way of addressing the problem if accurate phenotyping techniques are available [326] is by comparing patient cohorts which differ in the expression of the target biomarker. While this certainly raises the complexity and the costs associated with a clinical study, it also does not provide any significant advantages in the early evaluation of the NPs. In fact, at the critical step of assessing the clinical efficacy of a NP, it may be more rational to evaluate efficacy in homogenous, target-positive populations if such patients can be identified. Especially since in essence, the main criteria leading to failure or success of a drug is not the mechanistic aspects behind its performance, but how well it compares to standard treatments.

It is likely that the impact of active targeting might only be assessed in post-approval, phase 4 studies. Products are much less vulnerable to the outcome of mechanistic studies when they are commercially-available. This has been observed for approved non-targeted nanomedicines: in retrospective investigations establishing correlations between therapeutic response and biomarker expression [327] or in prospective pharmacokinetic studies trying to predict the performances of nanomedicine [64]. Since ligand-functionalized NPs rely on sufficient surface expression of specific makers to exert their effect, resistance could theoretically arise from the natural selection of target-negative cell populations less exposed to the drug. Nevertheless, there is currently no evidence suggesting that such resistance would appear at a faster rate for targeted NPs than for any other anticancer agent. In fact, in certain instances, actively-targeted NPs were shown to overcome multidrug resistance, at least *in vitro* {van der Meel, 2013 #337}. Mechanistic studies conducted once actively targeted NPs are commercialized and larger patient cohorts start to be treated with them should provide some understanding regarding potential resistance mechanisms and how to counter them. More importantly, a better understanding of how these nanomedicines work will also provide insights how to maximise the benefits for patients.

4. The near future of cancer nanomedicines

More than 30 years after the first observation of the EPR effect, we are just unravelling the mechanisms behind the distribution of macromolecules and nanomaterials inside the tumor. At the preclinical level, further investigation on how physicochemical properties affect the tumor deposition and intratumoral diffusion of nanomaterials might continue to influence the design of future cancer therapeutic NPs. The evolution of chemistry and material sciences constantly pushes back the limits of nanomaterial synthesis. As the tumor distribution of materials with different sizes, shapes, charges and physicochemical properties continue to be evaluated, the body of knowledge on the EPR effect can expand significantly. The key to improve our understanding lies in the adequate design of studies with respect to the current animal models and their limitations. The clinical development of future anticancer NPs also requires strict toxicological validation; it is therefore probable that biodegradable and biocompatible materials might continue to play a major role in the future development of cancer therapeutic NPs.

Thanks to the experience acquired on already approved nanomedicines, it is now appreciated that the susceptibility of patients to nanosized drug carriers depends on the type of cancer, but also on individual tumor biology aspects. Until now, the most successful treatments resulted from increased tolerability to the NPs (*e.g.*, the maximum tolerated dose of Nab-PTX is 1.5 that of solvent-formulated PTX). For PEGylated liposomal DOX, the major survival benefit was witnessed as a second-line treatment for recurring ovarian cancer where the altered toxicity profile of the drug allows repeated administrations over multiple cycles. In a clinical setting, relying on the EPR effect alone to significantly impact the efficacy of cancer nanomedicines in all patients seems increasingly unrealistic; strategies must therefore be investigated to improve the tumor distribution of the drugs and their efficacy.

To that end, screening patients *a priori* for their susceptibility to the EPR effect and preserving nanomedicines for those who would benefit the most from them might seem a viable, short term approach. It is also important to fully understand what dictates this increased predisposition to the EPR effect in humans. Ideally, clinical studies would evaluate nanomedicines in combination with biomarker screening and patient phenotyping to assess which characteristics are good predictors of efficacy. For this purpose, the clinical imaging community might provide useful, minimally-invasive tools. The ability to image in real-time what is happening in the tumor will probably prove very valuable in studying the tumor distribution of nanomaterials. Clinical initiatives trying to correlate tumor uptake of imaging nanomaterials with clinical efficacy of therapeutic NPs might answer many persisting questions. In parallel, recent advances suggest that tumor remodelling can be achieved with commercially-available drugs (*e.g.*, antiangiogenic bevacizumab or antifibrotic losartan). Therefore, minimal additional preclinical work could provide the bases for a pilot study to compare the combination of such treatment with cytotoxic nanocarriers in patients. If successful, these studies might provide viable clinical regimens to better fight tumors with the current available tools.

Active targeting is another tool that could modify the paradigm of our fight against cancer using nanotechnologies. Given the difficulties of immediately ascertaining the impact of

active targeting in humans, comprehensive work to really highlight the advantages of using NPs decorated with surface ligands might need to be conducted at the preclinical stage. To that end, the development of animal models which offer good predictive values for what is observed in the clinics is paramount and the standards of preclinical research might have to shift towards the use of orthotopic or genetically engineered models. Such models might help understand the impact of each physicochemical property on the cellular uptake and efficacy of the NPs. Since the tumor distribution of actively-targeted NPs seems strongly associated with the EPR effect, it will be interesting to see how insights obtained by studying both passive and active targeting strategies might converge to lead to more efficacious treatments. Active targeting strategies might possibly show added benefits as a means for active ingredients to cross physiological barriers, whether they are as simple as cell membranes, or as complex as the gastrointestinal epithelia or the blood brain barrier. In parallel, as tumor biology progresses to expose new molecular targets on cancer cells, improvements in ligand screening techniques should facilitate the isolation of highly specific ligands with strong affinities. While antibodies and proteins will probably continue to be beneficial to establish proof-of-concepts because of their availability, efficiency and applicability, aptamers, peptides and small molecules might play an increasing role in the development of more advanced NPs.

The next few years will hopefully demonstrate the clinical validation of actively-targeted NPs. Here again, in the context of personalized medicine, the challenge will be to clearly identify the patients who might benefit from actively-targeted treatments. When assessing the efficacy of a novel treatment in a cohort, it is crucial to make sure that receptive populations are not concealed by more abundant nonresponsive phenotypes. The risks of having the patients' characteristics impact response rates increases with the complexity of the given therapy. When treatments highly depend on specific metabolic pathways or the presence of a surface receptor to be efficacious, mutations and changes in phenotypes must be closely monitored to ascertain that optimal characteristics are maintained throughout the treatment. In this regard, advances in genotyping and phenotyping techniques should prove very insightful towards novel blueprints for more advanced clinical trials. Hence, there is good hope that the NPs currently under study might reveal very useful tools to fight cancer. Such breakthroughs might contribute to the blooming of cancer nanomedicines in general and actively-targeted platforms, in particular.

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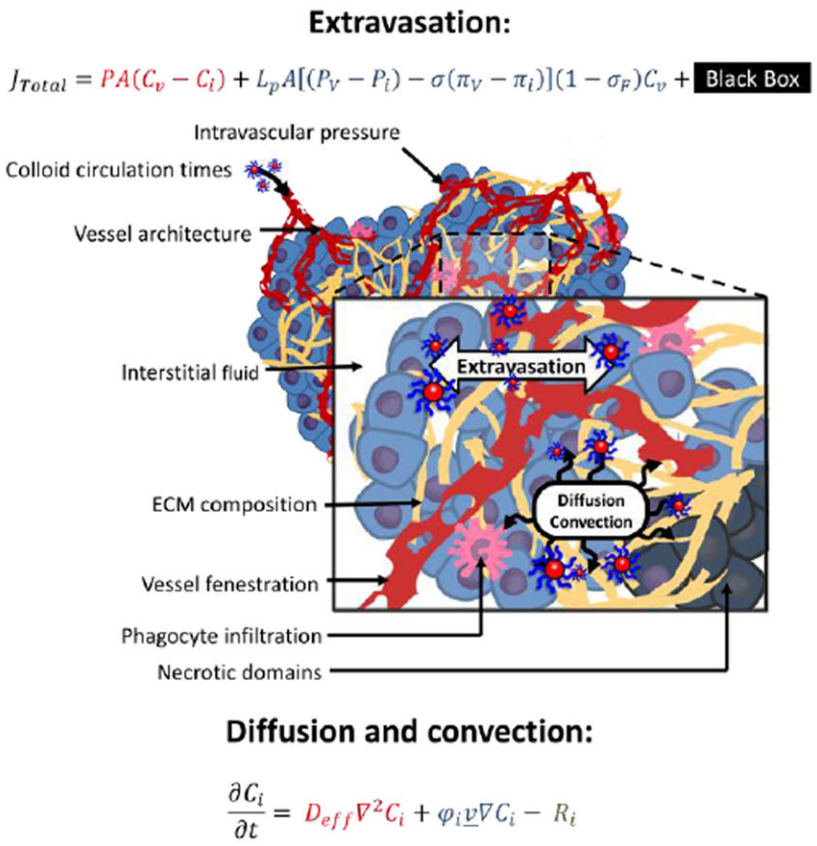

Figure 1.

The EPR effect results from 2 distinct phenomena: the extravasation of the colloid from the blood vessels and their subsequent movement in the tumor extracellular matrix (ECM) by diffusion and convection.

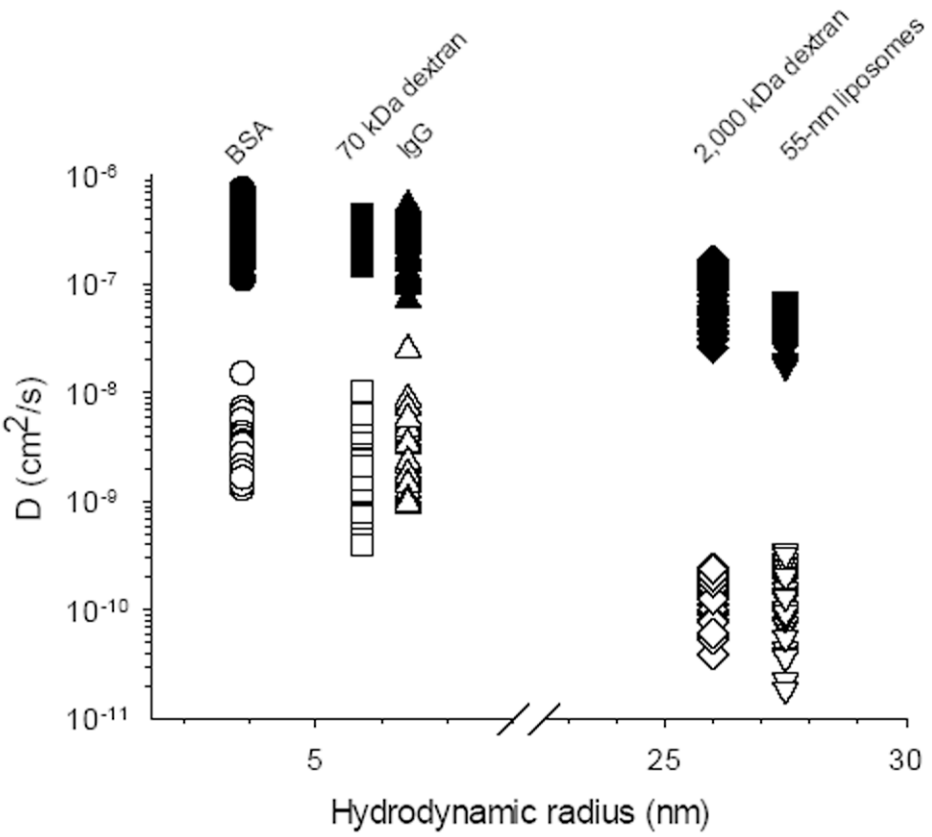

Figure 2.

After intratumoral injection in melanoma xenografts (Mu89), the diffusion coefficient of macromolecules and nanomaterial in the ECM is inversely proportional to the hydrodynamic radius. Diffusion of bovine serum albumin (BSA, circles), 70-kDa dextran (squares), immunoglobulins (IgG, triangles), 2-MDa dextran (diamonds) and liposomes (inverted triangles) follows 2 phases with fast (closed symbols) and slow colloid populations (open symbols). Adapted with permission from [61].

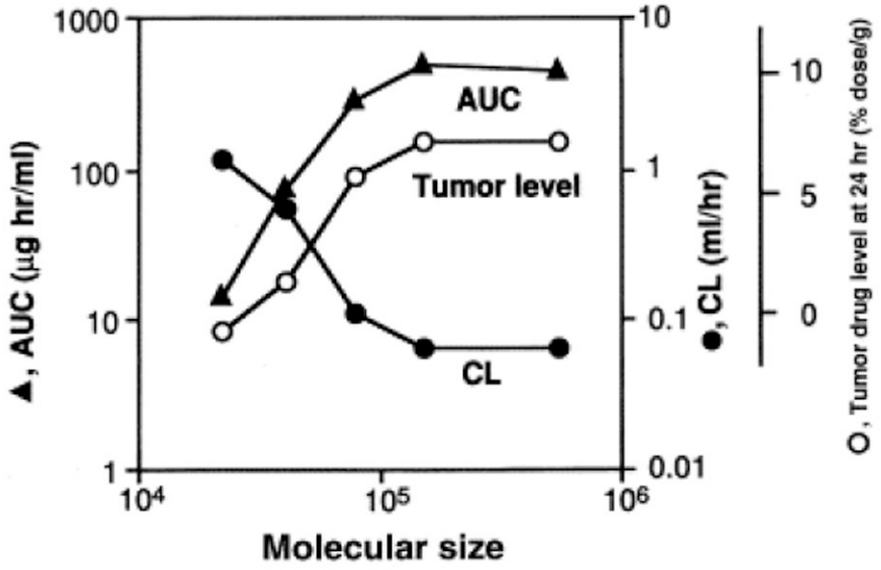

Figure 3.

In murine S-180 sarcoma, the tumor accumulation levels of macromolecules (○) are in direct relation with the total body exposure (AUC, ▲) and inversely proportional to their renal clearance (●). This holds true for other types of tumors and nanomaterials. Used with permission from [76]

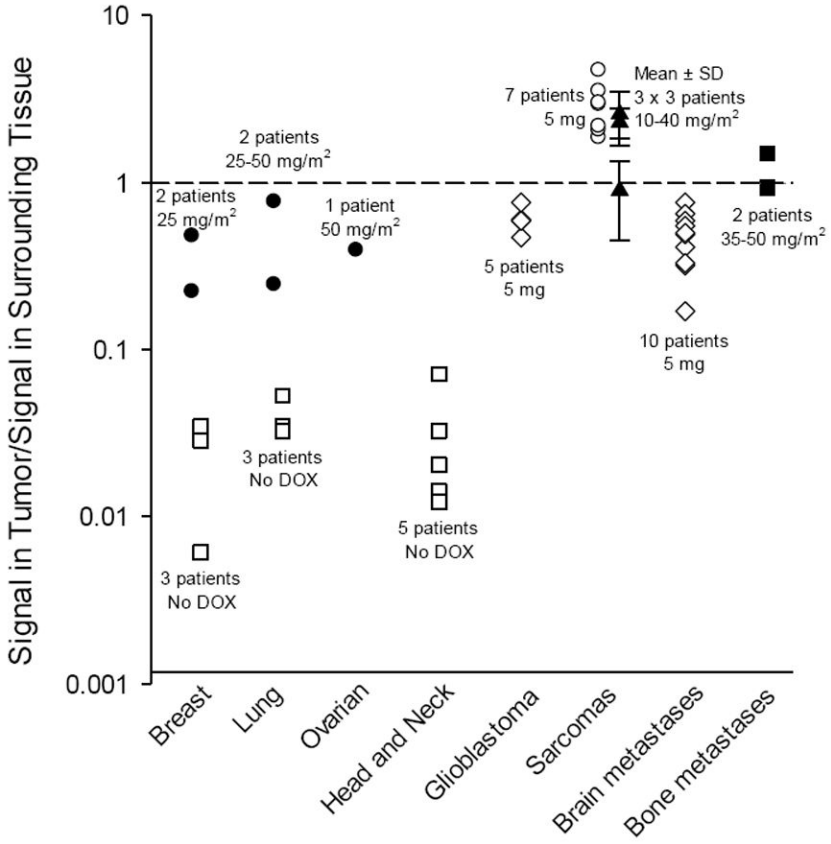

Figure 4.

In human, the accumulation of liposomes in tumors is different in each type of cancer. Sarcomas seem to be the sole cancers for which the accumulation of liposomes in tumor is superior to that in plasma or surrounding tissue. (●) from [116], DOX concentration in malignant infusion vs. plasma concentration, using Doxil®. (□) from [119], ^{111}In-DTPA-labelled liposomes in tumor ROI vs. plasma concentration. (◇) from [121], ^{99m}Tc-DTPA-labelled Doxil® tumor ROI vs. skull bone marrow. (▲) from [117] DOX concentration in tumor biopsies vs. plasma concentration, using DOX-containing PEGylated liposomes. (○) from [120], ^{99m}Tc-DTPA-labelled liposomes tumor ROI vs. surrounding tissue (■) from [118], DOX concentration in bone metastases vs. plasma concentration, using Doxil®. All ratios are given for concentrations measured at the same time-point and individual patients are presented when possible; the doses of DOX used are labeled on the figure (closed symbols); open symbols represent the use of empty liposomes.

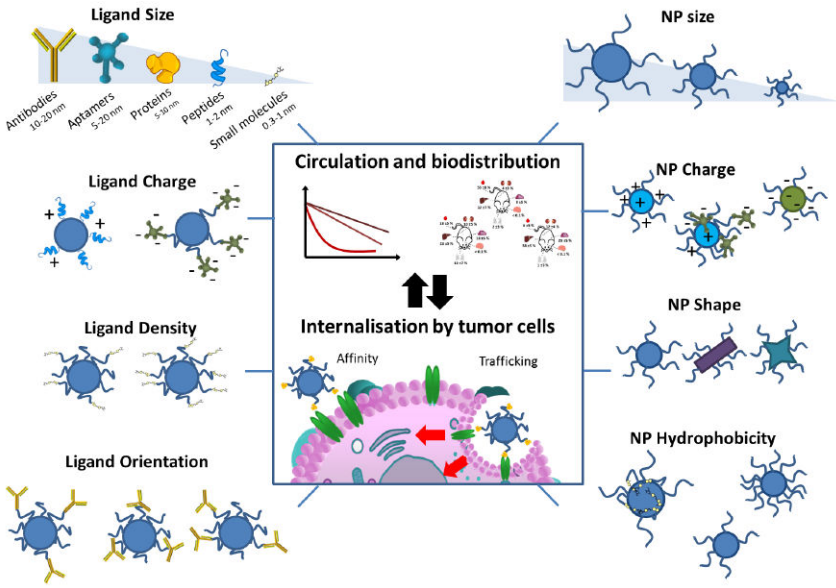

Figure 5.

The physicochemical properties of the ligand and the NP affect their blood circulation profiles, their biodistribution and their ability to be internalized by cancer cells.

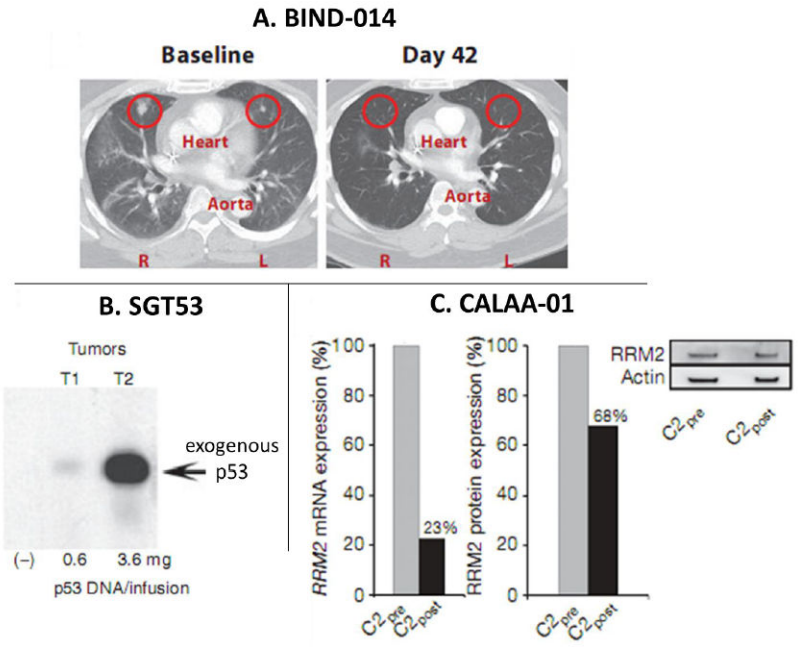

Figure 6.

Actively-targeted NPs have shown promises in early clinical trials. **A**. BIND-014, a PSMA targeted, docetaxel-containing polymeric NP has shown impressive anticancer response in heavily-pretreated patients; the regression of lung metastases experienced by one patient suffering from cholangiocarcinoma after two cycles of BIND-014 is evidenced here by CT scans. **B**. SGT53, a TfR-targeted liposomes containing plasmid DNA for the p53 gene were shown to allow expression of the exogenous p53 gene by DNA PCR; tumor biopsies were taken 100 (T1) and 26 hours (T2) after administration of different doses in 2 different patients. **C**. CALAA-01, a TfR-targeted polymeric NPs encapsulating siRNA was shown to reduce mRNA (77 %) and protein expression (32 %) compared to baseline ($C2_{pre}$) in one patient receiving 30 mg/m2 of siRNA ($C2_{post}$); mRNA expression was assessed by qRT-PCR while protein expression was evidenced by western blotting. Figures are used with permissions from [4], [310] and [2], respectively.

Table 1

Extravasation as well as interstitial diffusion and convection can be affected by the tumor biology and the various characteristics of the colloid.

Tumor biological properties	**Parameters potentially affected**
Vessel architecture (*e.g.*, fenestrations, blood flow)	P, A, P_v, L_p, σ, σ_F, **Black box** (endothelial uptake)
Interstitial fluid composition	π_i, **Black box** (protein adsorption)
Extracellular matrix composition	D_{eff}, R_i, π_i, **Black box** (adsorption)
Phagocyte infiltration (*e.g.*, TAM, dendritic cells)	R_i, **Black box** (cellular uptake)
Presence of necrotic domains	P_i, π_i, D_{eff}, R_i, **Black box** (protein adsorption, colloidal stability)
NP properties	
Blood circulation times	C_v, C_i
Size	P, L_p, D_{eff}, σ_F, ϕ_i, R_i, **Black box** (colloidal stability, cellular uptake)
Charge	P, L_p, D_{eff}, R_i, **Black box** (colloidal stability, cellular uptake)
Shape	P, L_p, D_{eff}, σ_F, ϕ_i, R_i, **Black box** (adsorption, cellular uptake)
Surface characteristics (*e.g.*, hydrophobicity, ligands)	P, L_p, R_i, **Black box** (adsorption, cellular uptake)

Table 2

Various examples of targeting ligands used in preclinical studies. EpCAM: epithelial cell adhesion molecule; EDB: extra-domain B.

Ligand type	System	Target	Indication	Ref
Antibodies and fragments				
Full antibody	Quantum dots	PSMA	Cancer (imaging)	[226]
$F(ab')_2$	Liposome	GAH	Gastric cancer	[227]
F(ab')	Liposome	HER2	Breast cancer	[228]
scFv	Liposome	HER2	Breast cancer	[158]
Proteins				
Transferrin	Polymeric NPs	Tf receptor	Cancer	[159]
Ankyrin repeat protein	siRNA complexes	EpCAM	Cancer	[229]
Affibodies	Polymeric NPs	HER2	Breast cancer	[230]
Peptides				
CGNKRTRGC (LyP-1)	Protein NPs	gC1qR (p32)	Cancer	[231]
F3 peptide	Iron oxide NPs	Nucleolin	Cancer (imaging)	[232]
iRGD	Iron oxide NPs	αvβ3/5	Cancer (imaging)	[233]
iRGD	Polymeric NPs	αvβ3/5	Cancer	[234]
KLWVLPKGGGC	Polymeric NPs	Collagen IV	Inflammation	[235]
KLWVLPK	Polymeric NPs	Collagen IV	Vascular wall	[236, 237]
Aptides	Liposomes	Fibronectin	Cancer	[238]
Nucleic acid-based ligands				
A10 aptamer	Polymeric NPs	PSMA	Prostate cancer	[161, 239]
A9 CGA aptamer	Gold NPs	PSMA	Prostate cancer	[240]
Small molecules				
Folic acid	Gold nanorods	FA receptor	Cancer	[241]
Folic acid	Polymeric NPs	FA receptor	Cancer	[242]
TPP	Polymeric NPs	Mitochondria	Various	[243]
ACUPA	Polymeric NPs	PSMA	Cancer	[4]

Table 3

Actively-targeted NPs currently in clinical trials. When possible, usual doses for the non-targeted drug payloads are given for comparison. CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease.

Name	System	Target (Ligand)	Active (Usual dose)	Doses (Recommended)	Available Phase I/II Results
BIND-014	Polymeric NPs	PSMA (small molecule)	Docetaxel (75 mg/m^2)	15-75 mg/m^2 (60 mg/m^2)	**28 patients** [4]: 1 CR (cervical cancer); 3 PR (NSLC, prostate, ampulary); 5 SD for > 12 weeks (pancreatic, colorectal, gall bladder, tonsillar, anal)
anti-EGFR-ILs-dox	Liposomes (Doxil®/ Caelyx®)	EGFR (Ab, cetuximab)	Doxorubicin (50-60 mg/m^2)	5-60 mg/m^2 (50 mg/m^2)	**29 patients (26 evaluable)** [253]: 1 CR (larynx); 1 PR (mesothelioma); 10 SD for 8 - 48 weeks
MM-302	Liposomes	Her2 (Ab scFv)	Doxorubicin (50-60 mg/m^2)	8-50 mg/m^2	**34 breast cancer patients (22 evaluable)** [312]: 2 PR (1 with 100% regression of lesion); 12 SD
MCC-465	Liposomes	Gastric cancer (Ab fragment)	Doxorubicin (50-60 mg/m^2)	6.5-45.5 mg/m^2 (32.5 mg/m^2)	**23 recurrent or metastatic stomach cancer patients (18 evaluable)** [309]: 10 SD for 6 - 20 weeks
MBP-426	Liposomes	Tf-Receptor (Tf)	Oxaliplatin (75-85 mg/m^2)	6-400 mg/m^2 (226 mg/m^2)	**39 patients :** 15 SD for 6 weeks; 3 SD for 12 - 24 weeks (colorectal)
SGT53	Liposomes	Tf-Receptor (Ab scFv)	p53 plasmid DNA (N/A)	0.6-3.6 mg (2.4 mg)	**11 patients (10 evaluable)**[310]: 7 SD at 6 weeks 3/3 patients with transgene

Name	System	Target (Ligand)	Active (Usual dose)	Doses (Recommended)	Available Phase I/II Results
					plasmid detectable in tumor
CALAA-01	Polymeric NPs	Tf-Receptor (Tf)	siRNA M2 subunit of ribonucleotide reductase (N/A)	18-30 mg/m^2	**3 patients** [2]: 3/3 patients with decreased mRNA expression in tumor
Rexin-G	Retroviral vector	collagen (viral envelope peptide)	Human cyclin G1 gene (N/A)	1 -6×10^{11} CFU per 1-week cycle [315] 8-24×10^{11} CFU per 4-week cycle [313, 314]	**12 metastatic pancreatic cancer patients** [315]: 1 SD for 4 weeks (symptomatic deterioration); 11 PD for 4-7 weeks **13 chemotherapy-resistant pancreatic cancer patients (9 evaluable)** [313]: 8 SD for 12-30 weeks; 1 PR for 52 weeks **42 metastatic sarcoma and osteosarcoma patients (37 evaluable)** [314]: 23 SD for 5-15 weeks