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Section V: Table of Contents

Section V: Tools and Resources to Accelerate Clinical Translation

Pre-Clinical Characterization of Nanomaterials

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The biggest challenge in preclinical characterization of nanomaterials is the diverse array of skills and knowledge required for a complete understanding of the formulation (**Figure 1**). A multidisciplinary team of experts including chemistry, immunology, toxicology, pharmacokinetics, pathology, and more is often required for an advanced evaluation of a nanomedicine, even and especially at the preclinical stage. Every data analysis and result depends on knowing exactly what the test material comprises. There have been numerous reported cases where toxicity was incorrectly assigned to a nanomaterial when in fact the toxicity stemmed from residual excipients, synthetic byproducts, biological impurities, undetected particle instability, or other anomaly[1–6].

The Nanotechnology Characterization Lab (NCL) was set up in 2004 as part of the NCI's Alliance for Nanotechnology in Cancer program to provide preclinical characterization services to oncology nanomedicine developers around the globe. The NCL staffs experts in a variety of fields who provide critical insight to organizations pursuing nanomedicine translation, but may not have the wide-ranging expertise or resources required for translational advancement. Having characterized more than 650 nanomaterial samples from nearly 100 different organizations, the NCL has had a unique opportunity to observe nanomaterial characterization challenges, including how the field has progressed over the years and insight into what lies ahead.

Challenges in Chemistry

It has been widely established that a nanomaterial's physical and chemical properties directly influence a variety of biological performances, including biodistribution, clearance, and immunotoxicity[7–10]. Therefore, a thorough characterization of these parameters is paramount to ensuring safe *in vivo* administration of the material. With this realization, the depth of routine physicochemical characterization performed on nanomaterials has increased dramatically. The recognition of the unequivocal importance of characterization and consistency is arguably the most significant advancement in this field.

The challenges associated with nanomaterial physicochemical characterization have shifted over the last decade. Initially, researchers grappled with proper ways to assess size, charge, or composition, including which measurement technique was most suited and what the most appropriate measurement conditions were. Now it is well accepted that materials should be analyzed by multiple orthogonal analytical techniques and under the appropriate biologically relevant conditions. However, with the evolution of more advanced nanotechnologies, new challenges in characterization are arising. One challenge at the forefront of physicochemical characterization of nanomaterials is surface analysis. It is imperative to know whether the surface ligands are covalently attached or simply physisorbed, which would allow their premature dissociation from the formulation. Furthermore, the density / coverage of the surface and the orientation and accessibility of the ligand(s) can also be important biological factors. As the number of surface modifications increases, so will the complexity in characterization. This is a particularly challenging area because techniques developed for one type of nanomaterial (e.g., liposomes) will not necessarily work for others (e.g., metals). Having realized the importance of surface properties for biological performance, there will be considerable advancements in tools to evaluate surface properties over the next few years[11]. Our laboratories and others have already begun to invest significant resources into this area.

Resources for scale-up and GMP manufacture of nanomedicines remain as another critical area of need for future development. The NCL is continually asked for advice on where to go for scale-up and / or GMP production services. There are limited establishments with the capabilities to meet this increasing demand for late-stage preclinical synthesis of complex nanomedicines. National efforts are underway now to address this critical gap in translation.

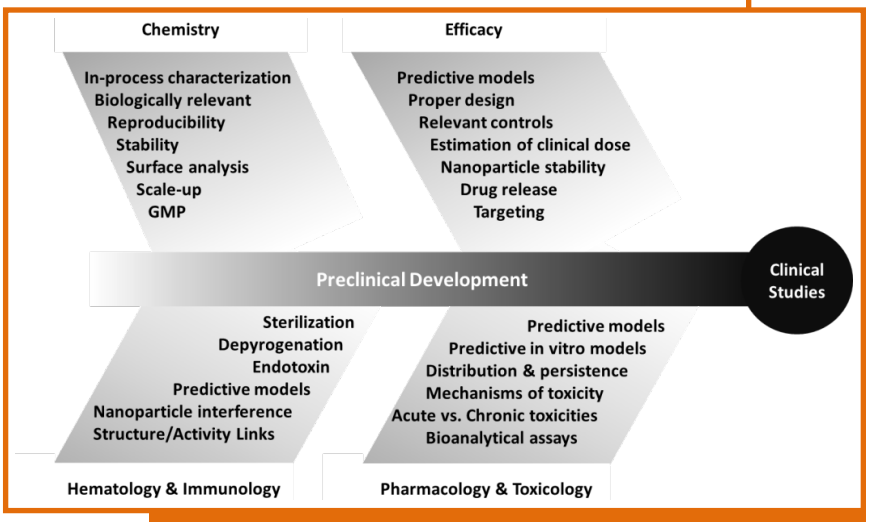

Figure 1. Challenges in Preclinical Characterization of Nanomedicines. Preclinical characterization of nanomedicines requires analysis in a variety of fields, each of which has their own set of challenges. Some of the most significant challenges associated with chemistry, immunology, efficacy and pharmacology/toxicology are noted.

Challenges in Immunology

Although, there has been increasingly more effort put into the early immunological evaluation of nanomaterials, immunology continues to be an underappreciated area during the preclinical stage. Structure-activity relationship studies have been an integral part of the early understanding of nanoparticle immunological influences. The association of nanoparticle physicochemical traits to immunotoxicities has afforded a significant knowledgebase to which the field needs to continue to build upon. However, many challenges associated with immunological evaluation of nanomaterials still remain, including sterility, sterilization, depyrogenation, biological contaminants (e.g., endotoxin and b-glucan), and accuracy and predictability of *in vitro* and *in vivo* methods.

Endotoxin detection and quantification is an area many researchers continue to struggle with. Nanoparticles are notorious for interfering with many of the traditional immunology assays, especially endotoxin quantification assays. A significant amount of research has been published on identifying and circumventing this interference, particularly as related to endotoxin, but educational efforts in this area need to continue[12–18]. Many researchers often avoid endotoxin evaluation until late in their preclinical development. This can be a costly oversight. Not only can the identification and elimination of the contamination source be expensive and time consuming, high endotoxin levels could adversely affect data interpretation.

Predictive *in vitro* and *in vivo* models for evaluating immunotoxicology continue to be one of the most important aspects of nanoparticle immunological characterization. Common immunological and hematological reactions to nanoparticles include hemolysis, complement activation, thrombogenicity, and cytokine storm. Many of these toxicities can be detected using *in vitro* assays, some of which are known to be predictive of corresponding *in vivo* toxicities. For example, a 5% hemolysis rate *in vitro* has been shown to correlate to hematocrit and hemoglobin changes *in vivo*[19]. Other hematotoxic effects, (e.g., myelosuppression) can also be studied *in vitro*, but knowledge of the *in vivo* nanoparticle biodistribution is needed for accurate data interpretation. In such situations, a systematic approach combining both *in vitro* and *in vivo* data is proven to be the most reliable characterization approach.

Future work in the immunological evaluation of nanomaterials will require monitoring the long-term effects of nanoparticles on the immune system. Delayed type reactions are triggered by nanoparticle influences of immune cell function and are often very complex, frequently involving many different cell types. Although specialized *in vitro* immune function tests have been developed and shown to be predictive of *in vivo* toxicities for small

molecules, applicability of these to nanoparticles is challenged by a distinct biodistribution profile and mode of transport across biological barriers. Many of these challenges have been reviewed in detail[20].

Challenges in Efficacy

Without question, the biggest challenge in preclinical assessment of efficacy is the availability of appropriate and predictive animal models. Most efficacy studies are conducted using human cancer cell lines in immune-deficient mouse strains that compromise the plausible interaction between immune cells and nanomaterials *in vivo*. Additionally, these xenograft models are unable to adequately recapitulate the tumor stroma, which plays an important role in tumor progression and can impede drug delivery.

There has been significant progress in the development of more suitable *in vivo* cancer models with the sequencing of cancer genomes and improved molecular biology tools. Several genetically engineered mouse models (GEMMs) have been generated to evaluate tumor growth and progression by utilizing noninvasive imaging modalities. Histopathological analysis of genetically engineered mouse tumors at different stages of disease progression has shown reasonable similarities to human disease. In addition to GEMMs, another focus has been on patient derived xenografts (PDX). PDX models implant human tumor cells in a mouse, providing a more relevant tumor microenvironment and genetic complexity that can better predict clinical outcomes. Future progress in this area will require further refinement of existing tumor models using improved understanding of cancer initiation and progression (e.g., most common genetic predictors of disease progression, signaling pathways, role of tumor stroma).

Experimental design issues also often plague *in vivo* efficacy analysis. Because of the cost of *in vivo* animal studies, it is not uncommon for researchers to forego some needed controls or preliminary analyses. For example, it may be necessary to run several small scale preliminary experiments to gain a better understanding of the maximum tolerated dose (MTD), nanoparticle stability, or drug release *in vivo*. Lack of the adequate controls is another common omission. A good efficacy evaluation should test materials at their respective MTDs and include controls of the platform, current standard of care, and the non-targeted particle where applicable.

Challenges in Pharmacology & Toxicology

Similar challenges exist for preclinical pharmacology and toxicology testing as with preclinical efficacy studies—the availability of appropriate models and proper experimental design. Development of predictive *in vitro* and *in vivo* models of toxicity would be big advancements

in the pharmacological and toxicological understanding of nanomaterials. There are differences in the mononuclear phagocytic system (MPS) between the animal species utilized that could affect accurate prediction of pharmacology and toxicology in humans. There have already been significant improvements in the development of bioanalytical assays in this area. For example, novel methods for analysis of drug release in biological matrix have allowed for a better understanding of nanoparticle stability, tendency for aggregation, drug release, and quantification of encapsulated and unencapsulated drug fractions.

Acute toxicities of nanomaterials are being well studied now; however, long-term chronic toxicities associated with nanomaterials should be further explored and will be an area of future development for this field. A better understanding of the mechanisms of nanomaterial toxicity (e.g., oxidative-stress, lysosomal dysfunction, inflammation) will aid these efforts, and research is ongoing now towards this goal. Additionally, bioanalytical challenges such as determination of dose linearity; estimation of clinical dose; and distribution and persistence of nanoparticles in tissues will be critical for the translation nanomedicine.

Preclinical characterization of nanomaterials has shown considerable advancement over the last decade.

Conclusion

Preclinical characterization of nanomaterials has shown considerable advancement over the last decade. Methods are being continually developed and optimized to meet the needs of the evolving complexity of nanomedicines. Detailed nanoparticle surface characterization, predictive immunotoxicity assays, and quantitative evaluation of the encapsulated vs. free drug fractions highlight the growth of this field. Continuing to pursue new methods development as well as conducting research directed at understanding the nano-bio interface will uncover additional relationships between nanoparticle structure and biological activity. This information will be invaluable for devising new strategies for using nanotechnology to improve upon existing pharmaceuticals and deliver novel therapies in the future.

Pharmacokinetics and Pharmacodynamics Characterization of Nanotherapeutics

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Introduction: Complex Pharmacology of Nanoparticles

Major advances in nanoparticles (NPs) have revolutionized drug delivery capabilities over the past decade. They provide numerous advantages, such as greater solubility, duration of exposure, less toxicity and delivery to the site of action over their small molecule counterparts, nevertheless NPs display substantial variability in systemic clearance and distribution, tumor delivery, and pharmacologic effects (efficacy and toxicity)[21]. NP research has historically focused on the development of NP formulations with less emphasis on evaluating the complex pharmacology and biology of NPs, which significantly influences the successful translation of these agents. This report is an overview of factors that affect the pharmacokinetics (PK) and pharmacodynamics (PD) of NPs in preclinical models and patients.

Major advances in nanoparticles have revolutionized drug delivery capabilities over the past decade.

The disposition of NPs is dependent upon the carrier, not the therapeutic entity, until the drug gets released from the carrier[22]. The nomenclature used to describe PK of NPs includes: encapsulated (the drug within or bound to the carrier), released (active drug that gets released from the carrier), and sum total (encapsulated drug plus released drug). After the drug is released from its carrier it is pharmacologically active (unless the released form is a prodrug) and subject to the same routes of metabolism and clearance as the non-carrier form of the drug. The pharmacology of NPs is complex and thus comprehensive PK studies must be performed in order to assess the disposition of encapsulated or released forms of the drug in plasma, tumor and tissues[23]. Considerable inter-patient variability exists in the PK/PD of NPs and appears to be associated with variability in the function of the mononuclear phagocyte system (MPS), which is the primary clearance pathway for NPs[24]. It is difficult to evaluate the factors that affect the PK and PD of NPs in animals and human patients, due to the fact that they are different and thus animal models may not be predictive of the effects displayed in patients[25].

NPs may be taken up by a wide variety of cells in the blood and in tissues; however, it has been discovered that NPs are primarily taken up by circulating monocytes and dendritic cells (DC) in blood, Kupffer cells in the liver, DC in the lymph nodes, and macrophages in the spleen all of which are components of the MPS[26,27]. Uptake mechanisms may occur through different pathways and are often facilitated by the adsorption of opsonins to the NP surface and subsequent phagocytosis by MPS cells. Although, the uptake of NPs by the MPS does appear to be the predominant factor that affects the clearance of NPs from the blood as well as the distribution of NPs to tissue and possibly even the tumor itself. Yet, it is currently unclear if the distribution of NPs from the blood and into tumor and/or tissues occurs by capture (i.e., the NP enters the tissue and then is taken up by the MPS cell) or hijacking (i.e., the MPS cell takes up the NP in the blood and carries it to the tissue)[28]. This complex issue complicates the optimal design of NPs and, moreover, the evaluation of the primary factors that alter NP delivery to solid tumors. **Figure 2** illustrates the complex interaction between NPs and the MPS. The following two sections will discuss, in more detail, these factors with respect to NP PK/PD and subsequent delivery to solid tumors.

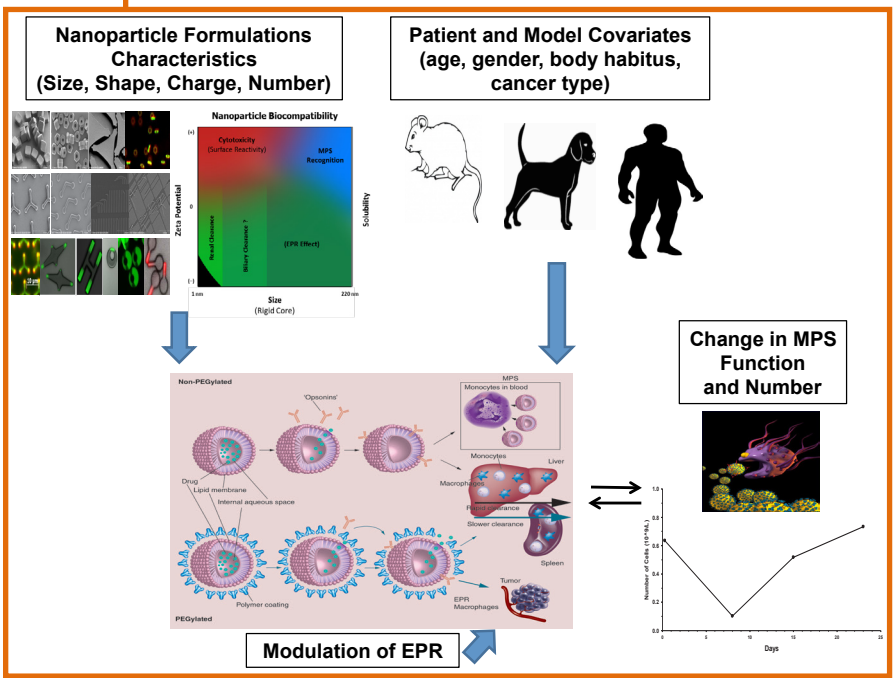

Figure 2. Summary of the complex bi-directional interaction between NPs and MPS. The factors affecting the PK and PD of NPs consist of the interactions between the characteristics of the NP carrier and host related factors. The NP characteristics consist of the size, shape, surface modifications, surface charge, and number of NPs administered. Several mediators (e.g., chemokines) and factors (e.g., age, gender, body habitus, tumor type and location, other drugs) have been reported to alter the PK and PD of NPs in animal models and in patients. The uptake of NPs by the MPS cells may also alter the function and number of MPS cells.

Factors Affecting the PK and PD of Nanoparticles

The factors affecting the PK and PD of NPs consist of the interactions between the characteristics of the NP carrier and host related factors. The NP characteristics consist of the size, shape, surface modifications, surface

charge, and number of NPs administered[22]. In an attempt to minimize opsonization and the subsequent uptake by the MPS, a commonly used strategy, although this is dependent upon the NP material type used, is to conjugate polyethylene glycol (PEG) onto the surface of the NPs. However, the optimal length, amount, and configuration of PEG or other surface coatings is unclear and is unique to each NP carrier[29,30]. There also may be hidden complications of PEGylating NPs. While PEGylation does prolong the circulation of NPs in blood compared to non-PEGylated NPs, the addition of PEG may increase the interpatient variability in the clearance of NPs[31]. Moreover, the number of NPs administered per dose significantly affects the clearance and distribution of NPs[32]. This affect is most likely due the non-linear or saturable uptake of NPs by the MPS.

Several mediators (e.g., chemokines) and factors (e.g., age, gender, body habitus, tumor type and location, other drugs) have been reported to alter the PK and PD of NPs in animal models and in patients[22]. One of the more clinically relevant issues to consider is that the type and location of the tumor may alter the PK of NPs and thus it may not be optimal to administer the same dose of a nanotherapeutic to patients with different types of tumors. The mechanisms of these interactions appear to all involve the MPS. MPS is highly promiscuous and thus takes up all types of particles (e.g., drug carriers, virus, antibodies, bacteria), but appears to have only a limited capacity to take up these particles. Thus, the presence of other natural or man-made particles in the body may alter the PK and PD of NPs. There also appears to be significant differences in the MPS function and PK of NPs across species and across different strains within a species[25,33]. Moreover, the PK and interaction of NPs with the MPS after repeated doses of NPs is opposite in some animal models compared to that of human patients[34,35].

Factors Affecting the Delivery of Nanoparticles to Solid Tumors

While conventional drugs encounter numerous obstacles *en route* to their target, in theory NPs can take advantage of tumor's leaky vasculature to extravasate into tissue via the enhanced permeability and retention effect (EPR)[36]. Furthermore, the poor lymphatic drainage in tumors leads to accumulation of the NPs for prolonged duration, allowing them to release the drug in tumor cells over time. Passive NP targeting exploits the classic features of tumor biology in order to increase exposure of NPs in the tumor.

In theory, EPR is the primary route of NP delivery to tumors (even for active, targeted nanotherapies), but heterogeneity of EPR between tumor types, location of the tumor (e.g., primary versus metastatic, organ, intracranial versus extracranial) and the inability to ensure homogeneous delivery to all regions of the tumor is forcing the need to understand the more fundamental aspects of EPR[37]. Variations in the distribution of blood flow, in vessel

permeability, in microenvironment density, and specific interactions of MPS cells within the tumor may all play an important role in the distribution and penetration of NPs to tumor[38]. It has been reported that the EPR effect is directly influenced by physiologic contributions such as vascular pore dimensions, vascular structure, surrounding stroma[36]. In addition, there appear to be interactions between macrophages and others immune system cells that influence tumor microenvironment factors[28].

In theory, active targeting of NPs may further improve tumor delivery and activity by allowing the NPs to bind to specific cells in tumors using surface-attached ligands capable of recognizing and binding to cells of interest[21]. Targeting strategies have consisted of the use of antibodies, nucleic acids, carbohydrates, peptides, aptamers, and vitamins. It is currently unclear if active targeting of NPs to factors on tumor cells can overcome the inherent barriers associated with the tumor matrix. With the notable exception in the treatment of hematological malignancies, whose use of active targeting strategies would, of course, avoid these issues and barriers[39].

While NPs are able to deliver more drug to solid tumors compared to small molecule drugs, the efficiency (e.g., % of drug) of NPs to penetrate from blood and into the tumor matrix is significantly less than small molecule drugs[38]. Thus, better and more effective NPs that exploit EPR are needed as well as employing methods to evaluate and address the structural and functional hindrances in the tumor microenvironment[40]. However, a major limitation to addressing these issues remains the lack of detailed studies comparing the EPR effect and NP delivery to tumors in preclinical tumor models and human patients.

Future Directions for Understanding PK/PD in Nanotherapeutics

The pharmacology of NPs is highly complex and the factors that alter the PK and PD of NPs, especially the clearance and delivery to solid tumors are highly variable and multifaceted. Future studies need to develop novel *in vivo* and high-throughput screening methods as well as experimental designs that can successfully evaluate how NP PK and PD are affected by the variable nanotherapy schemes, the MPS, and other immunologic factors and conditions. In addition, studies are needed to evaluate the factors influencing and inhibiting the efficient delivery of NPs to tumors as well as how these factors can be overcome[40]. However, before any of these issues can be addressed, we first need to identify and profile these factors in animal models and in patients to identify which preclinical model(s) optimally predict these effects in patients.

Preclinical Animal Models for NP PK and PD

It is currently unclear which animal model most accurately predicts the PK and PD (efficacy and toxicity) of NPs, especially after repeated dosing, in patients. For example, after repeated dosing of some NPs in animal models (e.g., dogs) there is higher clearance of NP after subsequent doses (accelerated blood clearance (ABC)); whereas, in patients the clearance of NPs is reduced after repeated dosing which results in accumulation of drug[34,35]. These differences may be due to differences in MPS function of animal models versus humans. However, the disconnect between ABC in animals and reduced clearance of NPs in human patients does not occur for all NP agents. The lack of consistent changes in clearance after repeated dosing of NPs in animal models and patients further complicates the determination of the optimal models and study design for all NPs. As the type and location of the tumor may also influence the PK and PD of NPs, studies in non-tumor bearing animals may not be as predictive as needed.

Nanoparticle Formulation Characteristics

Theoretical changes made to formulations to enhance or alter the PK and PD of NPs may not readily translate to changes *in vivo* and thus comprehensive *in vivo* studies are needed to evaluate these effects. The optimal size, shape and number of NPs dosed are currently unclear[21,22]. Studies suggest that smaller NPs may be better than larger NPs as a means to overcome potential barriers in solid tumors. However, the specifics of this parameter needs to be defined. Information from other carrier-mediated agents (polymer conjugates; antibody drug conjugates (ADC)) may be used to better define the size parameter of NPs. As the number of NPs dosed appears to be a critical parameter affecting NP PK this suggests that the dose of NPs should be based on the number of NPs administered instead of the mg of drug inside of the NP. It is also unclear if the optimal NP characteristics for the treatment of one type of cancer will be the same for other types of cancers.

Analytical and Biodistribution Studies

Based on the complexity and high variability in the PK of NPs, detailed methods and studies are needed to evaluate the PK of NPs in blood, tumor and tissues[22]. It is critically important to evaluate the PK of the NP encapsulated and released form of NP drugs. This has been evaluated for some NPs in plasma; however, these studies need to be extended to evaluate encapsulated and released drug in tumor and tissues in order to be of any relevance within acute and long-term PK studies. In addition, it may be important to distinguish the exposure of NPs in various cell types within tumor and tissues. It is also becoming apparent that circulating cells in the blood (e.g., MPS cells) act as a depot site for NP agents and thus

NPs may be detectable in circulating MPS cells for a longer period of time than in plasma. Understanding how the uptake of NPs by circulating cells in the blood influences the distribution of NPs to the tumor, liver and spleen, is also important. The ability to measure intracellular exposures (e.g., lysosome or nucleus) of the NP carrier and active-anticancer agent is also critically important for all NPs, but especially important for actively-targeted NPs[41]. In parallel to analytical PK studies, we also need to evaluate the biodistribution of NPs using imaging technologies, as this will be critical to comparing EPR and tumor delivery in animal models and in patients[40].

Interaction Between NPs and the MPS

Studies suggest that there is a bi-directional interaction between the immune system, especially the MPS, and NPs[28]. MPS cells are the primary pathway responsible for the uptake and removal of NPs from blood or plasma. In addition, the interaction or uptake of NPs by the MPS may alter the function of MPS cells and even be cytotoxic to the MPS. However, this bi-directional interaction is highly variable and is dependent upon the characteristics of the NPs and factors that affect MPS function in animal models and in patients[26,27]. The type of tumor, tumor burden and location of the tumor may alter MPS function and the PK and PD of NPs and thus the appropriate dose of NP may not be the same for all malignancies. As a result studies need to be performed to profile the sequence of events and interaction between NPs and the MPS (e.g., subject covariates, opsonization, complement activation, MPS recognition, phagocytic uptake by MPS, NP PK and PD, change in MPS function, cytotoxicity to MPS) after administration of single and repeated doses of NPs in animal models and in patients.

Tumor Delivery of NPs

There is a fundamental need for preclinical tumor models to accurately represent the types of tumors seen in patients in order to conduct informative profiling and developmental studies of NPs. It is thought that metastatic, orthotopic, and GEMM are better options for NP studies than flank tumor xenografts. However, systematic studies of several types of NPs in each tumor model have not been reported and are desperately needed to advance the field of NPs in the treatment of solid tumors. In addition, studies suggest that primary and metastatic intracranial tumors have enhanced delivery of NPs compared with small molecule anticancer agents. It is unclear if the mechanism(s) of the enhanced delivery NPs to intracranial tumors is the same as non-intracranial tumors. Studies of NPs should use valid preclinical tumor models of intracranial and non-intracranial solid tumors in patients to address these issues[22,36].

Historically, investigators have predominantly tried to improve the tumor delivery of NPs by altering the characteristics of the NP carrier. One potential NP factor that needs to be further evaluated is the potential for smaller NPs to achieve greater delivery and distribution throughout the tumor matrix[42,43]. However, changes to the NP carrier may only achieve incremental improvements in the delivery of NPs to tumors due to the inherent barriers within the tumor matrix. Thus, there is a need to develop treatment strategies, regimens, methods and devices to overcome or alter the tumor barriers. These plans could include pharmacological agents or non-invasive treatment modalities. For example, recent approaches to normalize both tumor vasculature and physical forces surrounding vessels have been explored[44]. Co-medications that effect stroma and blood pressure are also known to influence EPR effect. The use of non-invasive methods that apply external beams that alter tumor barriers also holds significant potential benefits[45]. Another fundamental problem with NPs is that, even when they are able to penetrate into tumors, the release of drug from the carrier is relatively low and highly variable[23]. Thus, there is a need to develop treatment strategies to increase the release of drug from the NP and into the tumor matrix.

...researchers could individualize treatment with NPs based on selection of tumors with high EPR, tumor targets and patient specific doses.

Milestones to address these critical areas that researchers should be able to be achieve over the next 5-15 year time frame include many aspects. In the next 5 years, researchers will identify animal models that predict the PK and PD (toxicity and efficacy) of NP agents; identify the factors affecting the tumor delivery and distribution of NPs in intracranial and non-intracranial models; and develop novel analytical methods and platforms to characteristic the pharmacology of NPs as part of high throughput screens, *in vivo* models and in patients. Looking further ahead over the next 10 years, researchers will define the bi-directional interaction between NPs and the MPS, as well as other parts of the immune system, in preclinical models and in patients; optimize NP carrier characteristics to avoid delivery to normal tissues and enhance delivery to intracranial and non-intracranial tumors; and develop treatment strategies, regimens, methods and devices to overcome or alter the tumor barriers to enhance the delivery of NPs to tumors. Looking further ahead over the next 15 years, researchers could individualize treatment with NPs based on selection of tumors with high EPR, tumor targets and patient specific doses.

Informative Assessment on Novel Oncology Therapeutics in Preclinical Cancer Models

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Introduction

It was not until the most recent decade that the tremendous complexity and diversity of molecular mechanisms, which underlie malignant transformation and cancer growth, became recognized. This new found knowledge fueling advanced efforts to dissect the cancerous pathways, pinpoint predictive biomarkers and promising drug targets and propose novel more efficacious therapeutic strategies to rein in the cancer disease[46]. As a significant component of the '*bench-to-bedside*' translational research arsenal, animal models of cancer occupy a capstone position and have become a broadly recognized mainstay in support of the preclinical phase for drug development's critical path[47,48]. In particular, mouse models have been constructed – either entirely surgically, by engrafting tumor cells/fragments into a judiciously chosen type of rodent recipients, or by using more 'cutting-edge' technologies via molecular engineering to edit the mouse genome in order to program selected sets of endogenous murine cells for oncogenic transformation (e.g., for the purpose of developing cancerous lesions of specific nature in pre-determined organs or anatomic locations). Presently, these models, which are reviewed in further details below, are broadly employed within a variety of experimental paradigms. The bulk, of which, are aimed at interrogating candidate therapeutics relative to their bioavailability, toxicity, mechanisms of systemic distribution, excretion and therapeutic action, as well as to their anti-tumor efficacy prior to moving these compounds into costly clinical testing workflows[49–51]. Such step-wise strategy has proven itself advantageous in preserving strained resources available to drug developers, while increasing scale and throughput of therapeutic testing; avoiding costly mistakes while mitigating the emotional burden of treating cancer patients; and, ultimately, accruing invaluable data to informatively guide clinical decisions in cancer disease management.

Patient-Derived Xenograft Models

Recognizing the heterogeneity and cellular complexity of cancer and the concomitant ability to reproduce the individual aspects of diverse malignancies in animal models is of critical importance for directing an informative preclinical assessment. This is of particular importance for evaluation of targeted and pathway-specific therapeutics, which display

efficacy only within a limited subset of the cancer patient population (e.g., that feature the appropriate molecular signature(s) of disease). Furthermore, individual (and not infrequently highly similar histo-morphologically) tumors may display acquired drug resistance to standard-of-care and first-line therapeutics; which mandates further evaluation of molecular content of the resistant disease's portion, followed by application of advanced next generation cancer therapeutics and/or combinatorial treatment regimens. With the purpose of attacking multiple components of the pro-oncogenic environment, which triggered the acquired resistance to mono-therapeutic intervention, in the first place. Last, many particularly aggressive tumor types reveal the notorious intra-tumoral heterogeneity, as evidenced by the presence in the same tumor mass of distinct sub-populations of transformed cells, all driven by divergent combinations of oncogenic drivers. This heterogeneity represents yet another tremendous challenge for selection of the most efficacious and durable therapeutic treatment available.

As such, patient-derived xenograft (PDX) models are constructed by grafting freshly dissected cancerous tissue (e.g., gained during tumor de-bulking surgeries or via diagnostic biopsies) either subcutaneously or orthotopically into carefully selected immunocompromised recipient mice. These can be reliably generated with a high take rate from a variety of tumor types[52–54]. Moreover, recent advances in the PDX modeling field have afforded preclinical drug developers the ability to derive models from metastatic or relapsed cancerous lesions as well as cancerous cells that have been deposited via tumor exfoliation or invasive growth into either ascitic fluid or blood circulation (e.g., circulating tumor cells)[55,56].

Among the myriad of substantial benefits PDX models' offer for preclinical

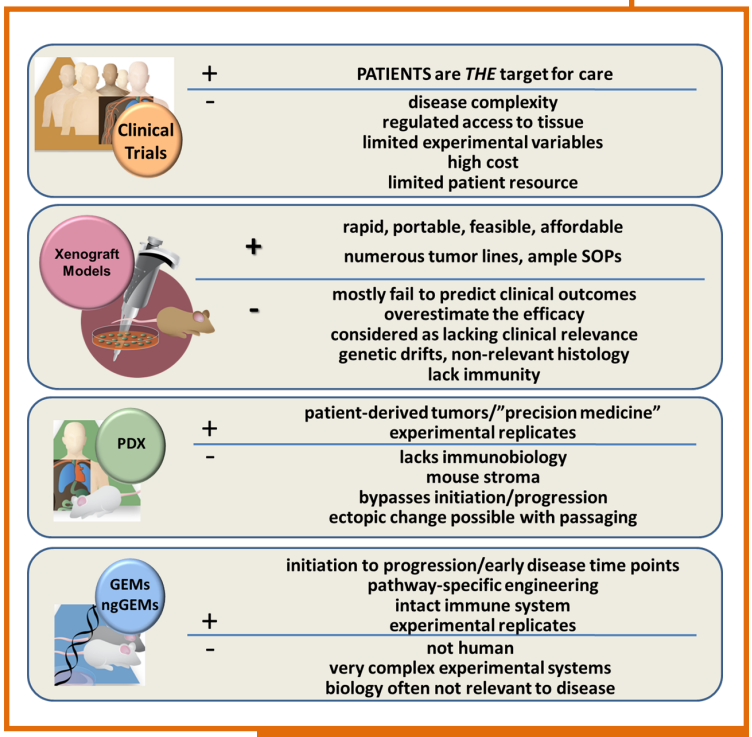

Figure 3. Comparative summary of cancer model types currently employed in preclinical evaluation vs. the clinical trials framework for oncology drug assessment. Various human-in-mouse grafted, mouse-in-mouse grafted and autochthonous/*de novo* models offer benefits for translational experimentation. All the while, featuring drawbacks limiting their applications and justifying integrated options of preclinical assessment in multiple relevant models.

assessment that should be highlighted when compared to the conventional established cell line-derived xenografts include, better preservation of original tumors' mutagenomes; the ability to mimic minimal residual and metastatic disease phases; and a faithful resemblance of therapeutic responses *vis-a-vis* those observed in parental tumors. Furthermore, the PDX models reveal histopathologic patterns and biomarker expression signatures closely approximating those of donor tumors. Also, they allow interactions between stroma or other tumor microenvironment components and the transformed tumor cells to be observed. Despite these advantages in employing PDX models for preclinical evaluation, several shortcomings should be mentioned limiting application of these models for broader use as a uniform testing platform. Mice bearing primary grafts of clinically obtained tissue specimens are immunocompromised – albeit efforts are underway in multiple organizations to reconstitute PDX recipient mice with a functional human immune system – thus largely excluding applications of PDX animals in the assessment of therapeutic strategies pursuing anti-tumor vaccination or activation of tumor immune surveillance mechanisms (e.g., immunomodulatory therapies). Furthermore, gradual passaging of PDX tumors, required to expand the pool of graft-bearing animals available for preclinical experimentation, is prone to substantial genetic and epigenetic drift, which is documented for several types of clinical malignancies. This is due to the fact that, although initially abundant at early passages, human stroma undergoes gradual replacement by its murine counterpart. This has the effect of disrupting the physiologic integrity of the tumor-stroma interaction and/or attenuating the signaling mechanisms required for sustained proliferation. The end result for the model is a misinterpretation of drug efficacy. Despite these challenges, as evidenced by rapidly growing interest and investments from multiple drug development organizations, PDX models have proven themselves as a superior predictive preclinical testing resource and are expected to gain further attention within the community of preclinical oncology experts.

Genetically Engineered Mouse Models

Genetically engineered mouse models (GEMMs), in the context of testing scientific hypotheses, have been extensively vetted as a strategy to elucidate a variety of biological mysteries, which range from developmental biology to mechanistic foundation of clinically challenging ailments. Albeit, it was not until recently when the GEMMs of oncogenic maladies started earning a widespread recognition as a predictive platform for assessment of cancer treatment options and discovery of novel diagnostic signatures, disease biomarkers, and promising drug targets. This could perhaps be best justified by the inherent complexity of cancer GEMMs, not infrequently requiring management of multi-allelic mouse inter-crosses and/or entailing implementation of tedious technologically complex workflows (e.g., inducing carcinogenesis by surgical application of infectious agents, monitoring tumor progression *in situ* via sophisticated imaging techniques, or statistically assessing

the whole gamut of disease histo-pathologic, cellular and molecular outcomes). However, once characterized and validated, the advantages of employing cancer-specific GEMMs for preclinical assessment are numerous. GEMMs provide virtually the only available experimental setting for cancer modeling that affords the cancer biologist and oncologists to monitor dynamics of autochthonous tumors from initiation through to late stage progression and metastatic spread. All the while, simultaneously capturing the disease's stochastic nature, molecular heterogeneity, and tumor-microenvironment interactions. Pending successful humanization of PDX models, the GEMM is, so far, the only experimental system featuring the presence of the fully intact immune system, an indispensable prerequisite for testing immunomodulatory therapies and anti-cancer vaccination strategies. Such models can be precisely engineered to activate a selected set of oncogenic drivers in a predefined cell sub-population or type, in the desired anatomic location. Finally, GEMMs could mimic important facets of cancer such as acquired drug resistance, incidence of minimal residual or metastatic disease, genomic instability, and heterogeneity. Although serving as a platform for numerous variables and multiple preclinical testing paradigms, genetically engineered mice remain undoubtedly the most laborious and expertise demanding preclinical asset. Of which, the application of GEMMs can be further limited by inconsistency in disease appearance, replicability, penetrance and latency, availability of robust colony management infrastructure, and the particular high-throughput options for genotyping and *in vivo* imaging. As a result, several dedicated and integrated Centers have been established. These Centers are tasked with developing optimized tractable strategies for preclinical assessment in GEMMs aimed at addressing these and other challenges impeding the broad application of GEMMs for preclinical drug development in oncology and other fields (e.g., autoimmune and neurodegenerative disorders). Such organizations are, not only expected to act as pivotal points of preclinical expertise, but are structured to offer contractual or partnership support to third parties as well as to be the hubs that disseminate best practices, optimized SOP's, and other resources. With the end goal of facilitating the application of cancer GEMMs for basic and translational purposes.

Non-Germline GEM and Syngeneic GEM-Derived Allograft Models

Despite the undeniable advantages GEMMs present for the preclinical drug evaluation arena; reaching the experimental throughput to match demand of drug developers and cancer translational biologists remains a formidable challenge. This is further amplified, today, by an almost exponential expansion of drug discovery pipelines propelling the demand for more robust preclinical assessment. This is particularly true for multiple promising and physiologically relevant models that display prolonged latency (e.g., in excess of one year from cancer disease initiation to detectable tumor), low penetrance, or significant attrition due to inconsistent or ectopic cancer incidence. A collection of

novel experimental approaches to model cancer disease in a more expedient, practical, flexible, standardized and ultimately cost-conscious way, designated non-germline GEMMs (ngGEMMs), has recently emerged and is gaining rapid adoption in both reputable academic labs and drug development organizations[57]. For example in one of the ngGEMM techniques, conventional GEMMs are bred to obtain preimplantation embryos that are converted into pluripotent embryonic stem (ES) cells, *ex vivo*, which contain the complete combination of desired oncogenic alleles (usually engineered as inducible mutations)[58]. The resultant ES cells undergo extensive genetic and karyotypic characterization prior to being employed for the production of chimeric animals according to well-established embryologic procedures. Such strategies afford the scalable, low cost maintenance of very broad portfolios of GEMMs to enable large synchronized experimental cohorts while simultaneously eliminating the need for costly step-wise interbreeding of multiple alleles and concomitant high volume genotyping. The end result is the models' improved clinical relevance[59]. Furthermore, in chimeric – but not in conventionally bred – models, a progeny of ES cells, genetically programmed for cancerous transformation, are intercalated into the hosts' embryo-derived tissue that lacks genetic alteration. Accordingly, this develops into non-pathogenic surrounding anatomic structures. This is to the contrary of oncogenic processes happening in tissues of conventionally bred animals, by which broad activation of oncogenic events in the entire target cellular subset or even whole tissue (e.g., the genetic field effect) result in either multiple "coalescing" lesions, not amenable to consistent longitudinal monitoring, or gives rise to overly aggressive tumors, limiting the therapeutic window beyond practicality. Some recently employed strategies utilizing modified ES-based chimeric ngGEMMs, have been used to rapidly assess systemically (i.e., in the context of the actual cancer disease) the biologic impact(s) of potential disease modifiers or putative drug target genes via targeted alteration of its expression in ES cells (e.g., using RNAi or CRISPR/Cas9 technologies) and subsequent tests of carcinogenicity *in vivo*[60]. The chimeric ngGEMM production technique carries only a few potential pitfalls that stem from intrinsic epigenetic instability of the pluripotent stem cells, risks of acquiring additional ectopic mutagenesis events, or undergoing loss of pluripotency in the course of ES passaging.

Yet another type of ngGEMM preclinical resource is referred to as mouse-in-mouse transplantation, or GEM-derived allograft (GDA), models. Construction of GDA animals entails dissection of cancerous tissues (either primary tumor or metastatic lesions, or even isolation of bloodborne CTC cells from murine circulation) and subsequent re-introduction of these cells – either as a dissociated single cell suspension, or as subcutaneously or orthotopically tissue fragments, – into a recipient mouse of identical genetic background[61,62]. Such syngeneic host animals, similar to conventional genetically engineered mice, harbor a fully intact immune system and thus are applicable for both investigation of

the immuno-oncology interface in cancer as well as testing of relevant IMT therapeutics. These GDA mice are generally characterized by a higher consistency and associated reproducibility in tumor appearance and histology, as well as shortened timeframe from implantation to development of enrollment-grade tumors ready for preclinical experimentation[63,64]. The dissociated cells derived from primary lesions can furthermore be genetically manipulated *ex vivo*, by established transfection or transduction techniques to, for example, visualize the grafted tumor or its derivative secondary metastatic lesions via expression of tracer markers such as fluorescent GFP/RFP proteins. Similar elegant approaches could be further extended to rapidly interrogate the functional implications of a suspected tumor modifier or candidate drugs' target genes with respect to their carcinogenic potential and/or sensitivity vs. resistance to pharmacologic challenges. This would be simply achieved via manipulating their expression level in tumor cells that will be subsequently tested in the GDA mice *in vivo*. **Figure 3** summarizes several of the aforementioned model types, also comparing them to conventional cell line-based xenograft models in a "strengths-weaknesses" format.

...efforts are underway in several organizations to assemble the proficient resources to advance the preclinical arena towards consolidated expertise in cancer disease modeling.

Conclusions and Future Directions: Integrated Strategies for Informative Preclinical Assessment in Predictive Animal Models

A common belief shared by a majority of the mouse modeling experts suggests that there is no "ideal" or "perfect", one-size-fits-all cancer model type. Or more specifically, that no single strategy of engineering the oncologic disease in mice will allow unambiguous and adequately granular recapitulation of all aspects of clinical malignances to facilitate straightforward predictions of disease progression path or deduction of unequivocally failure-proof treatment plans. To the contrary, an integrated multidisciplinary approach enabling simultaneous assessment of multi-dimensional data sets gathered from different cancer models that are subject to a battery of experimental assays presents itself as the most promising avenue in guiding clinical development and is strongly advocated for by preclinical science professionals. Although challenges still persist in identifying the best-fit robust, while sufficiently reproducible and portable, experimental frameworks. And more importantly, frameworks satisfying the unmet need criteria of the oncology field and attuned to current rigorous trends in precision medicine. Luckily, efforts are underway in several

organizations to assemble the proficient resources to advance the preclinical arena towards consolidated expertise in cancer disease modeling. The ultimate package of deliverables from such coordinated activities (e.g., pursued at the NCI Center for Advanced Preclinical Research, see https://ccr.cancer.gov/capr-about for further information) is anticipated to include collections of best practices and standard operating procedures; information on optimized materials, reagents, instrumental base, partnership business models and intellectual property mechanisms; and access to integrated enterprise quality information systems designed to accumulate, warehouse, evaluate, share and disseminate the full spectrum of preclinical data from multiple sources. But above all, such initiatives will offer tutelage and access (and whenever applicable or justified, sponsorship) to experimentally validated portfolios of preclinical modeling resources. Resources, of which, have been carefully selected to support flexible testing for the variety of novel diagnostic approaches, disease outcome monitoring and assessment methodologies, or improved oncology therapeutics. It is also both reasonable and enticing to argue that the current and projected progress in application of translational cancer models for preclinical drug development will galvanize and pave the way for collinear efforts in other clinical arenas – such as neurodegenerative or cardiovascular diseases, inflammation, and autoimmunity – to produce a similar toolkit of methodologies that explore relevant preclinical murine models for devising better treatment options.

...such initiatives will offer tutelage and access to experimentally validated portfolios of preclinical modeling resources.

Multiscale Modeling and Simulation to Guide Rational Nanomaterials Design

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Over the last decade, new nanomaterials, devices and systems have been developed for the diagnosis, imaging and treatment of multiple malignancies[21,65,66]. Nanoparticles with different geometrical and physico-chemical properties have been engineered, loaded with multiple agents, and systemically administered for the detection and treatment of primary and metastatic tumors[67,68]; nano/micro-fluidic chips have been presented for the rapid screening of potential medications and for the identification of cancer biomarkers[69,70]; and miniaturized devices have been designed for molecular imaging on patient-derived histological samples[71]. Although most of these nano-systems are developed following rather empirical approaches, mathematical modeling and computer simulation, over multiple biophysical scales, are crucial in understanding their *in vivo* behavior and optimizing their performance for clinical translation. As computational sciences have already had a profound impact across multiple disciplines of science and technology development, 'Computational Nanomedicine' could have an equally pervasive impact in our ability to rationally engineer novel and more efficient nanostructures, nanodevices, and nanomaterials for biomedical applications. Current efforts and future perspective in this field are discussed briefly below and in order of biophysical scale, from large to small.

Whole-animal scale modeling.

Multi-compartment mathematical models are now extensively used to understand, predict and compare, the *in vivo* pharmacokinetics (PK) of therapeutic and imaging agents[72]. In particular, based on anatomical and biological information, these models divide the whole-body in multiple compartments, which are interconnected via specific transport and adsorption parameters. Since PK models have been successfully applied for estimating the organ-specific absorption, distribution, and excretion of systemically injected small molecules; similar approaches are now being established for the biodistribution of nanoparticles (NPs). However, the predictive power of these PK models is still quite limited by empiricism and the lack of mechanistic information on the organ-specific deposition and sequestration of NPs.

Most recently, compartment-based models have been adopted for predicting the blood concentration of cancer biomarkers[73]. These models are extremely relevant to early cancer detection and aim at elucidating the correlation between blood biomarker concentration

and tumor size. Unfortunately, clinical data are not generally available to address such a question, thus this is an area where mathematical modeling can be helpful. Specifically, using a one-compartment model integrated with a conventional tumor growth law, it was possible to estimate the blood concentration of tumor biomarkers over time (**Figure 4**). Based on published data on ovarian carcinoma and considering CA125 as a tumor biomarker, the model computed that 8 years are required in order to detect a continuously growing malignant mass with the currently available clinical tools. These computational models clearly emphasize the need for developing more sensitive detection techniques, but also imply that increases to the blood concentration of biomarkers for facilitating earlier detection are necessitated[74].

Tumor and single-organ scale modeling.

Sophisticated multi-scale and multi-physics computational models have been developed for predicting the response of malignant masses to different treatments, including molecular and nano-based therapies as well as radiation and thermal ablation interventions[75]. These models have similarly been used for understanding and optimizing the vascular transport and tumor accumulation of NPs[76,77]. In particular, using an immersed finite element method, the vascular distribution of NPs was studied in whole blood (**Figure 5**). These computer simulations, supported by experimental intravital microscopy data, demonstrated that small NPs (≤ 100 nm) tend to distribute quite randomly within capillaries without interacting with red blood cells. Inversely, large NPs (> 500 nm) preferentially accumulate next to the vessel walls, in a size-dependent manner. This data suggests that sub-micron particles could be more efficiently employed for targeting the diseased vasculature as compared to conventional 100 nm NPs, whose tumor accumulation is primarily driven by the Enhanced Permeability and Retention (EPR) effect. Still focusing on the vascular deposition of NPs, computational models have been developed to predict the accumulation of systemically injected NPs in the tumor neovasculature[77]. By combining a mesoscale model for the vascular adhesion of NPs with a multi-dimensional tumor growth model, it was predicted that the fraction of NPs accumulating in the malignant tissue depends only on the vascularity. Additionally, it was observed that a moderate NP affinity for the tumor endothelium provided the optimal balance between spatial distribution and absolute tumoritropic accumulation. Clearly, this is another example where multi-scale

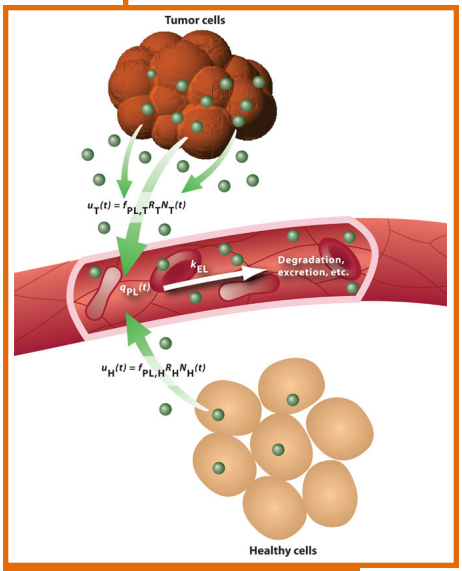

Figure 4. One-compartment model for plasma biomarker kinetics (*Reprinted with permission from Hori and Gambhir, 2011*)[73].

and multi-physics mathematical modeling provides input for rationally engineering NPs with enhanced tumoritropic accumulation.

Computational models can also be used to directly compare the therapeutic efficacy of a single bolus injection of drug molecules with an equivalent dose administered via NPs[78]. By modeling the interplay between mass transport in the microvasculature and blood perfusion in the extravascular volume, computer simulation allowed prediction of interstitial drug concentrations, rates of metabolization, and fractions of cell killing over time. These studies concluded that, for an equivalent injected dose, nano-based treatments ensure higher intratumor drug accumulation and longer exposure times as compared to single bolus injections, thus resulting in higher apoptotic indexes.

Cell and single nanoparticle scale models

Mathematical modeling has been fundamental in elucidating the biophysical mechanisms regulating NP transport dynamics within the vasculature and via internalization into cells[80]. For instance in vascular adhesion, numerical simulations suggested that oblate spheroidal particles would more avidly adhere to the vessel walls as compared to spherical particles of identical volume[81]. Also, mathematical models demonstrated that NP size and shape play a crucial role in modulating cellular endocytosis[82,83]. More recently, computational models for NP cell uptake and drug release were developed to characterize the multi-drug resistance in cancer cells[84]. Supported by experimental evidence, these models revealed that NP-mediated delivery increases both the total concentration and temporal exposure of chemotherapeutic molecules to the target cells. As a consequence, the respective IC_{50} values were improved upon as compared to free drug molecules.

Mathematical models can also be directly used to improve the performance of nanomaterials. For instance, by using molecular dynamics simulation, the diffusion of molecules within nanoporous structures, around nanoparticles, and proteins can be studied (**Figure 6**). Following this approach, the magnetic resonance imaging performance of mesoporous particles loaded with iron oxide NPs and Gd-macromolecules was predicted and optimized for future clinical use[79].

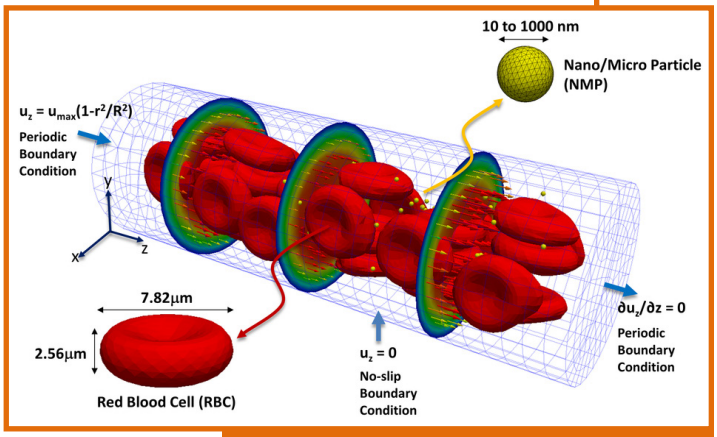

Figure 5. Modeling the transport of NPs into whole blood (*Reprinted with permission from Lee et al, 2013*)[76].

Future perspectives

'*Computational Nanomedicine*' could play a major role in facilitating and accelerating the clinical translation of nanotechnologies and in enabling what is often referred to as precision medicine. At the individual NP level, molecular dynamics simulation can be used to engineer NPs with new architectures enhancing the loading efficiency of drug molecules and contrast agents. This will allow us to reduce the injected doses and limit potential side effects; to improve upon imaging contrast agents for early disease detection; and enable combination therapies (i.e., polypharmacy) to be more rapidly correlated to efficacy. At the cell scale, mathematical models are needed to elucidate the role of thermal ablation therapies and mechanical stresses on cell proliferation and drug resistance. At the organ level, more sophisticated models of tumor growth. Those which account for the spatio-temporal heterogeneity of malignancies, occurrence of *de novo* and acquired drug resistance, presence of tumor initiating cells, and tissue deformability, known to modulate cell growth and migration, will have to be developed. The integration of cell scale and tumor growth models will help us designing new intervention strategies, where diseased cells and tumor microenvironment are coupled for synergistic and efficient targeting. Finally, more efforts should be devoted in developing truly multi-physics and multi-scale computational PK models for predicting patient-specific biodistribution of NPs. These mechanistic PK models should be derived by the hierarchical integration of cell/organ level mesoscopic models with conventional schemes for pharmacokinetic analyses. In this effort, the contribution of multi-modal imaging data will be crucial in the validation phase as well as in the actual clinical utilization for acquiring patient-specific information to be fed back into the computational models. In a near future, mechanistic PK models will help doctors to identify *a priori* the optimal *4S* – size, shape, surface properties and mechanical stiffness – NP properties for maximizing tumor accumulation; and the proper combination of therapeutic agents for eradicating the disease in each individual patient, allowing for eventual realization of 'precision medicine.'

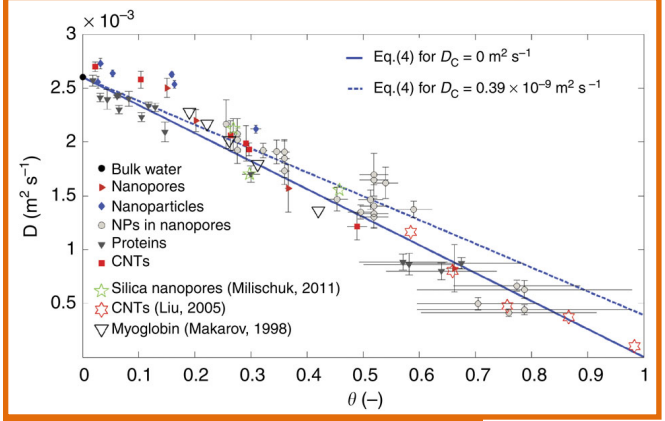

Figure 6. Molecular dynamics representation of a silicon nanopore containing iron oxide nanoparticles, a single walled carbon nanotube, a green fluorescence protein (top). Correlation between the diffusion coefficient of water molecules *D* and a geometrical parameter *Θ* (*Reprinted with permission from Chiavazzo et al, 2014*)[79].

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