

Chapter 8

Tumor Vasculature, EPR Effect, and Anticancer Nanomedicine: Connecting the Dots

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Abstract The progression of a tumor cell mass beyond 2 mm is critically dependent on neoangiogenesis. Angiogenic factors secreted by tumor cells, infiltrating macrophages, and stromal cells aggressively promote proliferation and migration of endothelial cells. The nascent primitive vasculatures are usually morphologically and functionally abnormal due to several features such as the lack of a vascular smooth muscle cell layer, abrupt change of the blood vessel diameter, tortuosity, and leakiness. Those characteristics which alter the blood flow and the transport of molecules in tumors led to the discovery of the enhanced permeability and retention (EPR) of nanosize molecules in tumor tissues. Following its discovery, various anticancer nanoconstructs have been developed with the EPR effect as a central mechanism for tumor targeting. However, the development of these nanodrugs has been hampered by a slow progress towards the clinic. Only nine nanomedicines have been approved for anticancer treatment for the last 26 years. In this chapter, we discuss various aspects that may explain the limited transition for an efficient anticancer nanomedicine. The specificity of the tumor vasculature, the discrepancy in tumor biology, the role of animal tumor models, and the physicochemical characteristics of nanoconstructs are closely examined. This chapter provides new considerations for successful development of EPR-based anticancer nanomedicine.

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Abbreviations

EPR	Enhanced permeability and retention
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
bFGF	Basic fibroblast growth factor
TGF	Tumor growth factor
MMP	Matrix metalloproteinases
NO	Nitric oxide
EBD	Evans blue dye
SMANCS	Styrene co-maleic acid conjugated neocarzinostatin
TDT	Tumor doubling time
HPMA	<i>N</i> -(2-hydroxypropyl)methacrylamide
RES	Reticuloendothelial system

Introduction

Angiogenesis is fundamental for many biological processes such as development, reproduction, and wound healing and has been implicated in the progression of a variety of diseases including diabetic retinopathy, rheumatoid arthritis, age-related macular degeneration (AMD), psoriasis, and tumor progression [1–4]. The early stages of the tumor development are characterized by the aberrant activation of oncogenes, inhibition of tumor suppressor genes, and modifications of genes that directly and indirectly control cell proliferation, all as a result of the accumulation of discrete genetic changes and epigenetic alterations [5]. Once the tumor has reached a certain size, the tumor propagation and progression will be dependent on the immediate environment. In 1889, Stephen Paget proposed the “seed and soil” hypothesis based on the concept that the microenvironment of a developing tumor is a crucial regulator of its growth and expansion [6]. The capacity of transplanted tumor cells to promote blood vessel formation was demonstrated by Greenblatt and Shubik [7] and Ehrmann and Knoth [8] who demonstrated that a diffusible factor produced by tumor cells can induce neovascularization. In the early 1970s, Folkman proposed that the tumor growth is essentially dependent on the establishment of its own vascular supply [9, 10]. Independent of the cellular origin of the cancer, angiogenesis is the critical step for the growth of tumors beyond 2 mm as well as the development of metastasis. The activation of tumor angiogenesis relies essentially on the balance between the pro-angiogenic factors and the anti-angiogenic factors.

The induction of the tumor vasculature growth is termed the “angiogenic switch” [11, 12] and is dependent on the increased expression of the pro-angiogenic genes and/or a decreased expression of anti-angiogenic factors. Many potential regulators of angiogenesis have been identified including acidic fibroblast growth factor

(aFGF), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), transforming growth factor- α (TGF- α), transforming growth factor- β (TGF- β), hepatocyte growth factor (HGF), tumor necrosis factor- α (TNF- α), angiogenin, interleukin (IL)-8, angiopoietins, angiotensin (ANG)-II, bradykinin, and prostaglandins [1, 13–17]. Negative regulators of angiogenesis were also identified and included factors such as thrombospondin [18], the 16 kDa fragment of prolactin [19], angiostatin [20], endostatin [21], and vasostatin [22].

The increased expression of these angiogenic factors has been demonstrated in several types of cancer and has been associated with increased permeability of the tumor vasculature compared to normal blood vessels [23–25]. Further, angiogenic factors have been associated with structural aberrations of the tumor blood vessels (for review see [26]). The higher permeability of the tumor blood vessels favors the accumulation of macromolecules and lipids in the interstitium of the tumor for extended periods of time. This feature of the tumor vasculature led to the characterization of the enhanced permeability and retention (EPR) effect of macromolecular drugs in solid tumors [27]. The EPR effect is the result of the distinctive vascular permeability of solid tumors [28] and inflammatory tissues [29]. The characterization of this phenomenon allowed the development of the first anticancer nanomedicine by Maeda: styrene co-maleic acid conjugated neocarzinostatin (SMANCS) for the treatment of hepatocellular carcinoma [30]. Following this discovery, several laboratories have developed EPR-based nanomedicine. The main advantage of the EPR-based anticancer nanomedicines is their altered pharmacokinetics caused by their hydrodynamic diameter as it exceeds 7 nm, a size sufficient to escape kidney filtration and urinary excretion [31, 32]. These nanoconstructs can exhibit prolonged circulatory half-life, high area under the concentration/time curve (AUC), and higher partition into tumor tissues [33–36]. Since the first nanomedicine was developed in 1986, the Food and Drug Administration (FDA) and several agencies worldwide have approved over 30 nano-therapeutics for clinical use, 11 of which are for the detection and treatment of various cancers. Despite the improvement in the design and targeting efficiency of these nanomedicines to the tumor site, the transition from the bench to the clinic is particularly slow. In this chapter, we will present an overview of the mechanisms involved in neovascularization, as well as the specific characteristics of the tumor vasculature. We will also discuss the critical considerations that might influence nanomedicine targeting efficiency to solid tumors utilizing tumor vasculature permeability.

Mechanism of Tumor Angiogenesis

The origin of the blood vessel formation is different depending on the biological process which it serves. During embryogenesis, the *de novo* formation of blood vessel originates from the differentiation of angioblasts into mature endothelial cells and their subsequent assembly into tubes, a process called vasculogenesis [37]. Several angiogenic factors such as VEGF, VEGFR-2, bFGF, and TGF- β

influence angioblast differentiation into mature endothelial cells [38–40]. Further development of these native vessels is the result of angiogenesis, a process in which new capillaries emerge by sprouting from existing ones [37]. Distinctive signaling mechanisms will promote either venous or arterial differentiation [41]. The recruitment of periendothelial cells such as vascular smooth muscle cells or pericytes is essential for the maturation of the blood vessel by inhibiting the endothelial cell proliferation and promoting the formation of extracellular matrix [42]. The periendothelial cells can also assist the endothelial cells to acquire specialized functions in different vascular beds [42]. In contrast, neovascularization taking place at postembryonic stage involves essentially angiogenesis as a result of the proliferation and migration of differentiated endothelial cells [13]. With the exception of few physiological processes such as wound healing and the female reproductive cycle where endothelial cells are transiently activated and proliferating, the endothelial cells are largely quiescent in mature vessels. The percentage of endothelial cells entering the cell cycle is only 0.45 % for arteries and arterioles and 0.11 % for capillaries [43].

Tumor expansion is marked by a constitutive activation of the “angiogenic switch” in most cases [11]. The newly formed blood vessels will emerge by sprouting from existing ones and sustain tumor growth [11]. However, recent studies have challenged these conceptions and identified several concomitant mechanisms contributing to the neovascularization of tumors. These mechanisms have been mainly characterized in the tumor vasculature but their contribution remains poorly understood. New blood vessels can emerge from vasculogenic mimicry where tumor cells can line and form a vessel-like structure [44] or putative cancer stem cells can differentiate into an endothelial cell lineage and contribute to angiogenesis [45]. Other studies have demonstrated the capacity of tumor cells to hijack an existing blood vessel, a process known as vessel co-option [46]. In other cases, new blood vessels can arise through intussusceptive angiogenesis where one existing vessel splits into two new vessels [47]. Several studies have also demonstrated the involvement of bone marrow-derived cells for the repair of adult vessels and the expansion of tumor ones. Endothelial cell progenitors can be mobilized from the bone marrow and transported through the blood circulation to become incorporated into the vascular walls of the growing blood vessels [48].

Tumor Vasculature as a Target for Selective Delivery of Nanomedicine

Normal vasculature networks consist of arterioles, capillaries, and venules and form a well-organized network with dichotomous branching and hierarchic order [49]. Newly formed tumor vessels are usually abnormal in form and architecture with narrowed, tortuous, and fragmented blood vessels. In addition, tumor vessels usually lack a smooth muscle layer and innervation, with defective endothelial linings and basement membranes [50]. Some structures are dilated, saccular, poorly aligned,

and heterogeneous [51]. Many vascular mediators such as bradykinin [52], prostaglandin [53], nitric oxide (NO) [54], peroxynitrite (ONOO⁻) [29], matrix metalloproteinases (MMP) [29], and VEGF [55] have been shown to play an important role in these alterations.

As a consequence of these defects, tumor vessels usually harbor wide fenestrations [56]. The blood flow is often irregular with vessels having different diameters and abnormal branching patterns. These tumor vessels are leaky and show increased permeability to large circulating molecules with fenestration sizes ranging from 300 nm to 4,700 nm [57–59]. Furthermore, lymphatic drainage of tumor tissues is generally deficient and limits the clearance of macromolecules [9, 27, 60–62]. Evidence for increased endothelial permeability of tumor vessels to large molecules was clearly demonstrated by Maeda, 26 years ago, using Evans blue dye (EBD) injected intravenously into rodents. After injection the dye bound to albumin in the bloodstream and the complex selectively concentrated into tumor tissue [28]. Additional studies using soluble tracers further demonstrated the extravasation of large molecules from the tumor vessel [63, 64]. EBD extravasation and accumulation in the tumor was the first demonstration the EPR effect concept [28]. The accumulation of nanosize drugs in the tumor tissue is time dependent ranging from several hours to several days [60, 65]. Maeda's work demonstrated that the rate of accumulation of macromolecules and lipids in the tumor was inversely proportional to their clearance rate. Following SMANCS, the first nanomedicine approved and used for the treatment of hepatocellular carcinoma in Japan (see Table 8.1), several laboratories have developed nanosize drug carriers. But, for the past 20 years, few nanomedicines were approved for the chemotherapeutic treatments of various cancers as well as for their detection (see Table 8.1). Among these formulations, liposome nanocarriers achieved significant success such as Doxil, DaunoXome, Depocyt, and Myocet (Table 8.1). Second- and third-generation types of micellar or polymeric drug carriers are currently being developed or evaluated in clinical trials (phase I–III).

To be efficient for cancer treatment, the size and shape of nanoparticles are critical for their accumulation at the tumor site [66]. Several studies have demonstrated that both criteria are essential for the longevity of the nanomedicine in the circulation, their distribution to different organs [66], as well as their recognition and elimination through the reticuloendothelial cells system (RES) [67]. The RES is composed of macrophages present in the liver, spleen, and bone marrow [67]. Generally, particles larger than 100 nm are rapidly eliminated from the circulation by the RES [68, 69]. To decrease their recognition by macrophages, several strategies have been developed, for instance, the addition of synthetic polymers such as polyethylene glycol (PEG) on the surface to sterically hinder interaction with plasma proteins [70] and reduce opsonization [71].

Overall, nanomedicine advantages over conventional drugs rely on the EPR effect and their improved pharmacokinetics that lower their systemic cytotoxicity. A schematic representation of this phenomenon is illustrated in Fig. 8.1. Furthermore drug delivery nanotechnology allows the controlled release of anticancer drugs and might partly circumvent multidrug resistance mechanisms that involve cell-surface protein pumps [72].

Table 8.1 Clinically approved nanomedicines for cancer treatment or imaging

	Name	Therapeutic agent	Approval year	Treatment	References
Conjugate	SMANCS	Neocarzinostatin	Japan (1993)	Hepatocellular carcinoma	[213]
Liposomes	Doxil/Caelyx	Doxorubicin	FDA (1995)	Late-stage ovarian cancer Advanced HIV-Associated Kaposi's sarcoma	[214] [215]
	DaunoXome	Doxorubicin	FDA (1996)	Advanced HIV-associated Kaposi's sarcoma	[216]
	Depocyt	Cytarabine	FDA (1999)	Malignant lymphomatous meningitis	[217]
	Myocet	Doxorubicin	Europe (2000) Canada (2001)	Metastatic breast cancer in combination with cyclophosphamide	[218]
	MEPACT	Muramyl tripeptide phosphatidylethanolamine	Europe (2004)	Osteosarcoma	[219]
Polymeric micelles	Oncaspar	PEG-L-asparaginase	FDA (2006)	Acute lymphoblastic leukemia	[220]
	Genexol-PM	Paclitaxel	South Korea (2006)	Metastatic breast cancer	[221]
Albumin Metal	Abraxane	Paclitaxel	FDA (2005)	Metastatic breast cancer	[222]
	Feridex	Superparamagnetic iron oxide	FDA (1996)	MRI contrast agent	[223]
	GastroMARK	Superparamagnetic iron oxide	FDA (1996)	MRI contrast agent	[224]

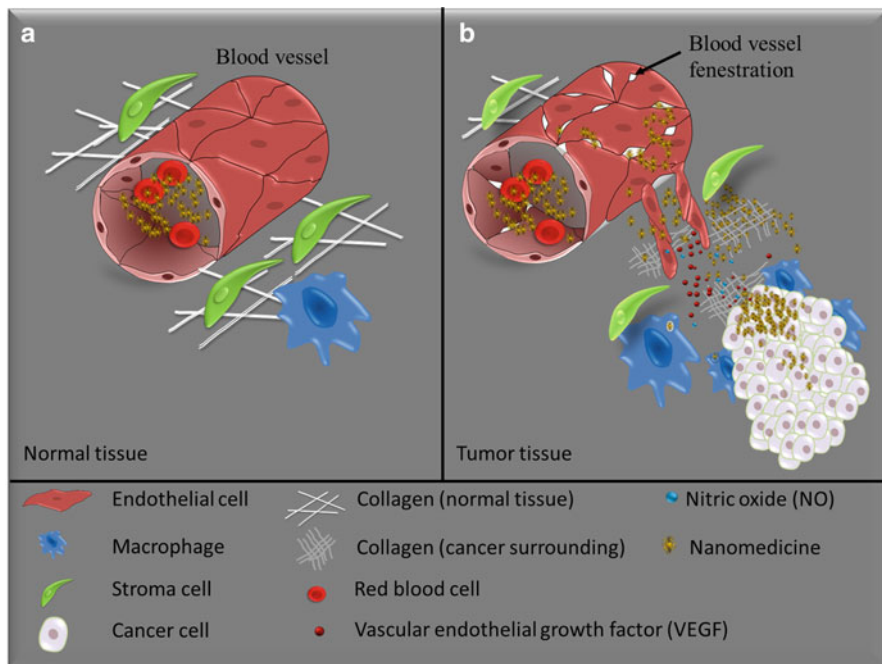


Fig. 8.1 Differences between normal and tumor tissue in relation to the targeting of nanomedicines by the enhanced permeability and retention (EPR) effect. (a) Normal tissue contains tightly connected endothelial cells which prevents the diffusion of the nanomedicine outside the blood vessel. (b) Tumor tissue contains large fenestrates between the endothelial cells allowing the nanomedicines to reach the matrix and the tumor cells by the EPR effect. VEGF and NO secreted by tumor cells, stromal cells, and macrophages increase permeability and stimulate angiogenesis and the migration of endothelial cells towards the tumor. A considerable proportion of the nanomedicine never reaches the tumor either due to entrapment or nonspecific interaction with collagen composing the matrix, or removal through macrophage endocytosis. Nanomedicines tend to concentrate at the periphery of the tumor, only a small proportion will diffuse to the center of the tumor

Factors Contributing to the EPR Effect

Several studies have demonstrated that the EPR effect is dependent on angiogenic factors produced from the tumor cells, stromal cells, or other cell types such as VEGF, bradykinin, nitric oxide, peroxynitrite, and other cytokines [73–75]. All these factors increase blood flow and promote diffusion and retention of nanomedicines inside tumors.

VEGF

The vascular permeability factor or vascular endothelial growth factor (VPF/VEGF) was originally characterized from guinea pig ascites as a secreted protein

inducing vascular permeability [76] and was later found in various human tumor cell lines [77]. The same protein was also later identified as a specific and potent vascular endothelial cell mitogen [78]. VEGF is highly expressed in most tumors (2–30-fold higher than normal tissue) and was shown to contribute to the tumor blood vessel structural abnormality [79]. The contribution of VEGF to the EPR effect was demonstrated by Claffey et al. who showed a greater extravasation of large molecules in tumors overexpressing VEGF [80].

VEGF is a homodimeric glycoprotein comprised of two identical subunits [78]. VEGF expression is regulated at the level of transcription by alternative splicing of the VEGF gene and the VEGF₁₆₅ isoform is the most abundant and assimilated as the native soluble heparin-binding endothelial mitogen activator [78]. Other VEGF isoforms have been identified and arise from different VEGF splicing such as VEGF₁₂₁, VEGF₁₈₉ and VEGF₂₀₆. The VEGF₁₂₁ isoform is secreted and fully soluble but lacks the heparin binding site, while VEGF₁₈₉ and VEGF₂₀₆ are largely sequestered at the cell surface and extracellular matrix and bind avidly to heparin and heparin-like moieties [78, 81]. In addition to the alternative splicing, a proteolytic activation of VEGF has been demonstrated for VEGF₁₆₅, VEGF₁₈₉, and VEGF₂₀₆ following plasmin [82, 83] and matrix metalloproteinase (MMP) activations [84]. These patterns of activation regulate bioavailability and bioactivity and also determine receptor specificities. VEGF acts mainly in a paracrine fashion binding to receptors expressed at the surface of endothelial cells. VEGF₁₆₅ binds to two receptor tyrosine kinases, VEGFR-1 (Flt-1), VEGFR-2 (KDR or Flk-1), as well as Neuropilin (NRP)-1 and NRP-2, transmembrane glycoproteins [85, 86]. In addition, several VEGF-related genes have been identified including VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF) [87]. These members of the VEGF gene cluster undergo alternative splicing with the exception of VEGF-C [87]. VEGF-C and VEGF-D were shown to bind to VEGFR-3 (Flt-4) and promote lymphangiogenesis [88].

The expression of VEGF is upregulated by multiple factors including hypoxia. Under hypoxia, hypoxia-inducible factor (HIF)-1 α dimerizes with the constitutive HIF-1 β to bind to the hypoxic response element (HRE) present in the promoter of the VEGF gene and stimulate its expression [89]. HIF-1 α is involved in the activation of transcription of many genes involved in the activation of angiogenesis and other physiologic processes (for review, see [89]).

Bradykinin

Bradykinin (kinin) is a peptide that causes vasodilatation and increased vascular permeability. Bradykinin is generated from its precursor kininogen by limited proteolysis by various serine proteases such as kallikrein, cathepsins, and collagenases [90]. Kininogens are multifunctional glycoproteins mainly synthesized in the liver but also in the kidneys, salivary glands, and endothelial cells [91] and circulate in human plasma in low (50–68 kDa) and high (80–120 kDa) molecular weight forms

[92]. Bradykinins' half-life is a few seconds in the plasma and is rapidly degraded by proteases such as kininase and angiotensin-converting enzyme (ACE) [92]. A greater synthesis of bradykinin has been reported in several types of cancer [52, 93] as well as the expression of bradykinin receptor B2 [94]. The effect of bradykinin appears to be direct as the release of bradykinin triggers vasodilatation and increased vascular permeability as well as indirect as mediated by the production of nitric oxide through the stimulation of the nitric oxide synthase [95], prostaglandins [96], and various cytokines such as interleukin-1, interleukin-6, and interleukin-8 [94]. The permeabilizing action of bradykinin was found to be similar to VEGF but mediated through a different pathway.

Nitric Oxide (NO)

NO is a signaling messenger and contributes to several pathways and biological processes. NO is produced from L-arginine by nitric oxide synthase (NOS) in the presence of oxygen. In pathological conditions such as cancer and inflammatory tissue, NO production is largely increased and plays an important role in the extravasation of large molecules and thus contributes to the EPR effect [54, 60]. Increased NO production was also associated with the overexpression of the inducible form of NOS (iNOS) in the tumor tissues due to the infiltration of leukocytes [97].

Moreover, NO to the same extent as oxidized NO products such as peroxynitrite contributes to the vascular permeability of solid tumor [54]. Peroxynitrite (ONOO⁻) is a reaction product of NO and anion superoxide O₂⁻ [98]. The increased production of peroxynitrite triggers the maturation of pro-matrix metalloproteinases (pro-MMP) into MMPs, which promotes remodeling of the extracellular matrix and contributes to the vascular permeability [29].

The identification of the factors contributing to the EPR effect has resulted in the development of anticancer nanomedicine. However, with the exception of a few clinically approved nanomedicines (see Table 8.1), many nanoconstructs failed to achieve a significant outcome in the clinic. The lack of complete understanding of the EPR effect and its specific biological implications has so far impaired EPR effect-based therapy as a paradigm for cancer treatment. The following describes some of the factors that could account for the slow transition of the EPR-based nanomedicine to an effective cancer treatment.

Animal Models for the EPR Effect

The EPR effect has been repeatedly proven in animal models through the use of large molecules such as the EBD. EBD binds instantly to plasma albumin which results in a large molecular weight complex of about 7 nm diameter that can simulate the effect of a nanomedicine. A diameter larger than 7 nm will escape renal

filtration and urinary excretion [31], due to the slit diaphragms at the level of the podocyte foot of the glomerulus which prevent the filtration of globular plasma proteins above this size [32]. Therefore, large particles can exhibit prolonged circulatory half-life, high area under concentration/time curve (AUC), and higher partitioning into tumor tissues [33]. After 6 h, there is usually a distinct accumulation in tumor lesions compared to surrounding tissues. Many nanomedicines have been observed to accumulate in tumor tissue from 2-fold and up to 27-fold more than free drugs depending on the nanocarrier, the drug encapsulated, and the xenograft tumor model used (Fig. 8.2a).

The question of whether the results of EPR-based drug targeting in animal models can be faithfully translated to the clinic remains unanswered. Macrophage infiltration has been demonstrated in a large cohort of cancers. The production of VEGF and NO by tumor-associated macrophages (TAM) and their role in cancer development is also well documented [99–101]. To determine the anticancer properties of a given nanomedicine against a specific human cancer, it is necessary to utilize immunocompromised mice to enable the use of human tumor xenografts. However human cancer patients are rarely immunocompromised. A change in macrophage activity in immunocompromised mice [102] can result in less VEGF and NO leading to a tumor with reduced vascular density, which in turn limits the access of the nanoconstructs to the tumor. Furthermore, the results obtained from immunocompromised models differ from results obtained in immunocompetent mice. In various drug delivery systems (conjugates, liposomes, and micelles), the tumor accumulation is a 2-fold higher in immunocompetent mice relative to immunocompromised ones (see Fig. 8.2b). Moreover, immunocompetent mice bear murine tumors and not human cancer cell lines which further complicate interpretation of *in vivo* animal data and jeopardize its value in predicting the performance of new drugs in clinical trials.

The expression of VEGF and its receptors between commonly used human tumor cell lines and their clinically isolated variants differs. It is clearly evident that tumor cell lines have pronounced expression of VEGF and its receptors with far less variability in comparison to clinical tumors. For example, human breast cancer cell lines MCF-7 and MDA-MB-231 expressed VEGFR-1, VEGFR-2, and VEGFR-3 as well as the ligands VEGF-A, VEGF-C, and VEGF-D with VEGF-B being found only in the MCF-7 cells [103–108]. In contrast, the expression pattern in breast cancer tumors collected from patients is more limited to one specific type of receptor and/or ligand and more importantly not all tumors tested within this cancer type expressed VEGFR and/or its ligand [109–111]. A similar observation was made with prostate cancer and lung cancer.

Relevant to this is the design of nanomedicine targeted to tumors which relies on the conjugation of target ligands that bind strongly to tumor cell-surface receptors to increase cell recognition, cell specificity, and cellular uptake. Galactosamine [112], transferrin [113], and folate [114] have been incorporated in nanomedicine based on the preferential expression of these molecules by cancer cells. Despite promising *in vitro* studies, these targeted nanomedicines failed to demonstrate significant benefit at the preclinical or clinical level [115]. The discrepancy between

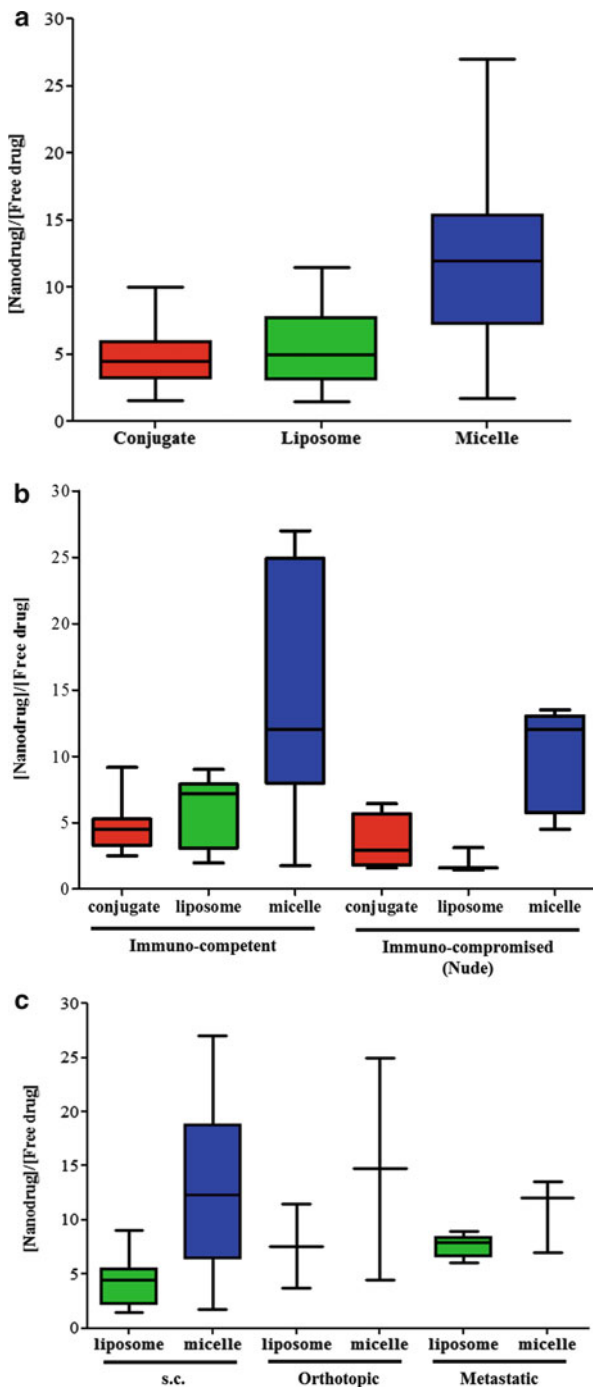


Fig. 8.2 Variability of the different nanocarriers based on their accumulation profile in the tumor, animal models used, and the site of tumor implantation and metastasis in animal model. **(a)** Comparison of the proportion of the different nanocarriers accumulation in the tumor [184–196]. **(b)** Comparison of the tumoral accumulation of different nanocarriers based on the animal model used [184, 189, 194, 197–201]. **(c)** Comparison of accumulation of different nanocarriers based on the site of the tumor implantation either subcutaneous (s.c.), orthotopic, or metastatic [184–197, 202, 203]

the results obtained from testing specific tumor cell lines in tumor models and the clinical trials points further to the sampling errors in generalizing the results of from specific cell line to that of relevant tumors [116].

A substantial difference between tumor models in animals and those of human patients is the progression rate. Animals usually develop a large, clinically relevant tumor (>5 mm) 1 week following subcutaneous (SC) tumor cell inoculation, while such a tumor volume can take years to develop in a human (Table 8.2). This rapid progression rate in animal models results in the overestimation of the targeting role of the EPR effect. Animal tumors developing quickly presumably produce a large quantity of VEGF and vascular mediators to support their rapid growth. In addition a 1 gram tumor mass in a 30 gram mouse is about 3 % of its total weight. In humans, a comparable tumor would weigh 2–5 kg, which is an advanced tumor stage that is not an ideal for utilization of anticancer nanomedicine. Finally, tumors are usually implanted SC in animal models, which allow the developing tumors to take advantage of the extensive cutaneous vascular network for extending their blood supply, a condition that is rarely encountered in human malignancy.

Data collected from available literature to date are plotted in Fig. 8.2c. Although there is a trend towards higher concentration of nanoconstructs in SC models, the results are not conclusive given the limited number of studies. Whether site of tumor development can influence the efficacy of the EPR effect remains an unanswered question.

Tumor Biology Diversity

Tumor Doubling Time (TDT)

Tumor doubling time (TDT) is an important factor to consider when designing EPR-based anticancer nanomedicine. Most cytotoxic drugs selectively target cancers by exploiting differential tumor characteristics such as high proliferation rates, hypoxia, and genome instability. The TDTs provide a selection trait that is exploited by chemotherapeutics that target DNA synthesis and cytoskeleton remodeling. Many chemotherapeutic agents fail to cope with rapidly dividing tumors as the amount of drug necessary to kill a given number of cells will double with each tumor doubling. However, the dose that will elicit dose-limiting toxicity will remain the same. A short TDT is well known to be associated with an unfavorable survival prognosis [117–122]. TDT is a highly heterogeneous, both within and between different tumor types, stages, and grades. There is a large degree of variation of TDT between tumors of different tissue origins. Pituitary adenoma, for example, has an extremely long TDT of 506–5,378 days and within the tumor type the TDT varies by ten times [123], while in meningiomas and neurinomas the TDTs are 6.5 days and 7.67 days, respectively [124]. Some tumor types have a high variation of the TDT, for instance, lung adenocarcinoma has an extremely high variation in TDT of

Table 8.2 Tumor doubling time (TDT) and microvascular density (MVD) in various cancers

	Grade	Tumor doubling time (TDT)	Patient number	References	Gleason's grade	MVD (mean)	Patient number	Marker	References
Prostate cancer		42–1,346 days	34	[225]	3–7	60	155	CD34	[226]
Hepatocellular carcinoma	Edmonson grade				Edmonson grade				
	I–II	118.2 (37–356)	23	[227]	I–II	254	52	CD34	[228]
	III–IV	70.1 (13–239)	39	[227]	III–IV	216	48	CD34	[228]
Brain cancer	WHO grade				WHO grade				
	I–II	14	9	[229]	I–II	15	30	CD31	[230]
	III	140	7	[229]	III	39	25	CD31	[230]
	IV	69.7	9	[229]	IV	50	22	CD31	[230]
Renal clear cell carcinoma	Fuhrman grade				Fuhrman grade				
	I				I	78.8	16	CD31	[231]
	II	486.4 (159–1,193)	17	[232]	II	56.4	69	CD31	[231]
	III	558.2 (253–987)	7	[232]	III	41.4	20	CD31	[231]
IV	321	1	[232]	IV	36.8	4	CD31	[231]	
Breast cancer					TNM stage				
		11–1,293	118	[126]	I–II	104	74	CD34	[233]
					III	111.1	33	CD34	[233]
				IV	131.9	13	CD34	[233]	

964-fold [125] followed by breast cancer with a variation of 117.5-fold [126] (see Table 8.2). TDT can also differ according to the specific cellular origin within a given tissue. Bronchoalveolar cancer, for example, has an extremely varied TDT of 36–1,092 days, a variation of 30.3-fold [125], while small cell lung cancer has a TDT of 61.9–120.4, a mere 1.9-fold difference [127]. In addition, TDT can range depending on tumor grade (see Table 8.2). Poorly differentiated hepatocellular carcinomas corresponding to the Edmonson grade III or IV are highly invasive and have a DT of 13–239 days [128], while well-differentiated tumors corresponding to Edmondson grade I or I–II has a significantly extended TDT of 54.7–1,508.3 days [128] (see Table 8.2). Interestingly, hepatocellular carcinomas are highly vascularized and the microvessel density (MVD) is not affected by the tumor grade (see Table 8.2). Astrocytoma also follows this trend with the TDT of grade IV astrocytoma, according to the WHO grading system, varying between 1.4 and 319 days, the TDT of grade III 30–472 days and the TDT of grade I–II tumors 138–1,045 [129, 130]. The tumor grade also correlated with the MVD, with higher grade having a higher MVD (see Table 8.2). The same trend was observed with prostate cancer and breast cancer where a high grade correlates with a lower doubling time and a higher MVD (Table 8.2).

The primary or metastatic status of a tumor can also cause large fluctuations. For example, primary melanoma may have a DT of 50–377 days [131], while metastatic melanoma may have a DT of 8–212 days [132].

EPR-based anticancer nanomedicine should consider doubling time variation when planning the release mechanism of active chemotherapeutic agents from its nanocarrier, as well as the internalization rate of macromolecular complexes into tumor cells. For example, a slow-releasing amide bond between the polymer backbone and the drug, or slowly internalized liposome, could both be a good choice for tumors with a slow DT. In contrast a fast-releasing micelle or an ester bond linkage can be a better fit for rapidly dividing tumors. Generally, EPR-based nanomedicine has a wider therapeutic window [133], an advantage that can be exploited to shape dose regimens based on individual patient conditions. A tumor's inherent sensitivity to specific chemotherapeutic agents as well as TDT is of the utmost importance in designing EPR-based anticancer nanomedicine.

Microvascular Density (MVD)

The EPR effect is strictly dependent on the vasculature of the tumor with theoretical assumption that all tumors independently of their origin, stage, and organs will behave identically. However, this concept is drastically challenged by a number of reports that show a high diversity in angiogenesis behavior [12, 51, 134, 135]. Nagy et al. have identified six structurally and functionally distinct types of blood vessels in human cancers [134]. Vascular density can provide, in most tumors, a

prognostic indication of tumor progression. As shown in Table 8.2, vascular density is largely dependent on the type of cancer and varies largely within each tumor type. For instance, renal cell carcinoma is highly vascularized [136], while the density of microvessels appears low in head and neck squamous cell carcinoma [137] or in ovarian carcinoma [138]. In addition, higher stages of cancer are well correlated with higher microvascular density as observed in astrocytoma and prostate cancer (Table 8.2), while in other types of tumors such as renal cell carcinoma, no direct correlation can be established between tumor stage and vascular density (Table 8.2). Furthermore, metastatic tumors tend to possess higher vascular density compared to non-metastatic tumors [139–143]. Another element regarding the EPR effect is the secretion of angiogenic factors such as VEGF by the tumor. Vascular permeability can be altered by VEGF as well as a wide array of inflammatory mediators [144], which can affect the extent of nanomedicine accumulation driven by the EPR effect and the penetration of the nanoconstruct into the tumor. As mentioned previously, there is a large heterogeneity in the expression of VEGF between different types of cancers. When designing a nanocarrier, the properties of the targeted tumor tissue such as the cancer type, the microvascular density, and the secretion of permeability factors such as VEGF should therefore be taken into account in order to take full advantage of the EPR phenomenon.

Optimization of Drug Nanocarriers for the EPR Effect

To optimize the engineering of nanoparticles for specific delivery, careful consideration should be undertaken regarding the biology of the tissue being targeted. In many instances, the nature of the nanomedicine itself has been a limiting factor that negatively impacted its chance of clinical success. The loading of active drug into a delivery system can be insufficient due to the physical or chemical limitations to achieve the critical dose needed to treat the tumor. For example, the HPMA copolymer-paclitaxel conjugate showed insufficient drug loading ($\leq 10\%$) with a particle size in the range of 12–15 nm [145] and lacked stability due to the use of an ester linker [146]. Consequently insufficient tumor tissue accumulation of the drug was evident in phase I clinical trials [147]. Another factor limiting the efficacy of nanomedicines is the fast release rate of drug in the circulation. For instance, low molecular weight HPMA copolymer-camptothecin conjugate showed a rapid release of drug and quick renal filtration and consequent bladder toxicity in phase I clinical trials [148]. The nanomedicine was designed with a labile ester linker, decreasing its stability and therefore its tumor accumulation [149]. In this example, a low molecular weight (below the renal excretory threshold of 7 nm) coupled to the toxicity associated with a fast release rate resulted in the drug failing to achieve EPR-based pharmacokinetics.

Following are a few considerations inherent to the design of nanomedicine that may significantly influence the outcome of EPR-based drug targeting (see Fig. 8.3).

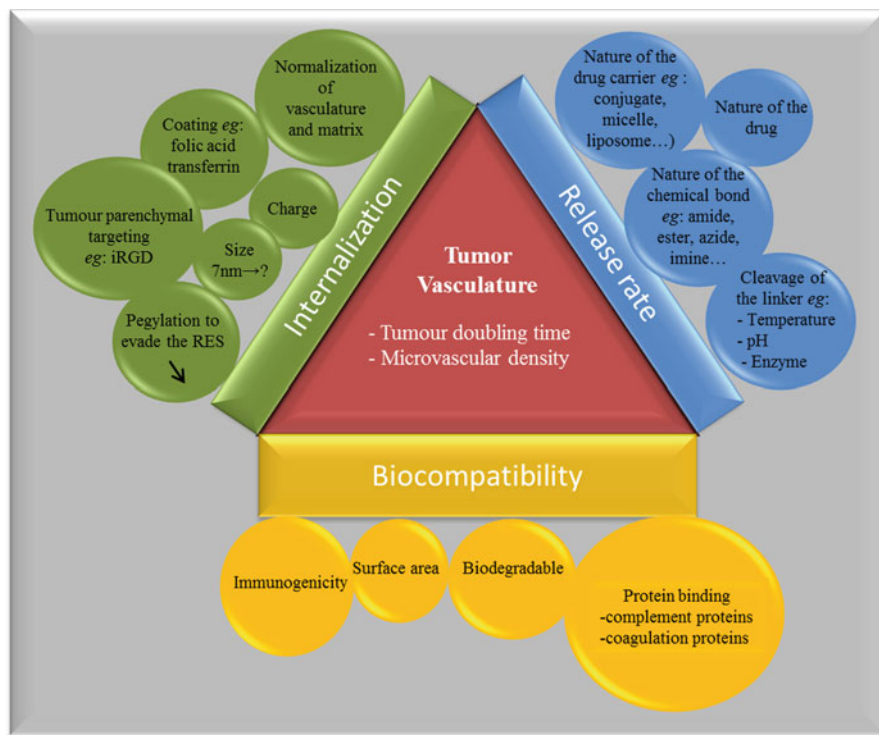


Fig. 8.3 Schematic representation of the variables influencing the clinical application of a nanomedicine. The biocompatibility, internalization, and release should be carefully considered when designing a nanomedicine utilizing tumor vascular abnormalities for targeted cancer treatment

Internalization of the Nanocarrier

The concentration of drug inside the tumor resulting from the EPR effect in a subset of highly vascularized tumors does not guarantee the efficient internalization of the drug within the tumor cells. Multiple factors can influence the cellular internalization process of the nanomedicine. Usually, nanoparticles and polymer-based drug delivery systems are internalized by endocytosis, a multistep process that culminates in the formation of a late endosome which finally fuses with a lysosome [150]. Malignant cells have an accelerated metabolism, a high glucose requirement, and an increased glucose uptake characterized by the elevated expression of glucose transporter proteins (GLUT) [151]. However, recent studies have shown that many cancer cell lines exhibit limited capacity for endocytosis compared to normal cells [152, 153].

Compared to tumor cells, macrophages usually exhibit a higher uptake of nanosized molecules [66, 154] as they can recognize nanomedicine either through

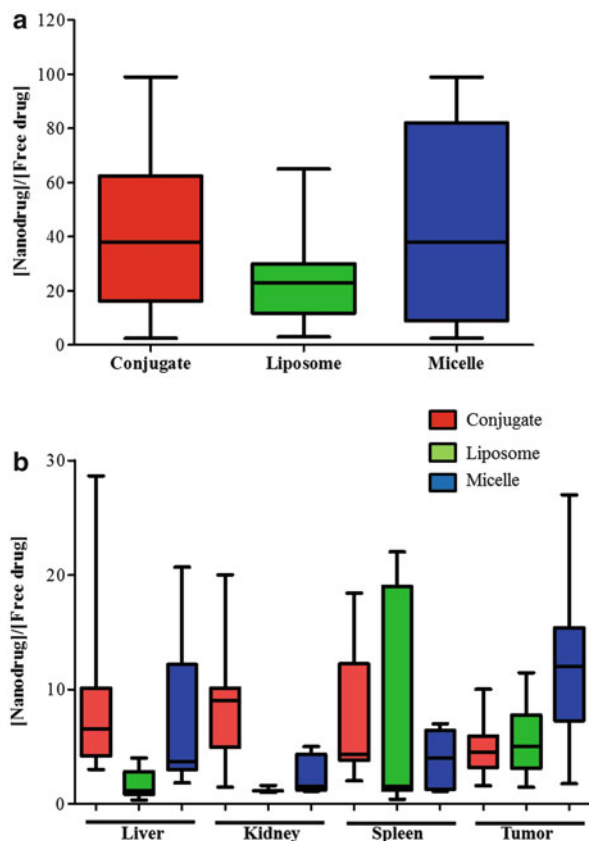
their Toll-like receptor 4 (TLR-4) [155] or through scavenger receptors [156]. Much work has therefore been devoted to the development of nanoparticles which can evade macrophage recognition, resulting in longer circulatory time and increased interaction with target tissue. On this basis, polyethylene glycol (PEG) is the polymer most commonly used to enhance in vivo circulatory half-life [157, 158]. Coating nanoparticles with PEG results in the formation of a polymeric layer which sterically hinders the interaction of nanoparticles with plasma proteins and cell membranes [159] preventing opsonization and phagocytosis by components of the RES [160, 161]. PEG-liposome-incorporated doxorubicin (Doxil[®]) is approved by the FDA for the treatment of ovarian cancer (see Table 8.1). Additional polymers such as N-(2-hydroxypropyl) methylacrylamide (HPMA), polyacrylamide, or poly(vinyl pyrrolidone) have also been used to improve the circulation time and steric hindrance of nanomedicines [162, 163]. The main disadvantage of this strategy is that it limits the interaction of (stealth) nanoconstructs with the tumor cell membrane and subsequently reduced internalization and uptake by tumor cells. To improve specific uptake by endocytosis, several nanoparticles have been coated with receptor ligands such as folate [164] or transferrin [165] to induce receptor-mediated endocytosis. These coatings increased the accumulation of drug inside tumor cells. However, the practical advantages in the management of human tumors in the clinic remain to be proven. Following intracellular internalization, active drug should be liberated from the lysosomal compartment to reach its cellular target. Mechanisms to escape the lysosomal compartment and improve intracellular targeted delivery have been described by Breunig et al. [166]. Another consideration relevant to relatively large sized macromolecular nanomedicine is their nonspecific interactions with the extracellular matrix; to reach tumor cells, nanoconstructs must move through the matrix, a highly interconnected network of collagen fibers that intermingle with proteins such as proteoglycans and glycosaminoglycans. This semisolid barrier could significantly reduce the amount of nanomedicine reaching tumor cells, either through nonspecific interaction (Fig. 8.1) or simply by impeding convection movements of relatively large sized nanoconstructs [167]. This could lead to nanomedicine being locally concentrated in proximity to the capillary that it leaked from without reaching the target tumor cells.

Recently, several studies have developed methods to circumvent these limitations such as using of the tumor penetrating peptide, iRGD, which has been shown to increase the delivery of nanomedicines in solid tumors by improving its interstitial transport [168]. Additional therapeutic strategies aiming to normalize the tumor vasculature and extracellular matrix in order to improve tumoral penetration of the drug have been described (for review [51, 169]).

Release Rate

Conjugates can be synthesized through covalent linking of drugs to polymeric carriers such as SMANCS (Table 8.1) [30]. In comparison, entrapment of drug inside a micellar structure requires either covalent or non-covalent bonds (ionic, hydrogen

Fig. 8.4 Variation of nanocarrier release rate and accumulation profile. (a) Determination of the release rate over a 24 h period of different nanocarriers across several studies [162, 204–212]. (b) Comparison of the proportion of the different nanocarriers accumulation in the tumor and various organs [184–197, 202, 203]



bonds, or hydrophobic) and involves a block polymer or copolymer. Various chemical bonds such as amide, ester, azide, imine, hydrazone, thioether, and urethane are currently used to prepare nanomedicines [29, 170]. Based on the nature of these chemical bonds, the release of drug from its carrier can depend on either pH, usually acidic pH of the lysosome [170], temperature [70], or on enzymatic cleavage [171]. Furthermore, the nature of this bond will determine the release rate; for example, an ester bond ensures a rapid release of drug due to an abundance of esterases in plasma, whereas an amide bond will show a slower release profile [29, 148]. The comparison of release rates between polymer conjugates, liposomes, and micelles nanomedicines after 24 h incubation (Fig. 8.4a) in different studies showed a distinctive profile. Overall, polymer conjugates and micelles have a comparable release rate which is higher than that of liposomes (Fig. 8.4a). The release profile of liposomes appears relatively homogeneous with a mean value of 24 % (3–39 %), while the release profile of conjugates and micelles appears heterogeneous across several studies with a mean value of 39 % (2.5–100 %) and 41 % (2.5–100 %), respectively.

In order for a nanocarrier to provide tumor targeting, the carrier should have a stable chemical bond with the cargo drug while in circulation. This prevents the rapid release of free drug and permits a therapeutic effect at the site of action. A rapid drug release from its delivery system in plasma can result in a biodistribution and toxicity profile comparable to its related free drug. In contrast, engineering a stable linkage between the drug and its carrier can result in a slow release rate at the target site and inability to reach the critical therapeutic concentration. The release rate of nanoconstructs needs to be tailored for the treatment of a specified tumor doubling time (see Table 8.2). Thus, the choice of a specific linker is critical for a favorable anticancer outcome of EPR-targeted nanosystems.

Biocompatibility

The EPR-based accumulation of active drug inside the tumor rarely exceeds 5 % of the total dose of nanomedicine administered by i.v. injection. The majority of the injected dose accumulates in various organs such as liver and spleen and to a minor extent kidneys and lungs [172]. As nanomedicines reach sizes of 7 nm, classical pharmacokinetics cannot be accurately applied due to two drastic changes. Firstly, nanosized drugs cannot be eliminated by renal glomeruli as they exceed the renal threshold of excretion dictated by the pore size in the glomeruli [32, 173]. Secondly, their organ distribution is limited to tissues that have capillaries with large enough endothelial fenestrations to allow macromolecular drugs to pass through [32, 33]. The EPR effect utilizes the unique characteristic of large gaps between endothelial cells that makes up tumor vessels. Usually these gaps can vary from few nanometers to up to 1200 nm in size [174, 175]. At this large size, nanomedicines can preferentially accumulate in tumor tissues. However, tumors are not the only organs with such large fenestrae as the spleen and liver show similar characteristics. Liver sinusoid can have fenestrae of around 100 nm in humans [176], whereas the spleen has large sinusoid lumina of ~ 5 μm that can support extravasation of aged red blood cells [32]. With such a large fenestration size, great amounts of nanosized drugs accumulate in these organs. As shown in Fig. 8.4b meta-analysis of 73 studies over the last 10 years revealed that with all the EPR effect-based nanoconstructs that were used, the liver and spleen were the two major organs competing with tumor for the nanoconstructs (conjugate, micelles, and liposomes). While spleen function can be compensated for by other lymphatic organs, liver damage due to the concentration of cytotoxic nanomedicine remains a challenge to successful anticancer drug targeting. For example, nanoconstructs of cis-platinum have reduced toxicity in the kidney compared to the free drug but result in a dose-limiting liver toxicity [177].

Surface modification of the nanomedicine, such as PEGylation, may increase their retention in the systemic circulation and favor tumor accumulation. However, more than 90 % of PEGylated nanoparticles will still be removed through liver clearance within several hours of administration. Studies have demonstrated that as

little as ~2 % of the total i.v. administered dose was found in the tumor after 4 h [172]. Thus there is a legitimate safety concern regarding the off target accumulation of the drug delivery system. Ideally, the drug carrier should be eliminated after drug release. But, unless the nanocarrier is biodegradable, it will remain in the body and be dealt with as a foreign body. The innate elements of the immune system could be stimulated nonspecifically by these foreign bodies through TLR-4 [155]. Activated macrophages will phagocytose and attempt to degrade the nanocarrier in its lysosomal compartment. Failure to do so may lead to the formation of foreign body giant cells caused by fusion of multiple macrophages or monocytes [178] and ultimately to the formation of lesions resembling granulomas [179]. This can potentially result in the pathological formation of a dense fibrous capsule replacing the original functional tissue. Another concern in relation to the accumulation of non-degradable materials is the induction of malignancy resulting from frustrated phagocytosis and prolonged inflammation [180].

To address these issues, recent work has focused on the development of biodegradable and nonimmunologic drug delivery systems containing either enzymatically or reductively degradable spacers such as poly(-D,L-lactide-co-glycoside) (PLGA) [181] or the star HPMA polymer carrier which enable a controlled degradation of the drug carrier [182, 183]. Some of these carriers demonstrate prolonged blood circulation and tumor drug accumulation but difficulties in the reproducibility of their synthesis could hamper further clinical development [182].

To summarize, EPR-related parameters of a nanoparticle delivery platform such as long circulatory half-life, reduced elimination, and altered distribution could be a double-edged sword. Careful consideration of these parameters is essential for effective, safe, and more personalized cancer treatments.

Conclusion

As a general concept of tumor vasculatures, vascular permeability has allowed the development of a variety of anticancer nanomedicines. In theory, the nanomedicine should decrease systemic toxicity and improve the delivery to the tumor site. However, despite high expectations for this targeting strategy, over the last 26 years, only a few nanomedicines have successfully exploited this concept and made the transition to the clinic. Possible reasons for this slow transition are the lack of control of essential parameters for a good delivery such as release rate, internalization, and biocompatibility. Moreover, the variability of tumors biology such as doubling time and microvascular density can influence the targeting potential of EPR-based nanomedicine. Consideration of these variables as well as the development of modular delivery systems of macromolecules can significantly hasten the transition of anticancer nanomedicine towards clinical application.

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