Toward Correlation in *In Vivo* and *In Vitro* Nanotoxicology Studies

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Introduction

Much of the focus of the published 2011 symposium that inspired this work focused on the question, "When have you reduced risk enough to move from bench/animal studies to 'first in-human' studies?" Building applied research ethics related to nanotherapeutics requires bench and clinical scientists to have a clear vision about how to test nanotherapeutic safety, and it is clear that there is still much to be considered at the steps before "in-human" assessment. Herein, the perspective of the bench scientist is brought to bear on using *in vivo* and *in vitro* models to assess the safety of nanotherapeutics. Much of this work falls under the purview of the field of nanotoxicology that aims to understand the toxicological impact of engineered nanoscale materials. Engineered nanomaterials include a wide variety of materials that are manipulated and controlled on the nanoscale level where, typically, the nanoparticle or nanomaterial has some dimension that is less than 100 nm. These materials are of interest for a wide variety of applications, including biomedical, due to the emergent properties of the materials, where emergent properties refers to the physical and chemical characteristics that are distinctive from both those of the atoms/molecules and the bulk of the same material.

The burgeoning use of nanoscale materials for biomedical applications has yielded many promising technologies for the treatment and diagnosis of diseases, particularly cancers. The development of nanotechnology as disease therapy agents, or nanotherapeutics, has accelerated because of their potential use as drug delivery vehicles. Preliminary evidence shows that nanotherapeutics efficiently traffic to the sight of treatment (e.g., a tumor) via enhanced permeability and retention and deliver a pharmaceutical payload with minimal side effects.[1] Additionally, nanoparticles can be functionalized in many different ways to simul-

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taneously enable drug delivery and imaging, propelling the personalized medicine trend forward.[2] Currently, there are 33 products that could be classified as nanotherapeutics approved for use by the Federal Drug Administration, with hundreds more in various stages of clinical trials.[3] With the development of nano-sized therapeutics, both the expanded use and the unique properties of nanomaterials, regulatory agencies are now faced with decisions regarding the regulation of such novel technologies.

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The FDA has begun to grapple with the regulatory implications of nanotherapeutics and now includes a nanoparticle size disclosure as an optional part of the approval process.[4] However, there are ongoing arguments about whether or not nanoparticles require different regulation to ensure safe use of these products or if the current mechanisms will be sufficient. This consideration is complicated by the ambiguity associated with nanoparticle characterization (e.g., how the size, surface reactivity, etc., of the nanoparticle is characterized).[5]

Guidance from regulatory agencies is especially lacking at the pre-clinical stages of nanotherapeutic development, in which a suite of *in vivo* and *in vitro* assessments must be carried out for products progressing from pre-clinical to clinical trials. *In vivo* studies are whole organism studies where nanotherapeutics are delivered via one of four pathways: inhalation, dermal, ingestion, or injection. Using a variety of techniques, typical *in vivo* assessments include the determination of physiological localization and the concentration of material in specific tissues, rate of excretion, and macroscopic tissue and organismal toxicity. *In vitro* assessments are the study of cells, either isolated from animals or an immortalized cell line, in a culture dish. In general, the use of primary cells (isolated directly from animals) will give a more realistic toxicity result because immortal cell lines transform over time; however, the use of immortal cell lines is often preferred simply because they do not require animal sacrifice. There is an abundance of *in vitro* assay options,[6] many of which allow researchers to probe a nanoparticle's mechanistic interaction with cells, that are fast and inexpensive to enable high-throughput cellular analysis. To be clear, *in vivo* and *in vitro* studies each have limitations (e.g., expense and dosing, respectively).[7] However, as both *in vivo* and *in vitro* studies provide necessary, and often complementary, information regarding the action of nanoparticle therapeutics, both *in vivo* and *in vitro* guidelines informed by oversight bodies have the potential to optimize technological progress.

Bench scientists are at the forefront of designing and creating new nanomaterials and are being pushed to assess the interaction of nanomaterials with cells, tissue, and organisms in pre-clinical studies, though these areas are outside their expertise. While contentious debate continues about whether new or additional regulation is required for nanotechnology, clear oversight guidelines will provide guidance for scientists in pre-clinical studies toward the type of toxicity testing and model systems that would enable quick and safe development of a products.[8] The caveat to implementing new regulation beyond the current oversight is that nanoscale therapeutics must initiate a clear and unique toxicity response, where a unique response is considered to be a cellular or organismal response that has not been observed in previous toxicity studies with exposures to molecules. Herein, following the four potential routes of biomedical nanoparticle administration (i.e., inhalation, dermal, ingestion, and injection), we examine the literature to correlate pre-clinical nanotoxicology studies where *in vivo* and *in vitro* testing is employed to determine any distinctive toxicity characteristics that should be considered in the oversight of nanotherapeutics. Due to the complementary results yielded from *in vivo* and *in vitro* studies, correlating the results enables a deeper understanding of the mode of nanoparticle toxicity so that nanoparticles can be designed for optimized disease treatment and minimal unintentional toxicity. Additionally, through these correlations, there is the potential to simplify pre-clinical evaluations because results from *in vitro* studies may enable generalization of the *in vivo* toxicity response, thus reducing time and cost of developing highly effective therapeutics by eliminating some of the animal testing. Figure 1 provides examples of results from *in vivo* and *in vitro* studies that are explored below. Within this comparison, *in vivo* studies are those where animals are exposed directly to nanoparticles, though cells/tissue may be extracted for analysis, whereas *in vitro* studies are those where the nanoparticle exposure is performed directly to isolated cells in a Petri dish. For clarity, this comparison is lim-

ited to nanotherapeutic drugs, rather than including devices and combination products.

Correlation of *In Vivo* and *In Vitro* Toxicity

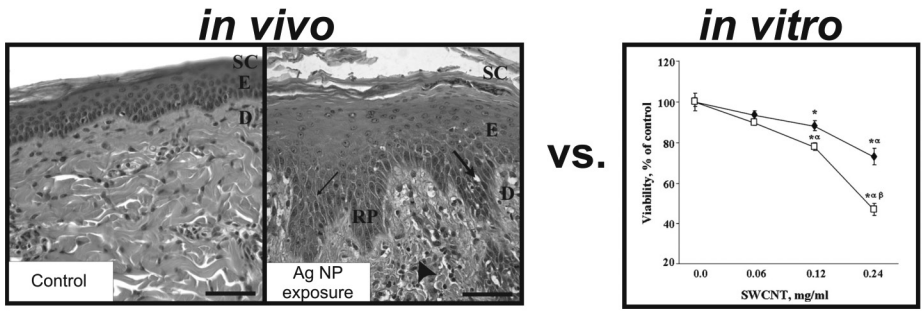
Inhalation

Currently, there is no nanotherapeutic on the market that has an inhalable delivery mechanism; however, there are a number of products in various stages of clinical trials for such diseases as bronchiolitis (i.e., severe airway damage/inflammation)[9] or lung tumors,[10] and it is conceivable that the treatment of lung diseases like asthma could include a nanoparticle-loaded inhaler. Though literature precedent is lacking regarding inhaled nanotherapies, there has been extensive work aimed at understanding the *in vivo* and *in vitro* correlation of inhaled nanoparticles from the occupational health perspective.[11] *In vivo* studies employ a variety of model animals, but primarily have focused on mice and rats, exposing the animals to varying concentrations of various nanoparticles using either the instillation (lung entry via the throat) or inhalation (lung entry through the nasal passage) mode of nanoparticle introduction. For *in vitro* studies, researchers commonly use the immortal (i.e., self-propagating) human lung cell lines A549 or BEAS-2B.[12]

While *in vitro* assays modeling inhaled nanoparticle toxicity use a wide variety of assays, *in vivo* studies focus on extracted bronchoalveolar lavage fluid (BALF), or fluid that is retrieved from the lungs, after a nanoparticle exposure, examining molecular markers of oxidative stress and/or inflammation. Often, markers measured within the BALF can be directly measured and compared to the same marker in the *in vitro* assays.[13] During oxidative stress, generated free radicals, or reactive oxygen species, overwhelm the system's innate ability to cope, which potentially leads to tissue damage. For example, Horie et al. studied instilled nickel oxide nanoparticles on rats and demonstrated that there were elevated levels of hydroperoxy octadecadienoate (tHODE), an indicator of oxidative stress within the lungs. This increase in tHODE was correlated to elevated levels of oxidative stress *in vitro* over a similar time (~24h) of exposure.[14] Warheit and coworkers demonstrated a similar correlation of *in vivo* and *in vitro* oxidative stress upon exposure to zinc oxide, though the magnitude of the oxidative stress response *in vitro* was smaller than *in vivo*;[15] this discrepancy may be a result of the difficulty in equating *in vitro* and *in vivo* dose. Biochemical markers for other lung cell/tissue damage can also be studied in the BALF, as was done by Nel and co-workers to identify fibrosis (i.e., lung damage) *in vivo* and *in vitro*.[16] Beyond direct comparisons of similar markers within the BALF after *in vivo* nanoparticle exposure and *in vitro* assays, *in vivo* studies also commonly utilize histology (see histology example in Figure 1 *in vivo* results) to examine tissue damage and compare

Figure 1

Examples of *in vivo* (histology) and *in vitro* (viability) toxicity data. *In vivo:* histological examination of porcine skin after application of silver nanoparticles (Ag NP). Abbreviations within image refer to parts of the skin that are measured as part of the histological analysis (SC-stratum corneum, E-epidermis, D-dermis, and RP-rete peg). Arrows and arrowheads indicate tissue damage (large arrows-intracellular epidermal edema, small arrows-focal areas of intracellular epidermal edema, arrowheads-perivascular inflammation). Adapted and reprinted with permission from reference 21. *In vitro:* measurement of murine epidermal cell viability after exposure to single-wall carbon nanotubes (SWCNT) as measured with the Alamar Blue assay. The SWCNT, partially purified (black circles) and unpurified (white squares), cause a dose-dependent decrease in viability. *p<0.05 vs control, $^{\alpha}$p<0.05 vs 0.06mg/ml SWCNT, $^{\beta}$p<0.05 vs 0.12mg/ml SWCNT. Reprinted with permission from reference 22.



to various *in vitro* assays that measure cell viability and/or markers of inflammation that would cause the observed histological damage.[17] The same biochemical markers are measured using similar methods (e.g., BALF and histology) to assess inflammation and oxidative stress upon exposure to inhaled molecular therapeutics.[18]

Dermal

As in the inhalation nanotherapies, there is yet to be a FDA-approved dermal or transdermal nanotherapeutic, though there are many commercially available products that utilize topical application of nanoparticles, namely sunscreens and cosmetics, which typically use nanoparticles for UV light protection, and wound dressings that use antibacterial nanoparticles. Porcine skin is the most common skin model used to test both traditional and nanoparticle products for human use and therefore, *in vivo* studies commonly use pigs. *In vitro* cell lines to model skin cells are much less common, with most studies isolating intact skin (i.e., an *ex vivo* model).[19] Alternatively, some studies that correlate *in vivo* to *in vitro* dermal toxicity make use of cell lines unrelated to the epidermis and assume generality across cell types.[20]

Histological investigation is the most common *in vivo* method for assessing toxicity, in all exposure pathways, and this is true for dermal nanoparticle studies. Skin characteristics monitored in the histological analysis after topical nanoparticle exposure have included skin thickness, abnormal tissue damage, and the presence of inflammatory lesions. Nancy Monteiro-Riviere and coworkers examined edema and erythema, signs of inflammation, upon *in vivo* exposure of pigs to nano-silver and correlated the response to inflammatory biomolecules generated by a human skin cell line *in vitro*. Results revealed that there was microscopic evidence of inflammation after 14-day exposure *in vivo* and this correlated with inflammation biomarkers after 24 h silver nanoparticle *in vitro* exposure.[21] Similarly, Ashley Murray et al. related the indicators of oxidative stress and inflammation *in vitro* (e.g., interleukin secretion and free radical generation) to increased levels of cell types *in vivo* that are known to migrate to sites of inflammation.[22] While these studies show correlation of an inflammatory response *in vivo* to *in vitro*, another study using silver nanoparticles has indicated minimal skin irritation *in vivo*.[23] The varied results shown between studies are likely a result of the parameters of the experiments such as length of exposure, nanoparticle dose, and nanoparticle surface chemistry. Similar disparities in toxicity results are found when exposure time and dose are varied for dermal application of molecular therapeutics, which commonly use similar models and evaluation criteria.[24]

Ingestion

Ultimately, the development of oral therapies to treat chronic disease, as an alternative to injected therapies, could be greatly facilitated by nanotechnology as nanoparticles lengthen the stability of drug molecules within the digestive system and enable favorable drug absorption in the intestines.[25] However, there again is no FDA-approved oral nanotherapy, though there are consistently more and more studies working to understand the relevant *in vivo* and *in vitro* toxicology correlation for this delivery mechanism. Models for *in vivo* study of ingestion are highly variable, from typical research rodents (i.e., mice and rats) to primates, but many *in vitro* studies utilize the immortalized colon cancer cell line known as Caco-2.[26]

While research to understand the toxicology of ingested nanoparticles is being actively pursued, there is no consistent assay or method used at this point. Some *in vivo* studies explore the distribution and localization of particles throughout an organism after oral nanoparticle exposure. For example, Michael Shuler and coworkers examined the distribution of iron from ingested iron-polymer composite nanoparticles in chickens and correlated iron amounts to *in vitro* iron transport and uptake within Caco-2 and other immortal cells.[27] In another example, Brice Moulari et al. demonstrated localization of aminosalicylic acid-coated silica nanoparticles within inflamed colon regions of a colitis mouse model and observed the therapeutic effect the nanoparticles had on the colitis-induced inflammation. However, the parallel *in vitro* studies only examined cell viability and therefore make toxicity correlations difficult.[28] One example of a better toxicity correlation for orally administered nanoparticles is work done with a polymer nanoparticle drug delivery vehicle for doxorubicin, a common chemotherapy agent.[29] In this study, the drug-loaded nanoparticles administered orally caused reduction of breast tumors in rats and similarly caused a decrease in Caco-2 cell viability in an *in vitro* assay, showing that these nanoparticles influence cancer cells in general rather than breast cancer cells specifically.[30] While both inhaled and dermal application of therapeutics are likely to act locally, ingested therapeutics must survive both the digestive system and be successfully distributed after absorption; this inherent difference makes nanoparticles especially promising but also make toxicity considerations significantly more complicated. So

far, the most likely difference between orally administered molecular and nanoparticle therapeutics lies in the excretion routes which, in either the molecular or nanoparticle case, can only be accurately assessed using *in vivo* studies.

Injection

The most widely investigated exposure pathway for nanotherapeutics is injection, and all 33 currently approved FDA nanomedicines fall in this category.[31] Nanoparticle injectables have been explored because they could potentially eliminate the negative side effects of traditional injectable drugs, particularly chemotherapy, where solubility and stability of drug molecules limit their use. In fact, most of the approved nanoproducts are aimed at cancer diagnosis or therapy as are many injectable nanomaterials in development. Therefore, many of the models for *in vivo* injection nanotoxicity are implanted cancer cells to stimulate tumor development (in a variety of animals), and *in vitro* studies generally use cancerous cell lines. Though it is possible to use similar cancerous cell lines as those implanted to create the *in vivo* tumor, few studies take this route.[32] Some *in vitro* work has examined primary culture cells from blood, such as platelets or red blood cells, to determine blood compatibility.

Since a major aim of injectable nanotherapeutics has been to treat cancer (i.e., be toxic to cancer cells directly or through targeted release of a drug payload), much of the *in vivo* toxicity assessments focus on the nanoparticle uptake into tumors followed by various characterizations of the progression of cancer (e.g., measuring tumor volume) along with the systemic biodistribution of administered nanoparticles to assess clearance and potential sites of unintentional toxicity.[33] These *in vivo* studies are correlated with *in vitro* viability assays that assess the percentage of cells, often cancer cells, that survive nanoparticle or drug-loaded nanoparticle exposure. For example, Harikrishna Devalapally et al. assessed tumor suppression in mice after intravenous administration of polymer nanoparticles loaded with tamoxifen and paclitaxel, FDA-approved molecular cancer treatments, and correlated a decrease in tumor size with *in vitro* studies of decreased cell viability with the same cancer cells used to implant the tumors. Sunil Singh et al. also examined nanotoxicity of nanoparticles after injection, though aimed at understanding unintentional consequences.[34] After injection of graphene oxide nanoparticles, *in vivo* pulmonary thromboembolism (i.e., damage due to clotting) was histologically observed in the lungs of mice, which correlated *in vitro* to a decrease in blood compatibility.[35] Injectable nanoparticles are the most advanced of the four therapeutic routes of administration, and accordingly, the effort to correlate *in vitro* and *in vivo* results are the most advanced and have been the most successful because of the aim to decrease cancer viability. As with molecular therapeutics, there is a distinct advantage because it is possible to draw human blood and assess blood compatibility without harming the research subject or having to use an animal model. Like orally administered nanotherapeutics, injectable nanoparticles may differ from molecular therapeutics in the excretion routes available; this possibility will have to be investigated using *in vivo* studies and will be an important part of in-human trials.

Based on the current state of the literature, it seems that, so far, there are no unique biomarkers or characteristics of nanoparticle toxicity, and thus, we see no justification for novel regulatory procedures at this point. The most likely candidate for distinct behavior lies in the fact that intact nanoparticles will likely be excreted through different routes than their molecular counterparts; this should be investigated systematically using appropriate animal models and then verified when first in-human trials are performed.

Conclusions and Perspective

Since that growth of nanotoxicology as a discipline (circa 2004), which has origins in the field of particle toxicology, there has been great concern that the emergent properties of engineered nanomaterials would cause novel biological responses upon exposure. Most would argue there are challenges in understanding nanoparticle toxicity that arise from the characterization of nanomaterials, both pre-treatment and during exposure to biological environments.[36] For example,

nanomaterials have been shown to elicit a different level of toxicity based on their size, but the molecular complexity of a biological environment can influence effective nanoparticle size. Based on studies in simulated biological environments, the nanomaterials are often transformed so that the effective size, or the size the cells or organism sees, is larger than originally intended.[37] Other characteristics, such as adsorbed molecules on the nanoparticle surface[38] and material integrity,[39] are also easily transformed within the body and have a significant influence on toxicity. The gap in measurement technology that makes dynamic, *in situ* measurements of nanoparticle characteristics currently impossible has been a great impediment to understanding and correlating *in vitro* and *in vivo* toxicity results.

The question still remains whether nanoparticles cause a unique biological response that should inform our regulatory actions. In examining the correlation between *in vivo* and *in vitro* studies above, there is yet to be evidence that the body's toxicity response to nanoparticles is different than other molecular toxicants or larger, micron-sized, particles that are already approved by the FDA. One agreement that seems to arise from these comparisons is that oxidative stress and/or inflammation can be correlated between Petri dish and whole organism studies, but this is also seen with other non-nanoparticle toxicants/therapeutics.[40] While nanotherapeutics have not yet induced a unique toxicity response, the lack of novelty may be an artifact of the discrepancies between *in vivo* and *in vitro* nanotoxicity comparisons, such as the dose and duration of nanoparticle exposure or the differences in model systems (i.e., cancerous cell lines versus healthy animals). That is, there may be nuanced toxicity modes induced by nanoparticles that are not observed because the dose *in vitro* is not relevant *in vivo* or the *in vitro* cancer cell line does not behave in a way that mimics a whole organism. These discrepancies are exacerbated by the fact that the field is relatively new. Many of the literature studies currently available, including some highlighted here, are aimed at showing promising results of a particular nanotherapeutic and therefore only perform standard preclinical toxicity evaluations. Generally, the standard toxicity evaluations do not attempt to achieve *in vivo* and *in vitro* correlation and are not aimed at elucidating mechanisms of nanoparticle toxicity. Clearly, more fundamental studies on this topic, for both nanoscale and molecular therapeutics, would benefit the field of toxicity at large.

It is clear that we, as bench scientists, are still struggling with the basic science in defining nanotoxicity and have left many gaps in correlating *in vivo* and *in vitro* data. However, striving for correlation among *in vivo* and *in vitro* data to achieve a better understanding of toxicity is not novel to nanoparticle toxicants. Molecular toxicology has been grappling with similar problems and have been working on solutions that could inform regulation.[41] This supports the idea of a "new toxicology" that has been introduced by Martin Philbert and coworkers, whose work is speaking directly to emerging, sophisticated materials,[42] but applies to molecular therapeutics as well. Based on the current state of the literature, it seems that, so far, there are no unique biomarkers or characteristics of nanoparticle toxicity, and thus, we see no justification for novel regulatory procedures at this point. The most likely candidate for distinct behavior lies in the fact that intact nanoparticles will likely be excreted through different routes than their molecular counterparts; this should be investigated systematically using appropriate animal models and then verified when first in-human trials are performed.

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