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3-D tissue culture systems for the evaluation and optimization of nanoparticle-based drug carriers

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Abstract

Nanoparticle carriers are attractive vehicles for a variety of drug delivery applications. In order to evaluate nanoparticle formulations for biological efficacy, monolayer cell cultures are typically used as *in vitro* testing platforms. However, these studies sometimes poorly predict the efficacy of the drug *in vivo*. The poor *in vitro* and *in vivo* correlation may be attributed in part to the inability of two-dimensional cultures to reproduce extracellular barriers, and may also be due to differences in cell phenotype between cells cultured as monolayers compared to cells in native tissue. In order to more accurately predict *in vivo* results it is desirable to test nanoparticle therapeutics in cells cultured in three-dimensional models that mimic *in vivo* conditions. In this review we discuss some 3-D culture systems that have been used to assess nanoparticle delivery and highlight several implications for nanoparticle design garnered from studies using these systems. While our focus will be on nanoparticle drug formulations, many of the systems discussed here could, or have been, used for the assessment of small molecule or peptide/protein drugs. We also offer some examples of advancements in 3-D culture that could provide even more highly predictive data for designing nanoparticle therapeutics for *in vivo* applications.

Introduction

Delivery of therapeutic agents using nanoparticulate formulations offers a number of advantages over delivery of the agents alone. For this reason, both small and macromolecular drugs are often incorporated into nanoparticle vehicles. These vehicles can potentially enhance drug efficacy by incorporating adjuvants, targeting agents, and multiple therapeutics within one carrier. The nanoparticles can also offer protection against degradation of therapeutic agents as well as facilitate the delivery of otherwise insoluble agents. An additional advantage is that the pharmacokinetic profile of drugs can be favorably altered by controlling the size and surface chemistry of the nanoparticle delivery vehicles. However, the significantly increased size of nanoparticle carriers compared to their therapeutic payload generally results in reduced tissue distribution after extravasation from the circulation. There are several therapeutic applications that benefit from efficient tissue penetration of the delivery vector. For example, some vectors, such as non-viral nucleic acid delivery vehicles that assist in intracellular trafficking, would ideally remain intact until internalization by the maximum number of target cells. For such systems, a key criterion for *in vivo* efficacy is that nanoparticle carriers must be able to overcome the various extracellular barriers encountered after extravasation to reach target cells.

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Currently, initial evaluations of nanoparticulate delivery formulations are usually completed in monolayer, or 2-D, cell culture systems. Often times, successful nanoparticle delivery observed in 2-D cell culture studies does not translate to similar results *in vivo*. This is in part due to limitations in monolayer cultures that fail to account for extracellular barriers that are present *in vivo* (1-3) (Fig 1). While monolayer cultures produce extracellular matrix materials, they are less dense and incomplete on the monolayers apical side compared to cells in a 3-D environment (4-7) and thus present a less significant barrier for transport and cell-binding of delivered agents compared to cells in 3-D. Thus, whereas nanoparticles delivered in a bulk solution to a 2-D cell culture typically reach and bind to cells relatively unimpeded, the same nanoparticles delivered *in vivo* will be hindered by extracellular factors such as the relatively small pore sizes within the extracellular matrix (ECM) (8) and the tortuosity of the interstitium. The surface properties of the nanoparticles can also lead to interactions with charged ECM components, such as proteoglycans, thereby affecting the fate and stability of nanoparticles (9,10). In addition, cell monolayer studies are typically performed under static culturing conditions, whereas living tissues are mechanically dynamic systems and are constantly subjected to mechanical forces such as tension, compression and interstitial fluid flow. The direction and magnitude of fluid flow can alter the bioavailability of the delivery vehicle by redirecting nanoparticles in the direction of flow and potentially away from the target sites.

Cell culture conditions also affect the phenotype of cells and thereby affect the cellular response to delivered drugs. For example, hepatocytes lose some liver-specific functions, such as albumin production, when maintained under monolayer single cell culture conditions but retain the functions when cultured in 3-D perfusion models (11,12) or when co-cultured with supporting cell types such as fibroblasts (13-15) or epithelial cells (16,17). These phenotypic differences may be due in part to cells residing in materials that are more compliant than on 2-D plastic substrates typically used for monolayer cultures (18-21). Cell phenotype is also determined by interactions between cells and the supporting extracellular matrix (ECM), as well as cell-cell communication that results in changes in biological activities, including gene and protein expression, proliferation, migration, remodeling and signal transduction (13, 22-25). Cells in 2-D monolayer cultures are generally exposed to a uniform environment under culture medium whereas cells in tissues, particularly in solid tumors, are exposed to gradients of pH, nutrients, and waste products that exert both stimulatory and inhibitory influences on proliferation and malignant properties of the cells (26). Furthermore, multicellular drug resistances can result from conditions in the 3-D tissue microenvironment that may not be present in monolayer cultures, such as the occurrence of hypoxic tissue regions, altered cell proliferation, and poor availability of delivered drugs in deeper tissue layers (27).

For these reasons, researchers are utilizing and developing 3-D culture systems with the goal of more closely mimicking the human clinical setting. Realistic and controllable *in vitro* 3-D tissue culture systems that more closely mimic *in vivo* human tissues in terms of functionality, morphological and mechanical architecture could be applied during the development and evaluation of delivery vehicles to offer a more predictive platform for assessing *in vivo* delivery efficiencies. Such systems can serve to bridge the considerable gap between traditional 2-D monolayer experiments, animal studies and clinical trials since nanoparticle formulations can be more effectively optimized in these 3-D *in vitro* models (28).

In this review, we will summarize the evaluation of nanoparticle carriers in several prevalent 3-D cell culture models currently used in nanoparticle delivery studies. In addition, we will highlight several advanced 3-D systems designed to account for additional factors in the complex biochemical and biomechanical environment that cells experience *in vivo*. Finally, we will discuss existing challenges in the adoption of such systems and some future directions in the development of 3-D systems for the assessment of nanoparticle delivery to tissues.

3-D Culture Systems

Hydrogels

Acellular hydrogels have been used to study nanoparticle diffusion in a tissue-like environment. These systems offer perhaps the simplest model of a “tissue” for assessing nanoparticle behavior. Although these models lack cells and generally employ simplified ECM compositions, they offer a practical method for probing the stability, diffusion rates, and binding interactions of nanoparticle carriers in cross-linked networks. For example, investigations using iron oxide particles have shown that diffusion through isolated ECM is highly restricted even for particles as small as 130 nm in diameter (29). The importance of particle surface chemistry was highlighted by Valentine and co-workers who demonstrated that the addition of the hydrophilic polymer poly(ethylene glycol) (PEG) to the nanoparticle surface, an approach known as PEGylation, led to significant reductions in particle binding to fibrin networks and subsequently increased diffusion rates compared to unmodified particles (30). Other studies have confirmed that the PEGylation of particles can increase movement through hydrogels, but that the rate of movement is still size dependent (29). The effectiveness of PEGylation is highly dependent on the type of matrix and the composition of nanoparticle formulations. For example, charged matrices can lead to the aggregation or unpackaging of polyplexes even after PEGylation (31). The implications of particle shape have also been probed in hydrogels with results suggesting that extending nanoparticles into a flexible worm-like structure may allow for more effective tissue penetration compared to the traditional spherical carrier (32). To partially alleviate the restricted movement of particles, covalent conjugation of an ECM disrupting enzyme, collagenase, to nanoparticles has been carried out, which led to significant improvements in magnetically-driven nanoparticle movement through isolated ECM (33).

In summary, evaluations of delivery vehicles in acellular constructs have provided valuable insight into the stability of carriers in the presence of ECM components as well as the effect of size, shape, and surface chemistry on the mobility of vectors through the ECM. Still, the usefulness of such systems is limited by their inability to offer information about the interactions of carriers with cells and the efficacy of therapeutics on the target cells.

Multicellular Spheroids

Perhaps the most widely used 3-D tissue model for the assessment of nanoparticle delivery is the multicellular spheroid. Multicellular spheroids are formed by culturing cells in spinner flasks or agar coated culture plates (34). Under these conditions, cells form spherical clusters that can survive for weeks and can reach sizes of up to several millimeters. Multicellular spheroids offer a simple and highly reproducible model that contains many of the features of natural tissue *in vivo* including some patterns of protein production (35), and the production of an ECM (36). In the later stages of growth, larger spheroids mimic properties of avascular regions of solid tumors including regions of differential growth such as a proliferating region, a quiescent region, and a necrotic core (35). As spheroids are relatively easy to handle, they are amenable to confocal analysis, cryosectioning, and commonly-used fixing and tissue processing methods. They can also be disaggregated by trypsin treatment and individual cells collected for analysis by flow cytometry. Exposure of the spheroids to a cell permeable dye prior to flow cytometry allows for determination of the original radial location of the cells within the spheroid (37). These properties make spheroids a very useful and widely used model for investigating nanoparticle-tissue interactions.

Multicellular spheroids have been used to probe some of the major barriers to effective drug delivery to solid tumors, including the effect of hindered vehicle diffusion in the ECM and the effect of non-specific binding between nanoparticles and tissue components. The

heterogeneous nature of the spheroids has also allowed for the study of the effect of the proliferative state of cells on delivery efficacy.

Hindered diffusion of delivery vehicles due to restrictive pore sizes and high tortuosity in the interstitial space was demonstrated in experiments that highlight the correlation between nanoparticle carrier size and tissue penetration into multicellular spheroids. Studies investigating the penetration of carboxylated polystyrene beads in spheroids have shown that particles with diameters less than 100 nm can infiltrate spheroids more effectively than particles ≥ 100 nm, but that particle penetration for all tested sizes (20-200 nm) was significantly hindered (3). Enger and co-workers demonstrated that adeno-associated virus (AAV), with effective diameters of ~ 25 nm can more effectively penetrate multicellular spheroids compared to adenovirus with average diameters of 100 nm (38). Differences in viral penetration were attributed at least in part to these differences in particle size.

In addition to size, vector surface charge affects distribution in multicellular spheroids. The penetration of liposomes with different surface charges was analyzed in this model, and liposomes with neutral or low surface charge were shown to penetrate to the central regions of the spheroids while liposomes with higher surface charge were confined to the outer cell layers of the spheroid, demonstrating an example of a binding site barrier (39). One approach to reduce strong binding interactions is PEGylation. Indeed, PEGylation of polyplexes results in a reduction in toxicity and improved penetration of polyplexes in multicellular spheroids (40, 41). Interestingly, in studies by Han et al., toxicity profiles of PEGylated polyplexes differed for spheroid cultures compared to monolayer cultures, with spheroids exhibiting an increased sensitivity to non-PEGylated polyplexes. Oishi et al. have also demonstrated that the effectiveness of siRNA delivery using lipoplexes and PEGylated polyplexes is affected by the method of cell culture, with cells in monolayer showing increased sensitivity to therapy compared to spheroid cultures (42). These studies further demonstrate the importance of 3-D screening for nanoparticle carriers as important toxicological data could be missed with only monolayer screening (Table 1).

Multicellular spheroids that reach a certain size contain different regions that are also present in solid tumors, including an outer proliferating region, an intermediate quiescent region and a central necrotic core. For certain carriers, such as non-viral gene delivery vectors, cells must be dividing in order to observe effective therapy; therefore quiescent regions of spheroids are not efficiently transfected even if vectors reach these target cells (43). The effect of cell proliferation on cell killing by antineoplastic agents has also been well documented in the literature (44).

The limitations in nanoparticle penetration due to some of the aforementioned physiochemical properties of the nanoparticles can be partially overcome by modulation of the ECM. Several studies have demonstrated the potential of this approach for improving delivery efficiency. Addition of the matrix metalloproteinase (MMP) collagenase, either in solution or immobilized on the surface of nanoparticles, increases the penetration of vectors up to 100 nm in size (3) (Fig. 2). Improved viral particle penetration has also been accomplished in spheroids through the use of the peptide hormone, relaxin (45). Relaxin, which can down-regulate collagen production and increase collagenase production (46), was engineered into an oncolytic adenovirus and delivered to multicellular spheroids. Viruses that included the relaxin gene were able to transduce almost all areas of spheroids while control virus transduction was confined to the outer few cell layers. The ability to incorporate these penetration enhancers into nanoparticle drug carriers offers a promising method for improving treatments, such as gene therapy for solid tumors, which rely on effective access to the majority of the target tissue.

From these studies, it is clear that the 3-D tissue environment affects the mobility of nanoparticles through tissue, which in turn affects therapeutic efficacy of delivered nanoparticles. These factors also affect the sensitivity of tissues to toxic therapeutics. Studies using multicellular spheroids have been used to understand key challenges for *in vivo* delivery to tissue and to demonstrate the importance of 3-D screening for nanoparticle carriers.

Multilayer Cell Cultures

Multilayer cell cultures have been used for the assessment of small molecule drug delivery (47), and even though they have not been used extensively for nanoparticle delivery applications, they merit brief mention because they have potential use in assessing nanoparticle delivery across tissue layers. Multilayer cultures are most commonly formed by allowing monolayer cultures to continue to grow beyond confluence in Transwell™ cell culture dishes. The Transwell™ dishes allow isolation between the apical and basal layer of the multilayer cell growth so that any compound delivered to one side must pass through the cell layers to reach the opposing side. This allows for easy assessment of transcellular drug delivery by incubation of drugs on one side of the growing culture followed by collection of media on the opposite side to assess the concentration of drug that has passed through the cell layer. Studies have been carried out to assess the diffusion of anti-cancer drugs through multilayer cultures (47), and could easily be extended to nanoparticulate drug carriers.

Limitations of Aforementioned Culture Systems

Despite the valuable information that can be gained from the 3-D culture systems mentioned above, deficiencies remain in the systems that limit their *in vivo* predictive power. These weaknesses include, in the case of acellular models, the inability to predict how interactions with cells affect nanoparticle distribution and therapeutic efficacy, and, in the case of the other models, an inability to recreate outside forces such as mechanical stress and convective flow. These forces could dramatically affect nanoparticle distributions. Therefore, advanced 3-D systems that incorporate mechanical forces and multiple cell types and that more closely mimic the *in vivo* architecture are desirable. In order to address the limitations of the previously described 3-D systems, more advanced 3-D models have been developed that mimic physiological conditions even more realistically. These include (a) co-culturing of 2 or more cell types in a biological matrix to mimic the complex cell-cell communications in addition to cell-ECM interactions, (b) using tissue explants, and (c) incorporating mechanical forces such as fluid flow into culture platforms to mimic the mechanical environments that cells experience *in vivo*, or combinations of these methods.

Co-culture Models

Co-culture models, wherein two or more different cell types are grown together (Fig. 3a), are useful as *in vitro* models because tissues *in vivo* consist of more than a single cell type, and cell-cell interactions are important in determining and maintaining the normal cell function and phenotype (14,16,17,48,49). Applications of co-culture models are useful for investigating the specificity of nanoparticle carriers. For example, Garnett and coworkers developed a brain tumor co-culture model consisting of cancerous cell aggregates and organotypic brain slices (50). Evaluation of the uptake of poly (glycerol-adipate) nanoparticles revealed that in 3-D co-cultures, nanoparticles were predominantly taken up by cancerous cells rather than normal cells. In contrast, in monolayer cultures, nanoparticles were taken up more readily by normal cells. This conditional specificity of the nanoparticle uptake suggests that the nanoparticles may be useful for anti-cancer formulations with reduced toxicity to normal cells, an observation apparent only through studies with 3-D co-culture. A multicellular spheroid co-culture was also successfully used to demonstrate the specificity of a viral vector targeted towards cancer cells (51).

Co-culture models are particularly important when attempting to mimic a tissue containing many types of specialized cells whose function determines the tissue microenvironment. Along these lines, a 3-D co-culture that contains differentiated cells that are similar to *in vivo* lung tissue has been constructed (52,53). The co-culture of bronchial epithelial cells seeded on a human lung fibroblast-embedded collagen matrix contained a pseudostratified epithelium consisting of basal cells, mucus-secreting cells, and ciliated columnar cells with beating cilia. The cilia and mucus are important *in vivo* barriers to nanoparticle delivery to airway cells as they are specifically adapted to resist and remove foreign particles. Only in the 3D co-cultures are well-differentiated ciliated epithelial cells observed *in vitro*; cells in monolayer culture don't express these cilia. Furthermore, the airway wall co-culture model was placed in a dynamic cyclic strain device to mimic compressive effect observed during bronchoconstriction in asthma in order to study the rate of viral infection of the epithelial and subepithelial cells (54). Previous studies with cyclic stretch in monolayer culture have suggested that intracellular processes are affected that in turn affect transfection (55), and in the bronchial 3-D culture model, dynamic compression substantially enhanced transduction of epithelial cells and underlying fibroblasts compared to static co-culture controls (54). This increased transduction was attributed, in part, to enhanced transport through the matrix due to the mechanical compression. This finding, not observable in 2-D static monolayer cultures, may be useful for asthma pathophysiology as well as for designing delivery strategies of viral gene therapies to the airway.

From these studies, it can be concluded that co-culture models can promote differentiation and cell behavior of the cultured cells that resembles *in vivo* conditions more realistically than single-cell type culture models. Co-culture models thus offer a better predictive platform for nanoparticle uptake and therapeutic efficacy in complex tissue with specialized cells. An additional attribute of these models is the ability to evaluate the cell specificity of nanoparticle formulations, an important parameter for applications that must target diseased tissue while sparing surrounding healthy tissue.

Ex-vivo Models

Ex-vivo models involve harvesting tissue from human or animal sources followed by culturing on substrates such as culture wells and coverslips (56-59). Although *ex vivo* models require the use of animals, multiple data points may be obtained from a single animal source in most cases. The main advantage of *ex vivo* models is that they generally preserve the native complex and differentiated 3-D cell-matrix architecture, cell phenotype, and cell-cell interactions of the tissue of interest, thus providing a more accurate mimic of cell behavior and transport of particles to these primary cells. The *ex-vivo* model also allows for direct delivery of nanoparticle formulations to target tissue along with the ability to more easily process tissues for analysis after exposure. Near real-time monitoring through confocal microscopy is also possible. *Ex vivo* models are especially beneficial if human tissue can be obtained. For example, human tissue slices have been used to screen adenoviral gene therapy agents for specificity and efficiency. In these studies, vectors were identified that effectively transduced cancerous cells while sparing normal cells (60,61). For specificity studies such as this, the maintenance of cell phenotype, which is facilitated by the *ex vivo* model, is necessary for results that are likely to be predictive of *in vivo* results.

Ex vivo studies have also been used to demonstrate the possibility of gene delivery to fetal somatic cells for the treatment of monogenic disorders such as cystic fibrosis. Human fetal tracheas, maintained in *ex vivo* cultures, were shown to be highly susceptible to adeno-associated virus transduction (62). The high efficiency of transduction was attributed to reduced physical barriers to target cells in the prenatal tissue compared to developed tissue, which is resistant to transduction (63,64). Again, preservation of the tissue in its near-native

state during these studies may result in a higher probability of predicting *in vivo* efficacy and specificity.

Other *ex vivo* studies have been useful in developing methods to overcome physical barriers to therapeutic nanoparticle penetration. Quinlan et al. developed an embryonic mouse intestinal epithelium model that permits the maintenance of the gut tube and enables the introduction of exogenous genes to study the normal development of the intestinal epithelium and pathogenesis of intestinal neoplasia (58). These researchers found that high efficiency adenoviral vector transduction of the intact mesenchymal and epithelial layers required the addition of an ECM modulating agent for effective adenoviral transduction.

As with other static models, results from *ex vivo* studies may not reflect the actual biodistribution of the delivery vehicles *in vivo* because some of these studies involve incubating the explanted tissue in a bulk solution with media containing the nanoparticles of interest (Fig. 3b), while *in vivo* delivery is typically administered intravenously or injected at a specific site. Furthermore, mechanical forces are typically absent during static *ex vivo* culturing, and a lack of mechanical forces, perfusion, and surrounding tissue results in remodeling and changes in cell behavior compared to the original *in vivo* environment. Long term studies with *ex vivo* models may, therefore, be difficult, as the tissue may exhibit significant changes over the course of the experiment. Another major drawback of the *ex vivo* cultures is that they require harvesting of tissue from animal or human subjects. However, the advantage of maintaining native tissue structure makes *ex vivo* models unique.

Perfusion Models

The majority of the studies discussed to this point have been carried out under static culture conditions. As mentioned previously, cells in tissues are typically subject to fluid flow, which can be an important determinant of nanoparticle distribution in tissues as convective transport can often dominate diffusion. Nanoparticles diffuse very slowly in biological tissues and may take several months to diffuse a millimeter's distance, whereas the time to cover the same distance aided by convective transport can be much shorter. Therefore, convective transport can either greatly aid or greatly hamper the delivery of nanoparticles depending on the direction of fluid flow. In addition, it has also been shown that fluid flow can influence cellular functions. For example under long term culture, perfused hepatocytes maintained their liver-specific functions such as albumin secretion substantially closer to native liver compared to single monolayer or 3-D static cultures (12,65,66). Finally, static cultures can result in gradients of waste and nutrients within larger tissue constructs. Although this may be useful in studies of tissues like avascular regions of solid tumors, it can be detrimental to the preservation of larger 3-D cultures designed to model normally vascularized tissue. Thus, a model incorporating fluid flow may be beneficial for the optimization of delivery vehicles that encounter convective forces that influence their interstitial distributions.

There have been many recent advances towards the development of 3-D culture systems (Fig. 3c) that allow for perfusion of tissue cultures, with particularly useful contributions from the fields of microfluidics and microfabrication (67-74). Some of these systems have the potential for higher throughput screening due to the ability to simultaneously establish various conditions such as pH, flow rate, or drug formulation concentrations on different portions of the array. Many of the systems also allow for *in situ* visualization of the cultures and, therefore, potential real-time imaging of nanoparticle delivery. One such promising perfusion system has been developed by Griffith and coworkers, who have constructed a microfabricated multi-well chamber that allows for growth of isolated hepatocyte cultures in wells under flow conditions (65). A broad comparison to other standard *in vitro* models, including static ones, revealed that the perfusable system more closely mirrored *in vivo* liver function with respect to gene and protein expression as well as the ability of the cultured cells to metabolize delivered drugs

(11). Although perfusable systems are apparently useful for nanoparticle delivery studies, there are limited examples as many of the systems are relatively new or their development has focused primarily on cellular and tissue engineering applications rather than gene/drug delivery applications.

We have recently developed a perfusable 3-D cell-matrix gel culture model and demonstrated its applicability for *in situ* observation of nanoparticle transport in cell-gel matrices (75). In this model (Fig. 3d), a cell-matrix culture is loaded and immobilized in a PDMS-glass chamber which allows for real-time imaging of particles in the 3-D structure over time. This potentially avoids any artifacts that would occur if tissue processing was necessary for visualization. Nanoparticles can be introduced laterally to media compartments situated in each side of the gel. We found size-dependent penetration of model fluorescent polystyrene nanoparticles similar to that observed in other 3-D systems such as multicellular spheroids (3). In addition, we observed deeper penetration of nanoparticles under interstitial flow compared to static controls using the same chamber. Currently we are applying this simplified perfusion model to evaluate the penetration and cellular uptake of therapeutic nanoparticles, such as polyplexes, under various flow conditions. Besides the ability for real time imaging, other advantages of this system include ease of manufacture, control over components of the initial cell-gel composition, and an ability to recover cells for flow cytometry analysis. The system can also be used for calculating the permeability of different cell-gel matrix compositions.

In summary, perfusion systems provide an additional advancement over static cultures by mimicking flow conditions in vascularized tissues. These perfusion chambers also offer the ability to recreate *in vivo* situations where convective flow is a driving force in determining nanoparticle distributions.

Implications for Nanoparticle Carrier Design

The results from previous studies of nanoparticle delivery to *in vitro* 3-D tissue models offer insight into a number of parameters that need to be considered during nanoparticle design. In particular, they highlight the importance of nanoparticle size, charge, surface chemistry, and stability when designing a drug delivery vehicle that can efficiently penetrate tissue. The effect of these design parameters on delivery may be understated or even ignored when nanoparticle vehicles are simply analyzed in monolayer cultures. The *in vitro* 3-D models also offer a more favorable platform for optimization compared to *in vivo* studies in terms of cost, ease of use, and time requirements.

First, and perhaps foremost, the nanoparticle carrier size must be considered. This is important for nanoparticle therapeutics that rely on diffusion or convection through interstitial spaces in order to reach cells in the tissue interior. A key consideration is that particle size should be smaller than the characteristic pore size of the extracellular space. Because tissue architecture varies greatly depending on tissue type, it is impossible to determine a global cutoff diameter for tissue penetration, but the results from the studies summarized here suggest that nanoparticles with sizes less than 150 nm experience less hindered transport.

Nanoparticle surface charge and chemistry provide an example of the balancing act that must often be played when designing a nanoparticle carrier. Effective cell surface binding is necessary for uptake of therapeutics, but if the charge density is too high then the carrier will experience hindered diffusion (76). Addition of steric shielding molecules, such as PEG, have in several instances proven to increase particle penetration in tissue (29,40,41), but can also hinder cellular uptake of particles, thus decreasing delivery efficacy (77,78). The use of cleavable shielding molecules, or shielding molecules with terminal targeting groups may provide a method for overcoming this reduced cell uptake (31,79,80). It is also important that

targeted nanoparticles are assessed in culture models where phenotypes of cells are similar to *in vivo* situations in order to more accurately predict specificity.

Three-dimensional culture models have also helped demonstrate the usefulness of including components in nanoparticle carriers that can disrupt or digest ECM components in order to improve particle penetration. Similar to strategies employed by some pathogens to increase tissue distributions by stimulation of MMPs production to locally degrade tissue, nanoparticles that incorporate such enzymes could lead to much more favorable distributions of therapeutics in tissues (81).

It is important to keep in mind the high degree of variability in tissue architecture and composition. The results from studies in these 3-D tissue models offer insight into the most important parameters to consider when designing nanoparticle carriers, but specific nanoparticle behavior will vary greatly depending on the type of target tissue under consideration as well as vehicle administration method. However, 3-D models, overall, will provide a more accurate prediction of *in vivo* outcome compared to simple monolayer cultures.

Challenges and Future Directions

While researchers are increasingly turning to 3-D cell-matrix cultures for studying gene expression and other biological activities (82), the use of these cultures in the nanoparticle delivery field has not been widespread. This could be due, in part, to the higher degree of difficulty for setup and use compared to 2-D systems. In addition, many advanced systems are not as appealing because they are still in prototypical stages and could benefit from improvements in ease of manufacture and use. Also, the exact determinants of cell differentiation, such as cell-cell signaling, cell-ECM signaling, tissue architecture, and mechanical forces are different for each tissue and are often not well elucidated. Therefore, accurate advanced 3-D models will have to be refined on a tissue-to-tissue basis. Despite the difficulties associated with the use of 3-D culture, many of the studies reviewed here have reinforced the need to move from 2-D to 3-D culture in order to more accurately predict the *in vivo* efficacy of drug delivery with nanoparticle formulations.

Refinement of advanced systems will most likely result through combinations of elements from many of the models discussed here. An ideal 3-D culture would allow for the introduction of multiple cell types grown in a chamber that allows for recreation of as many of *in vivo* forces that act on the cells as possible, including mechanical forces that affect cell behavior. To accomplish this, current systems may need be modified to include (i) surface modification of cell chambers to allow for long term perfusion culture without matrix detachment due to cell contraction, (ii) more precise characterization of flow rates and profiles, (ii) the incorporation of appropriate cell types and extracellular materials to better mimic the heterotypic cell interactions, tissue architecture, and fluid flow stresses to obtain the full possible spectrum of tissue-specific functions, and, for some applications (iv) chamber flexibility to allow for the application of mechanical forces to the tissue. With the addition of high-throughput capabilities, such systems could lead to improved screening and evaluation of nanoparticle formulations, resulting in more accurate predictions of *in vivo* outcomes and a smoother transition into clinical trials.

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Fig 1.
3-D culture systems include additional extracellular barriers encountered by delivery vehicles that are not accounted for in 2-D monolayer cultures.

Fig 2.

Penetration of model nanoparticles into multicellular spheroids. Phase contrast of multicellular spheroids shows intact spheroids prior to treatment with 40 nm fluorescent polystyrene nanoparticles (A). Cryosections of untreated spheroids (B) showed poor particle association compared to spheroids co-incubated with collagenase (C). Scale bar is 200 μm .

Fig 3.

Examples of advanced 3-D models. (a) Some variations of co-culture models: (1) spheroid co-culture models, (2) cell-cell co-culture in ECM and (3) cell-monolayer on 3-D cell-ECM co-culture model; (b) Ex-vivo models: one version where samples of tissues such as the liver were harvested, sectioned and cultured on 2-D substrates for screening; (c) A typical set up of 3-D perfusion cell-ECM culture models where media flow is driven over the cell-ECM by a pump from a reservoir; and (d) A short-term perfusion system developed by Ng and Pun for nanoparticle penetration studies.

Table 1

Examples of differences in nanoparticle-based drug delivery observed in 2D versus 3D cell culture systems.

Deliverable	Difference between 2D and 3D systems	Reference
Cytotoxic small molecules drugs	Multicellular resistance (lower cytotoxicity) observed in spheroid cultures and polystyrene scaffolds compared to monolayer cultures.	23,83,84
PEI polyplex delivery of plasmid DNA	PEI-mediated transfection was limited to cells at the spheroid periphery, even with electroporation. This spatially-dependent transgene expression is not observable with monolayer cultures.	38
P[Asp(DET)] polyplex delivery of plasmid DNA	Difference in cytotoxicity of PEGylated compared to non-PEGylated polyplexes observed in spheroids but not detected with the conventional monolayer cultures. Specifically, PEG-b-P[Asp(DET)] polyplex micelles did not induce the destruction of spheroids even at N/P=40, where the MCTS structures were destroyed by the transfection with P[Asp(DET)] polyplexes. Difference in the timecourse of transgene expression observed in 3D spheroid cultures vs. monolayer cultures. Peak transgene expression occurred at 6 days after transfection in spheroids whereas difficulties were encountered in maintaining viable monolayer cultures beyond 4 days.	36
Lipoplex delivery of siRNA	Differences in growth-inhibitory effect between spheroid and monolayer cultures. OligofectAMINE-siRNA lipoplexes had no inhibitory effect on HuH-7 spheroids compared to strong effects on HuH-7 monolayer culture.	42
Poly (glycerol-adipate) nanoparticle delivery of DNA	Differences in cellular uptake of nanoparticles in co-cultures. Nanoparticles were predominantly taken up by cancerous cells rather than normal cells in 3D co-cultures whereas nanoparticles were taken up more readily by normal cells in monolayer cultures.	45
Adeno-associated vectors (AAV) and adenovirus vector (ADV)	Differences in penetration efficiencies between AAV vs ADV vectors. AAV penetrated glioblastoma spheroids and xenografts more efficiently compared to ADV vectors; biodistribution not observable with monolayer cultures.	34
Lentivirus	Enhanced transduction of epithelial cells and underlying fibroblasts compared to static co-culture controls in airway wall model; this transgene expression profile was not observable with monolayer cultures.	49