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A Perfusable 3D Cell–Matrix Tissue Culture Chamber for In Situ Evaluation of Nanoparticle Vehicle Penetration and Transport

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Abstract

A key factor in gene or drug therapy is the development of carriers that can efficiently reach targeted cells from a distal administration. In many gene/drug delivery studies, results obtained in 2D cultures fail to translate to similar results in vivo. In this work, we developed a perfusable 3D chamber for studying nanoparticle penetration and transport in cell–gel soft tissue cultures. The compartmented chamber is made of a polydimethylsiloxane (PDMS) top layer with the chamber features, created using micromachined lithography, bonded to a bottom glass coverslip. A solution of cells embedded in a hydrogel is loaded in the chamber between PDMS posts that serve as anchors to the cell–matrix at the gel-media interface. The chamber offers the following unique features: (i) rapid fabrication and simplicity in assembly, (ii) direct in situ cell imaging in a plane normal to the direction of flow or action, (iii) an easily configurable and controllable environment conducive cell culture under static or interstitial flow conditions, and (iv) facile recovery of live cells from chambers for post-experimental analysis. To assess the chamber, we delivered fluorescently labeled nanoparticles of three distinct sizes to cells-embedded Matrigels in the 3D chamber under flow and static conditions. Penetration of nanoparticles were enhanced under interstitial flow while live cell imaging and flow cytometry of recovered cells revealed particle size restrictions to efficient delivery. Although designed for delivery studies, the chamber is versatile and can be easily modified. Thus it may have broad applications for biological, tissue engineering, and therapeutic studies.

Keywords

gene delivery; drug delivery; interstitial flow; convective transport; cell culture

Introduction

Drug and gene delivery vehicles are frequently used to enhance the therapeutic value of their deliverables by providing altered biodistribution, bioavailability, and/or toxicity profiles (Cheng and Pun, 2006). A key aspect of this approach is the development of effective nanocarriers that can overcome the many extracellular barriers post-administration and reach the targeted cells that often reside in the interstitium of tissues. Current modus operandi of gene or drug delivery studies involves first performing in vitro studies of nanoparticles of interest on 2-dimensional (2D) cell monolayer cultures before directly proceeding to animal studies in vivo. Oftentimes, successful delivery observed in 2D cultures fails to translate to similar results in vivo. A possible reason for this is that cells in monolayer cultures on 2D substrates do not realistically mimic the 3-dimensional morphologies of cells in tissues nor the dynamic and

complex interactions of cells with their supporting extracellular materials; these factors result in gene expression and biological activities that differ from their 2D monolayer counterparts (Albrecht et al., 2006; Bhatia et al., 1998; Cukierman et al., 2001; Griffith and Swartz, 2006; Liu and Bhatia, 2002). In addition, 2D culture systems do not capture the *in vivo* barriers to delivery of macromolecules or nanoparticles resulting from transport limitations to matrix interactions (Goodman et al., 2007; Netti et al., 2000; Pluen et al., 2001). Furthermore, 2D cell monolayer cultures are generally exposed to a uniform environment whereas cells in tissues such as solid tumors are exposed to pH and concentration gradients that exert both stimulatory and inhibitory influences on proliferation and malignant properties of the cells (Heldin et al., 2004). Thus, the use of an *in vitro* culture system that more closely mimics the 3D tissue architecture *in vivo* during the development and evaluation of delivery vehicles could provide a more predictive platform for assessing *in vivo* delivery efficiencies. Increasingly, scientists and bioengineers are turning to 3D cell–matrix cultures that mirror *in vivo* conditions more closely than *in vitro* 2D models for studying gene expression and other biological activities (Abbott, 2003); these models typically involve culturing cells in biohydrogels such as collagen, fibrin, and Matrigel (Abbott, 2003; Griffith and Swartz, 2006; Vailhe et al., 2001). However, the use of cell–matrix cultures in the gene and drug delivery field is minimal, mostly because culture models tailored to investigate nanoparticle penetration and delivery to cells in 3D microenvironments are generally lacking. In addition, most cell–gel culture set-ups are conducted under static conditions and lack dynamic mechanical forces such as interstitial fluid flow that cells in tissues experience under physiological and pathological conditions.

The key to successful penetration and delivery of therapeutic agents such as drug and gene carriers to targeted cells in the interstitium depends on their carrier's abilities to overcome physical factors and barriers that influence their interstitial distribution. One such important yet often overlooked factor is the flow of interstitial fluid in porous extracellular spaces of tissues where cells reside. In healthy adult tissues, interstitial flow is driven by pressure difference between the two capillary networks, causing plasma to leave a blood capillary through its wall and drain into the initial lymphatics (Fig. 1). In addition, reabsorption of plasma in post-capillary venules can result in some interstitial flow. Interstitial flow may especially be of interest in drug/gene delivery research since it can play an important role in the transport of drug/gene carriers, many of which are typically larger-sized (scale in nanometers) and of high Peclet number ($Pe > 1$, meaning convective transport dominates over diffusion), to cells in the extracellular matrices. For example, in the case of transmural convection, experimental studies using radiolabeled tracers such as albumin, sucrose, and drugs have shown that interstitial flow promotes macro-molecular transport across the arterial wall from blood to tissue (Baldwin et al., 1997; Tedgui and Lever, 1985,¹⁹⁸⁷; Wilens and McCluskey, 1954). Furthermore, the delivery of these nanoparticles to interstitial cells is likely affected by the magnitude, direction and pattern of flow, which in turn can be altered by subtle organ movement such as skeletal motion, arterial pulsation, respiration (Rutkowski and Swartz, 2007) and enhanced in the event of injuries such as inflammation, endothelium damage or atherosclerosis (Baldwin et al., 1997; Penn et al., 1992). In solid tumors, high interstitial pressure (Flessner et al., 2005; Heldin et al., 2004) combined with the lack of functional lymphatics (Leu et al., 2000) can result in a pressure gradient that reverse flow towards the leaky tumor blood capillaries and enhance plasma reabsorption, thereby hindering macromolecular transport. Thus it is clear that the incorporation of interstitial flow in penetration and uptake studies will offer a more realistic assessment on the effectiveness and efficiency of the gene or drug vehicle that is designed to target interstitial cells.

Currently there are no suitable *in situ* models for studying nanoparticle penetration in 3D matrix architecture under interstitial fluid flow. More recently, a 3D tumor spheroid model cultured by growing mammalian cells in spinner flask has been used to study nanoparticle penetration after the spheroid are grown to a certain size (Goodman et al., 2007; Grill et al., 2002;

Kostarelos et al., 2004). However, drawbacks of the model includes the high costs for culture at this scale, time required for spheroid growth, initial non-controllable environment and the inability for in situ studies. There are also recent developments in microfluidic 3D cell or cell-gel culture models where cell-biohydrogel cultures are plated in the chambers and flow is induced on top of cell-matrix culture (Frisk et al., 2005; Powers et al., 2002; Tan and Desai, 2003). This configuration however makes these models unsuitable for penetration studies in situ since the view of the penetration depth should be normal to the plane of penetration for direct observation to be possible. Furthermore, devices at this scale are impractical for cellular analysis such as flow cytometry to assess the efficiency of nanoparticle uptake and transfection. There are very few existing interstitial flow chambers designed to truly study interstitial flow in cell-matrix cultures (Goldman et al., 2005; Ng and Swartz, 2003; Wang and Tarbell, 2000). In one such model designed for direct observation, the soft cell-gel 3D culture was supported laterally by thick porous polyethylene materials (Ng and Swartz, 2003); this makes it unsuitable for penetration studies since the particles have to navigate through this hydrophilic porous material to reach the gel interface. Furthermore, this model has multi-component parts that are time-consuming to prepare and assemble.

Here, we present the design, fabrication and evaluation of a new cell-gel culture device that can be used to study nanoparticle penetration and delivery to cells in a 3D matrix architecture under pressure gradients and interstitial fluid flow. We also demonstrate that the design criteria for this device, including (i) viable cell culture in a 3D matrix, (ii) imposition of fluid flow through the cell-gel culture, (iii) live cell imaging during perfusion, and (iv) facile live-cell harvesting after culture, are met. In addition, a proof-of-concept study is reported that illustrates the feasibility of using this device to investigate nanoparticle penetration and delivery to cells cultured three-dimensionally. The optically clear chamber is made by bonding glass and a PDMS layer with features such as array of posts that support the culture and give the cell-gel a definable geometry. The posts are spaced apart as possible to minimize their effects on flow and macromolecular transport on the cell-gel culture. We were able to induce flow through cell-seeded Matrigel, one of the most compliant tissue materials, in the chamber for up to 6 h. In addition, the milli-scale chamber created from microfabrication techniques of laser micromachining and soft lithography is low-cost, simple to assemble and can be rapidly fabricated. To demonstrate the application of the chamber, we introduced nanoparticles of various sizes into one of the media compartments and assessed penetration in the gel via fluorescent intensity over a period of 4–5.5 h under flow or static conditions. In addition, live cells were recovered intact from the chamber for further biochemical or cellular studies such as flow cytometry. The design of the chamber is versatile enough to be used for other cell-gel culture studies and thus may have broad applications in biology and engineering beyond gene and drug delivery research.

Materials and Methods

Micromachined PMMA Lithography

The chamber is comprised of a glass coverslip base with a PDMS layer that includes channels and a compartmentalized main chamber area separated by posts. The PDMS layer is created from a modified microfabrication technique known as Micromachined PMMA Lithography. In this technique, a PMMA template with the negative chamber features was created from a 1/16" extruded acrylic sheet (McMaster Carr, Elmhurst, IL) using a CO₂ laser cutting system (Universal Laser Systems, Inc., Scottsdale, AZ) described previously (Hatch et al., 2001; Klank et al., 2002). This PMMA template was then bonded to a PMMA disc using superglue to create the PMMA master. The polydimethylsiloxane (PDMS) layer of the chamber was prepared by mixing the base and the curing agent of the Sylgard-184 elastomer kit (Dow Corning, Midland,

MI) in the weight ratio of 10:1 and then casting the elastomer on the PMMA master. The PDMS layer with chamber features was allowed to cure in a 658°C oven for at least 2 h.

Chamber Features

The device consists of a main chamber area, two media reservoirs and four microchannels. The main chamber area is comprised of a gel compartment and two media compartments on opposite ends of the gel compartment. An array of PDMS posts defines each interface between the gel and media compartments, and also serves as supports to the cell–matrix gel retained in the gel compartment. Each media compartment is connected to two 1.1 mm-wide microchannels that facilitate the introduction of media from the ports; one port allows air release while the compartment is being filled with media from the other port so that air is not forced through the gel thereby affecting its integrity. This design also allows the chamber to be easily configured to create various flow conditions that mimic different aspects of the tissue microenvironment. The main gel compartment of the chamber is 3,000 μm (length) $\times$ 4,000 μm (width) $\times$ 1,600 μm (height). Each PDMS post-measures 660 μm (length) $\times$ 170 μm (width) $\times$ 1,600 μm (height); posts are spaced 660 mm from center to center of the posts.

Chamber Assembly

The PDMS layer and a 50 mm $\times$ 24 mm #1.5 thickness glass coverslip (Fisher Scientific, Pittsburgh, PA) were surface-modified by a 1 min oxygen plasma treatment (600 W, 90 kPa vacuum and 35 psi oxygen, Plasma-Preen II-973, Plasmatic Systems, Inc., North Brunswick, NJ) for cleaning and chamber bonding. NIH 3T3 fibroblasts grown in DMEM media (Mediatech, Inc., Herndon, VA) or GFPactin transfected MDCK cells (used in situ studies) grown in MEM media (Mediatech Inc.) supplemented with 10% Fetal bovine serum and 1% Antibiotic and Antimycotic (ABAM) in monolayer flask cultures were trypsinized, centrifuged down into cell pellet and mixed with Phenol Red-free Matrigel (12 mg/mL, BD Bioscience, San Jose, CA) at 4°C. This cell–matrix mixture (2 million cells per mL solution, $\sim$ 50 μL) was then loaded into a syringe and injected into the space between the PDMS posts about 3–4 h after the plasma bonding of the chamber. When loading the cell–matrix solution, overshoot solution can be rescued by gently pulling the viscous solution back into the syringe. The cell–matrix solution was allowed to polymerize and covalently bind to reactive hydroxyl (–OH) groups generated by the plasma treatment on the posts and the walls of the chamber in an incubator for 30 min. Media reservoirs made of 1,000 mL pipette tips were attached to the ports and media was loaded in the side compartments adjacent to the cell–gel culture to the same height level to keep the culture hydrated and under static condition. The chambers were incubated for at least 12 h to allow the cells to adjust to their new environment before flow experiments. All cell loading steps were completed under sterile conditions.

Short-Term Flow Studies

A simple and compact perfusion system was used to induce short-term pressure-driven fluid flow. From the static configuration described above, media in one of the reservoirs was removed and the reservoir (refer to as the outlet reservoir) was replaced with a bent metal tubing made from an 18G needle to divert flow from the port to a waste container. The level of the media in the reservoir attached to other port (refer as the inlet reservoir) was adjusted to the necessary pressure difference. To revert to static culture, the outlet reservoir was reattached to the outlet port and media was raised to the same level of the media in the inlet reservoir.

Trypan blue dye-containing media (0.04% v/v solution in phosphate-buffered saline, MW 960.8, MP Biomedicals, Solon, OH) was used to ascertain if there was any flow bypass around the gel and to demonstrate the effects of perfusion on the cell–gel matrix. For the nanoparticle penetration studies, the 40, 100, 500 nm fluorescently labeled carboxylic acid-modified nanoparticles (Invitrogen, Carlsbad, CA) were suspended in experimental media (at 10 μL

nanoparticle stock solution per mL media, giving concentrations of 1.4×10^{13} , 3.6×10^{11} , 2.9×10^9 beads per mL, respectively) were loaded into inlet reservoir and side compartment in cell culture media as described above. For flow cytometry studies, concentration mixtures of 40 and 100 nm nanoparticles (4.7×10^{11} and 1.2×10^{10} , respectively) were delivered to the cells for 5.5 h under pressure gradient of (2.3 ± 0.1) mm Hg prior to cell recovery.

Estimation of Darcy Permeability

The Darcy permeability (K_p) of the Matrigel was determined from the Darcy's equation (Levick, 1987; Wang and Tarbell, 2000):

$$K_p = \frac{\mu (J_v/A)}{(\Delta P/L)}$$

where μ is the viscosity of the perfusing medium (1 cp), J_v is the measured volumetric flow rate, A is the cross-sectional area of the gel in the chamber (4 mm wide and 1/16" deep) and ΔP is the imposed pressure difference over the length ($L = 3$ mm) of the Matrigel.

The volumetric flow rate was measured by two methods. First with the simple perfusion system set-up described above, the small volume loss in the reservoir (by taking the difference in the media level multiplied by the cross-sectional area of the reservoir) was measured over time. The pressure difference was estimated by taking the difference between the average pressure at the inlet media reservoir and the pressure at the outlet. We found this method of estimation acceptable with respect to this study since the pressure level drop was minimal (1–2 mm of media) over a period of hours as flow is very slow (0.1–3.6 $\mu\text{L}/\text{min}$). Second, a more complex perfusion system using pump-driven flow with the incorporation of the fluid flow sensor to measure the volumetric flow rate was employed (see detailed description in the Supplementary Materials). In this system, the pressure difference across the matrix was determined by taking the difference between the steady-state pressure at the pressure manometer set up at the other inlet port and the pressure at the outlet.

Cell Recovery From Matrigel for Flow Cytometry

To recover and isolate the cells from the chamber, media from adjacent compartments was removed and replaced by a loading wash of phosphate buffered saline (PBS) applied through the ports. The metalloprotease dispase (BD Biosciences) at 50 caseinolytic units per mL in Hank's Balanced Salt Solution (HBSS), pH 7.4, was then loaded to the side compartments and the chamber incubated at 37°C for 2 h for complete Matrigel dissolution. EDTA (5 mM) was then loaded to dilute and stop the dispase dissolution action. The cell suspension was removed from the chamber through the ports. In addition, the PDMS layer around the gel compartment was removed using a scalpel and any cells lodged to the posts or compartment surfaces was dispersed and collected by pipetting up and down a few times with EDTA. The cell suspension was washed once with PBS before labeling with 1 μM propidium iodide in PBS for 5 min at room temperature. The cell suspension was washed twice before flow cytometry by centrifugation at 1,300 rpm for 5 min. Nanoparticle uptake and cell viability was assessed by flow cytometry using a FACSCanto cytometer (BD Biosciences). Live cells were selected by forward and side scattering profiles and by gating for lack of propidium iodide fluorescence. Uptake of dark red fluorescent (Ex/Em 660/680) 40 nm and yellow-green fluorescent (Ex/Em 505/ 515) 100 nm nanoparticles was assessed using FL4 and FL1 channels, respectively, with analysis using the FACSDiva software.

Results and Discussion

Micromachined PMMA Lithography

The perfusion chamber developed by this work is composed of a PDMS layer with channels and post-features bonded to a glass coverslip. The PDMS layer with chamber features was made by casting the elastomer on a master template that was created using micromachined PMMA lithography, a novel modification of conventional photolithography (Fig. 2a). In contrast to the SU8 photoresist photolithography typically used in the microfabrication of miniaturized chambers, this method offers several advantages. Micromachined PMMA lithography can be used to create features with depths greater than 500 μm , unlike SU-8 photolithography with has depth limits of around 350 μm . Preparation of master templates by micromachining also takes less than 30 min while preparation of similar masters by SU-8 photolithography takes several hours. Additional processing time is saved because the PMMA masters can be used directly, unlike SU-8 masters that require surface modification to prevent sticking to PDMS. Because master preparation by this new method is fast and inexpensive, it was easy to modify and optimize the design of the chambers such as the post geometry in the development phase (Fig. 2b).

Chamber Features and Characterization

The perfusion chamber, which measures about 50 mm (length) $\times$ 24 mm (width) $\times$ 5 mm (height) (Fig. 3a–d), was designed to consist of only two major components, in part for simple assembly and in part because multicomponent perfusion chambers have more potential locations for fluid leakage. The device was also designed for direct in situ cell observation in 3D morphology (Supplementary Fig. 1) in a plane normal to flow. Thus, the chamber has to be optically transparent under low or high magnification (Fig. 3f–g). In situ observations eliminate possible artifacts associated with washing, fixing, handling, and freezing for immunohistology. In addition, the media compartments were situated at the sides, rather than above and below the gel compartment which may prevent high magnification imaging due to the limits in the working distance of the microscope lens.

The chamber consists of a center area for tissue culture bounded by PDMS posts. The PDMS posts guide and retain the viscous cell–matrix solution within the gel compartment during loading, thus giving the cell–gel a definable geometry after gelation, and also serve as lateral but porous supports for the cellular gel (Fig. 3f). Furthermore, spacing between posts was selected so that the posts could serve as anchoring points for the cell–matrix without significantly affecting the media–gel interface since it was important for the nanoparticle penetration studies. Without the posts, severe movement and compaction of the cell–gel matrix was observed with imposed flow (See Supplementary Movie 1 and contrast to flow in Supplementary Movie 2).

The simple and compact perfusion system was designed for easy set-up and maintenance. Matrix integrity was evaluated under various conditions by permeability measurements and by delivery of trypan blue-containing media that can be easily visualized (Fig. 4). These studies revealed that Matrigel matrices, one of the most compliant biohydrogels known in the literature (Discher et al., 2005; Yeung et al., 2005), could support up to 3 million cells per mL of gel and remained intact for up to 6 h under flows driven by pressure gradients up to 6 mm Hg without any loss of matrix integrity. Static cultures have also been conducted without loss in matrix integrity for at least a week in duration. Besides Matrigel, we have also successfully loaded and imposed flow on reconstituted rat tail collagen matrix (2 mg/mL) under similar pressure gradients. Cells in the chamber were viable during the course of the experiments as assessed by GFP-fluorescence for GFP-transfected MDCK cells (Supplementary Fig. 2a) and by Calcein AM/propidium iodide fluorescence using Live/Dead Assays for non-GFP transfected NIH 3T3

cells (Supplementary Fig. 2b). For longer term flow studies, a more complex system with pump mechanisms and further optimization of the chamber (such as post-configurations and surface modification) may be necessary to maintain the integrity of the matrix.

The anchored cell–gel matrix has a definable shape and dimension as described previously that allows for the mathematical characterization such as the determination of permeability of the matrix. For example, the Darcy permeability (K_p) of the matrix was determined by imposing a known pressure gradient of (3.0 ± 1.6) mm Hg ($P_{in} = 3.0$ – 6.4 mm Hg, $P_{out} = 1.1$ – 1.9 mm Hg) and measuring the flow rate across the fixed volume gel. The pressure conditions used were comparable to reported interstitial pressures ranges in normal tissues (from -8 to 6 mm Hg, Sven and Josipa, 2007). In addition, the interstitial velocity for the Matrigel of (3.8 ± 3.7) $\mu\text{m/s}$ estimated here was similar to that of rabbit ear tissue (0.6 $\mu\text{m/s}$) determined by fluorescence photobleaching (Chary and Jain, 1989). The K_p of Matrigel in the perfusion chambers was estimated to be $(2.44 \pm 2.24) \times 10^{-10}$ cm^2 . Comparing values from literature, the K_p of the Matrigel system measured falls within physiologically-relevant values (Levick, 1987) and is similar to that of vitreous humor tissue (Fatt, 1977). Furthermore, using a different methodology where pump-driven flow is employed (Supplementary Fig. 3a), we determined the Matrigel permeability at 1.52×10^{-10} cm^2 , (Supplementary Fig. 3b), which was within the range and order estimated by the earlier method.

Nanoparticle Penetration

Nanoparticle delivery to cells grown in a 3D matrix-containing culture was evaluated as an application of the developed chamber. Existing nanoparticles for drug delivery show poor penetration in solid tumors due to diffusional restrictions, non-specific binding and interaction with extracellular materials/matrix (ECM, Flessner et al., 2005; Goodman et al., 2007; Grill et al., 2002; Heldin et al., 2004; Jain, 2001; Pun et al., 2004). These transport barriers of nanoparticle delivery in tumors cannot be replicated with 2D cell monolayer cultures that are generally exposed to a uniform environment and lack the extracellular components for cell–ECM interactions. Thus, a cell culture system that more accurately reproduces *in vivo* delivery conditions would be useful in evaluation and testing of drug delivery systems. Here, fluorescently labeled nanoparticles of various sizes (40, 100, 500 nm) were introduced by pressure-induced flow or under static conditions to NIH 3T3 fibroblast cells cultured in Matrigel in the perfusion chambers. Experiments were conducted using conditions similar to the earlier permeability studies to mimic the delivery of extravasated nanoparticles to normal tissue by interstitial flow.

First, confocal imaging was performed on the flow-imposed and static chambers to ascertain the extent of nanoparticle penetration into the cellularized Matrigel (Fig. 5). In the absence of convective flow, penetration of 100 and 500 nm-sized beads into the gel was severely diffusion-limited; fluorescence of these nanoparticles was limited to the matrix boundary (Fig. 5d and f, respectively). Delivery of nanoparticles with imposed fluid flow for 5.5 h increased the penetration of all beads into the Matrigel compared to the static gel, even when a small pressure gradient of (2.3 ± 0.1) mm Hg was imposed (Fig. 5a, c and e, for flow-induced delivery of 40, 100, and 500 nm particles). This is reflected in the fluorescence line profiles as the bead penetration fronts were more than two times deeper into the matrix for the flow condition compared to the static controls. From intensity line profiles such as Figure 5i and j, for example, the 500 nm nanoparticle front penetrated about 58.6 ± 37.6 μm ($n = 7$) under flow compared to 5.8 ± 4.8 μm ($n = 6$) under static culture while 100 nm beads penetrated about 97.2 ± 56.2 μm ($n = 7$) under flow compared to 43.9 ± 20.7 μm ($n = 6$) under static culture. In addition, only 40 nm beads were detected in the bulk matrix for both flow (Fig. 5a and g) and static conditions (Fig. 5b and h). Cellular uptake of 40 nm nanoparticles was observed for both flow and static conditions (Fig. 5a and b, respectively) and the fluorescence intensity in the cells

was noticeably higher than in the bulk matrix. There were very few cells associated with red fluorescent, 100 nm nanoparticles detected and these were typically observed near the gel-media interface at the upstream end. This sharp difference between delivery of 40 and 100 nm nanoparticles had also been observed in a spheroid cell culture model and was hypothesized to result from differences in particle diffusivity as well as steric hindrance from matrix components (Goodman et al., 2007). This also suggested a size cutoff for efficient transport of nanoparticles in the matrix and further reinforced the matrix as a barrier to successful delivery and might account for the difference in transfection observations between the 2D cultures and in vivo studies.

In addition, live time-course imaging was performed to visualize the penetration of 100 nm nanoparticles into GFP-actin expressing MDCK cells seeded in Matrigel in situ in a modified version (see Supplementary Fig. 4) of the chamber under flow and static over a period of 4 h on a epifluorescent microscope with environmental control chamber (see Supplementary Movie 2). To perform these studies, minimal modifications were made to lengthen the channels connecting the ports to the media compartments so that the chamber could mount comfortably on the stage of our microscope with the media reservoirs out of the way of the condenser lens (Supplementary Fig. 4). Similar to the confocal studies, fluid flow was observed to enhance nanoparticle penetration in the cell-gel model in contrast to the static controls, which exhibit minimal penetration. Furthermore, cell migration was interestingly observed in the direction of imposed interstitial flow. This phenomenon was previously reported although in those studies, quantification was performed by counting the trans-migrated cells across the inserts post-fixation since the authors used a modified Boyden chamber assay flow system that cannot be imaged in situ (Goldman et al., 2005; Shields et al., 2007).

Cell Recovery for Flow Cytometry Analysis

A key attribute of cell culture systems is the collection of cells or cell lysates for post-culture analysis. Thus, a method to recover live cells from the perfusion chamber was developed. Matrigel within the chamber was degraded by infusion of dispase, a metalloprotease that gently cleaves ECM proteins such as fibronectin, collagen IV, and minimally collagen I but do not degrade cell surface proteins for 2 h at 37°C. Using this treatment, fluorescent (Calcein AM)-labeled or GFP-transfected cells were recovered without the loss of fluorescence signals (data not shown). Cells collected can be replated for further growth (Fig. 6b) or be used for analysis such as flow cytometry (Fig. 6c). For example, to quantify delivery efficiencies of nanoparticles to 3D cultured cells, mixtures of dark-red fluorescent 40 nm nanoparticles and yellow-green-fluorescent 100 nm nanoparticles were first introduced to NIH 3T3 cells cultured by perfusion for 5.5 h. Following nanoparticle delivery, cells were recovered as described above for flow cytometry analysis. Dead cells (<15%) were excluded from analysis by propidium iodide staining. As shown in Figure 6c (bottom right graph), the entire dark-red fluorescence profile shifts, indicating association and/or uptake of 40 nm nanoparticles; in contrast, no major increase in fluorescence was detected in the yellow-green fluorescence channel (Fig. 6c, bottom left graph), reflecting low uptake of 100 nm nanoparticles. Flow cytometry therefore confirmed our previous confocal observations that 40 nm nanoparticles are efficiently delivered throughout the matrix and are associated with cells whereas 100 nm nanoparticles are not accessible for cellular uptake. This suggests a diffusion-convection limited size-cut off between 40 and 100 nm. A similar particle size cutoff for efficient nanoparticle delivery to cells grown in 3D was determined using a multicellular spheroid model (Goodman et al., 2007). Overall, the results here, combined with the nanoparticle penetration studies demonstrate the feasibility of the perfusable tissue culture chamber for evaluation of gene and drug delivery vehicles.

While the initial studies here appear to demonstrate that cellular uptake is a function of nanoparticle size, it is important to note that the efficiency of nanoparticle uptake in cells may

also be dependent on other properties of the particles, such as the surface charge and particle-matrix interactions. The nanoparticle penetration is also related to matrix density and pore size, which can vary considerably depending on the composition of the matrix. Tissues and organs where the studied nanoparticles will be eventually used are likely to differ in terms of composition, density, and average pore size since they usually consist of many extracellular material types such as basement membrane ECMs, collagen, glycosaminoglycans, and proteoglycans in varying composition. Tissue and organs are typically denser due in part to many extracellular material types and thus their pore sizes in the range of 1–100 nm (Leuschner, 2007) are usually 1–2 orders of magnitude smaller than gels in the range of 1–10 μm (Pedersen and Swartz 2005; Zaman et al., 2006). It is highly likely that the penetration depths in tissues will be less than observed in hydrogel systems although data and information obtained from gel cultures provide insights and may be used in computational models and analysis to predict nanoparticle behavior in tissues.

One of the advantages of the perfusable system developed here is that the density and composition of nanoparticles, cell and matrix types in the chamber can be easily controlled by changing the concentration and type of loaded material initially. However, while the material loaded might not resemble the dense, oriented and organized ECM typically observed *in vivo*, we expect that with the incorporation of mechanical factors such as interstitial flow, the cells will eventually organize and remodel the extracellular matrix into a more physiological state in the longer term. With the development of this perfusable chamber, future studies can be performed to ascertain the effects of the nanoparticles properties, cell densities and matrix compositions to penetration and delivery to tissue cultures. These studies will contribute toward the development of a more efficient nanoparticle delivery vehicles for *in vivo* applications.

Applications for the Perfusion Chamber

As demonstrated, the developed chamber offers an *in situ* tool for more realistic nanoparticle transport and uptake studies under *in vitro* conditions. Nanoparticles can be further studied and optimized in the model rather than performing the optimization and troubleshooting procedures in animals, thus reducing the use and cost associated with these *in vivo* studies. For example, the major limiting extracellular and intracellular barriers to successful delivery can be identified by elucidating the fate of fluorescently labeled therapeutic agents and their carriers in the cell–gel culture, whether they are bound or unpackaged in the extracellular materials before reaching to the cells or if they are successful in achieving cellular internalization but were degraded in the cells before nuclear delivery. Thus, the device could serve as an intermediate system and bridge the experimental gap between 2D studies and *in vivo* delivery studies.

Although the chamber is designed to study, optimize and troubleshoot the delivery of therapeutic nanoparticles, the design is versatile and easy to implement, and therefore may have relevance to a broad scientific audience interested in employing cultures that mimic *in vivo* physiological and pathological conditions more realistically. For example, biologists and bioengineers can utilize the chamber to study and understand cell processes, such as migration and adhesion, or/and gene or cytokine expression of cells subjected to various magnitude of fluid force in a 3D architecture, since the system offers the advantage of being able to control and vary the pressure gradient and flow rates across the chamber. Furthermore, static controls (Fig. 7a) can be employed for comparison to the flow conditions. Studies with concentration gradients are also possible with the device by loading the media compartments with solutions of different concentration or composition (Fig. 7b). Upon further optimization or modification, the chamber could also be configured to mimic circulation (Fig. 7c and d) in various tissue microenvironments (such as artificial liver, kidney) due to the four loading media ports.

Conclusions

In this work, a transparent and versatile soft tissue culture chamber amenable to imposed flow is presented for the study of nanoparticle penetration, transport and uptake in a more realistic 3D environment. The device offers the following unique features: (i) direct in situ cell observation in a plane normal to the direction of flow or action, (ii) an easily configurable and controllable flow environment, and (iii) a recoverable cell–gel mixture that is usable in post-experimental analysis such as flow cytometry. In addition, the chamber is simpler to set-up compared to previous multi-component perfusion chambers. Nanoparticle penetration studies demonstrated the versatility (imaging clarity and post-experimental cell recovery) of the chamber and confirmed a particle size cutoff for efficient nanoparticle delivery to cells living in a surrounding 3D extracellular matrix. Taken together, this chamber offers an in situ tool for more realistic studies under in vitro conditions prior to in vivo and clinical studies. Furthermore, the chamber may have broad applications beyond gene and drug delivery in diverse fields as cell biology, biomechanics to tissue engineering since improvements and modifications to the system may lead to more complex and integrated in vitro systems that mimic in vivo conditions more closely and will enable investigations of physiological and pathological processes of interest in a more realistic tissue architecture.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Nanoparticle transport in the interstitium. In healthy normal tissues, the pressure difference between blood and lymphatic capillaries drives inward interstitial flow, thereby aiding nanoparticle transport into the interstitial space. The presence of lymphatic vessels and reabsorption into the post-capillary venules clear nanoparticles from the spaces. In solid tumors, high interstitial pressure combined with the lack of functional lymphatics result in a general outward flow, preventing deep penetration of nanoparticles.

Figure 2.
a: Micromachined PMMA lithography. **b:** Stereo micrographs of various PDMS post-configurations created from micromachined PMMA lithography.

Figure 3.

a: Schematic of the soft tissue culture chamber design. **b:** Image of flow (front) and static (back) chambers in their compact set-up. **c** and **d:** Respective side-view schematics of flow and static system set-up. **e:** Estimation of Darcy's permeability via measuring the volumetric flow over average pressure difference across the gel. **f** and **g:** Phase contrast low magnification (10 $\times$) and high magnification (136 $\times$) micrograph of cells in the Matrigel that have been cultured in static conditions for a week, demonstrating the visual clarity of the chambers.

Figure 4.

Time course trypan blue front (bright gray from the left) through Matrigel. A pressure gradient of (2.3 ± 0.1) mm Hg was imposed for the flow condition. This time-course images also demonstrated that there was no leakage at the start of the flow or through the experiment since a uniform front was observed rather than a low resistance leak path.

Figure 5.

Induced flow (a, c, and e) even at a very low pressure gradient of (2.3 ± 0.1) mm Hg improves penetration of 40 nm (blue), 100 nm (red) and even 500 nm nanoparticles (green) into the cell–Matrigel compared to delivery under static conditions (b, d, and f). Overlay images (g and h) of the nanoparticle (blue for 40 nm, red for 100 nm and green for 500 nm) penetration and their respective intensity profiles (i and j) on the yellow arrow lines in the images. The confocal micrographs of 4.5 μm optical sections also showed a contrast in penetration between the smaller size particles (40 nm), which can access cells within the matrix by diffusion, and the larger size particles (100, 500 nm) which require flow to drive their penetration into the gel. Cellular uptake of blue-fluorescent, 40 nm particles is observed under both flow and static conditions.

Figure 6.

Recovery of cells from Matrigel in chamber for flow cytometry. **a** and **b**: Epifluorescent phase-contrast images of cells recovered immediately after Dispase treatment and 3 days after replating. **c**: Flow cytometry plots of cells that are labeled with dead cell marker propidium iodide (PI) and with associated 40 and 100 nm fluorescent nanoparticles.

Figure 7. Various possible experimental configuration for the soft tissue culture chamber. Static cell-gel soft tissue cultures with (a) one media of interest, (b) two media of interest to generate a gradient. Flow configurations focusing on the (c) local interstitium and (d) circulatory system (such as artificial liver, kidney, microcirculation).