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***In vitro* Microfluidic Models of Tumor Microenvironment to Screen Transport of Drugs and Nanoparticles**

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Abstract

Advances in nanotechnology have enabled numerous types of nanoparticles (NPs) to improve drug delivery to tumors. While many NP systems have been proposed, their clinical translation has been less than anticipated primarily due to failure of current preclinical evaluation techniques to adequately model the complex interactions between the NP and physiological barriers of tumor microenvironment. This review focuses on microfluidic tumor models for characterization of delivery efficacy and toxicity of cancer nanomedicine. Microfluidics offer significant advantages over traditional macro-scale cell cultures by enabling recapitulation of tumor microenvironment through precise control of physiological cues such as hydrostatic pressure, shear stress, oxygen and nutrient gradients. Microfluidic systems have recently started to be adapted for screening of drugs and NPs under physiologically relevant settings. So far the two primary application areas of microfluidics in this area have been high throughput screening using traditional culture settings such as single cells or multicellular tumor spheroids, and mimicry of tumor microenvironment for study of cancer-related cell-cell and cell-matrix interactions. These microfluidic technologies are also useful in modeling specific steps in NP delivery to tumor and characterize NP transport properties and outcomes by systematic variation of physiological conditions. Ultimately, it will be possible to design drug-screening platforms uniquely tailored for individual patient physiology using microfluidics. These *in vitro* models can contribute to development of precision medicine by enabling rapid and patient-specific evaluation of cancer nanomedicine.

Graphical Abstract

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INTRODUCTION

Recent advances in nanotechnology have enabled development of numerous types of nanoparticles (NPs), such as liposomes, polymeric micelles, carbon nanotubes and nanohorns, colloidal gold, silver, and iron oxide NPs and quantum dots^{1–3}. Therapeutic/diagnostic strategies using these NPs, so-called nanomedicine, aims to transform cancer treatment by enabling targeted delivery of therapeutics to the cancerous tissues and resulting in early diagnosis and effective treatments with minimal toxic side effects⁴. In addition to numerous NP formulations showing promising pre-clinical efficacy, only several NPs have been approved by the FDA and are being translated into clinical settings, including AbraxaneTM, DoxilTM and gold nanoshellsTM.

Despite the promising outcomes of several NP systems, the delivery and therapeutic efficacy of the majority of NPs are still quite limited^{5–7}. This under-achievement is mainly attributed to current NP designs, which do not adequately address complex transport barriers around tumors. Delivery of NPs to tumor is a multi-step process where each step involves inter-related multiple physiological barriers that hinder the transport and shows the potential to be the bottleneck for effective delivery of NP^{3, 6, 8}. Current delivery strategies of NPs involve tailoring of NP properties as well as modulation of the tumor microenvironment to alleviate the physiological barriers. A considerable literature has grown up around the theme of NP design landscape that investigates the relationships between NP design parameters, such as size, shape, surface charge, and functionalization, and NP *in vivo* behavior including specific interactions of NPs with individual physiological barriers^{9–14}. If quantitatively established, these relationships can be utilized to perform predictive design of NPs for delivery to specific targets. Unfortunately, there is growing amount of evidence indicating that there is no one-size-fits-all solution for NP design since conditions imposed on NP for optimal transport vary significantly and often conflicting with each other between different stages of delivery and it is extremely difficult to optimize the NP design to go through the *in vivo* journey and effectively reach their target.

The problem is also complicated by intra- and inter-tumoral heterogeneity, which signifies the variations in the tumor microenvironment depending on the disease type and stage as well as unique characteristics attributed to individual patients. Tumor heterogeneity introduces a great degree of uncertainty to relationships between the NP design parameters and NP *in vivo* behavior¹⁵ and results in transport and partitioning characteristics being phenotype- and microenvironment-dependent¹⁶. As a result, the relationship between NP properties and ultimate tumor delivery remains to be vague, precluding rational design of nanomedicine for effective delivery.

Characterization of NP transport properties such as diffusivity and permeability are rare, and when present, are often not measured under physiologically relevant conditions, thus fail to fully explain NP-surrounding interactions³. Traditional use of *in vitro* model systems, involving 2D and 3D culture of cancer cells under static environment provides only limited information and offers little translational capability to *in vivo* NP behavior. Microfluidic systems offer opportunities for evaluation of NPs under physiologically relevant conditions by simulating dynamic tissue-tissue interfaces and fluid flow, chemical gradients, and control of local cell-cell and cell-ECM interactions by partitioning of culture.

Therefore, microfluidic tumor models are well suited for modeling specific steps in transport pipeline and characterize transport properties and outcomes by systematic variation of biological, physical and chemical conditions. Fine-tuning of device transport properties to match those of unique patient characteristics has the potential to enable personalized testing of drugs.

In this review, we first summarize the physiological barriers that affect the NP transport to tumor, followed by the exploration of NP design landscape. Later, current delivery strategies are reviewed. Finally, newly emerging transport-oriented perspectives based on mathematical principles of NP transport are introduced together with a survey of *in vitro* tumor models currently used to evaluate NP performance with an emphasis on microfluidic systems.

DESIGN CHALLENGES OF CANCER NANOMEDICINE FOR EFFECTIVE DELIVERY

Physiological Barriers

In order to reach their target in cancerous tissue, NPs undergo a complex and multi-stage transport process. Treatment schemes commonly involve intravenous infusion of NPs during which NPs will need to circulate through the blood stream for extended periods of time and interact with the endothelium in tumor vasculature to selectively extravasate into the tumor interstitium. NPs then need to effectively penetrate into the tumor interstitium. To reach an intracellular target, they also need to be uptaken by cancer cells and retained within for periods long enough to perform their therapeutic action³. Each of these steps involves pathophysiological barriers that potentially hinder, or completely prevent delivery of NPs to their target.

Main physiological barriers affecting the delivery of nanomedicine to the tumor are illustrated in Figure 1. Plasma clearance is the first barrier encountered by NPs during their *in vivo* journey. In the absence of specific precautions, circulating NPs are generally eliminated from blood stream within minutes, or few hours of injection^{17–19}. NPs smaller than 5.5 nm undergo rapid renal filtration and have minimal plasma lifetime^{20, 21}. In addition, NPs can be tagged by the serum proteins, allowing them to be recognized as foreign substances and removed from bloodstream phagocytic cells such as macrophages in blood, liver and spleen. Macrophages internalize NPs with wide range of sizes from 20 nm^{22, 23} to 1–2 μ m^{24, 25}. In the absence of surface modification, uptake of NPs by

macrophages is size dependent with NPs with smaller sizes, 20–80 nm, uptaken at a significantly higher rate than NPs with sizes 100 nm and above^{23, 25, 26}. However, surface functionalization and specific interactions of protein corona with the NP core often alters size dependence trends²². NP distribution also shows organ specificity. NP with 10 to 20 nm diameter, uptaken by liver where they are either internalized and withheld by Kupffer cells or transferred to bile and removed from body by hepatocytes²¹. Larger particles, with sizes up to 1–2 μm , often end up in spleen²⁴.

²⁴The immune response against NPs is highly effective and the amounts of NPs accumulated in liver and spleen can be more than an order of magnitude greater than the amount accumulated in the tumor^{18, 27, 28}. Rapid plasma clearance of NPs is a significant obstacle to targeted delivery and accumulation of the NPs at distant sites is a prominent cause of toxic side effects. In the tissue level, long-term residence of NPs in liver, spleen and lungs results in formation of fibrotic lesions^{29–31}. Small NPs are thought to be largely inert suggesting that aggregation or microparticulates could be responsible for the disease states in those organs³². However *in vitro* cell-level studies indicate that silver NPs have induced cytotoxicity on macrophages³³ and fibroblasts³⁴ where the extent of NP cytotoxicity in both cell types was found to be size-dependent with smaller NPs causing largest cytotoxic effect^{33, 34}. The differences observed *in vitro* and *in vivo* studies indicate a need for new technologies for *in vitro* evaluating biodistribution and toxicity of NPs in physiologically relevant environments.

Tumor vasculature is composed of a highly disorganized network of blood vessels with larger-than-normal intercellular gaps and incomplete formation of basement membrane^{35–39}. Interestingly, those functional defects render tumor vasculature hyper-permeable to physiological fluids, macromolecules, and NPs, allowing those to selectively accumulate in the tumor interstitium⁴⁰. However, the penetration of NPs into the tumor interstitium still remains limited since tumor tissue architecture is marked by a tightly packed compartment of cancer and stromal cells and an interstitium that features a relatively stiff extracellular matrix (ECM), high in collagen, proteoglycan and hyaluronic acid content^{41, 42}. Dense tumor microenvironment is the leading barrier for the penetration of NPs into tumor interstitium^{43–45}. Functional characteristics and specific barrier functions of various ECM components are summarized in Table 1.

In addition to dense ECM compartment, tumors are associated with elevated levels of interstitial fluid pressure (IFP) ranging from 20 to 53, 54 130 mmHg^{49, 53, 54}. High and relatively uniform IFP diminishes the perfusion and associated convective transport of NPs within the tissue^{55, 56}. In the lack of a transvascular pressure gradient, the extravasation of NPs is limited by diffusive processes.

Uptake and detainment of NP by cancer cells is critical for their therapeutic action. However, cancer cells often develop multi drug resistance (MDR) by utilizing membrane transport proteins to pump the internalized chemotherapy drugs out of the cell. Then, the intracellular concentration of therapeutic agents is governed by relative rates of cellular uptake of NP carrier and MDR mediated efflux^{57–59}. MDR is one of the main barriers against the intracellular accumulation of free chemotherapy drugs, motivating research on

alteration of membrane biophysical properties, including membrane fluidity and permeability to enhance the uptake of drugs by cancer cells^{60, 61}. Co-administration of the anticancer drug with MDR suppressants (or chemosensitizers) such as verapamil or tariquidar is another approach^{62, 63} where NPs are considered to be suitable platforms for carrying and releasing both agents simultaneously. Improved tumor-specific targeting of NPs is also a desirable feature since ABC transporter proteins have vital functions in normal physiology of various tissues, e.g. blood-brain barrier, kidneys and intestines; where the suppression of the drug efflux mechanisms can lead to loss of that functionality⁵⁸. Recently, various NP-based multi-drug delivery systems have been proposed to overcome MDR and have shown promising results^{62–65}.

Nanoparticle Design Landscape

Studies in the past decade have provided important information on how variations in certain NP characteristics such as size, shape, charge and surface functionalization affect the interaction of NPs with physiological barriers. Based on existing research, a general understanding is being established to develop guidelines for fine-tuning the NP properties to maximize transport of NPs in individual stages of delivery to tumor. Detailed reviews in these topics can be founded elsewhere^{3, 10, 12, 66, 67}. Here, we provide a brief summary to illustrate key properties of NPs to be designed for cancer therapy.

NP size has been identified as one of the primary factors affecting the plasma clearance and associated blood circulation times. Many studies have been performed about liposomes¹⁷, gold NPs⁹, and micelles⁶⁸. While the results of these studies might seem to favor intermediately sized particles, 60 to 100 nm in diameter, there is little consensus on optimal size for long circulating NPs. For an example, in a study that studied polymeric micelles between 30 and 100 nm, the greatest tumor accumulation and anticancer activity was observed for the smallest 30 nm particles⁶⁹. Tumor accumulation was also found to be highly sensitive to particle size between 111 and 166 nm⁷⁰. While the former accumulated in the tumor, the latter was predominantly located in liver. Taken together, experimental evidence indicates that the plasma half-life of NPs and the associated tumor accumulation amounts indeed depend on the particle size but identifying the relationships among those three is still elusive. Some studies suggest that particle shape is also an important factor affecting the plasma half-life⁷¹.

Interactions of NPs with the serum proteins are also important. It is now well known that charged particles are readily opsonized by the plasma proteins of opposite charge, and as a result have a significantly reduced plasma half-life when compared their neutral counterparts^{72–74}. When a NP in the bloodstream attains a corona of native proteins by opsonization, it develops a biological identity that is different from its synthetic counterpart. Apart from the known role of opsonization during NP clearance by RES, protein corona can also change the effective size and surface charge of the NP. The thickness of the protein corona has been reported to vary between 20 and 35 nm for metal and polymer NPs of 30 to 200 nm sizes⁷². Coating of NP surface by poly(ethylene)glycol (PEG) significantly reduces the extent of opsonization and help NPs escape from RES^{17, 75, 76}. In particular, PEGylation has been shown to improve the plasma half-life of many types of NPs^{9, 17, 28, 70, 73, 77}.

However, PEGylation can also have adverse effects on the rate of uptake of NPs by cancer cells⁷⁸.

Particle surface charge and functionalization have significant effects on NP transvascular transport. Positively charged macromolecules, such as cationized BSA and IgG, have been shown to extravasate approximately two times faster than their negatively charged counterparts⁷⁹. While it is not yet clear whether this mechanism holds for NPs, cationic liposomes are known to preferentially bind to tumor vascular endothelial layer^{80–82} resulting in improved extravasation.

Shape is another factor that affects the interactions between the NP and vessel wall. *In vitro* experiments indicate that spherical asymmetry needs to be considered for enhancing tumor accumulation due to improved deposition of particles on vessel walls. In addition, disk shaped particles associated with larger surface area for adhesion and less drag within bloodstream can have increased deposition on the vessel wall when compared to rods and spheres^{83, 84}.

The efficiencies of transvascular and interstitial transport of NPs are often represented by congregate properties such as tumor accumulation^{68, 70} since extravasation and interstitial penetration of NPs into tumor interstitium is highly heterogeneous, making identification of representative metrics from local distributions difficult^{27, 69, 85}. Surface charge-wise, there is substantial evidence that neutral and negatively charged NPs penetrate into tumor interstitium more effectively than their positively charged counterparts^{27, 74, 81, 82, 86}.

CURRENT DELIVERY STRATEGIES

The central question of nanomedicine has been how to design and synthesize NPs with the desired physiochemical properties. In the following sections, prominent delivery strategies that involve tailoring of NP properties to improve tumor accumulation are discussed.

Enhanced Permeability and Retention

Leaky tumor vasculature combined with poor drainage from compressed tumor lymphatics⁸⁷ can enable preferential accumulation and prolonged presence of anti-cancer drugs in the tumor when compared to the rest of the body^{40, 88, 89}. The so-called *enhanced permeability and retention* (EPR) effect has been the primary guiding principle to explain tumor accumulation of macromolecular drugs and has extended as the design paradigm of NPs for selective delivery to tumor tissues. Although the improvement of drug accumulation at the tumor by NPs utilizing EPR effect has been repeatedly reported in small animal models, there is still room to improve as only about 1 to 10% of the administered drugs are delivered to the intended site^{20, 23, 64}. The degree of EPR effect is also variable between tumor types and the fact that tumor tissue is poorly perfused with vast regions without functional vasculature limits the utility of EPR effect in certain cases^{90, 91}. As a result, many current NP designs that rely on EPR effects often fail to be translated to clinical use^{92, 93}. Therefore, NP design based on EPR effect alone does not sufficiently address the delivery challenges and NP designs based on EPR effects will need to be supplemented by alternative means of enhancing delivery. An example to this latter approach comes from a recent report

featuring artificial augmentation of the EPR effect by dilating tumor vasculature using NO-releasing agents that resulted in effective treatment of highly advanced prostate cancer⁹⁴. Additional discussion of EPR effect as a NP design paradigm is available in recent reviews in the literature^{90, 91}.

Functionalization and Targeting

Another delivery strategy is so-called “active targeting” in which the NP surface is functionalized by ligands such as antibodies and growth factors that can bind to specific receptors overexpressed by cancer cells or tumor-associated endothelial and stroma cells. Improved accumulation of NPs by active targeting has been reported in various tumor xenograft studies^{14, 68, 95–97}. The functionalization of block-copolymer micelles by endothelial growth factor could improve the tumor accumulation of the NPs⁶⁸. In addition, it was reported that iRGD, which is one of the tumor-homing CendR (C-End-Rule) peptides⁹⁵, could be a new targeting ligand. iRGD targets the tumor-associated endothelial cells and also results in an increase of vascular and interstitial permeabilities within the tumor. iRGD has been shown to significantly improve the penetration of both conjugated and co-administered drugs into the tumor^{95, 98}. However, there are also reports of targeting modalities that didn't work as expected, and the use of some tumor-specific markers resulted in even lower tumor accumulation than untargeted controls⁹⁹. One of the reasons for the poor performance is suggested to be the binding-site barrier¹⁰⁰ that describes the lack of mobility and tumor penetration of antibody conjugated NPs since they directly bind to their intended target upon extravasation. The unexpected outcomes can also be considered in the context of “targeting dilemma”¹⁰¹ stating that selecting between passive and active targeting for NP design requires a compromise, as the functionalization of NPs by targeting ligands for active targeting usually conflicts with PEGylation that is used to enable long circulation. Detailed review of current status in active targeting modalities is provided elsewhere^{102, 103}.

Environment Responsiveness

As mentioned above, NP design landscape is complicated due to conflicting needs imposed on NP properties during individual stages of delivery. As a result, NP needs to evolve to effectively overcome each and every physiological barrier encountered during its *in vivo* journey. Based on this understanding, new types of NPs are being proposed, whose configurations change in response to tumor environmental cues and/or external stimuli^{104–106}. One of the recent studies in this line of thought proposes multi-stage delivery system composed of 100 nm degradable gelatin NPs embedded with 10 nm quantum dots. The agent, thanks to its large size, remains in the blood stream for extended periods of time, enabling preferential accumulation of the agent at tumor site through EPR effect. Enhanced interstitial transport is achieved by disassociation of the gelatin structure upon encounter with matrix metalloproteinases to release quantum dots into the tumor interstitium¹⁰⁷. Another example of this design approach is polystyrene nanoparticles conjugated with collagenase on their surface. This formulation achieved enhanced penetration into multicellular spheroids *in vitro* when compared to unconjugated controls⁴⁵. Other applications include the delivery of therapeutic payload by NPs that are sensitive to tumor-specific microenvironment such as low pH⁶⁴. External stimuli like light¹⁰⁸, heat^{109, 110}, and pressure waves generated by ultrasound¹¹¹ have also been studied to trigger drug delivery or

mediate targeting for cancer. Guidance of magnetic nanoparticles to tumor site under the action of a localized external magnetic field have also shown promising results¹¹². An example to environment responsiveness is the shape-shifting polymeric NPs that can change between spherical and needle like (elongated) configurations in response to elevated heat or decreased pH. Those particles can be effective in accumulating in tumor cells by initially having the elongated configuration to escape the clearance by RES and then change to the spherical configuration for faster cellular uptake¹¹³. While these examples provide demonstrations of promising ideas, the design of environment and stimuli responsive NPs that function *in vivo* will also need to be based on the knowledge of spatiotemporal dynamics of tumor microenvironment. A recent detailed review of environment-responsive NPs is provided elsewhere¹⁰⁶.

TRANSPORT-ORIENTED APPROACHES FOR RATIONAL DESIGN OF CANCER NANOMEDICINE

The decade long work has proven that one-size fits all concept does not apply to the case of cancer nanomedicine and it has become clear that a rational design approach is necessary to significantly advance the field to render NPs advantageous and practical to use in cancer clinics.

However, despite the tremendous amount of research by numerous research groups that feature development of novel NP formulations and bulk of case-by-case data gathered through their characterization by using mostly xenograft models, successes of newly developed cancer nanomedicine often remain limited to a patient subpopulation and the resulting development of personalized treatments still remain to be a pressing challenge in this area^{92, 93}. Ironically, cutting-edge nanomedicine is still being developed via trial-and-error, and lacks systematic and predictive design practice¹¹⁴.

Rational design of NP for effective delivery is possible but will require quantitative relationships between NP design parameters and their spatiotemporal penetration and accumulation in tumor tissue. Rational design of NP clearly needs to be transport-oriented and involve analytical performance metrics that are easily quantifiable and have universal meaning. Continuum models of species transport in porous medium define certain “transport properties”, that are ideal for use as such performance metrics. Transport properties such as effective diffusivity or microvascular permeability are commonly available for small molecules but are rarely characterized for NPs. This lack of NP transport property data is partly due to relative infancy NP-focused research but also closely related to lack of proper evaluation methods for the *in vivo* NP transport characteristics. Existing tumor models to test NPs are not adequate to establish a mechanistic understanding of their transport characteristics around tumors^{3, 6, 92, 115}. In particular, the lack of *in vitro* models that will allow characterization of NPs under physiologically relevant environments is a critical bottleneck in development of effective nanomedicine^{3, 6, 92, 115}.

Physical Principles

Transport of NPs within tumor vasculature and interstitium is largely determined by pore-level interactions of NPs with the surrounding fluid and structures. Part of the movement of NPs within the pores of microvascular wall and ECM occurs through diffusion by random Brownian motion often dampened by collisions and electrostatic interactions with surrounding structures. NPs are also mobilized by convection where the drag generated on the NP by fluid flow carries NPs along, but often at significantly slower speeds than fluid flow itself due to retardation effects similar to ones observed in diffusion. These pore-scale transport phenomena are quantified in terms of transport properties that are used in continuum modeling of biological fluid and solute transport as illustrated in Figure 2 and described below. These NP transport properties include effective diffusivity, microvascular permeability and retardation coefficients that depend on both NP design parameters i.e. size, shape, charge, functionalization, as well as tumor microenvironmental parameters such as interstitial cell and ECM density. Modeling of NP transport behavior also requires the knowledge of hydraulic conductivity parameters that govern the transvascular and interstitial fluid fluxes, which are independent from NP design but depend on tumor microenvironment.

Transvascular transport in tumors is often mathematically described by the Kedem-Katchalsky formulation, a phenomenological law originally developed to model the transport of species across a semipermeable membrane¹¹⁶.

In simple form, transvascular solute flux, J_s , is:

$$J_s = J_v \underbrace{(1 - \sigma_f)}_{f_s} \bar{C}_s + P \Delta C \quad (0)$$

where, P is the microvascular permeability, f_s is microvascular retardation coefficient and $\bar{C}_s$ is the logarithmic mean of capillary and interstitial concentrations of drug or NP defined by:

$$\bar{C}_s = \frac{C_v - C_i}{\ln(C_v/C_i)} \quad (0)$$

with C_v and C_i being the capillary and interstitial concentrations of drug or NP. The plasma concentration, C_v , is time-dependent due to NP's pharmacokinetics as discussed in previous sections.

The transvascular fluid flux, J_v , is determined by Starling's Law¹¹⁷:

$$J_v = L_p (\Delta p - \sigma_s \Delta \pi) \quad (1)$$

With p and π being the hydrostatic and osmotic pressure differences across the vessel wall and σ_s is the osmotic reflection coefficient.

The effectiveness of tumor targeting by the EPR effect, also referred to as “passive targeting”, is significantly affected by the microvascular permeability (MVP) of tumor vasculature⁹⁷. MVP relates the rate of extravasation of particles to the concentration difference between the vascular and interstitial spaces. MVP is a characteristic of both the tumor microvascular wall and the drug molecule or NP under consideration. Early studies for molecular drugs have reported that the MVP of tumor vasculature does not depend on the particle size as long as the particle is much smaller than the pore cut-off diameter of vascular wall. Pore cut-off diameter is reported to be between 300 to 700 nm while in rare occasions, can be up to 1.2 to 2 μm ^{118, 119}. Therefore, P for small macromolecules such as BSA and IgG do not differ significantly from each other¹¹⁸. However, as the molecular weight of agent is further increased, P decreases about two orders of magnitude⁸⁵. This is supported by earlier findings that the dominant mode of molecular transport across microvascular wall is diffusion, which is a molecular weight-dependent process, rather than convection⁵⁵.

In the interior of tumor, transport of drugs and NPs is modeled reasonably well by species transport equation^{120, 121}:

$$\frac{\partial C}{\partial t} + \vec{f} \cdot \vec{u} \cdot \nabla C = \nabla \cdot (D_{\text{eff}} \nabla C) + Q_s - Q_L \quad (2)$$

Where C is the concentration of drug or NP, $\vec{u}$ is the velocity field D_{eff} is the effective diffusivity of NPs within tumor interstitium, which is a measure of mobility of particles and small values of D_{eff} indicate high hindrance of diffusion in interstitial space. Q_s and Q_L are generic volumetric source and sink terms that represents the rate of net extravasation and drainage/cellular uptake, respectively.

Flow through tumor interstitium is often modeled by Darcy's Law¹²² where interstitial fluid velocity, $\vec{u}$, is related to the interstitial fluid pressure through hydraulic conductivity, K :

$$\vec{u} = -K \nabla p \quad (3)$$

A summary of above governing equations for the continuum-based modeling of NP transport phenomena is provided in Table 2. It is important to note that, the simple models presented here by no means fully address the complex NP transport problem. These models do not take into account the variation in the vessel pore size and its effect on transvascular permeability. The depletion of mobile NPs in interstitium by cellular uptake or binding site barrier is not considered either. For more advanced modeling of transport in tumors, the reader is referred to other works on this topic^{13, 123–128}.

Physiologically Relevant Characterization by *In Vitro* Models

Figure 3 illustrates the scientific publication trends for tumor spheroid models and microfluidic tumor models. It is seen that tumor spheroid remains to be the mainstream *in vitro* model for cancer research possibly due to its ability to mimic the densely packed three-dimensional configuration of tumor cellular compartment¹²⁹, proven track record in

reproducibility, ease of production. Research on microfluidic tumor models started on second half of last decade and interest in microfluidics has been steadily growing since. Microfluidic systems receive major interest as cell culture platforms aimed toward study of angiogenesis and metastasis. Microfluidics are also useful in testing drug candidates produced in small quantities efficiently with minimal use of reagents^{130–135}. Ultimately, microfluidic platforms can help close the gap between the current static culture systems and animal models by enabling systematic and independent control of tumor environmental parameters while maintaining a realistic representation of tumor environment such as the vascular and interstitial compartments and the presence of interstitial fluid pressure driven flow.

Massive Parallelization—The microfluidic systems are suitable for development of multiplexing assays for high throughput drug screening operations. This has been a major focus of recent research with numerous studies exploring ways to rapidly test drug efficacy on isolated single cells¹³⁶ cell monolayers^{137–140}, and tumor spheroids^{141–147}. For example, a digital single-cell assay introduced by Wang et al.¹³⁶ used a microfluidic hydrodynamic trapping system for trapping individual cells without the need for surface modification, external electric force, or robotic equipment. The device enabled parallel testing of isolated single cells for apoptosis-inducing effects of mainstream chemotherapeutic drugs. Cunha-Matos et al.¹⁴⁸ have developed a single cell trapping system to investigate delivery of ovalbumin-conjugated gold nanorods to primary dendritic cells. Using this microfluidic platform, it was possible to isolate cells alone, or in groups of 2 or 3 cells in using an array of approximately 1500 traps with efficiency greater than 80 percent. A linear gradient of nanorod concentration was generated across the trap array using a diffusive mixer, enabling testing of their uptake and intracellular processing with a range of concentrations simultaneously and at real-time. Apart from studies featuring single-cell assays, there have been efforts to develop microfluidic assays involving cells cultured on two-dimensional substrates. An et al.¹³⁸ developed an automated high throughput screening for screening and optimization drug combinations in combinatorial chemotherapy. The device incorporated microfluidic diffusive mixers with programmed micropumps to generate an sequential combinatorial concentrations of two different drugs in 64 different cell culture chambers and enabled testing of drug combinations on cell monolayers simultaneously and with small amount of reagents. Similarly, Li et al.¹⁴⁰ developed a microfluidic assay that generated linearly or exponentially changing concentrations of drug by varying lengths of microchannels to control the relative flow rates from reagent reservoirs. The assay was considered to be useful to analyze multidrug resistance in cancer cells at single-cell level. The drug testing was conducted on monolayers of HepG2 hepatocellular carcinoma cells, followed with an assay to determine the drug efflux from those cells.

Significant effort has been on extending the high-throughput screening technologies on single cells and two-dimensional culture settings to multicellular tumor spheroids. For example, Patra et al.¹⁴⁷ introduced a microfluidic platform that allowed on-chip formation, culture and drug treatment of large number (approximately 5000) of uniformly sized tumor spheroids. The device featured a microarray of rectangular cavities positioned under a main flow channel where cells settle down and form spheroids through strong cell-cell

interactions. Similar cell trapping technologies have also been utilized to perform in situ drug screening against common chemotherapy drugs such as doxorubicin, paclitaxel,¹⁴³ and cisplatin^{143, 145}. Formation of large number of homogenously sized specimens based on small amount of source cells using microfluidic technologies will be particularly useful in development of personalized treatment schemes where the amount of source cells originating from patients is often scarce¹⁴⁵.

Biomimickry—The ability of microfluidic tumor models to evaluate NPs under physiologically relevant settings no doubt depends on the degree by which they recapitulate the tumor microenvironment. There has been significant progress in this area where microfluidic systems have been developed to generate specific tumor tissue composition, architecture and interface. For this purpose, there have been large body of research on microfluidic cell culture platforms that feature connected microchannels inhabited by living cells and configured to simulate/mimic tissue- and organ-specific physiological environments¹⁴⁹. These microfluidic platforms often contain cell culture within a three dimensional ECM environment. While single-cell assays enable rapid evaluation of anti-cancer drugs based on response of isolated cells and multicellular tumor spheroids¹⁴³, such culture settings does not reflect the *in vivo* environment and lack the complex interactions that are present between cells and the ECM. ECM-mediated factors within the tumor microenvironment are particularly important as ECM plays a major role in transmission of traction forces, transport of interstitial fluid and distribution of soluble factors. In particular, there are cell- and tissue-dependent differences observed in drug sensitivity of cancer cells cultured in two-dimensional and three-dimensional environments. Mammary carcinoma cells cultured within ECM show significantly higher resistance to doxorubicin compared to assays conducted with 2D monolayers¹⁵⁰. In addition, drug sensitivity of leukemic cells cultured in microfluidic devices featuring 3-D culture settings showed a decrease compared to those conducted in two-dimensional culture¹⁵¹.

Microfluidics enable realistic generation of local tissue-tissue interfaces by micro-scale patterning of different three-dimensional ECM where surface tension, hydrophobic interactions and boundary geometries are often controlled within the microfluidic channels for separate containment of precursor solutions¹⁵². Microfluidic platforms involving culture of cells within three dimensional extracellular environments are increasingly being used to study specific cell-cell and cell-matrix interactions. A cell-microenvironment-on-a-chip platform involving culture of MCF-7 mammary carcinoma cells within a collagen-polyethyleneglycol matrix was used to delineate the effects of matrix stiffness and interstitial flow on MCF-7 phenotype by control of interstitial fluid pressure¹⁵³.

In the current state, applications of microfluidic assays mimicking the tumor microenvironment for drug and NP evaluation are rare. While majority of microfluidic platforms that are designed to recapitulate certain features of tumor microenvironments are aimed towards study of cancer-related processes such as cancer cell invasion^{152, 154}, intravasation¹⁵⁵ or immune cell-endothelial interactions¹⁵⁶, it is possible to repurpose and utilize these technologies for drug and NP testing. *In vitro* microengineered capillary vessels mimic endothelial barrier and basement membrane^{155, 157–161}. Similar techniques are also being utilized to generate ductal lumen structures and are used to investigate drug response

in early stage malignancies such as ductal carcinoma in situ¹⁶². Formation of 3D cell cultures densely packed with tumor cells and ECM components under dynamic flow conditions enable testing of penetration and accumulation of drugs and NPs^{163, 164}.

Several examples of microfluidic technologies that can be utilized in evaluation of NP transport during various steps of delivery to tumor are presented in Figure 4. In order to investigate NP interactions with vasculature during blood-borne transport, endothelial cell monolayers were cultured within microfluidic channels. This configuration allowed characterization of adhesion dynamics of nanoparticles to endothelium under systematic variation of flow conditions and associated shear stress (Figure 4A)¹⁶⁵. Transvascular transport of NP can be investigated using *in vitro* vessel networks formed within microfluidic devices by vasculogenesis and/or angiogenesis. The barrier function of these *in vitro* vessels against fluorescent macromolecules has also been demonstrated (Figure 4B)¹⁶⁶. Microfabrication allows the architecture of such lumen structures to be precisely defined (Figure 4C)¹⁶⁷. For investigation of NP penetration and accumulation in tumor interstitium, cancer cells were allowed to form densely packed aggregates in a microfluidic channel and was exposed to adjacent flow of macromolecular and NP formulations of doxorubicin. Using this platform it was possible to dynamically change drug perfusion conditions and simulate the effects of plasma clearance on drug accumulation (Figure 4D)¹⁶⁴.

Microenvironment Control—Microfluidic devices are attractive for generation of tumor microenvironmental cues through formation of gradients of reagents, oxygen and fluid pressure with a high level of spatial and temporal control. Small diffusion distances result in rapid equilibration of gradients and enable time-varying profiles of drugs and other macromolecules^{138, 140, 146, 168–171}. Gas permeable nature of poly (dimethylsiloxane) (PDMS) allows development of varying levels of oxygen tension by utilization of adjacent channels separated by thin PDMS membranes^{139, 169, 172, 173}. Since flow within microfluidic channel networks is most commonly laminar, it is possible to reliably generate fluid pressure gradients and to control convective flow through cell culture compartments¹⁷⁴. Laminar flow within microchannels also enable accurate predictions of wall shear stress that are useful to investigate processes related to microvascular capillary wall. For example, Soroush et al¹⁵⁶ developed a microfluidic device featuring culture of human umbilical vein endothelial cells (HUVEC) within an anatomically accurate convoluted microchannel network where it has been possible to study the correlation between the wall shear stress and neutrophil adhesion to HUVEC based on inlet and outlet pressure conditions to the network.

Integrated Sensing and Actuation—Finally, by integrating sensor and actuator layers to microfluidic systems it has become possible to perform non-invasive assessment of drug efficacy^{175, 176}, and cell metabolism monitoring^{172, 177}. Recent technologies developed in this area also include on-chip generation of localized heat by focused ultrasound to study triggering of stimuli responsive targeted NP formulations¹⁷⁸.

A summary of microfluidic systems discussed above grouped by innovation area and focus is provided in Table 3.

CONCLUSIONS

An increasing number of studies have reported that NP transport behavior is significantly different from that of small molecules. A deeper understanding can only result from understanding their complex biological, chemical as well as mechanical interactions with their surroundings. Moreover, considering the highly diverse nanomaterial designs being explored, as well as the heterogeneous tumor microenvironment, it is critical that experimental approaches be integrated with predictive computational approaches.

Evaluation of NP transport properties and their efficacy in environments physiologically relevant environments to tumor tissue has been receiving growing interest but still remains to be a challenge. Developing Human on chip devices that incorporate together multiple organ mimetic functional compartments will be extremely useful to evaluate biodistribution and toxicity of NP, however currently remains to be an ambitious goal due to inherent difficulties in recapitulating proper *in vivo* function while simulating different scales of each organ or tissue. While there are recent efforts to address those scaling issues¹⁷⁹, primary benefits of microfluidic in terms of microenvironment control will be best achieved if the complexity of the device architecture is limited by modeling only a single organ or tissue structure. Then, additional features of tumor microenvironment can be incorporated such as co-culture with stromal and immune cells to improve the degree of biomimicry.

Finding ways to properly interpret and translate the results from studies with microfluidic systems to towards those in preclinical animal and clinical studies remains to be another open challenge. Use of patient derived cellular end ECM components and fine tuning of device architecture to simulate individual patient's tumor microenvironmental properties will significantly improve the translational capabilities of microfluidic platforms.

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Figure 1.

Physiological barriers of tumor microenvironment affect delivery of nanoparticles.

Figure 2. Modeling of Multi-scale Biophysical Phenomena for Mechanistic Understanding of NP Transport. Pore-level interactions of NPs with their surroundings determine the transport properties that are used as inputs of microvasculature- and tissue-level continuum models for predictive simulation of NP transport. Parameters arising from tumor microenvironmental conditions and NP pharmacokinetics are also inputs to the continuum models.

Figure 3.

Scientific publication trends for contemporary *in vitro* tumor models. Records of original research publications matching “tumor spheroid model” and “microfluidic tumor model” keywords in their topics were retrieved from Web of Science citation index and plotted according to publication year. The cumulative number of records are indicated by gray-scale symbols while percentage of number of publications is indicated by colored symbols. The graph shows the years between 2000 and 2015.

Figure 4.

Microfluidic technologies to evaluate transport of NPs during various steps of delivery to tumor. Blood-borne transport: (A) endothelial cell monolayers in cultured within microfluidic channels allow characterization of adhesion dynamics of nanoparticles to endothelium under systematic variation of flow conditions and associated shear stress. Adapted from¹⁶⁵. Transvascular transport: (B) *in vitro* vessel networks can be formed within hydrogels by vasculogenesis and/or angiogenesis within microfluidic devices and fulfill barrier function against extravasation of macromolecules. Adapted from¹⁶⁶. (C) The

architecture of lumen structures can be precisely defined by casting hydrogels against microstructured molds. Adapted from¹⁶⁷. Interstitial transport: (D) Cancer cells culture in microfluidic side channels adjacent to flow simulate penetration of macromolecular drugs and NPs within dense tumor interstitium and enable comparison of drug accumulation under dynamic perfusion with plasma clearance. Adapted from¹⁶⁴

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Table 1

Tumor Extracellular Matrix Functional Composition

Component	Functional Characteristics	Relevance to transport	Concentration (mg/g wet tissue)	Reference
Collagen I	• Denser collagen matrix than the originating normal tissue.	• Increasing interstitial fluid pressure ⁴⁶	1.7±0.4	Mammary carcinoma (murine) MCa1V ⁴²
	• Linearly re-organizing	• Causing leaky vasculature and impractical lymphatics ⁴⁶	4.6 ±0.7	Mammary carcinoma (DMBA induced) ⁴⁷
Fibronectin	• Increasing fibronectin rigidity ⁴⁶	• Reducing drug sensitivity by promoting adhesion with cells ⁴⁶	0.275 ⁴⁸	Human Pancreatic cancer ⁴⁸
	• Binding collagen and remodeling fibrils		N/A	
Hyaluronic Acid (HA)			0.22±0.02	Mammary carcinoma (murine) MCa1V ⁴²
	• overproduced by many tumors	• Playing critical role to increased IFP ⁴⁹	1.9±0.3	Mammary carcinoma (DMBA induced) ⁴⁷
	• known to promote cell invasiveness and EMT		9.59 0.388 ± 0.032 (normal pancreas: 0.029 ± 0.003)	Pancreatic (Human) MIA PaCa-2 ⁵⁰ Pancreatic carcinoma (30 months of 12 patients) ⁵¹
			0.16±0.04	Mammary carcinoma (murine) MCa1V ⁴²
Proteoglycan	• Known for a role in organizing collagen fibrils ⁵²		1.6±0.9	Mammary carcinoma (DMBA induced) ⁴⁷

Table 2

Governing equations for the continuum-based modeling of NP transport phenomena within tumor microenvironment.

Tumor Microenvironment	Nanoparticle Design + Tumor Microenvironment	
Darcy's Law (Interstitial fluid transport)	Species Conservation Law (Interstitial NP transport)	
$\vec{u} = -K_{\text{hyd}} \nabla p$	$\frac{\partial C}{\partial t} + f \cdot \vec{u} \cdot \nabla C = \nabla \cdot (D_{\text{eff}} \nabla C)$	
K_{hyd} : Hydraulic conductivity $\vec{u}$: Interstitial fluid velocity p : Interstitial fluid pressure	D_{eff} : Effective diffusivity f : Interstitial retardation coefficient C : NP concentration	
Starling's Law (Transvascular fluid transport)	Kedem-Katchansky Formulation (Transvascular NP transport)	
$J_v = L_p (\Delta p - \sigma_s \Delta \pi)$	$J_s = J_v \underbrace{(1 - \sigma_f)}_{f_s} \bar{C}_s + P \Delta C$	
L_p : Microvascular hydraulic conductivity J_v : Microvascular fluid flux π : Osmotic pressure ¹¹⁷ σ_s : Osmotic reflection coefficient	P : Microvascular permeability f_s : Microvascular retardation coefficient J_s : Microvascular NP flux C_v : Capillary concentration C_i : Interstitial concentration	$\bar{C}_s = \frac{C_v - C_i}{\ln(C_v / C_i)}$

Table 3

Innovative applications of microfluidic systems for *in vitro* evaluation of cancer nanomedicine.

Innovation Area	Promise	Focus
Massive Parallelization	<ul style="list-style-type: none"> Development of multiplex assays for high-throughput screening 	<ul style="list-style-type: none"> Single cell,¹³⁶ 2D culture:^{137–140} Spheroid:^{141–147}
Biomimicry	<ul style="list-style-type: none"> Recapitulation of physiological barriers. By mimicking specific tissue composition, architecture and interface 	<ul style="list-style-type: none"> Vascular endothelium^{154–161} Interstitial penetration^{163, 164} Ductal structure/gland¹⁶²
Microenvironment Control	<ul style="list-style-type: none"> Generation of controlled gradients of fluids and reagents 	<ul style="list-style-type: none"> Drug/macromolecule^{138, 140, 146, 168–171} Oxygen^{139, 169, 172, 173} Fluid pressure/Flow Perfusion^{151, 156, 174}
Integrated Sensing and Actuation	<ul style="list-style-type: none"> Non-invasive monitoring of cell response On-chip triggering of stimuli responsive targeted nanomedicine 	<ul style="list-style-type: none"> Viability assessment:^{175, 176} Metabolism monitoring:¹⁷⁷ Hypoxia¹⁷² Mild hyperthermia¹⁷⁸