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Stromal Barriers and Strategies for the Delivery of Nanomedicine to Desmoplastic Tumors

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Abstract Nanoparticle based delivery formulations have become a leading delivery strategy for cancer imaging and therapy. The success of nanoparticle-based therapy relies heavily on their ability to utilize the enhanced permeability and retention (EPR) effect and active targeting moieties to their advantage. However, these methods often fail to enable a uniform NP distribution across the tumor, and lead to insufficient local concentrations of drug. Oftentimes, this heterogeneous drug distribution is one of the primary reasons for suboptimal treatment efficacy in NP delivery platforms. Herein, we seek to examine the biophysical causes of heterogeneous NP distribution in stroma-rich desmoplastic tumors; namely the abnormal tumor vasculature, deregulated extracellular matrix and high interstitial hypertension associated with these tumors. It is suggested that these factors help explain the discrepancy between promising outlooks for many NP formulations in preclinical studies, but suboptimal clinical outcomes for most FDA approved nanoformulations. Furthermore, examination into the role of the physicochemical properties of NPs on successful drug delivery was conducted in this review. In light of the many formidable barriers against successful NP drug delivery, we provided possible approaches to mitigate delivery issues from the perspective of stromal remodeling and NP design. In all, this review seeks to provide guidelines for optimizing nanoparticle-based cancer drug delivery through both modified nanoparticle design and alleviation of biological barriers to successful therapy.

Keywords Nanoparticle; Tumor microenvironment; Extracellular matrix; Cancer associated fibroblasts; Abnormal Vasculature

Abbreviations α -SMA, alpha smooth muscle actin; BM, basement membrane; CAFs, cancer associated fibroblasts; ECM, extracellular matrix; EPR, enhanced permeability and retention; FAP, fibroblast activation protein; GAG, glycosaminoglycans; HSV, herpes simplex virus; IFP, interstitial fluid pressure; TAM, tumor associated macrophages; MMP, matrix metalloproteinase; MSNP, mesoporous silica nanoparticles; NP, nanoparticles; TME, tumor microenvironment; VEGF, vascular endothelial growth factor

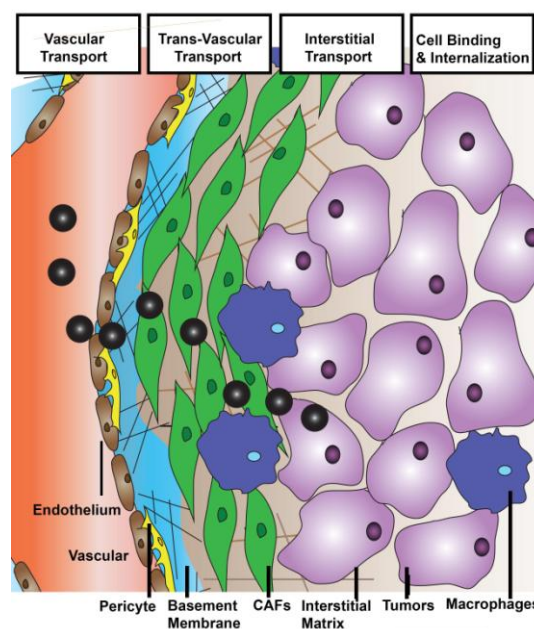


Figure 1. Scheme of NP intratumoral transport

1. Introduction

The field of nanomedicine has recently attracted tremendous attention, particularly for applications in cancer drug delivery. Owing to the advancements in material science and new manufacturing methods, nanoparticle (NP) drug delivery platforms can now be fabricated to an almost unlimited number of configurations with respect to size, shape, and payload, allowing for versatile applications for the detection, prevention and treatment in oncology. Nanotherapeutics such as liposomes, polymeric micelles and inorganic NP possess a distinct functional advantage over conventional small molecule chemotherapy regimens by overcoming severe systemic toxicities that limit the clinical application of most chemotherapy drugs [19]. Furthermore, nanoparticle drug delivery platforms permit significantly prolonged circulation when compared to small molecule drugs alone. Most importantly, the leakiness of vessels established during angiogenesis and the impairment of lymphatic drainage, constituting the so called *EPR effect*, provides the primary driving force for the extravasation of NP, improving the intratumoral accumulation and distribution and resulting in enhanced therapeutic outcome [4]. Examples of applications include both the preclinically investigated nanoformulation (i.e. liposomes, polymeric micelles) and FDA approved NP of liposomal doxorubicin (Doxil®) and albumin-bound paclitaxel (Abraxane®). Despite demonstrating outstanding antitumor efficacy in preclinical studies and promising outlooks for multifunctional use in clinical trials, nanoparticulate anti-cancer therapeutics have only provided modest survival benefits overall [34]. Factors such as the fundamental design of the NP, abnormal tumor microenvironment, and the heterogeneity across tumors can compromise the EPR effect and lead to treatment failure. Clinically, the tortuous tumor vasculature and abnormal basement membrane limit both the trans-vascular and interstitial transport of NP. Additionally, the high level of extracellular molecules, increased solid stress and high interstitial pressure limit successful NP extravasation. In addition, off-targeted distribution to non-tumor stromal cells induced by the heterogeneity of the tumor microenvironment (TME) result in diminished tumor cell accumulation, induced drug resistance and compromised clinical outcomes [39]. These serve to explain the discrepancies between the promising results obtained from preclinical studies, and the subpar performance in clinical trials.

Herein, we first present mathematical models for intratumoral NP transport to achieve a better understanding of this complex process. We then discuss the key physiological barriers for NP transport, and analyze the design of NP for enhanced intratumoral transport. Finally, we summarize the strategies to overcome delivery barriers through remodeling the TME and designing the TME-responsive NP.

2. Mathematical Modeling and *In Vitro* Models of NP's Intratumoral Distribution

The intratumoral delivery of macromolecules and NP requires several steps in transport, including vascular transport, transvascular transport, interstitial transport, cellular binding, internalization and metabolism (**Figure 1**) [19]. All these steps are generally limited by pathophysiology of tumors. To better understand the biophysical underpinnings of these transport barriers, Jain and his colleagues have developed several mathematical models to simulate the intratumoral behaviors of NPs [47-49].

For the modeling of vessel and trans-vasculature transport, the tumor vasculature was represented by a two-dimensional percolation network with one inlet and one outlet that resembles the

vascular structure and function of tumors [50]. Vessel transport is mainly dominated by convection (flow rate governed by pressure gradient) and is quantified based on the perfusion rate of blood flow (Q). Poiseuille's law was used to simulate vessel transport, suggesting that blood flow is proportional to the vascular pressure gradient and blood viscosity [50]. The transvascular flow was set proportional to hydraulic conductivity of the vessel wall, the surface area of the vessel and also the influence of interstitial fluid pressure. All parameters can be measured using standard intravital microscopy, multiphoton microscopy and optical frequency domain imaging [19, 51-54]. This modeling formula emphasizes the potential influence of blood vessel area, pore size and interstitial fluid pressure (IFP) on NP transport.

Interstitial transport, mainly indicating the diffusion of NP through the extracellular matrix (ECM) toward tumor cell targets, is another significant step in determining NP penetration. Interstitial transport follows the Darcy's theory, which requires calculation of the diffusion coefficients (D_{eff}) of NP in the ECM [50, 55]. Diffusivity of NP in the ECM was modeled *in vitro* using matrigel or collagen confined diffusion chamber models. Diffusion coefficients were determined using these *in vitro* ECM models by non-linear fits of intensity gradients to a diffusion model (e.g. Fickian model) [34, 55, 56]. Diffusion coefficients of macromolecules and liposomes can also be quantified *in vivo* using either single-photon fluorescence recovery after photobleaching or two-photon fluorescence correlation microscopy. These measurements are more clinically relevant, but limited by equipment requirements and cost [56, 59]. A recent report by Lu et al. indicated that cellular density is another factor affecting interstitial transport [60]. Densely packed tumor cells induce solid stress and reduce the interstitial space for NP transport. Therefore, parameters including cell volume, density and spacing should be taken into consideration when modeling the sophisticated interstitial transport.

For the majority of the aforementioned short time scale transvascular transport models or long-term NP penetration models, neither the binding of NP to cancer cells, nor cell uptake was included [61]. However, these two factors play a substantial role in the process of NP transport. In light of this, Mok et al. are credited for the development of mathematical models considering rapid cell surface binding, internalization and degradation through an intratumorally infused Herpes Simplex Virus (HSV). In addition to diffusion coefficient, second-order binding rate constants, first-order dissociation constants and internalization constants were included in the differential equation to distinguish the free interstitial virus, bound virus and internalized virus respectively [61, 62]. Using a similar mathematical model, Kim et al. discriminated the interstitial diffusion, cellular uptake and intracellular release of fluorescein-labelled gold NP (6 nm overall with ligand) on a three-dimensional multicellular tumor cylindroid model [63]. The intracellular release kinetics of fluorescein from NP were further included into the differential equation. However, these mathematical equations are all confined to *in vitro* 3D tumor models or intratumoral injection of NP, and do not consider the effect of vascular transport and plasma clearance. In another study, Schmidt et al. modeled the cellular binding affinity of the targeted molecules along with the dynamic plasma clearance of macromolecules into the mechanistic compartmental model, and avidly studied the effect of molecular size and binding affinity on tumor targeting [64].

Although, most of the mathematical and *in vitro* models are based on assumptions and limitations, the overall modeling of intratumoral transport of NP still provides a semi-quantitative

method for the extrapolation of parameters such as NP physicochemical properties and tumoral barriers on NP transport. This can then be extended so as to predict dynamics of NP transport and the therapeutic outcomes of NP delivering chemotherapy and gene therapy.

3. Enhanced Permeability and Retention Effect and Anti-cancer NP in the Clinical Trials

The mathematical models discussed in the previous section emphasize the importance of the tumor vasculature on vascular and transvasculature transport of NP. New vessels formed during angiogenesis are known to have a leaky and tortuous morphology, permitting NP extravasation [1]. On a different note, rapid unconstrained proliferation is coupled to an IFP and solid stress, which results in the compression of lymphatic vessels and impairs NP clearance [65]. Together, these characteristics comprise the enhanced permeability and retention (EPR) effect. The EPR effect describes how the leaky vasculature of tumors permits enhanced NP permeability, while the lack of a functioning lymphatic network promotes NP retention in the tumor. The EPR effect states perhaps one of the most fundamental advantages for NP-based drug delivery. Clinically, NP have been applied to treat a broad range of cancers. Abraxane is an albumin-stabilized NP designed for the delivery of paclitaxel [66]. Along with other platforms such as a PEGylated liposome based doxorubicin delivery system (Doxil®), both share the ability to exhibit enhanced tumor localization through the EPR effect.

4. Tumor Microenvironment Barriers for Intratumoral NP Distribution

Ironically enough, the mechanistic basis for the EPR effect also comprises one of the primary barriers to NP delivery. Namely, the elevated IFP [67] and increased solid stress act to inhibit successful NP extravasation into the tumor. This paradoxical observation explains the discrepancy between promising preclinical research and the subpar clinical outcomes for NP application. Therefore, successful NP drug delivery relies heavily on the balance of these two competing aims.

4.1 Abnormal Tumor Vasculature Plays Paradoxical Roles in NP-based Delivery

Tumor vessels are known to be heterogeneous and dilated, leaving avascular spaces of various sizes. In addition, abnormal vessel-wall structures with heterogeneous basement membranes, wide inter-endothelial junctions and large pore sizes contribute to the irregularity of the tumor vasculature [4, 68-71]. These factors therefore compromise NP transport and undermines the efficacy of therapeutic agents. The dynamic formation process of abnormal tumor vasculature structure and its role in compromising NP delivery was discussed in details as follows:

Genetic and epigenetic changes drive tumor and mesenchymal cells to produce pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) [72]. Due to over-activation of these pro-angiogenic pathways, tumor vessels become tortuous and leaky. As mentioned in the previous section, leaky vasculature then facilitates transient NP extravasation through the EPR effect. However, the mechanistic basis behind the EPR effect also contributes to excessive fluid extravasation, inducing increased IFP, fluid viscosity and therefore impairs NP vascular transport [73]. The tortuous nature of tumor vessels further contributes to this elevated geometric resistance through decreasing blood flow [1, 74]. Meanwhile, solid stress resulting from unconstrained tumor growth and an abnormal ECM, further compresses vessels, blocking blood flow and leading to vessel collapse [1]. The decrease in blood flow, in turn, has a great effect on the viscous resistance of the blood. Slow blood-flow rates and high blood viscosities govern the vascular and transvascular transport of small molecules, macromolecules and NP.

Furthermore, unlike normal tissues, blood velocity in tumors is independent of vessel diameter and unevenly distributed. The poorly perfused or even unperfused blood supply leads to hypoxia and acidic conditions, which bolsters drug resistance and further limits NP diffusion [4]. Aside from blood vessel constriction, the lymphatic vessels in the tumor are also compressed by proliferating cancer cells, causing collapse. The inefficient drainage of fluid from the tumor center coupled with fluid leakage from tumor vessels contributes to interstitial hypertension, which further limits NP perfusion deep into the tumor core [39].

4.2 Acidic and Hypoxia Limit Nanotherapeutic Approaches to Necrotic Areas

Acidic and hypoxic conditions are distinctive features of most solid tumors. Acidification of tumor microenvironments primarily arises from the Warburg effect, which describes the tumor's shift in energy metabolism from pyruvate oxidation to glycolysis. The excess lactic acid produced through this pathway remains in the tumor due to poor lymphatic drainage as described previously, a characteristic of most solid tumors [75]. Acidic conditions lead to the ionization of weakly basic drugs and compounds, limiting their ability to diffuse in the ECM [76]. On the other hand, hypoxic conditions primarily arise due to unconstrained cell proliferation that outgrows the blood supply in the tumor. As a result, it is well documented that hypoxic regions most commonly surround the necrotic center of the tumor [77]. Due to the altered blood-flow in hypoxic regions, particles often fail to localize in regions of hypoxia [78]. Even if delivery of NP is successful to hypoxic regions, cells deficient of oxygen show surprising resilience to chemotherapy. As hypoxia reduces cell proliferation, chemotherapy drugs targeting rapidly proliferating cells are rendered useless in these regions [79]. Furthermore, hypoxic regions are hotspots of hypoxia-inducible factor (HIF) production. The stabilization of HIF-1A, a normally labile transcription factor, is known to induce drug resistance through up-regulation of genes such as MDR1 [79] [80]. Therefore, hypoxic and acidic environments are a barrier to nanoparticle-based chemotherapy options from both a delivery and therapeutic standpoint. However, many novel treatment regimens seek hypoxic and acidic environments as a tumor drug target. Specifically, some treatments use the acidic environment to induce drug release from polymeric nanoparticles [81].

4.3 High Interstitial Fluid Pressure Limits NP Convection and Accumulation

Another notable contradiction that arises when discussing the mechanism behind the EPR effect is the elevated IFP. The balance between elevated IFP and the increased NP uptake via EPR effect influences successful NP delivery. High IFP is known to be the result of a variety of factors. Firstly, the dense surrounding collagen matrix of the TME is rich in cancer associated fibroblasts (CAF), which contract and tighten the collagen network by secreting ECM associated molecules and integrin dependent binding [67, 82]. This first barrier physically limits the expansion of the tumor cavity in response to growth. Continuous unregulated tumor proliferation in this enclosed space then compresses blood and lymphatic vessels due to growth induced solid stress, which in turn prevents the efficient discharge both NP and interstitial fluid from the tumor [1, 83, 84]. An elevated IFP is known to be particularly detrimental for large molecule/nanoparticle delivery, which rely primarily on convection for their extravasation [70]. High IFP acts against convection and force NP to enter via passive diffusion, a kinetically slower process. Furthermore, IFP induced vessel constriction has been shown to cause tumor hypoxia, where the increased precedent of angiogenic and growth factors contribute to lymph node

metastasis and drug resistance. These secreted factors are then relocated from the tumor periphery toward the outer invasive front due to the IFP gradient where they communicate with fibroblasts to induce resistance and metastasis [85-87]. A high IFP therefore obstructs the therapeutic efficacy of NPs and leads to heterogeneous drug distribution in the tumor stroma.

4.4 High Tumor Cell Density Influences the Interstitial Diffusion of NP

While aforementioned discussions outlined the role of IFP modulation on convective transport, a study conducted by Kuh et al. demonstrated the influence of high tumor cell density on NP diffusion into the tumors [88]. The study performed *in vitro* using tumor fragments in the absence of a functioning blood supply avoided the impact of IFP and convection on NP transport. It therefore demonstrated that high tumor cell density is a key determinant for the diffusion process. This observation was further confirmed *in vivo* by Lu et al, showing that cell density was crucial for both convection and diffusion of NP to the tumor [60]. As discussed earlier in this review, rapid tumor cell growth in a limited area imposes solid stress and compresses blood vessels, thus, limiting convection [1]. High cellular density also reduces the interstitial space and limits NP diffusion. Further coherent studies using apoptosis-inducing drugs (paclitaxel and doxorubicin) to reduce tumor cell density led to increased NP perfusion and highlight the importance of cell density on the modulation of NP interstitial transport [89].

4.5 Abnormal Extracellular Matrix Interferes with NP-based Drug Delivery

The non-cellular components of the tumor ECM is another key element that imposes solid stress [1]. Particularly, the high cellularity of tumors compresses the interconnected collagen network and space-filling hydrogel-like glycosaminoglycans (GAG), the two major components of the ECM. The compressed network in turn results in the accumulation of solid stress and dictates interstitial transport [90]. At the cellular level, ECM is localized at two different intratumoral sites, the basement membrane (BM) and the interstitial matrix.

The BM functions as a scaffold for endothelial and mural cells. In particular, the matrix of the BM contains highly compact, sheet-like dispositions formed from fibronectin, laminin and type IV collagen, linked via nidogen and heparin sulfates [91, 92]. More than 99% of non-cancerous blood vessels are covered by a thin layer of BM, which regulates vessel development through paracrine secretion of pro-inflammatory and pro-angiogenic cytokines [93]; Whereas the BM of tumor microvessels are primarily continuous but conspicuously abnormal [93]. Heterogeneous BM morphologies were observed in different tumors or different regions of the same tumor. MCA-IV breast carcinomas and 4T1 breast cancer are characterized by a lesser amount of collagen in the vessel wall, which are loosely associated with the underlying endothelia [16]. Separately, breast cancer 3LLL and pancreatic cancer BxPC3 are distinguished by a second type of BM, with a distribution of more condensed collagen nodules overlapped with the capillary. The BM does not induce the elevation of IFP, yet functions as a sieve to modulate extravasation of free drug and NP from capillaries into the tumor microenvironment. Free DOX, FITC-tagged dextran and 80 nm PEGylated, DOX loaded liposomes (DOX-PLD) were used as tracers to evaluate the extravasation of small molecules and large NP into the tumor microenvironment [16]. Yokoi et al's study showed that the extravasation pattern of small molecules (DOX, FITC-tagged dextran) in the type I BM model 3LLL and type II BM Model 4T1 were comparable [16]. However, the extravasation of NP (PLD) from the same two tumor types was very

different, as suggested by their *in vivo* NP diffusion model and *in vitro* collagen-sleeve model. Diffusion based transport of NP was severely hindered by the thickness of collagen fibers, fiber mesh pore size and fiber density. Moreover, the BM is not static, but dynamic as angiogenesis of blood vessels requires degradation of collagen IV by matrix metalloproteases MMP2 and MMP9 [94, 95]. Therefore, degradation of collagen IV provides a transient niche with leaky tumor vasculature and a low/thin BM, a beneficial window for NP delivery. Identification of this window is thus, important to achieve improved NP extravasation. Besides the collagen meshwork, some earlier works also proposed that the extensively charged heparan sulfate chain, which is attached to the laminin/collagen IV network, is essential for the microscopic filtering of positive charged particles. Simultaneously, the nidogen molecules and the protein core of the perlecan complex geometrically hinder the negatively charged particles [18]. Overall, BM characteristics such as the matrix density, presence of proteoglycans and angiogenesis mediated BM remodeling limit the extravasation of NP from blood vessels into the interstitium of tumors.

While the interstitium is also rich with ECM, it limits NP penetration differently from the BM in three ways. Firstly, high stromal fraction and large matrix molecules are restricted to a limited interstitial volume, compressing the matrix into a dense network with increased solid stress and IFP; limiting convection of NP. Secondly, fibrillar structure, mesh size and collagen thickness directly limit the diffusion of NP. Since the molecular components of interstitial ECM are more confluent than BM, the interactions between these parameters are both more pronounced and more complicated. The tortuous nature of the interstitial space is the third primary barrier for NP since it elongates the diffusion path of both NP and macromolecules from blood vessels to target cells. In tumors with a lower amount of interstitial matrix, such as melanoma and colorectal cancers, NP can easily diffuse across the interstitial barrier, access tumor cells and therefore induce growth inhibition [6]. However, this process proves more difficult for tumors with a thick interstitial matrix. Collagen content is the major determinant of interstitial transport [4]. Unlike BM, collagen I rather than collagen V is the major component of the collagen matrix [96]. The fibril orientation of collagen I influences particle diffusion rates. During cancer development, collagen-remodeling enzymes convert the orientation of collagen scaffolds from thin and relaxed collagens (curly fibrils) to thick, aligned fibrils. A study done by Stylianopoulos et al. suggested that this alignment of the collagen fibrils stiffens the ECM, narrows the inter-fiber spacing, and retards the movement of particles [23, 97]. The crosslinking of collagen fibers also causes disparate NP diffusion. Crosslinking of collagen via lysyl oxidase (LOX), regulated by fibronectin and organized by SPARC (secreted protein acidic and rich in cysteine) increased the stiffness of collagen fibers [97, 98]. These molecules are therefore target candidates to inhibit stromal stiffness and improve delivery. With this aim, Kanapathipillai et al. designed a PLGA loaded LOX inhibitory antibodies to decrease collagen crosslinking and improve therapy [99].

The contribution of interstitial GAGs toward macromolecular diffusion is controversial. As one of the major non-sulfated GAG, hyaluronan is a linear polysaccharide with repeating disaccharide units of β -d-glucuronic acid and N-acetyl- β -D-glucosamine [23]. In most cases, the elimination of hyaluronan inhibits nanoparticle transport, opposite to the effect of eliminating collagen [59]. The polymerization of HA has been shown to partition the collagen matrix into aqueous and viscous compartments.

Hyaluronidase treatment increased the proportion of slow-diffusing compartments and decreased the diffusion rate for NP transport [17, 100]. On the other hand, for certain tumors such as pancreatic cancer, in which more than 70% ECM consists of HA, the degradation of HA resulted in increased drug diffusion [24]. The sulfated glycosaminoglycan, similar to that in the BM, carries a highly negative charge, which can inhibit the transport of macromolecules or NP by forming aggregates [4, 101].

4.6 Stromal Cells Regulate the Interstitial Distribution of NP

Tumor stroma contains modified ECM attached to multifaceted stromal cells, including fibroblasts/myofibroblasts (carcinoma-associated fibroblasts), mesenchymal cells such as pericytes/mural cells, endothelial cells and immune cells [1, 39]. Apart from the unconstrained proliferation of tumor cells, the high cellular density of stromal cells also contribute to the solid stress, compressing the matrix into a disorganized network and limiting NP penetration. On the other hand, stromal cells compromise the internalization of therapeutic NP in cancer cells through nonspecific depletion of NP that extravasate from adjacent microvessels.

Cancer associated fibroblasts are a major player of tumor fibrosis. CAF inhibit the interstitial transport of NP via the secretion of ECM, construction and stiffening the fibrillar structure and also secretion of paracrine growth factors for tumor resistance and metastasis. For example, lysyl oxidase (LOX) synthesized by CAF could be stimulated via the TGF- β signaling pathway and contributed to increased stromal stiffness through crosslinked collagen fibers [102]. As a major stromal cellular component, CAF also nonspecifically internalizes therapeutic NP and compromises NP's association with tumor cells in turn; resulting in sub-optimal clinical outcomes [103]. The ratio of off-target NP distribution into fibroblasts is regulated by the spatial distribution of blood vessels in relation to tumor cells and other stromal components (i.e. fibroblasts) [104]. Two dominant phenotypes based on tumor stromal architecture delineate tumor types into either a tumor vessel phenotype (with vessels embedded throughout tumor cells) or a stromal vessel phenotype (vessels distributed in proximity to fibroblasts). While the tumor vessel phenotype is commonly applied in xenograft models, the stromal vessels are more clinically relevant and seen in orthotopic tumors (4T1 breast cancer or pancreatic cancer) and other primary cancers. Off-target distribution of NP into CAF is in fact, more prominent in the stromal vessel type. This was demonstrated in an experiment with 120 nm Docetaxel-conjugated NP which distributed to fibroblasts in 4T1 and MDA-MB-231 orthotopic breast cancer to deplete CAF content by 70% [28]. Given that the tumor-fibroblast transition is a major source of CAF, surface receptors that are highly expressed in tumor cells are not surprisingly also found on respective fibroblasts [105]. Because of the increased binding affinity between fibroblasts and targeted NP, the binding site barrier of CAF is stronger for the targeted NP, rendering off-target distribution more likely. Although therapeutic NP delivered off-target to fibroblasts could deplete fibroblasts and synergize with chemotherapy in some tumor models [27], the therapeutic effect of anti-cancer therapies are also likely to significantly deviate from initial predictions in CAF, due to the different sensitivities and resistance mechanisms of benign fibroblasts and tumor cells [104]. For example, CAF attacked with chemotherapy could secrete survival factors such as Wnt16 that induced the formation of resistant phenotypes of prostate cancer [103]. Overall, the off-target delivery of NP in CAF severely hinders NP penetration and induced convoluted anti-tumor efficacy.

Immune cells, including B cells, T cells, granulocytes, dendritic cells, myeloid derived suppressor cells and macrophages, are indispensable constituents of the TME that modulate the intra-tumoral immune response [106, 107]. Owing to the partial peri-vasculature localization of infiltrated immune cells (other immune cells are likely to distribute in inflammatory hypoxia and necrosis area)[108] and phagocytic properties of some of the immune cells (e.g. macrophages) [109], off-target internalization of NP is inevitable. Using an orthotopic model of melanoma and fluorescently labeled PRINT nanoparticles, Roode et al demonstrated that association between tumor-associated macrophages (TAM) and NP were 4-fold greater than that of cancer cells despite TAM constituting only 1% of all cells in tumors [110]. In another study, the correlation between increased delivery and release of CKD-602 from S-CKD602 liposomes, and increased expression of CD11c-positive dendritic cells in a SKOV3 ovarian xenograft suggested that the NP disposition may be associated with the phagocytic cells (i.e. DC and TAM) [111, 112]. Although, the off-target association of NP in leukocytes and the general immune compartment may modulate the immune pathway (i.e. Stat-3, ERK) and modify the suppressive tumor microenvironment to synergistically improve cancer vaccines (Data not shown), direct phagocytosis of NP by phagocytic cells may deplete the NP and will limit accumulation into the tumor. Overall, the cellular components of the tumor stroma deplete NPs through multiple mechanisms and interfere with the therapeutic outcome of anti-cancer agents.

Pericytes are another group of stromal cells that regulate intratumoral NP transport. Pericytes are primarily characterized as periendothelial mesenchymal cells embedded within the vascular basement membrane[113], which can be identified by pericyte markers such as alpha smooth muscle actin (α -SMA), PDGFR- β , NG2, RGS5 and XlacZ4 [114]. The establishment of pericytes starts with recruitment, which is primarily mediated by endothelial cells through PDGF- β /PDGFR- β signaling during physiological angiogenesis [113]. On the other hand, VEGF- α acts as a negative regulator of pericyte function and vessel maturation [115, 116]. The structure of newly established pericytes is loosely associated with the endothelial cells, and has cytoplasmic processes that penetrate deep into the tumor parenchyma. However, heterogeneity of tumors determines the spatial arrangement pericytes. Work by Kano et al recently classified cancers into two categories based on pericytes coverage: those with less coverage of tumor neovasculature (e.g. colon cancer, CT26) by pericytes and those with more (e.g. pancreatic cancer and diffuse-type gastric cancer) [31]. Unlike the previously discussed stromal cells which compromise NP delivery by depletion, pericytes influence NP transport by regulating blood flow along with controlling the stabilization and maturation of tumor vasculature. Neither leaky, immature blood vessels with little coverage, nor over-matured vessels with abundant pericyte coverage are suitable for NP delivery and should be optimized for better NP perfusion [2]. For example, NP extravasation was severely impeded in the high pericyte coverage (BxPC3) model. Yet, treatment by TGF- β inhibitors or PDGF- β inhibitors could slow down pericyte recruitment, inhibit endothelial pericyte associations and therefore, improve NP extravasation. While the low pericyte subtype showed better NP perfusion, modulation of the pericytes still offered an improvement in NP perfusion with respect to the unmodified group. Specifically, through diminishing non-functional microvessels with low pericyte coverage while increasing pericyte coverage in the normalized tumor vasculature [2]. For example, the VEGF inhibitor,

Sorafenib, which negatively controls the regulations of pericytes, increased extravasation of 2 MDa dextran in the CT26 model [5].

5. Physicochemical Properties of NP influences NP transport in Stroma-rich Tumors

Though the biophysical properties of tumors present prominent barriers for successful NP delivery, the physicochemical properties of NP govern the extent and limitations of such barriers. Steric small particles (with size <50 nm), such as PEGylated NP (i.e. polymeric micelles, gold nanoparticles or quantum dots), can penetrate poorly permeable hypovascular tumors such as BxPC3, better than the larger NP (> 50 nm) [6]. The inverse relationship between diffusion rate and NP size was also observed *in vitro* on the multicellular spheroid models [117]. One potential explanation is that the vascular pore size and the cross-linked collagen fiber mesh form pores that are in between the size of the large and small particles. Therefore, both transvascular and interstitial transport of smaller NP occur rapidly [118]. However, one should note that NP smaller than 10 nm are likely to be excreted from the kidney, at least partially. Therefore, NP below the 10 nm limit exhibit compromised pharmacokinetic profiles and an increase in collateral damage toward normal organs. Increasing the size of NP will provide selectivity, but at the cost of limiting extravasation and diffusion.

Aside from particle size, surface charge also affects intratumoral transport by regulating NP's diffusive mobility in the ECM. Both PEGylated NP and neutrally charged liposomes exhibit quasi-free diffusive motion in ECM hydrogel and have the advantage of deep penetration into tumors. Cationic NP (e.g. DOTAP liposomes), on the other hand, were entrapped in the hydrogel [18]. However, cationic NP have been shown to exhibit optimized transvascular transport by preferential targeting to the tumor endothelial cells and electrostatic attraction with the negatively-charged vessel pores [119, 120]. Furthermore, positively charged NP are more likely to be taken up by proliferating cells (e.g. tumor cells) compared to neutral and negatively charged NP, which is an additional advantage for effective drug delivery [63].

As far as the shape of NP is concerned, research has shown that NP or macromolecules with linear, rod-like semi-flexible configurations diffuse and penetrate more efficiently into the interstitial matrix compared with solid spherical particles of similar size. The shape of therapeutic NP also affects their circulation time in the blood stream. For example, rod-shaped micelles have a circulation lifetime ten times longer than their spherical counterparts [4, 47, 121].

Table 1. Summary of Stromal Barriers and Strategies

Major Barriers	Type	Barriers Constituent	Barriers Mechanism	Strategy Mechanisms	Strategy Agents	Applied Tumor Models	Ref
Vasculature		Angiogenesis Tortuosity Low blood flow; High viscosity	IFP increase Solid stress increase <i>EPR effect</i> decrease	Normalization Leakiness Improvement	DC101 (VEGF-R mAb); bevacizumab, ranibizumab (VEGF mAb); SST0001 (Heparanase inhibitor); Trastuzumab (HER2-R mAb) Imatinib (PDGF antagonist); LY364947 (TGF- β inhibitor); Thrombin (vasoactive agents) Angiotensin II (transient vessels pressure raising agent)	Hypervascularity: colon carcinoma, myeloma, melanoma Hypovascularity: pancreatic, lung and breast carcinoma	[1-4] [2, 5, 6]
	BM	Collagen IV	Thickness, mesh size, orientation, and density limit NP penetration	Degradation	Collagenase; MMPs	Lung and breast carcinoma	[16]
Stroma		Nidogen Perlecan	Crosslinking Collagen Network, and hinder the anionic NP	-	Modulating the surface charge and antifouling effect of NP	-	[4, 17, 18]
	GAG	Trap the cationic NP	-	-	-	-	-
	ECM	Collagen I	Same as Collagen IV	Degradation	Collagenase; Relaxin	Lung carcinoma, melanoma	[20-22]
		HA	Partitioning collagen matrix into aqueous and viscous compartments	Degradation (Paradox)	Hyaluronidase; PEGPH20	Osteosarcoma, PDA	[23, 24]
Stromal Cells	CAF		Secrete ECM molecules, stiffen ECM	Reprogram to Normal Fibroblasts	Losartan (inhibit collagen synthesis); VDR inhibitor (inhibit CAFs activation)	Breast carcinoma, PDA	[25, 26]
			Off target depletion NP	CAF Depletion (Paradox)	cisplatin NP and Doxetaxel conjugates; pFAP (anti-FAP vaccine); FAP substrate-drug Conjugates; FAP targeted liposomes	Stroma Rich Bladder Cancer; 4T1 breast cancer; colon	[27-30]
	Pericytes		Regulate vasculature maturation and limit NP penetration		TGF beta inhibitors PDGF- β inhibitors VEGF inhibitors	Pancreatic and breast carcinoma	[5, 31, 32]

Note: MMP, matrix metalloproteinase; PDA, pancreatic ductal adenocarcinoma

Surface modification of NP with targeting ligands is another concern for enhancing NP intra-tumoral transport. High binding affinities between NP and the target site are generally seen as an advantage by increasing the internalization of NP. However, the use of targeted NP with high binding affinity may elicit a binding site barrier. This regards a phenomenon where NP binding to target cells paradoxically reduces diffusion deep into tumors. The binding site barrier was first observed during antibody delivery into tumors and later found to be present for NP based delivery as well. High avidity may also comprise the selectivity, since particles may also inadvertently bind to non-tumor cells expressing low levels of tumor specific determinant and depleted accordingly. After all, targeting ligands exclusive to tumor cells are unlikely to exist [122, 123].

Though surface charges and targeting moieties modulated intra-tumoral NP distribution, a growing field of evidence suggests that nanomaterials interacting with biological fluids (e.g. plasma) are likely to adopt a “new” identity through acquiring a surface corona of biomolecules (such as lipids and proteins). This phenomenon, in turn, may comprise the influence of surface properties and dictate the *in-vivo* biodistribution of NP [124]. The composition of this dynamic corona reflects properties of the nanomaterial such as size, surface curvature and hydrophobicity [125]. Among the identified compositions, opsonins (including immunoglobulin (IgG), complementary factors and fibrinogen) were reported to promote receptor mediated phagocytosis, leading to rapid clearance and limited tumor

accumulation of NP [124]. The attachment of antifouling polymers, in particular PEG, has widely been used to prevent protein binding, and consequently modulate the pharmacokinetic profiles of NP [125]. On the other hand, strongly bound monolayer bio-corona could be exploited to prolong NP circulation. For example, albumin complexes with negatively charged NP to promote prolonged circulation times. Abraxane™, the FDA approved albumin-bound form of paclitaxel, is a prime example of using an albumin based drug carrier for efficient anti-tumor therapy [126]. Furthermore, one may consider exploiting the bio-corona for targeting purposes [124]. Kim et al demonstrated specific ApoE binding to NP for delivery across the BBB into the brain [127]. The aforementioned study focused on bio-corona formed in plasma and discussed the pros and cons of this bio-corona for NP delivery. However, NPs approaching the tumor cells must first pass through a dense stromal matrix. How the bio-corona coat interacts with the stroma and whether covered biomolecules will exchange with the stroma are key in determining the ultimate fate of NP disposition. This poorly understood phenomenon requires further investigation into the optimal use of bio-corona coated nanoparticles.

Overall, the physiological properties of NP greatly influence the pharmacokinetics, transvascular transport, intratumoral penetration and cellular internalization in paradoxical manners. This helps to explain the inconsistency between preclinical animal studies and clinical outcomes and therefore, physicochemical properties of NP need to be optimized for each tumor.

6. Strategies to Improve NP extravasation and Penetration

Examination of the barriers that hinder NP delivery has opened doors for new treatment regimens that seek to mediate these factors. Generally, these approaches involve restoring the abnormal tumor vasculature and interstitial stress towards that of normal tissue, and modifying NP with environmentally responsive modifications to enhance delivery (**Table 1**).

6.1. Normalization of Tumor Vasculature Benefits NP Extravasation

To counter the detrimental effects of abnormal tumor vasculature on NP transport, strategies to restore normal vasculature have been proposed [31, 123]. Since irregular BM and pericyte coverage hinders the maturation of tumor vasculatures, promoting BM and pericyte recruitment has been proposed to normalize blood vessels [31]. Collagen IV, the major constituent of BM, have been shown to be degraded by MMPs. Therefore, the application of MMP inhibitors such as the peptide inhibitor TIMP-1, or a non-peptide inhibitor, AG3340, could inhibit BM remodeling and is considered a promising method for vessel normalization [128]. Another method involves the knockdown of the VEGF signaling pathway. VEGF receptor-2 blocking antibody DC101 can prune immature vessels and recruit pericytes. Proangiogenic molecules, including VEGF, fibroblast growth factor (FGF) and PDGF are overexpressed in tumors and favor angiogenesis [4]. Therefore, VEGF inhibitors can also be applied as an anti-angiogenic agent to revert the vasculature toward a more normal phenotype. A variety of monoclonal antibodies (mAb) against VEGF and other angiogenic signaling factors have been designed. For example, bevacizumab (Avastin), the first approved anti-angiogenic mAb, and its derivative, ranibizumab have been applied in the treatment of metastatic colorectal cancer. Furthermore, the inhibition of heparanase, which plays a major role in angiogenesis, has also been considered as a promising tumor priming strategy [3]. The resulting modifications reduced size of pores in the vessel

walls and decreased IFP, allowing NP extravasation to occur through convection rather than diffusion, a much faster process for free molecules and small NP (<12 nm) [50].

Despite these improvements, the smaller vessel pores established through normalization may compromise the advantages gained from enhanced convection for large NP, since the increased steric and hydrodynamic hindrance inhibit the extravasation process [48, 50]. As an additional consideration, the anticancer agents should be administered during the window of normalization to obtain improved delivery, since vascular normalization is a transient process. Furthermore, this strategy may not lead to desirable results against cancer with compressed and less permeable tumors as seen in the case of pancreatic tumor BxPC3 and breast cancer 4T1. Owing to the thick pericyte coverage that limits diffusion of NP in these compressed hypovascularity tumor models, strategies to inhibit angiogenesis may lead to diminished NP accumulation. While somewhat counterintuitive, tumors of the hypovascularity phenotype should be remodeled to improve the leakiness of blood vessels by decreasing pericyte coverage and BM thickness. TGF- β receptor antagonists are the most frequently used therapeutic agents, since low dose TGF- β inhibitor inhibits pericyte recruitment without affecting the function of tumor cells and endothelial cells. Consistent with this finding, various types of TGF- β inhibitors, including small kinase inhibitor and its nano-formulation in addition to siRNA and antibodies, have been used to improve the intratumoral penetration of sub-100 nm NP, such as liposomes, polymeric micelles and PEI-PEG-coated mesoporous silica nanoparticles (MSNP) in hypovascularity tumor models [2, 32, 129]. Another approach to improve NP penetration in the hypovascular models involves the administration of vasoactive agents such as thrombin, lipopolysaccharide endotoxin, to initiate a cascade of cellular events that lead to the disruption of endothelial cell junction and increase vascular permeability. Transiently raising the systemic pressure by infusing vasoconstrictors (e.g., angiotensin II) can also increase the vascular permeability and consequently increase NP extravasation. A combined treatment might also be beneficial for hypovascularity model, with one treatment to alleviate solid stress through depletion of stromal cells or extracellular matrix, and a subsequent or concurrent vascular normalization treatment to improve perfusion [130]. Overall, the approaches for remodeling tumor vasculatures to improve NP delivery vary with regards to vasculature contents and abnormalities, as well as the size of the therapeutic NP. One should be cautious when choosing strategies and agents since the efficacy of treatment depends largely on the nature of each specific tumor [2].

6.2 Normalization of the Extracellular Matrix Improves NP Penetration

Apart from activation of chemical signaling, interstitial stromal barriers in desmoplastic tumors physically restrict diffusion of macromolecules and nanotherapeutics within the tumor parenchyma. Attesting to this, Dellian et al observed heterogeneous distribution of stealth liposomal-clusters in the perivascular ECM of a xenograft tumor, indicating the role of ECM in limiting the particle diffusion [131]. In another study, Eikenes et al. showed that the degradation of the structural collagen network is more important than the degradation of the GAG and hyaluronan when attempting to increase diffusion of NP and macromolecules [17]. Jain and his colleagues further confirmed this finding [59, 132, 133]. Based on these findings, numerous studies have focused on the subsequent or concurrent delivery of collagenase alongside nanotherapeutics to enhance the intratumoral transport of NP. For example, co-intratumoral injection of oncolytic HSV vector along with bacterial collagenase increased the viral

vector distribution by nearly a 3-fold difference in a human melanoma xenograft [20]. In another example, intravenous injection of collagenase-I resulted in higher gene expression of lipoplex in xenograft tumors, further confirming the usefulness of collagen degradation in improving NP distribution [22]. Enhanced diffusion after modification of collagen production was also demonstrated by the use of hormone relaxin, which was reported to both stimulate collagenase synthesis and down-regulate collagen production [21, 134]. Additionally, relaxin is safer compared with bacterial collagenase for *in vivo* application and proposed for long term use [1].

Similar to collagen, GAG are also key matrix element that induces vascular collapse; among which, hyaluronan (HA) is a major component [133]. HA polymerizes into cage-like structures, partitioning the interstitial space into aqueous and viscous compartments as previously investigated. The use of hyaluronidase to improve the tumor permeability is controversial. High doses of hyaluronidase collapse the HA-based water swelling cage, increase the ECM viscosity and thereby reduces the diffusion coefficient of NP [135]. Notably, elevated expression of tumor-derived hyaluronidase has been used as a diagnostic cue for high-grade bladder cancer and limited perfusion, suggesting the negative outcome of combining hyaluronidase with therapeutic NP [100, 136]. However, in pancreatic ductal carcinoma (PDA), with HA overpowering collagen and constituting 70% of the ECM, the opposite is observed [23]. In a genetically engineered mouse model of PDA, PEG-PH20, a PEGylated recombinant human PH20 hyaluronidase, can effectively improve vascular perfusion of doxorubicin and gemcitabine [23]. Intratumoral administration of bovine hyaluronidases has also shown promise in several xenograft models [137, 138]. A recent study in a human osteosarcoma xenograft model indicated that hyaluronidase induced a 4-fold increase in the distribution of liposomal doxorubicin [24]. Therefore, the application of HA is not limited to small molecules and can also be a promising combinatory component to improve the delivery of NP with larger particle size [23].

However, the aforementioned ECM modifiers may produce collateral damage in healthy tissues (e.g., bacterial collagenase) or increase the risk of tumor progression (e.g., relaxin, matrix metalloproteinases, and hyaluronidase) [25]. Therefore, rather than systemic delivery of these modifiers, site-specific degradation of ECM was preferred and conducted by coating NP with specific ECM enzymes [139, 140]. Goodman et al indicated that collagenase coated 100 nm gold NP showed a 4-fold increase in the number of particles delivered to the spheroid core compared with normal stealth particles [139]. The major concern for enzyme coated NP is maintaining the enzymatic activity during, and after conjugation with the NP. Also, the pharmacokinetic profile of the coated NP proves important as well. Recent work by Ji et al examined these major concerns with enzyme coated NP platforms and confirmed the applicability of the design for improved NP diffusion [141].

6.3 Disruption of Stromal Cells Improves the Intratumoral NP Delivery

In addition to high ECM concentrations, desmoplastic tumors are usually characterized with high levels of stromal cell density. As discussed earlier in this review, this abundance of stromal cells secretes interstitial matrix molecules (i.e. collagen, fibronectin, etc.) that constituent the high interstitial solid stress. Simultaneously, they act as strong binding site barriers for interstitial NP delivery. CAF has been considered as the major component of tumor stroma. The dense ECM associated with CAF also obstructs the intratumoral vasculature, preventing small molecule and NP delivery. These findings

suggest that CAF represents a potential target for both therapeutic efficacy and NP delivery. Directly eliminating fibroblasts from the tumor can increase the interstitial transport and distribution of nanotherapeutics by decreasing the tortuosity of the interstitium. For example, off-target distribution of therapeutic NP through platforms such as cisplatin NP and Docetaxel conjugates greatly improved the outcome of stroma rich bladder and breast cancer models through depletion of fibroblasts [27, 28]. To improve the therapeutic outcome, targeted depletion of fibroblasts was investigated in detail. One example is the oral DNA vaccine that targets fibroblast activation protein (FAP), which is commonly overexpressed on CAF [142]. The DNA vaccine specifically depleted fibroblasts and improved the delivery of the therapeutic agents [142]. Later on, CAF targeted NP, such as FAP antibody conjugated immuno-liposomes and FAP substrate conjugated drugs, were designed to specifically deplete CAF [29, 30]. Though depletion of CAF undoubtedly induced an increased vessel perfusion and enhanced NP diffusion, two recent studies also suggest that eliminating stroma by targeted deletion of fibroblasts results in undifferentiated progression of aggressive pancreatic cancer, suggesting the paradoxical effect of CAF depletion [143]. One explanation for the paradox is that these depletion strategies run the risk of eliminating key stromal components needed for tissue homeostasis. Furthermore, the off-target distribution of chemotherapy in fibroblasts may induce senescence in CAF, a procedure that will induce the growth and resistance of neighboring tumor cells and ultimately stiffen ECM, limiting NP perfusion in the long run [144]. To prevent this paradox, alternative approaches were adopted to remodel fibroblasts and ECM. Note that resting fibroblasts (normal fibroblasts) can be transdifferentiated into CAF in response to cytokines (i.e. TGF- β , PDGF and sonic hedgehog (SHH) [145-147]), growth factors, oxidative or metabolic stress, and synthesize abundant ECM proteins to support tumor proliferation. Therefore, the pharmacological means to reprogram CAF back to this quiescent state would be a promising strategy to inhibit tumor growth and improve NP delivery [26, 148]. Recent studies by Diop-Frimpong et al indicated the downregulation of TGF- β signaling in CAF by an angiotensin receptor inhibitor, Losartan, could reprogram and reduce the activity of CAF, decreasing the synthesis of collagen I and thus improving the intratumoral penetration of NP [25]. Success from this formulation has led to a phase II clinical trial of Losartan along with FOLFIRINOX in patients [123, 149]. Vitamin D Receptor (VDR) has also been shown to be overexpressed in the pancreatic stellate cells (PSC), an activated form of CAF in PDA. Sherman et al suggested that the VDR ligand acts as a master transcriptional regulator of PSC to reprise the quiescent stroma, reduce the fibrotic content and increase intratumoral vasculatures; which is promising for enhancing NP delivery [26]. Evidence presented above emphasizes the advantages of stromal cell reprogramming over depletion. Despite diluting the ECM components for increased NP perfusion in the reprogramming strategy, high cell densities still act as a *binding site barrier* for NP with high cell binding affinity, which limits the outcome of this strategy. Considering the spatial intratumoral distribution of CAF and blood vessels as discussed in the earlier sections, fibroblasts are a more likely target for NP and regarded as a therapeutic target. Instead of depletion and transdifferentiation back to noncancerous fibroblasts, CAF can be reprogrammed into killer cells that secrete toxins or other factors that inhibit the proliferation of neighboring tumor cells. Fibroblasts can be engineered into macrophage like cells, providing us hope for *in situ* engineering using NP delivered agents to convert fibroblasts into natural killers [150].

6.4 Design of Extracellular Matrix Responsive NP

Specific characteristics of the TME can be utilized to design the stimuli-responsive NP to improve intratumoral NP drug delivery (**Table 2**). Strategies can be divided into two categories. In terms of the first strategy, stimuli-responsive NP can be designed to release the free drug in the tumor interstitium. This method is advantageous as it overcomes the limitation of barriers in the ECM for large sized NP. Stable hydrophobic drugs with membrane permeability could be used as candidate drugs, for example, doxorubicin (DOX). The hydrophobic drug could be directly conjugated to a hydrophilic polymer through an ECM cleavable linkage, or the segments of block copolymers could be connected by stimuli-cleavable linkers and further assembled into micellar NP [10]. Another strategy is based on the premise that the *binding site barrier* hindered NP distribution. To improve the penetration, stealth materials, such as PEG, were linked to the NP with stimuli-responsive linkers as a shielding layer. The degradation and cleavage of this shield triggered by the TME resulted in the exposure of targeting ligands, which lead to the efficient internalization by neighboring cells [151]. The aforementioned ECM stimuli, distinct from intracellular stimuli, are usually referred to as extracellular stimuli, including temperature, acidic pH and enzymes. When designing the responsive structure, the structure should be smart enough to distinguish the extracellular signals from intracellular signals, and thus achieve the original designed purposes.

6.4.1 Temperature Responsive NP. Temperature is originally considered as an external stimuli generated using external forces [152]. Temperature responsive materials was first found during hyperthermia treatment of tumors, dated back to 1970s, and lately became a major constituent of chemotherapy and used as a complementary strategy for hyperthermia treatment [152, 153]. However, in early 1990s, studies revealed that the core temperature of breast cancer was higher than the peripheral tissues, suggesting that temperature is not only applied as an external force but also an internal physicochemical feature of the ECM [154]. Abnormal temperature gradients were also observed later in brain tumors and melanoma [155, 156]. It is suspected that the elevated temperature in tumor regions is due to glycolysis degradation and reaction, energy exhaustion [157]. Temperature dependent response is usually governed by a sharp nonlinear change in the conformation and physicochemical properties of at least one component of the nanoformulations across their phase transition temperature. The sharp response triggers the release of free drugs from the cargos. Ideally, materials exhibiting relatively sharp thermal phase transitions around body temperature would be utilized to form the thermo-response NP [152]. The commonly used chemicals are summarized in Table 2, among which the thermo-responsive polymer, including poly(N-isopropylacrylamide) (PNIPAM), poly(vinyl chloride) (PVC) and their derivatives were most widely investigated. Thermo-responsive NP were designed according to the aforementioned two strategies. Besides polymer micelles, liposome and peptide conjugates were also designed with thermal responsive components [158]. For liposomes, temperature responsiveness usually arises from a phase transition of the constituent lipids and the associated conformational variations in the lipid bilayers [152]. However, since the tumor temperature is hard to detect, most of the studies in this field focus on the *in vitro* characterization.

6.4.2 Tumor acidity Due to the production of lactic acid converted from glucose during the aerobic glycolysis (also known as the Warburg effect, the hallmark of tumors), the extracellular pH of most

tumor tissues is mildly acidic in the range of 6.5-6.8, which is in between that of blood (7.4) and lysosome pH (6.4) [159, 160]. pH variations between pathological situations and the normal tissues have been exploited to trigger the release of therapeutic drugs and imaging agents [10]. Notably, pH-dependent delivery systems designed on the basis of this subtle pH difference require that the systems possesses ultrasensitive pH-responsiveness [44]. In the past decades, pH sensitive polymers that are synthesized to meet the requirement include tertiary amine-containing methacrylate copolymers, poly(β -amino ester) (PAE) and poly(sulfadimethoxine) (PSD), as well as those containing pH-sensitive chemical bonds (e.g., imidazole functionality of histidine, 2,3-dimethylmaleic anhydride (DMMA) modified amine moieties) (Table 2) [10]. Accordingly, designs of nanoformulations can be classified into two categories. One is the use of polyacids or polybase with ionizable groups that undergo conformational changes in response to environmental pH variations; the other is the design of polymeric systems with acid-sensitive bounds whose cleavage enables the release of molecules anchored at the polymer backbone. For example, a series of micellar NP possessing ultrasensitive pH-activatable fluorescence emission were formulated by Gao's group [42, 44]. The pH-activatable nano-probe was composed of a PEG conjugated poly(2-(hexamethyleneimino)ethyl methacrylate) copolymer and showed a micellization-disintegration transition within a narrow pH range of 0.25 units. The release of fluorescent dye from micelles in response to extracellular pH can nonlinearly amplify tumor microenvironment specific signals for imaging purposes [44].

6.4.3 Extracellular Enzymes In the tumor tissue microenvironment, specific enzymes, including MMPs, phospholipases, glycosidases and esterases, etc., are present with high concentrations and abnormally high activity [10]. Therefore, they have been extensively exploited to modulate targeted drug delivery and release. The first approach is the direct conjugation of lipophilic drugs to a hydrophilic polymer via enzyme cleavable linkage, and further self-assembled into micelles for both anti-tumor drug delivery and tumor imaging. One such linker is the MMPs specific cleavable peptide Gly-Pro-Leu-Gly-Val (GPLGV) [161]. The PEG-peptide-DOX micelle formulation improves the release and delivery of DOX both *in vitro* and *in vivo* [161, 162]. The second approach is to use the enzyme-cleavable linkers to link segments of polymers or to use enzyme-degradable materials to loaded chemotherapeutic drugs or imaging agents and release them via enzyme degradation. Enzyme-cleavable linkers crosslinked with hydrogel is among one of the most commonly used formulations. For example, cisplatin was loaded in PEG-diacrylate hydrogel wafers linked with an MMP substrate. The controlled release of cisplatin from the hydrogel upon MMP degradation improved the therapeutic outcome for the treatment of Glioblastoma multiform (GBM) [163, 164]. Gelatin is another frequently used nanocarrier that can be degraded by MMP-2, which is overexpressed in the ECM. A recent study by Wong et al. indicated that a multistage NP composed of 100 nm gelatin core covered with 10 nm quantum dots showed deep tumor diffusion. The triggered release of smaller NP upon MMP degradation lowered the diffusional hindrance in the interstitial matrix compared to larger NP. In the meantime, the lymphatic clearance rate for 10 nm NP was significantly lower in comparison with free small molecular drugs [34, 61].

7. Conclusions and Future Perspectives

Table 2. Design of ECM targeted NP

Stimuli Factors	Major Stimuli Structure	Formulation	Materials	Stimuli Criteria	Disease Model (Cell line)	Drug	In Vitro& In Vivo	Ref.
Thermo Sensitive	PNIPAAm	Polymeric Micelle	PNIPAAm- <i>b</i> -PLA	LCST 36°~40°	-	Dox	<i>in vitro</i>	[7]
			P(NIPAAm- <i>co</i> -NDAPM)- <i>b</i> -PCL	LCST 36.5°	-	Prednisone Acetate	<i>in vitro</i>	[8]
			PNAS- <i>b</i> -PNIPAAm- <i>b</i> -PCL	LCST 36.5°	Hela	Dox	<i>in vitro</i>	[9]
	PNVCL		DHBCS, PEG- <i>b</i> -PNIPAM	LCST 32.0°	PKH26	Dox	<i>in vitro</i>	[10]
			Chitosan- <i>g</i> -PNVCL	LCST 38.0°	L929, MCF7, PC3, KB	Curcumin	<i>in vitro</i>	[11, 12]
			P(mNVCL)- <i>co</i> -PNVCL	LCST1 20~24°; LCST2 30~42°	B16-F10 melanoma	Dox	<i>in vitro</i>	[13]
	DPPC, DSPC	Liposome	DPPC, DSPC, CHOL, DSPE-PEG2000	Tm 41°	SK-BR-3, MDA-MB-435 breast cancer; U87-MG glioma; B16F10	Dox	<i>in vivo</i> & <i>in vitro</i> (external)	[14, 15]
	Leucine Zipper Peptide	Lipid-peptide NP	Leucine zipper peptide; DPPC, DSPC, MSPC, HSPC	Tm 40°	B16F10, SW480	Dox	<i>in vivo</i> & <i>in vitro</i>	[33]
	Elastin & Elastin mimetic	NP	Elastin/DNA aggregation	Tm 50-60°	-	-	-	[35]
pH Sensitive	PHis	Micelle	PEG-PHis-PLL or PLL- <i>b</i> -PEG and PHis- <i>b</i> -PEG Mixed	pKa ~6.5; disassembling	4T1 breast cancer	Dox	<i>in vivo</i>	[8, 36, 37]
			PHEMA- <i>b</i> -PHis		HCT116 human colon	Dox	<i>in vivo</i> & <i>in vitro</i>	[38]
	PAE		PEG- <i>b</i> -(PLA- <i>co</i> -PAE) PEG-PAE	pKa ~7.0	HepG2 BT-20, B16F10 melanoma	Dox Paclitaxel	<i>in vitro</i> <i>in vitro</i>	[40] [41]
	PC7A		PEG- <i>b</i> -PC7A	pKa ~6.9	A549	Fluorescence	<i>in vivo</i>	[42-44]
	PSD	Polyplex	PEG-PSD/PEI NP	pKa ~7.0	A2780, human ovarian carcinoma	Gene	<i>in vitro</i>	[45]
	Chitosan	NP	Chitosan-silica nanospheres	pKa~6.3; swelling	MCF-7 Breast cancer	TNFα	<i>in vitro</i> & <i>in vivo</i>	[46]
Enzyme Sensitive	MMP2-cleavable octapeptide gelatin	liposome	PEG-MMP cleavable peptide-PE (Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln)	MMP	4T1 breast cancer	Fluorescent probes	<i>in vitro</i> & <i>in vivo</i>	[57]
		NP	gelatin-gold/quantum dots fabricated multistage NP		C6 glioma cells	Gold NP, quantum dots	<i>in vitro</i> & <i>in vivo</i>	[34, 58]
	GPA peptide sequence	Drug Conjugate	Peptide-FAP cleavable substrate-promelittin protoxin	FAP	MCF7 breast cancer	promelittin protoxin	<i>in vitro</i> & <i>in vivo</i>	[29]

Note: LCST, the lower critical solution temperature; PNIPAAm, poly(*N*-isopropylacrylamide); NDAPM, *N*-(3-(dimethylamino)propyl)methacrylamide; PNAS, poly(*N*-acroyloxysuccinimide); PNVCL, poly(*N*-vinylcaprolactam); DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSPC, 1, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; PAE, polyacrylic ester; PSD, polysulfonamide

The treatment of tumors requires efficient delivery of therapeutic agents to the target cells through a series of transport steps, including vascular transport, transvascular transport and interstitial transport. The dense ECM structure and aberrant tumor vasculature constitute the physiological barriers that hinder the transport of NP through these steps, and subsequently limit therapeutic outcome. The physicochemical properties of NP, including size, shape, surface properties would also regulate the intratumoral NP distribution. In this review, we summarized the barriers and provided strategies to overcome these barriers for improved NP delivery.

However, penetration alone is not the only criteria to evaluate therapeutic response. Firstly, the approaches to remodel the TME for enhanced NP transport may controversially promote tumor cells migration and metastasis, compromising or even reversing the therapeutic effects. As seen, degradation

of collagen improves the NP diffusion, but also increases the risk of tumor cell migration by eliminating barriers that usually prevent it. Using cell-based delivery systems such as stem cells with homing properties, may overcome the delivery barriers without adversely changing the TME. Secondly, heterogeneous distribution of NP in the interstitium and disparate internalization of NP to stromal cells may result in acquired resistance from TME and eventually lead to the treatment failure. Therefore, the current challenge is to design multifunctional NP to target both tumor and stroma cells, blocking the resistant tumor-stroma crosstalk [165]. Thirdly, the dynamic change of TME during cancer development or during the remodeling process using priming agents require the efficient nanotherapeutics delivery within the optimal modeling “window”. Therefore, time-dependent dosing in accordance with time-dependent TME changes are beneficial for enhanced NP delivery. To tackle this problem, time-dependent TME change should be first monitored either using imaging tools to visualize the TME or monitoring the levels of certain secreted factors in the blood that could change in response to the TME modeling process. Upon determining the therapeutic window based on monitoring TME changes using the aforementioned methods, therapeutic strategies described in the review can be applied to each specific situation. This *monitoring followed by therapy* strategy could also provide guidance for the possibility of individualized therapy, considering the heterogeneity of the TME across different cancers or even individuals is a prime deterrent to successful therapy. Fourthly, in addition to delivering therapeutic and diagnostic NP to solid tumors, the delivery of NP to metastatic sites for cancer therapy is even more difficult considering the difference in TME between primary tumors and the metastatic niche. Recently, Swami et al. approached this challenge by formulating NP to target myeloma and the bone metastatic microenvironments [166]. Furthermore, a recent study on the relationship of melanoma-derived exosomes, which induce vascular leakiness at pre-metastatic sites, may provide a means of passively targeting NP to metastatic sites [167]. More systemic cancer treatments may require the combination of immune therapy, and other methods.

Overall, the advancement of NP mediated cancer drug delivery has been fueled by discoveries in material science and understandings in the pathology of tumor TME. This knowledge allows the development of nanotherapeutics that can cater to a seemingly endless number of situations. As examined, this versatility is crucial considering the differences between each tumor and across different patients. As each specific case demands its own specific treatment approach, the diversity of investigated and available nanotherapeutics proves promising to the advancement of cancer treatment.

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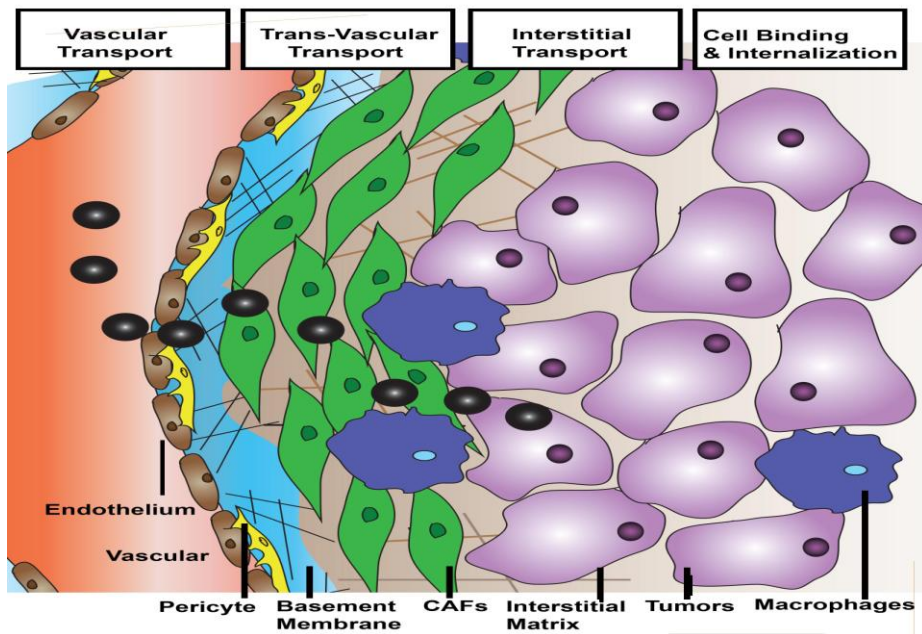
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Graphical abstract