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3-D Tumor Model for *In Vitro* Evaluation of Anticancer Drugs

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Abstract: The efficacy of potential anticancer drugs during preclinical development is generally tested *in vitro* using cancer cells grown in monolayer; however, a significant discrepancy in their efficacy is observed when these drugs are evaluated *in vivo*. This discrepancy, in part, could be due to the three-dimensional (3-D) nature of tumors as compared to the two-dimensional (2-D) nature of monolayer cultures. Therefore, there is a need for an *in vitro* model that would mimic the 3-D nature of tumors. With this objective, we have developed surface-engineered, large and porous biodegradable polymeric microparticles as a scaffold for 3-D growth of cancer cells. Using the MCF-7 cell line as model breast cancer cells, we evaluated the antiproliferative effect of three anticancer drugs: doxorubicin, paclitaxel and tamoxifen in 3-D model vs in 2-D monolayer. With optimized composition of microparticles and cell culture conditions, a density of 4.5×10^6 MCF-7 cells/mg of microparticles, which is an 18-fold increase from the seeding density, was achieved in six days of culture. Cells were observed to have grown in clumps on the microparticle surface as well as in their interior matrix structure. The antiproliferative effect of the drugs in 3-D model was significantly lower than in 2-D monolayer, which was evident from the 12- to 23-fold differences in their IC_{50} values. Using doxorubicin, the flow cytometry data demonstrated ~2.6-fold lower drug accumulation in the cells grown in 3-D model than in the cells grown as 2-D monolayer. Further, only 26% of the cells in 3-D model had the same concentration of drug as the cells in monolayer, thus explaining the reduced activity of the drugs in 3-D model. The collagen content of the cells grown in 3-D model was 2-fold greater than that of the cells grown in 2-D, suggesting greater synthesis of extracellular matrix in 3-D model, which acted as a barrier to drug diffusion. The microarray analysis showed changes in several genes in cells grown in 3-D, which could also influence the drug effect. In conclusion, the cells grown in 3-D are more resistant to chemotherapy than those grown in 2-D culture, suggesting the significant roles of cellular architecture, phenotypic variations, and extracellular matrix barrier to drug transport in drug efficacy. We propose that our model provides a better assessment of drug efficacy than the currently used 2-D monolayer as many of its characteristic features are similar to an actual tumor. A well-characterized 3-D model can particularly be useful for rapid screening of a large number of therapeutics for their efficacy during the drug discovery phase.

Keywords: Polymer; scaffold; cancer cells; anticancer drugs; prescreening; drug discovery

Introduction

During the initial drug development and discovery phase, the efficacy of anticancer drugs is usually tested in two-dimensional (2-D) monolayer in culture plates. However, these drugs do not work as effectively *in vivo* as in 2-D

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monolayer. This discrepancy in drug efficacy, in part, could be due to the three-dimensional (3-D) nature of tumor vs the 2-D nature of monolayer culture. Further, it is known that the cells grown in 3-D have different cell surface receptor expression and proliferation,[1,2] extracellular matrix synthesis,[3] cell density,[4] and metabolic functions[5] than cells grown in 2-D monolayer. It has been suggested that many important signals, key regulators, and tissue phenotypes are lost when cells are cultured on 2-D substrates such as culture plates.[6] Therefore, it would be useful to develop an *in vitro* model of tumor that, in many aspects, would resemble an actual tumor to obtain a realistic assessment of drug efficacy prior to their testing in animal models or patients.

The field of tissue engineering has been traditionally developed for the repair or reconstruction of damaged or lost tissues or organs but has not been explored for developing a tumor model for *in vitro* evaluation of drug efficacy. For this purpose, we have been investigating large, porous, biodegradable microparticles as a scaffold to grow cancer cells into a 3-D tumor-like structure.[7] Microparticles offer a larger surface area for cell attachment than a regular slab-type matrix; therefore the cell growth on microparticles could be more optimal.[8] Further, the large, porous internal matrix structure of microparticles could allow the infiltration and anchoring of cells as well as the diffusion of nutrients to sustain the cell growth inside the matrix structure.[9,10] Of the various polymers, biodegradable poly(hydroxy acids) such as the copolymers of poly(lactic acid) (PLA) and poly(lactic-*co*-glycolide) (PLGA) are extensively used because of their biocompatibility and high mechanical strength.[11–13]

In our previous study, we optimized parameters for the formulation of large, porous microparticles using PLA and PLGA polymers and carried out preliminary studies to determine the effect of various physical parameters (porosity, hydrophilicity, size, etc.) and conditions for cell culturing (cell seeding, frequency of medium change, etc.) on microparticles.[7] One of the goals of our study was to achieve the cell density on scaffold closer to that present in an actual tumor ($\sim 2 \times 10^8$ mm^3).[14] With this objective, we further investigated the effect of polymer molecular weight and interfacial properties of microparticles on cell growth. Chitosan and poly(vinyl alcohol) (PVA) were selected to modulate interfacial properties of microparticles to enhance cell adhesion and growth on the scaffold structure.

We hypothesized that the drug response in 3-D model would be different than in 2-D monolayer because of the structural, molecular and genetic variations in cells grown in two different structural configurations. To determine the drug efficacy in 2-D monolayer and 3-D model, we selected three model anticancer drugs (doxorubicin, paclitaxel and tamoxifen), which are currently used in clinical practice for the treatment of breast cancer but act via different mechanisms of action. We studied drug diffusion and cellular uptake to understand the discrepancy in the drug efficacy in 2-D monolayer vs 3-D model. Our overall results demonstrated significantly lower drug activities in 3-D model vs in 2-D monolayer.

Experimental Section

PLA polymers of different intrinsic viscosity (0.17 dL/g, 0.44 dL/g and 0.66 dL/g) were purchased from Birmingham Polymers, Inc. (Birmingham, AL). PVA (average MW 30,000−70,000 Da), bovine serum albumin (Fraction V) (BSA), chitosan from crab shells (85% deacetylated) and tamoxifen were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). RPMI-1640, trypsin EDTA, fetal calf serum (FCS), fetal bovine serum (FBS) and Dulbecco's phosphate buffered saline (DPBS) were purchased from Gibco BRL (Grand Island, NY). Doxorubicin was a gift from Dabur India

(1) Wang, F.; Weaver, V. M.; Petersen, O. W.; Larabell, C. A.; Dedhar, S.; Briand, P.; Lupu, R.; Bissell, M. J. Reciprocal interactions between beta1-integrin and epidermal growth factor receptor in three-dimensional basement membrane breast cultures: a different perspective in epithelial biology. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 14821–6.

(2) Anders, M.; Hansen, R.; Ding, R. X.; Rauen, K. A.; Bissell, M. J.; Korn, W. M. Disruption of 3D tissue integrity facilitates adenovirus infection by deregulating the coxsackievirus and adenovirus receptor. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 1943–8.

(3) Beningo, K. A.; Dembo, M.; Wang, Y. L. Responses of fibroblasts to anchorage of dorsal extracellular matrix receptors. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 18024–9.

(4) Ng, K. W.; Leong, D. T.; Hutmacher, D. W. The challenge to measure cell proliferation in two and three dimensions. *Tissue Eng.* **2005**, *11*, 182–91.

(5) Rhodes, N. P.; Srivastava, J. K.; Smith, R. F.; Longinotti, C. Metabolic and histological analysis of mesenchymal stem cells grown in 3-D hyaluronan-based scaffolds. *J. Mater. Sci. Mater. Med.* **2004**, *15*, 391–5.

(6) Lee, G. Y.; Kenny, P. A.; Lee, E. H.; Bissell, M. J. Three-dimensional culture models of normal and malignant breast epithelial cells. *Nat. Methods* **2007**, *4*, 359–65.

(7) Sahoo, S. K.; Panda, A. K.; Labhasetwar, V. Characterization of porous PLGA/PLA microparticles as a scaffold for three dimensional growth of breast cancer cells. *Biomacromolecules* **2005**, *6*, 1132–9.

(8) Hacker, M.; Tessmar, J.; Neubauer, M.; Blaimer, A.; Blunk, T.; Gopferich, A.; Schulz, M. B. Towards biomimetic scaffolds: anhydrous scaffold fabrication from biodegradable amine-reactive diblock copolymers. *Biomaterials* **2003**, *24*, 4459–73.

(9) Cukierman, E.; Pankov, R.; Stevens, D. R.; Yamada, K. M. Taking cell-matrix adhesions to the third dimension. *Science* **2001**, *294*, 1708–12.

(10) Cukierman, E.; Pankov, R.; Yamada, K. M. Cell interactions with three-dimensional matrices. *Curr. Opin. Cell Biol.* **2002**, *14*, 633–9.

(11) Wu, L.; Ding, J. In vitro degradation of three-dimensional porous poly(D,L-lactide-co-glycolide) scaffolds for tissue engineering. *Biomaterials* **2004**, *25*, 5821–30.

(12) Webb, A. R.; Yang, J.; Ameer, G. A. Biodegradable polyester elastomers in tissue engineering. *Expert Opin. Biol. Ther.* **2004**, *4*, 801–12.

(13) Mikos, A. G.; Lyman, M. D.; Freed, L. E.; Langer, R. Wetting of poly(L-lactic acid) and poly(DL-lactic-co-glycolic acid) foams for tissue culture. *Biomaterials* **1994**, *15*, 55–8.

(14) Zinkernagel, R. M. On natural and artificial vaccinations. *Annu. Rev. Immunol.* **2003**, *21*, 515–46.

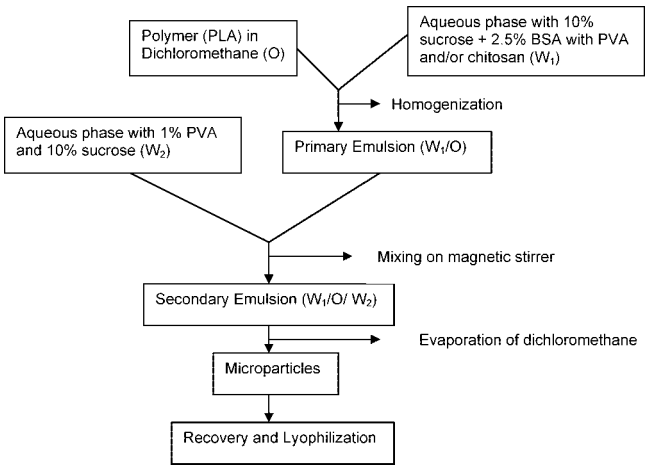

Figure 1. Flow diagram for the preparation of microparticles using a multiple emulsion solvent evaporation method. Sucrose was added in the internal phase to form porous microparticles whereas bovine serum albumin (BSA) was added to improve the stability of the primary emulsion.

Limited (New Delhi, India). Paclitaxel was purchased from Shanghai 21CEC Pharmaceuticals Ltd. (Shanghai, China). All other chemicals and organic solvents used were of analytical grade obtained from Fisher Scientific (Pittsburgh, PA).

Preparation of Microparticles. All formulations of microparticles were prepared with PVA present in the external phase as an emulsifier; in some formulations, PVA and/or chitosan were added in the internal phase of the primary emulsion to create microparticles with an internal matrix structure interface coated with these hydrophilic polymers (Figure 1). For the preparation of these microparticles, chitosan solution prepared in 5% (v/v) lactic acid or an aqueous solution of PVA was added into the internal aqueous phase along with sucrose, which in our previous study was shown to create large, porous microparticles.[7] To formulate microparticles with both PVA and chitosan in the internal structure of microparticles, different proportions of chitosan and PVA solutions were mixed to achieve the desired concentration of each component. We used PLA polymer as it demonstrated better cell growth than PLGA polymer in our previous study.[7] In this study, we further determined the effect of molecular weight of PLA polymer and optimized the composition of scaffold interface with PVA and chitosan to further improve the cell growth. Briefly, 200 mg of PLA was dissolved in 4 mL of dichloromethane to which 800 μL of aqueous phase containing 2.5% (w/v) BSA, 10% (w/v) sucrose, 1.25% (w/v) chitosan and 5% (w/v) PVA were emulsified using a homogenizer (Biospacte Product Inc., Butler, PA) at 1,000 rpm to form a water-in-oil primary emulsion. The primary emulsion (2.5 mL) was added dropwise into 250 mL of 1% PVA (w/v) solution containing 10% (w/v) sucrose; the emulsion was stirred overnight on a magnetic stir-plate to evaporate organic solvent. Microparticles were recovered by centrifugation at 4,000 rpm for 15 min at 10 °C (Dupont Sorvall, Creative Technology Systems, Inc., Newark, DE), washed three times with distilled water and then lyophilized for 48 h (VirTis Company, Inc., Gardiner, NY).

Determination of PVA and Chitosan Associated with Microparticles. A colorimetric method was used to determine the amount of PVA associated with microparticles.[15] Briefly, 2 mg of lyophilized sample of each formulation was treated with 2 mL of 0.5 M NaOH for 15 min at 60 °C. The samples were neutralized with 900 μL of 1 N HCl, and the volume was adjusted to 5 mL with distilled water. To each sample, 3 mL of 0.65 M solution of boric acid, 0.5 mL of a solution of I_2/KI (0.05 M/0.15 M) and 1.5 mL of distilled water were added. Following incubation for 15 min at room temperature, the absorbance of each sample was measured at 690 nm using a spectrophotometer (model UV-10601PC, Shimadzu Scientific Instruments, Columbia, MD). A standard plot of PVA was prepared under identical conditions.

To determine the amount of chitosan associated with microparticles, 2 mg of each formulation was treated overnight at 37 °C with 1 mL of 0.01% (v/v) lactic acid solution. Samples were centrifuged at 1,000 rpm, and the supernatant from each sample was used to determine chitosan content. A stock solution of Cibacron brilliant red 3B-A dye (Sigma-Aldrich) was prepared (1.5 mg/mL) in water, and the pH of the dye solution was adjusted to 3.2 with 0.1 M glycine hydrochloride so that the dye concentration is 0.075 mg/mL.[16] To each sample (500 μL), 3 mL of the dye solution was added, and after incubation for 30 min at room temperature, the absorbance of each sample was measured at 575 nm. A standard plot of chitosan was prepared under similar conditions. Similarly, PVA and chitosan associated with microparticles formulated with both the agents were assayed as above. There was no interference of chitosan in the analysis of PVA and vice versa.

Characterization of Microparticles. Surface morphology of microparticles was characterized by scanning electron microscopy (SEM) (JEOL JSM-T220A, Briarcliff Manor, NY) operating at an accelerating voltage of 30 kV. A sample of microparticles was sputter-coated with gold palladium at a thickness of about 70 nm prior to acquisition of SEM images. To view the internal structure, microparticles were cut with a fine edged blade and gold coated as described above. The NIH ImageJ software (http://rsb.info.nih.gov/ij/) was used to calculate the mean particle size and the internal pore diameters from the SEM photomicrograph.

Cell Culture and Cell Seeding for Scaffold Composition Optimization. MCF-7 cells were maintained in RPMI-1640 media supplemented with 10% FBS and 1% penicillin−streptomycin (cRPMI) in T-75 cm^2 flasks in an incubator (Thermo Electron Corporation, Asheville, NC) at 37 °C and

(15) Sahoo, S. K.; Panyam, J.; Prabha, S.; Labhasetwar, V. Residual polyvinyl alcohol associated with poly (D,L-lactide-co-glycolide) nanoparticles affects their physical properties and cellular uptake. *J. Controlled Release* **2002**, *82*, 105–14.

(16) Muzzarelli, R. A. Colorimetric determination of chitosan. *Anal. Biochem.* **1998**, *260*, 255–7.

5% CO_2. Each formulation of PLA microparticles was incubated with 50% FBS in RPMI-1640 (2 mg/mL) for 3 h at 37 °C and 5% CO_2 in 15 mL Falcon tubes. Microparticles were centrifuged at 1,000 rpm for 15 min at 20 °C, and then resuspended in RPMI-1640 (2 mg/mL). For studying the effect of microparticle composition on cell growth, 1 mL of the microparticle suspension prepared as above was transferred to separate wells of 6-well plates. The medium from each well was aspirated carefully, leaving microparticles in the wells immediately prior to cell seeding. A 500 μL aliquot of MCF-7 cell suspension (1×10^6 cells/mL) in cRPMI medium was added directly onto microparticles in each well. After 3 h of incubation at 37 °C and 5% CO_2, an additional 1.5 mL of cRPMI was added to each well, and cells were allowed to grow in an incubator. The medium was changed on alternate days, and cells were counted at 7 days postseeding to determine the effect of composition of microparticles on cell growth. In a separate experiment, the growth curve was followed with the microparticles prepared either with PVA or chitosan alone or in combination at optimized concentration and the cell growth was followed up to 10 days postseeding. Separate wells were used for each time point in the above experiment. Cells were detached from microparticles by treating them with 1 mL of 0.1 M citric acid containing 0.1% crystal violet for 1 h at 37 °C. The detached cells were counted using a hemocytometer.

Apart from the quantitative measurement of cell number, microparticles were observed for cell growth under a light microscope (Nikon, Osaka, Japan). Further, the growth of cells inside the scaffold was observed using transmission electron microscopy (TEM). For this purpose, microparticles (5% PVA and 1.25% chitosan) with cells grown for 10 days in culture were harvested for analysis. The microparticles with cells were fixed in 2.5% glutaraldehyde solution (Sigma-Aldrich) in Sorensen's phosphate buffer (0.1M, pH 7.4), postfixed in 1% osmium tetroxide solution, dehydrated in a graded ethanol solution and finally passed through 100% propylene oxide three times. Thin sections of microparticles were stained with 2% uranyl acetate and then with Reynolds lead citrate, and were visualized by TEM (Philips 410 LS, Philips/FEI, Briarcliff Manor, NY).

Antiproliferative Effect of Anticancer Drugs. The above protocol of cell culturing was slightly modified for drug efficacy studies in 2-D monolayer and 3-D model, in which Petri dishes were used instead of 6-well plates. For 2-D monolayer cell growth, tissue culture-treated Petri dishes (100 mm × 20 mm, Becton Dickson #353003, Franklin Lakes, NJ) were used that provided more surface area for cells to grow without reaching confluency, a problem that was noticed in 6-well plates when cells were allowed to grow for a longer period of time. For the 3-D model, non-tissue culture Petri dishes (100 mm × 15 mm; Fisher Scientific #08-757-13) were used which enabled better cellular attachment and growth onto microparticles rather than onto the surface of Petri dish. In 6-well plates, occasionally cultured microparticles required detachment (sometimes using a flat spatula) to prevent cell growth on the surface of culture plates. This step was not required when microparticles were cultured in the above non-tissue culture Petri dishes because the surface of these Petri dishes is not activated to favor cell attachment. The remaining protocol was similar to that used above for 6-well plates except that the cell seeding in the case of 2-D monolayer was carried out the second day postseeding on scaffold at a cell density of 0.5×10^6 cells/mL. This protocol for cell seeding and growth was optimized to obtain approximately the same cell count in both sets of experiments at the time of drug treatment for a better comparison of drug efficacy. Three model anticancer drugs, which are currently used in the treatment of breast cancer but act via different mechanisms, were selected to determine their IC_{50} in 3-D model and 2-D monolayer. Doxorubicin is a DNA intercalating anticancer agent,[17] and paclitaxel works by stabilizing microtubules,[18] whereas tamoxifen is an orally active selective estrogen receptor modulator and primarily acts as a cytostatic rather than cytocidal drug.[19] Stock solutions of paclitaxel and tamoxifen were prepared in ethanol and diluted appropriately in tissue culture medium to obtain the desired drug concentration. On 5 days postseeding, media was carefully removed and replaced with 5 mL of media containing different concentrations of drugs. After 24 h of drug treatment, cells were detached by treating them with trypsin-EDTA for 5 min in the case of microparticles or for 2 min in the case of 2-D monolayer. The time required for treatment with trypsin-EDTA was optimized for complete detachment of cells from microparticles without affecting cell viability. The detached cells were stained using 0.4% (w/v) trypan blue in deionized water, and viable cells (unstained) were counted using a hemocytometer.

Flow Cytometry. Doxorubicin was used for this study because of its inherent fluorescent property. Fluorescent intensity as a measure of drug uptake by cells in 3-D model and 2-D monolayer was determined using a FACSCalibur flow cytometer (Becton Dickson) at 488 nm excitation and a 585/42 filter (564−606 nm) to match the emission spectra of doxorubicin. On 5 days postseeding, media was carefully removed and 5 mL of doxorubicin solution (2,500 ng/mL) was added to each Petri dish and placed at 37 °C for 4 and 8 h. Cells were trypsinized from microparticles and monolayer as described above, centrifuged and resuspended into a single cell suspension in DPBS + 2% FBS at concentrations of 2×10^6 cells/mL and 1×10^6 cells/mL, respectively. The cell suspensions containing microparticles were filtered twice through a 35 μm nylon mesh tube cap into a 12 × 75 mm round-bottom tube (Becton Dickson #352235) to remove

(17) Myers, C. E.; McGuire, W. P.; Liss, R. H.; Ifrim, I.; Grotzinger, K.; Young, R. C. Adriamycin: the role of lipid peroxidation in cardiac toxicity and tumor response. *Science* **1977**, *197*, 165–7.
(18) Schiff, P. B.; Horwitz, S. B. Taxol stabilizes microtubules in mouse fibroblast cells. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 1561–5.
(19) Ishii, Y.; Waxman, S.; Germain, D. Tamoxifen stimulates the growth of cyclin D1-overexpressing breast cancer cells by promoting the activation of signal transducer and activator of transcription 3. *Cancer Res.* **2008**, *68*, 852–60.

microparticles from the cell suspension. Cells were initially analyzed using propidium iodide to ensure cell viability. There was no cell death either in 2-D monolayer or in 3-D model experiments following 4 h of doxorubicin treatment (2,500 ng/mL), whereas at 8 h, 1.1% of the cells grown in 2-D monolayer were nonviable, compared to 0.5% of cells grown in 3-D model (data not shown).

Confocal Imaging. To determine the extent of drug (doxorubicin) diffusion in cells grown in 3-D model vs in 2-D monolayer, images were obtained using a Zeiss LSM 410 (Goettinger, Germany) with an argon krypton laser, an excitation of 488 nm and a long pass 515 nm filter following exposure to the drug. Individual microparticles with 5 day cell growth were harvested from Petri dishes and placed into Delta TPG culture confocal dishes (Bioptechs, Butler, PA) approximately 3 h prior to imaging to allow the 3-D model to anchor to the dish. Cells for 2-D monolayer were seeded in confocal dishes approximately 24 h prior to imaging to ensure proper cell attachment. The 3-D model and 2-D monolayer were treated with doxorubicin (2,500 ng/mL), and the images were taken at selected time points. We selected this concentration of the drug because at a lower concentration (100 ng/mL) the fluorescent intensity was not sufficient to monitor the drug uptake by cells.

Collagen Assay. Cell preparation followed a previously reported protocol,[20] and the collagen content was assayed with the Sircol Soluble Collagen Assay kit (Biocolor Ltd., Northern Ireland) according to the manufacturer's protocol. Cells grown in 2-D monolayer were scraped from Petri dishes in DPBS whereas those in 3-D model were suspended in DPBS. The preparations were centrifuged at 1,000 rpm using an Eppendorf microcentrifuge (5417R, Eppendorf-Netheler-Hinz-GmbH, Hamburg, Germany), and the sediment was treated with 1 mL of 0.5 M acetic acid for 18 h at 4 °C. One milliliter of the dye reagent supplied with the assay kit was added to 100 μL of the acid extract in 1.5 mL eppendorf tubes and mixed gently for 30 min at room temperature. The collagen-bound dye complex was recovered after centrifugation at 10,000 rpm for 10 min as above. The complex was solubilized in 1 mL of the alkali reagent provided with the assay kit. The absorbance of the samples was measured at 550 nm using a microwell plate reader (Fisher Biotech, Pittsburgh, PA). A standard plot of collagen was prepared under similar conditions. Microparticles without cells or DPBS were used as the respective controls. There was no interference of the polymer used in microparticles in the assay.

Recovery of Cells from 3-D Model Following Freezing. To determine whether the cells grown in 3-D model could be rescued after freezing, microparticles were seeded (seeding density = 0.5×10^6 cells/mg of microparticles) and cultured as above in non-cell culture Petri dishes for 5 days. The 3-D models were washed with incomplete RPMI-1640 medium, resuspended in 1 mL of cryopreservation media (Synth-a-Freeze, Cascade Biologics, Portland, OR), transferred into cryovials, and then frozen in a −80 °C freezer. After 24 h of freezing, the 3-D models were thawed and the entire content of each cryovial was transferred into Petri dishes and recultured for 48 h in cRPMI medium. The 3-D model cultured as above for the same time period, but not subjected to the freezing step, was used as the control. Cells cultured in 2-D monolayer (0.5×10^6 cells/mL of cryopreservation media) were also frozen and recultured as above. Cell viability for each sample was determined using trypan blue as above. The percents of cells rescued for 3-D model and 2-D monolayer were calculated from the cell numbers with and without the freezing step.

Microarray Analysis. As described above, cells were grown in Petri dishes either as a 2-D monolayer or on a 3-D scaffold, and detached after 6 days of growth by treating with trypsin-EDTA. The cells were centrifuged at 1,000 rpm for 10 min at 4 °C and resuspended into a single cell suspension in 1X DPBS at a concentration of 1×10^6 cells/mL. The experiment was carried out in triplicate. The cell suspension from microparticles was filtered twice through a 35 μm nylon mesh tube cap into a 12 × 75 mm round-bottom tube as described above to remove microparticles. The cell suspension was then submitted for RNA isolation and gene array analysis at the Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, NE. All DNA microarray gene expression studies used human oligonucleotide arrays custom-printed by a dedicated core facility within the Eppley Institute, as described previously[21–23] (details in the Supporting Information).

Statistical Analysis. Statistical analyses were performed using Student's *t* test. The differences were considered significant for *p* values of <0.05.

Results

Formulation and Characterization of Microparticles. Modification of the solvent evaporation technique with sucrose added in the internal phase of the primary emulsion

(20) Luppen, C. A.; Leclerc, N.; Noh, T.; Barski, A.; Khokhar, A.; Boskey, A. L.; Smith, E.; Frenkel, B. Brief bone morphogenetic protein 2 treatment of glucocorticoid-inhibited MC3T3-E1 osteoblasts rescues commitment-associated cell cycle and mineralization without alteration of Runx2. *J. Biol. Chem.* **2003**, *278*, 44995–5003.

(21) Batrakova, E. V.; Kelly, D. L.; Li, S.; Li, Y.; Yang, Z.; Xiao, L.; Alakhova, D. Y.; Sherman, S.; Alakhov, V. Y.; Kabanov, A. V. Alteration of genomic responses to doxorubicin and prevention of MDR in breast cancer cells by a polymer excipient: pluronic P85. *Mol. Pharmaceutics* **2006**, *3*, 113–23.

(22) Chan, C. Y.; Salabat, M. R.; Ding, X. Z.; Kelly, D. L.; Talamonti, M. S.; Bell, R. H., Jr.; Adrian, T. E. Identification and in silico characterization of a novel gene: TPA induced trans-membrane protein. *Biochem. Biophys. Res. Commun.* **2005**, *329*, 755–64.

(23) Ding, Y.; Xu, L.; Chen, S.; Jovanovic, B. D.; Helenowski, I. B.; Kelly, D. L.; Catalona, W. J.; Yang, X. J.; Pins, M.; Ananthanarayanan, V.; Bergan, R. C. Characterization of a method for profiling gene expression in cells recovered from intact human prostate tissue using RNA linear amplification. *Prostate Cancer Prostatic Dis.* **2006**, *9*, 379–91.

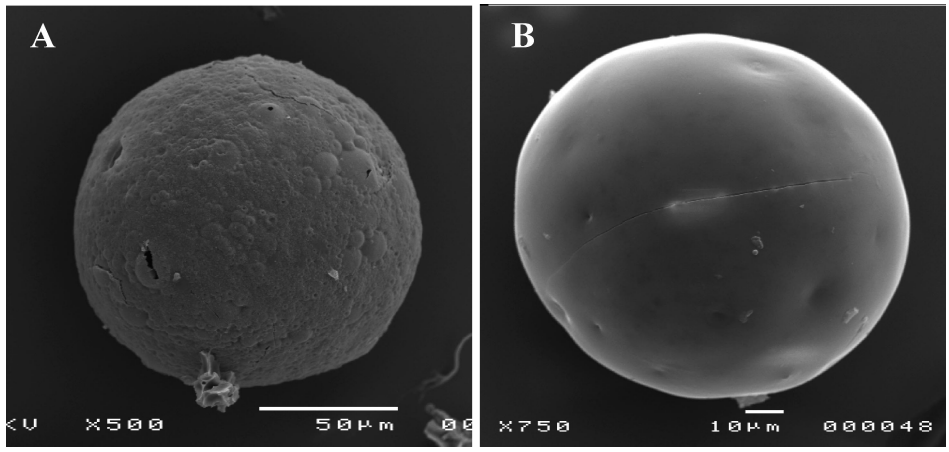

Figure 2. Scanning electron micrograph of PLA microparticles with (a) chitosan or (b) PVA−chitosan added in the internal phase during emulsification. Microparticles formulated with chitosan alone in the internal phase have a rough surface whereas those prepared with PVA alone or PVA−chitosan have a relatively smooth surface.

Table 1. Effect of PLA Molecular Weight on MCF-7 Cell Growth on Microparticles at 7 days post-seeding

		PVA (% w/v)				cell concn (1×10^6 cells/mg of microparticles)[b]	
formulation	intrinsic viscosity (dL/g) [MW]	in internal phase	in external phase	internal pore diameter (μm)[a]	PVA content (μg/per mg) of microparticles[b]	initial	at 7 days
PLA[c]	0.14 [11 kDa]	5	1	16.2 ± 4.2	4.85 ± 0.08	0.25	1.26 ± 0.12
PLA	0.44 [56 kDa]	5	1	14.8 ± 2.4	4.65 ± 0.12	0.25	0.86 ± 0.14
PLA	0.66 [85 kDa]	5	1	7.4 ± 1.2	4.57 ± 0.09	0.25	0.83 ± 0.08

[a] Data as mean ± SEM ($n = 20$). [b] Data as mean ± SEM ($n = 3$). [c] Data in this row represent optimized composition of microparticles from this set of experiments.

formed large, porous microparticles with interconnected void structures. The average diameter of different formulations of microparticles prepared in this study ranged from 160 to 182 μm. In general, the microparticles formulated either with PVA alone or with PVA plus chitosan in the internal phase demonstrated smoother surfaces whereas those formulated with chitosan alone demonstrated a rough surface (Figure 2). The wetting property of microparticles was determined by measuring the average time required for them to settle down in water at room temperature. The microparticles formulated with PVA and/or chitosan in the internal phase demonstrated better wetting as they settled down within 5 min in cell culture medium whereas the microparticles formulated without these polymers required 6 to 12 h to settle. The longer time required for wetting could be due to the entrapped air in the internal matrix structure of these microparticles which is not easily displaced.

Microparticles prepared with lower molecular weight PLA polymer demonstrated larger pores as compared to those prepared using higher molecular weight PLA (Table 1). This is apparent from their surface characteristics as well as from the internal matrix structure (Figure 3). The SEM pictures of microparticles formulated with lower molecular weight polymer demonstrated large pores which are covered with a thin film of polymer (Figure 3A). The presence of such pores was confirmed from the SEM pictures of broken microparticles (Figure 3A1). The molecular weight of PLA polymer used for making microparticles does not seem to affect the amount of PVA associated with microparticles (Table 1).

The total amount of PVA associated with microparticles increased with the increase in PVA concentration used in the internal phase during emulsification; however, this increase was insignificant beyond 5% PVA concentration (Table 2). The increase in concentration of chitosan in the internal phase of the emulsion from 1.25% to 2.5% did not significantly change the amount of chitosan associated with microparticles, hence we continued the study with 1.25% chitosan concentration (data not shown). Addition of PVA along with chitosan in the internal phase of the emulsion did not seem to significantly affect the amount of chitosan or PVA associated with microparticles, suggesting their noncompetitive binding to the microparticle matrix structure at the interface (Table 3).

MCF-7 Cell Growth on Different Formulations of Microparticles. First, we studied the effect of molecular weight of PLA polymer used in the fabrication of microparticles on cell growth. Based on the initial assessment of the effect of PVA on cell growth, microparticles formulated with 5% PVA in the internal phase were used. It was seen that the microparticles formulated with lower molecular weight demonstrated better cell growth than those formulated with higher molecular weight polymer (Table 1). Therefore, in the subsequent studies, we used PLA polymer with lower

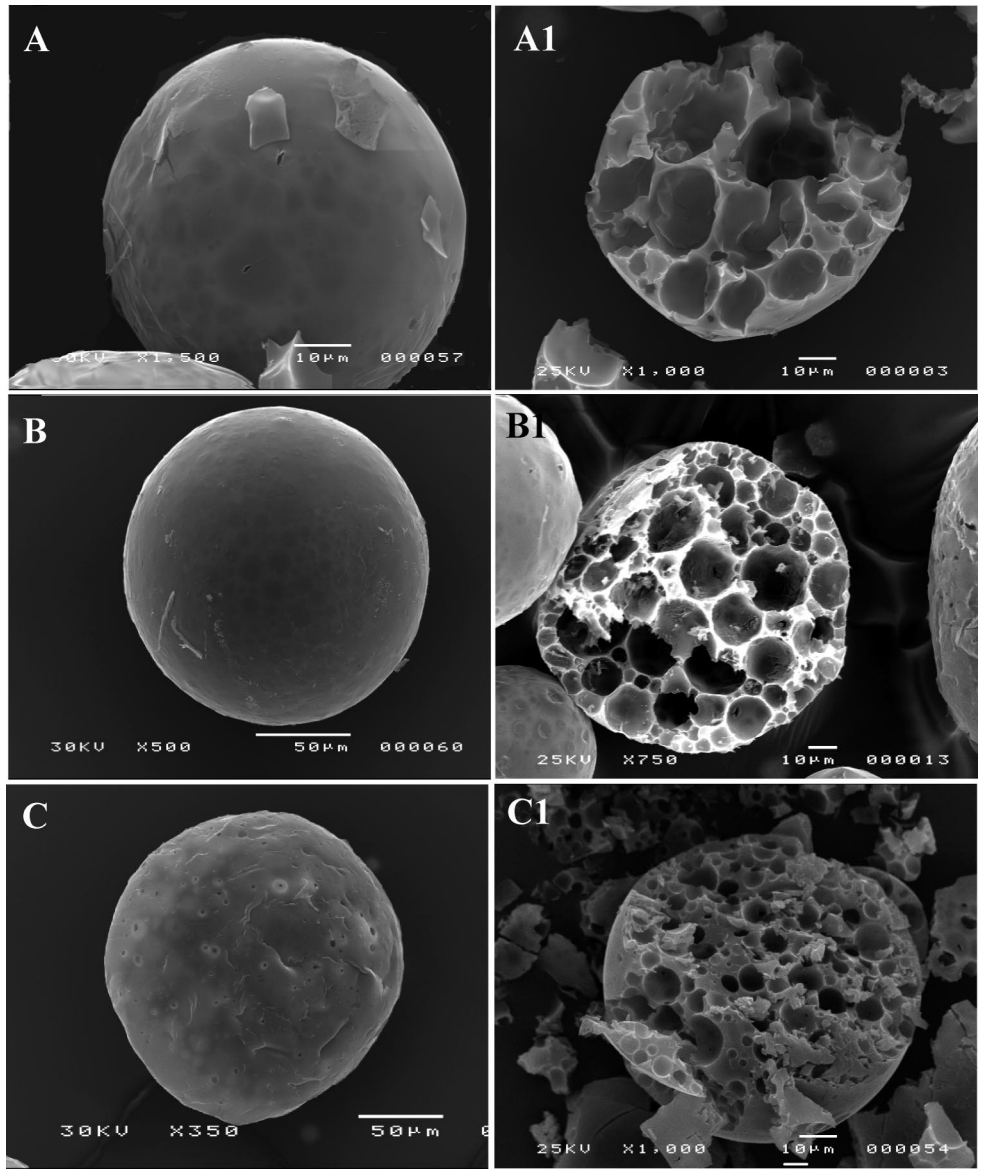

Figure 3. Scanning electron micrograph of outside (A, B and C) and inside (A1, B1 and C1) of typical PLA−PVA microparticles prepared with PLA of different intrinsic viscosities. Intrinsic viscosity = 0.17 dL/g (A and A1), intrinsic viscosity = 0.44 dL/g (B and B1) and intrinsic viscosity = 0.67 dL/g (C and C1). Microparticles prepared with low molecular weight PLA (intrinsic viscosity = 0.17 dL/g) are more porous than those prepared with high molecular weight PLA (intrinsic viscosity = 0.67 dL/g).

molecular weight (0.17 dL/g) for the fabrication of microparticles. Microparticles formulated with PVA in the internal phase demonstrated better cell growth than those formulated without it. Increasing the concentration of PVA in the internal phase of microparticles exhibited an increase in cell growth; however, the effect was insignificant beyond 5% PVA concentration (Table 2). Further, the microparticles formulated with only 5% PVA demonstrated better cell growth than those formulated with only 1.25% chitosan, but the combination of the two polymers in the above concentration showed better cell growth than either PVA or chitosan alone (Table 3). The cell growth on microparticles was slow initially but increased more rapidly 3 days postseeding and then reached a steady state at 7 days postseeding (Figure 4).

The optimized composition of microparticles, which consisted of 5% PVA and 1.25% chitosan formulated with low molecular weight PLA (0.17 dL/g), demonstrated cell growth from an initial seeding cell density of 0.25×10^6 to 4.5×10^6 cells/mg of microparticles, which is an 18-fold increase in cell density following 6 days of culture. This cell density is higher than that seen with the microparticles cultured in 6-well plates. Cells favored attachment to the microparticles rather than to the surface of the non-tissue culture Petri dish. Initially, cells were seen attached to the microparticle surface, and with time cells engulfed the microparticles completely, forming a 3-D tumorlike structure (Figure 5A). Either a single microparticle or a cluster of 2−3 microparticles were witnessed to form the 3-D tumorlike

Table 2. MCF-7 Cell Growth on Microparticles Formulated with Different Concentrations of PVA in the Internal Phase of Primary Emulsion at 7 Days Postseeding

	PVA (% w/v)			cell concn (1×10^6 cells/mg of microparticles)[a]	
polymer intrinsic viscosity (0.14 dL/g)	in internal phase	in external phase	PVA content (μg/mg) of microparticles[a]	initial	at 7 days
PLA	0	1	3.41 ± 0.08	0.25	0.67 ± 0.09
PLA	2	1	4.11 ± 0.05	0.25	1.12 ± 0.11
PLA[b]	5	1	4.84 ± 0.08	0.25	1.25 ± 0.15
PLA	10	1	4.99 ± 0.04	0.25	1.22 ± 0.09
PLA	20	1	5.01 ± 0.05	0.25	1.11 ± 0.08

[a] Data as mean $\pm$ SEM ($n = 3$). [b] Data in this row represent optimized composition of microparticles from this set of experiments.

Table 3. MCF-7 Cell Growth on Microparticles Prepared with Different Concentrations of PVA and Chitosan at 7 Days Postseeding

	composition of internal phase (% w/v)		PVA or chitosan content (μg/mg) of microparticles[a]		cell concn (1×10^6 cells/mg of microparticles)[a]	
polymer intrinsic viscosity (0.14 dL/g)	PVA	chitosan	PVA	chitosan	initial	at 7 days
PLA	0	0	3.35 ± 0.09		0.25	0.68 ± 0.08
PLA	5	0	4.79 ± 0.12		0.25	1.26 ± 0.11
PLA	0	1.25		2.42 ± 0.15	0.25	1.13 ± 0.13
PLA[b]	5	1.25	4.65 ± 0.08	2.24 ± 0.13	0.25	1.47 ± 0.08

[a] Data as mean $\pm$ SEM ($n = 3$). [b] Data in this row represent optimized composition of microparticles used for drug effect studies.

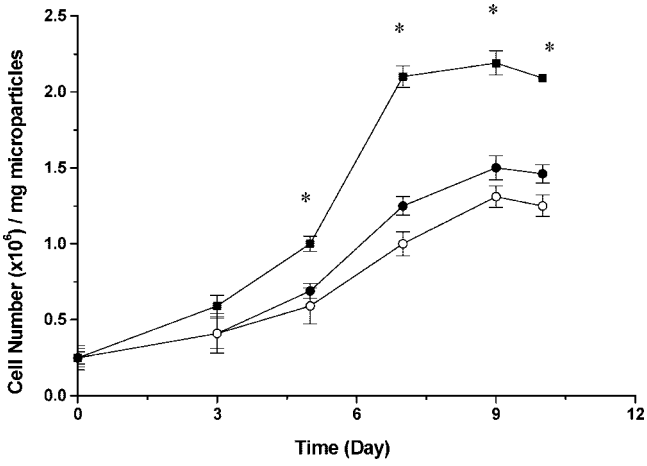

Figure 4. Growth kinetics of MCF-7 cells on composite microparticles formulated using PLA polymer with either PVA or chitosan alone or in combination. The cell growth was higher with the combination of 5% (w/v) PVA and 1.25% (w/v) chitosan used in the internal phase than with either chitosan or PVA alone. Initial seeding density = 0.25×10^6 cells/mg of microparticles. (●) PLA−chitosan, (○) PLA−PVA, (■) PLA−PVA−chitosan. Data as mean $\pm$ SEM, $n = 3$. *$p < 0.05$ vs other groups.

structure. The TEM of the cultured microparticles demonstrated infiltration of cells inside the matrix structure, indicating that cell growth occurred both outside and inside the microparticle matrix structure (Figure 5B).

Antiproliferative Activity of Drugs in 2-D Monolayer vs in 3-D Model. Cytotoxicity of model anticancer drugs was determined in cells grown in 2-D monolayer and in 3-D model following a 24 h drug treatment. Cell numbers were similar in both models at the time of treatment, and the inhibition in cell growth was calculated according to the respective untreated controls. It could be seen that IC_{50} values of drugs are significantly higher in 3-D model than in 2-D monolayer (Figure 6). The differences in the IC_{50} values observed in 2-D monolayer vs 3-D model were 12- to 23-fold depending upon the drug. This difference in IC_{50} values was lower for paclitaxel than for doxorubicin and tamoxifen (Table 4).

Drug Uptake by Cells in 2-D Monolayer vs in 3-D Model. After 4 h of treatment with doxorubicin (2,500 ng/mL), significantly fewer ($p < 0.05$) cells in 3-D model exhibited drug uptake than cells grown in 2-D monolayer, and the cells in 3-D model demonstrated 2.4-fold less drug uptake ($p < 0.05$) (Figure 7). The majority of the cell population was gated in 2-D monolayer (84%) treated for 4 h with doxorubicin, but only 26% of the cells grown in 3-D model were within the same fluorescent intensity range. Further, the difference in fluorescence intensity of the drug in the cells from 2-D monolayer and 3-D model was 3.2-fold at 4 h (R2 in Figure 7A) which increased to 4.7-fold at 8 h (R2 in Figure 7B).

The confocal studies further confirmed slower diffusion of drugs in the cells grown in 3-D model compared to the cells grown in 2-D monolayer. Within 15 min of incubation, doxorubicin was visualized inside the cytoplasm of the cells grown in 2-D monolayer; at 1 h the drug localized into the nucleus, and within 24 h the majority of cells had undergone cell death (Figure 8, columns A and B). In contrast, the cells grown in 3-D model did not show accumulation of doxorubicin in the cytoplasm, until 1 h and thereafter remained localized in the cytoplasm until 8 h. The drug was seen

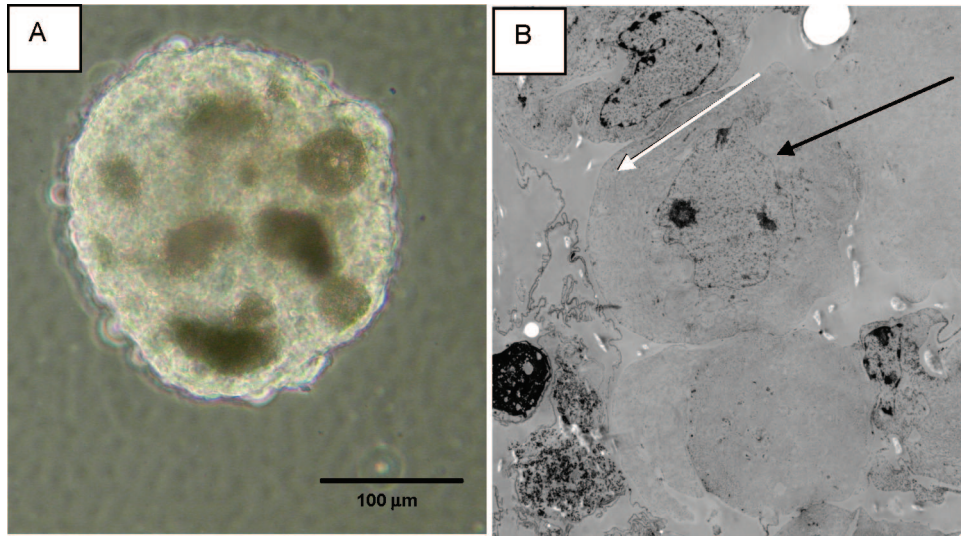

Figure 5. Cell growth on microparticles in culture. (A) Representative photographs of MCF-7 cells grown on PLA−PVA−chitosan microparticles at 5 days in culture. (B) TEM of a microparticle demonstrating infiltration of MCF-7 cells (black arrow) inside the microparticle matrix structure (white arrow).

localized within the nucleus at 24 h (Figure 8, columns C and D) and, at that time, some of the cells were detached from the 3-D model structure.

Extracellular Matrix Synthesis. After 5 days of growth, cells in 3-D model synthesized 57.2 ± 1.3 μg of collagen per 1×10^6 cells, which was significantly higher than the 27.7 ± 3.0 μg of collagen synthesized for the same number of cells in 2-D monolayer ($p < 0.05$, $n = 6$).

Rescue of Cells from 3-D Model Following Freezing. Following the freezing step, cell viability was slightly higher in 3-D model compared to that in 2-D monolayer (38% vs 33%, $n = 4$).

Microarray Analysis. Results of the cDNA microarray revealed changes in the expression of 702 genes (overexpression of 694 genes; down-regulation of 8 genes) in cells grown in 3-D model compared to those grown in 2-D monolayer (Figure 9). Among the overexpressed genes, 2 genes showed a 2-fold increase and 184 genes showed greater than 1.5-fold increase. We classified the altered transcripts based on their biological roles. Our classification showed that genes in categories such as protein binding (30%), cell membrane (23%) and signal transduction (19%) were significantly (Fisher's exact test) altered in cells grown in 3-D model. The data also identified several novel candidate genes like MEIS 1 (mouse homologue of homeobox 1) and villin as being significantly increased (~2 fold; $p < 0.05$) in cells from 3-D model compared to those from 2-D monolayer. MEIS 1 expression is vital to keep cells in undifferentiated and proliferative states. Villin is responsible for cell migration and motility. (See tables in the Supporting Information for the list of genes which are altered in 3-D model).

Discussion

It is essential to have three basic properties for the scaffold materials: mechanical strength, cell attachment capacity and cytocompatibility.[24] In addition, the wetting property and porosity of the scaffold matrix play a direct role in cell attachment and subsequent growth.[25] In this study, microparticles were made from PLA polymer of low molecular weight with PVA and/or chitosan, thus forming an interface that provides an adequate substrate for cell adhesion and growth, resulting in the formation of a 3-D tumorlike structure within 1 week in culture. The lower molecular weight PLA was preferred as it formed microparticles with greater void structures than with higher molecular weight PLA. This could be due to the weaker emulsifying property of the low molecular weight polymer, thus forming large-sized water droplets in the primary emulsion (water-in-oil: W_1/O). Once the organic solvent evaporated and microparticles were dried, the large-sized water droplets formed void structures. The porous structure of these microparticles may have allowed more cells to infiltrate and proliferate inside the matrix structure (Figure 3).

The incorporation of PVA into the internal phase formed void structures, with interface covered by hydrophilic PVA that improved the wetting property of microparticles, thus favoring improved cell attachment and hence cell growth as opposed to that observed on the microparticles formulated without PVA (Table 2). Due to its amphiphilic nature, PVA anchors at the interface with its hydrophobic portion embed-

(24) Bacakova, L.; Filova, E.; Rypacek, F.; Svorcik, V.; Stary, V. Cell adhesion on artificial materials for tissue engineering. *Physiol. Res.* **2004**, *53*, S35–45.

(25) Guan, J.; Fujimoto, K. L.; Sacks, M. S.; Wagner, W. R. Preparation and characterization of highly porous, biodegradable polyurethane scaffolds for soft tissue applications. *Biomaterials* **2005**, *26*, 3961–71.

(26) Lee, S. C.; Oh, J. T.; Jang, M. H.; Chung, S. I. Quantitative analysis of polyvinyl alcohol on the surface of poly(D, L-lactide-co-glycolide) microparticles prepared by solvent evaporation method: effect of particle size and PVA concentration. *J. Controlled Release* **1999**, *59*, 123–32.

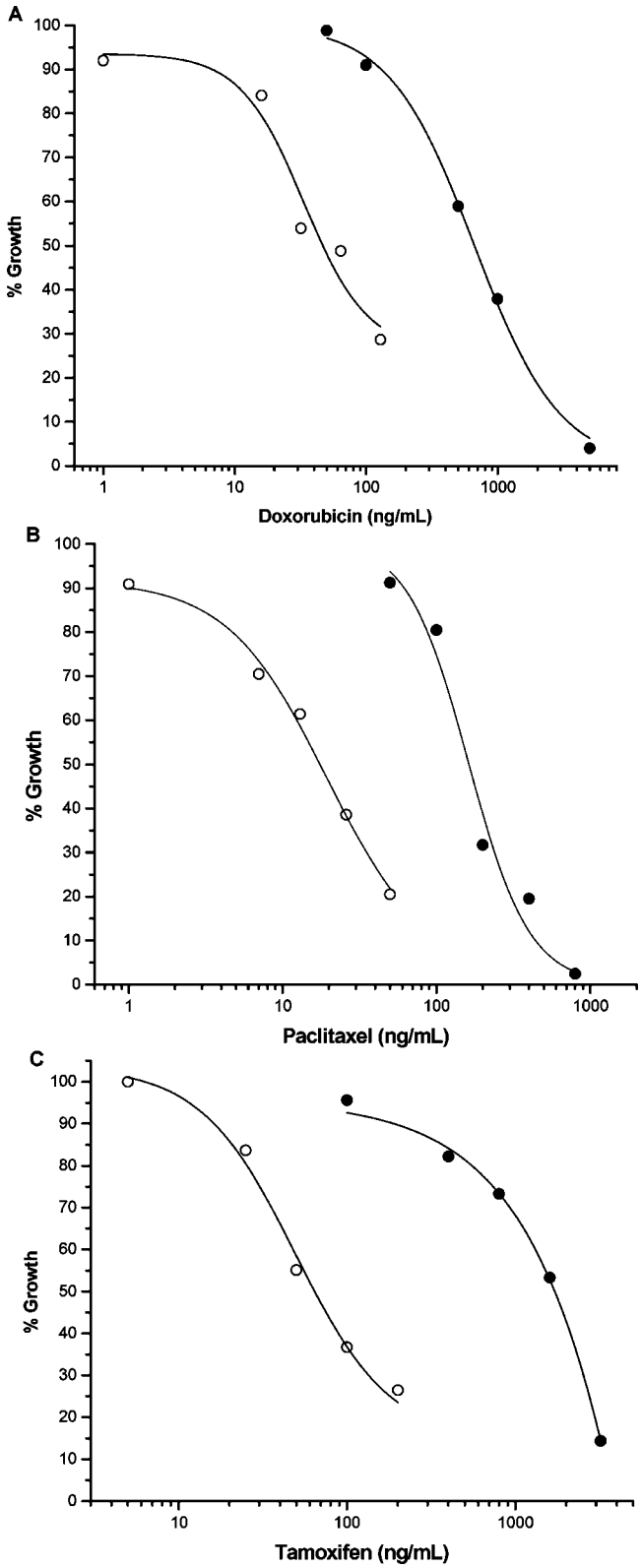

Figure 6. Antiproliferative effect of doxorubicin, paclitaxel, and tamoxifen in (●) 3-D model and (○) 2-D monolayer (n = 6). Cell numbers were determined after 24 h of drug treatment.

ded in the PLA matrix structure and its hydrophilic portion available for hydration in cell culture medium.[15,26] Our strategy of anchoring hydrophilic polymer at the interface is better than incorporating it into the polymer matrix as it could weaken the strength of the matrix structure, which could prevent the diffusion of nutrients into the infiltrated cells. Further, the anchored PVA provides functional OH groups which have been reported as being helpful in cell attachment.[27] The increase in cell growth with increasing PVA concentration used in the internal phase, and the amount of PVA that remains associated with microparticles, correlate with the cell growth thus signifying its role in cell attachment and growth (Table 2). Hydrophilicity of scaffold is believed to be a factor that affects surface free energy (surface tension) and has been directly related to the degree of cell adhesion.[27,28] Greater than 5% PVA concentration did not significantly change cell growth further, possibly because of saturation at the interface with PVA. This is evident from the insignificant change in the PVA associated with microparticles beyond 5% concentration (Table 2).

Microparticles formulated with chitosan in the internal matrix structure also demonstrated better cell growth than those formulated without it; however, the effect of chitosan on cell growth was lower than that with PVA (Table 3). Chitosan is probably adsorbed onto the matrix interface because of its ionic interaction, as the microparticle polymer interface is anionic and chitosan is cationic.[29] The ability of chitosan to support cell attachment and survival has been attributed to its chemical structure which resembles glycosaminoglycans, an extracellular matrix component.[30] Chuang et al. have demonstrated that PVA membranes modified by blending with chitosan promoted fibroblast adhesion.[31] In our studies, improved cell attachment with the composite PVA and chitosan interface can be attributed to the combined effect of both PVA and chitosan, with PVA primarily contributing toward hydrophilicity and chitosan providing the functional amino groups and surface charge to achieve better wetting and cell attachment.[31,32] A maximum of 4.5×10^6 cells/mg of microparticles observed at 6 days is higher than what had been reported previously with

(27) Oh, S. H.; Kang, S. G.; Kim, E. S.; Cho, S. H.; Lee, J. H. Fabrication and characterization of hydrophilic poly(lactic-co-glycolic acid)/poly(vinyl alcohol) blend cell scaffolds by melt-molding particulate-leaching method. *Biomaterials* **2003**, *24*, 4011–21.

(28) Yang, J.; Bei, J.; Wang, S. Enhanced cell affinity of poly (D,L-lactide) by combining plasma treatment with collagen anchorage. *Biomaterials* **2002**, *23*, 2607–14.

(29) Mao, J. S.; Cui, Y. L.; Wang, X. H.; Sun, Y.; Yin, Y. J.; Zhao, H. M.; De Yao, K. A preliminary study on chitosan and gelatin polyelectrolyte complex cytocompatibility by cell cycle and apoptosis analysis. *Biomaterials* **2004**, *25*, 3973–81.

(30) Mei, N.; Chen, G.; Zhou, P.; Chen, X.; Shao, Z. Z.; Pan, L. F.; Wu, C. G. Biocompatibility of Poly(epsilon-caprolactone) scaffold modified by chitosan-the fibroblasts proliferation in vitro. *J. Biomater. Appl.* **2005**, *19*, 323–39.

(31) Chuang, W. Y.; Young, T. H.; Yao, C. H.; Chiu, W. Y. Properties of the poly(vinyl alcohol)/chitosan blend and its effect on the culture of fibroblast in vitro. *Biomaterials* **1999**, *20*, 1479–87.

(32) Koyano, T.; Minoura, N.; Nagura, M.; Kobayashi, K. Attachment and growth of cultured fibroblast cells on PVA/chitosan-blended hydrogels. *J. Biomed. Mater. Res.* **1998**, *39*, 486–90.

Table 4. Antiproliferative Effect of Anticancer Drugs in 2-D Monolayer and 3-D Model

drug	IC_{50} (ng/mL) 2-D monolayer	IC_{50} (ng/mL) 3-D model	fold change in IC_{50}	log *P*	IC_{90-100} (ng/mL) in 3-D model[a]	max serum concn ($C_{max,av}$) (ng/mL)
doxorubicin	32	677	21.2	−0.25	~4,000	4,183[b]
paclitaxel	13	155	11.9	4	~1,000	1,460[c]
tamoxifen	60	1,377	22.9	7.9		

[a] Data obtained from Figure *6*. [b] Doxorubicin administered intravenously at doses of 60 or 75 mg/m^2 from ref 39. [c] Paclitaxel administered intravenously at a dose of 100 mg/m^2 from ref 40.

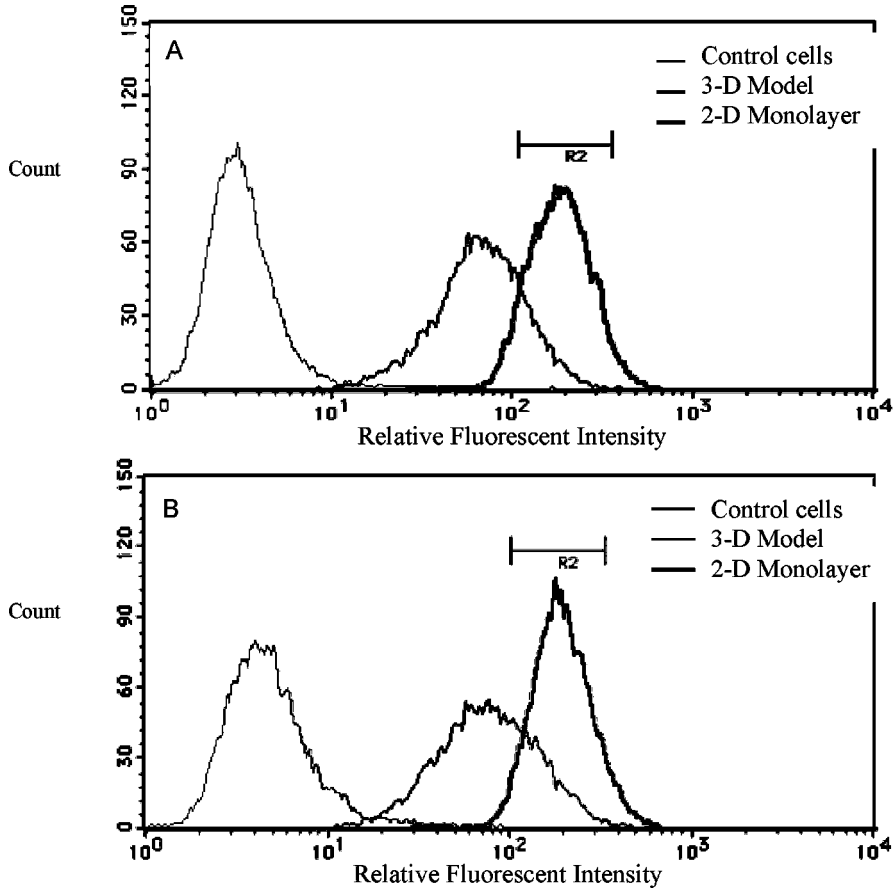

Figure 7. Flow cytometry histogram representing the fluorescence of cells grown in 3-D model vs in 2-D monolayer after (A) 4 h and (B) 8 h of treatment at 2,500 ng/mL of doxorubicin. R2 represents the gated cell population. Only one-fourth of the cell population in 3-D model contained the concentration of drug that is present in the cells cultured as 2-D monolayer.

chitosan (maximum 0.6×10^6 in 5 days);[33] however, it is still ~1.5 orders of magnitude lower than the cell density present in a typical tumor mass.[14] Microparticles seem to degrade and their fragments are visible in 3-D model at 5 days in culture (Figure 5A), but the study could not be continued beyond 10 days to determine the time it takes for microparticles to completely degrade as cell growth starts declining thereafter (Figure 4). The flow cytometry data from 3-D model cultured for 5 days show that 99.5% of cells are viable. Therefore, we selected this time point for drug efficacy and other studies in 3-D model.

Our results clearly demonstrated the difference in the drug efficacy in cells grown in 2-D monolayer vs in 3-D model (Figure 6). For all three anticancer drugs, the IC_{50} values in 3-D model were 12- to 23-fold higher than in 2-D monolayer, with paclitaxel demonstrating relatively less difference than the other two drugs (Table 4). This could be attributed to the ability of paclitaxel to perpetuate cell apoptosis, thus creating channels for drug diffusion through the 3-D model structure.[34] The difference in the IC_{50} values does not seem to depend upon the drug lipophilicity alone because tamoxifen is more lipophilic than paclitaxel but still has a higher

(33) Dhiman, H. K.; Ray, A. R.; Panda, A. K. Characterization and evaluation of chitosan matrix for in vitro growth of MCF-7 breast cancer cell lines. *Biomaterials* **2004**, *25*, 5147–54.

(34) Jang, S. H.; Wientjes, M. G.; Au, J. L. Determinants of paclitaxel uptake, accumulation and retention in solid tumors. *Invest. New Drugs* **2001**, *19*, 113–23.

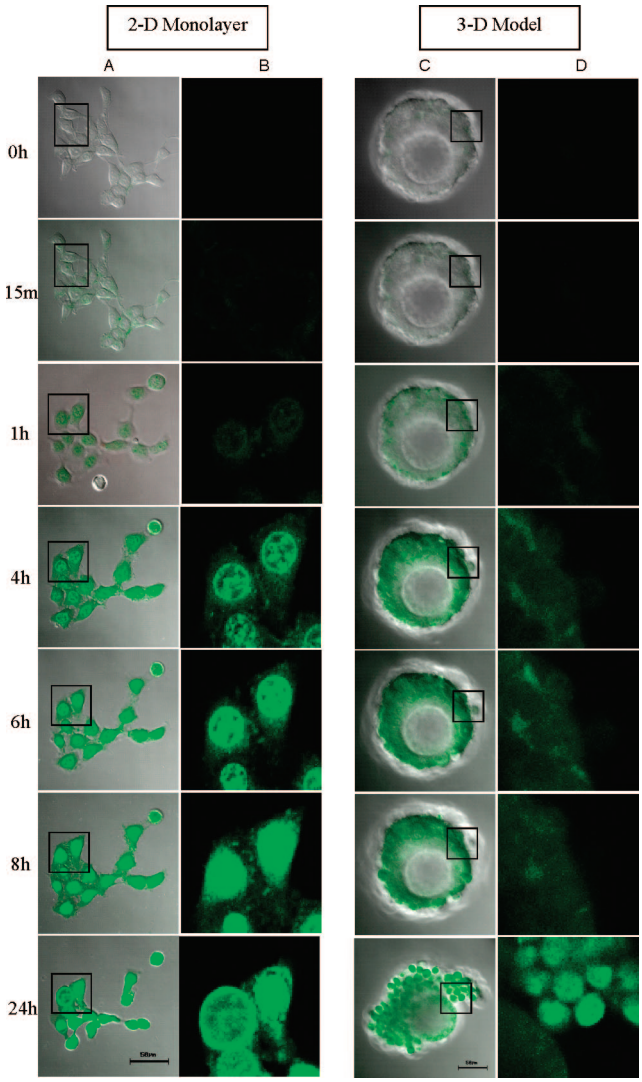

Figure 8. Time series confocal images of MCF-7 cells grown in 2-D monolayer (A and B) and in 3-D model (C and D) following treatment with 2,500 ng/mL of doxorubicin. B and D are the enlarged fluorescent images of cells located within boxes of the respective images in A and C. The drug uptake was slower in cells grown in 3-D model vs in cells grown as 2-D monolayer. Bar = 50 μm.

difference in the IC_{50}. This could be because tamoxifen is cytostatic,[19] unlike paclitaxel which is cytocidal,[34] and thus cannot create channels for its penetration into 3-D model structure. For better correlation between drug lipophilicity and drug efficacy, it would be ideal to test the same series of drugs that vary in their functional groups, influencing the lipophilicity/hydrophilicity but not the basic mechanism of action (e.g., cisplatin or paclitaxel and their derivatives). Such a structure−activity correlation can be useful in developing drugs with enhanced diffusion characteristics through the tumor mass, which is also one of the major issues in developing effective cancer chemotherapeutics.[35] It is known that in tumor masses not all the cancer cells are exposed to the same concentration of drugs because of poor drug diffusion through the extracellular matrix of the tumor,[36] causing the tumor to relapse or develop drug resistance.[37,38] Our study also demonstrated significantly slow diffusion and heterogeneous distribution of drugs in the cells of 3-D model compared to that of 2-D monolayer (Figures 7 and 8). It would be interesting to determine how different types of cancer cells grown in 3-D model respond to anticancer drugs and whether that information could be used in deciding the drug of choice to treat a particular type of cancer.

The important application of the 3-D tumor model would be to predict the efficacy of drugs *in vivo*. The correlation could be between the efficacy in 3-D model and therapeutic serum drug levels or tumor drug levels that are effective in regressing the tumor growth. With limited data from two drugs (paclitaxel and doxorubicin; tamoxifen was excluded from the comparison as it is given orally), we find a good correlation between the IC_{90-100} of the drugs in 3-D model and that with the C_{max} plasma drug levels in cancer patients for doxorubicin[39] and paclitaxel[40] following their intravenous administration (Table 4). Similarly, a correlation between other pharmacokinetic parameters (e.g., steady state plasma drug levels, treatment time, etc.) and the efficacy in 3-D tumor model would be extremely useful for optimizing the effective doses for the treatment of cancer patients. However, a systematic study with a large number of drugs would be required prior to generalizing the above correlation as many variables are involved in drug treatment such as continuous infusion vs bolus injection and the frequency of dosing intervals. In addition to the above application, our 3-D model could be used to test some of the innovative approaches of drug delivery such as coating nanocarriers with collagenase[41] to overcome the extracellular matrix barrier to facilitate the intratumoral delivery of drugs.

(35) Lu, D.; Wientjes, M. G.; Lu, Z.; Au, J. L. Tumor priming enhances delivery and efficacy of nanomedicines. *J. Pharmacol. Exp. Ther.* **2007**, *322*, 80–8.

(36) Minchinton, A. I.; Tannock, I. F. Drug penetration in solid tumours. *Nat. Rev. Cancer* **2006**, *6*, 583–92.

(37) Kyle, A. H.; Huxham, L. A.; Yeoman, D. M.; Minchinton, A. I. Limited tissue penetration of taxanes: a mechanism for resistance in solid tumors. *Clin. Cancer Res.* **2007**, *13*, 2804–10.

(38) Vijayaraghavalu, S.; Raghavan, D.; Labhasetwar, V. Nanoparticles for delivery of chemotherapeutic agents to tumors. *Curr. Opin. Invest. Drugs* **2007**, *8*, 477–84.

(39) Overmoyer, B.; Fu, P.; Hoppel, C.; Radivoyevitch, T.; Shenk, R.; Persons, M.; Silverman, P.; Robertson, K.; Ziats, N. P.; Wasman, J. K.; Abdul-Karim, F. W.; Jesberger, J. A.; Duerk, J.; Hartman, P.; Hanks, S.; Lewin, J.; Dowlati, A.; McCrae, K.; Ivy, P.; Remick, S. C. Inflammatory breast cancer as a model disease to study tumor angiogenesis: results of a phase IB trial of combination SU5416 and doxorubicin. *Clin. Cancer Res.* **2007**, *13*, 5862–8.

(40) Ichiki, M.; Gohara, R.; Fujiki, R.; Hoashi, S.; Rikimaru, T.; Aizawa, H. Phase I and pharmacokinetic study of carboplatin and paclitaxel with a biweekly schedule in patients with advanced non-small-cell lung cancer. *Cancer Chemother. Pharmacol.* **2003**, *52*, 67–72.

(41) Goodman, T. T.; Olive, P. L.; Pun, S. H. Increased nanoparticle penetration in collagenase-treated multicellular spheroids. *Int. J. Nanomed.* **2007**, *2*, 265–74.

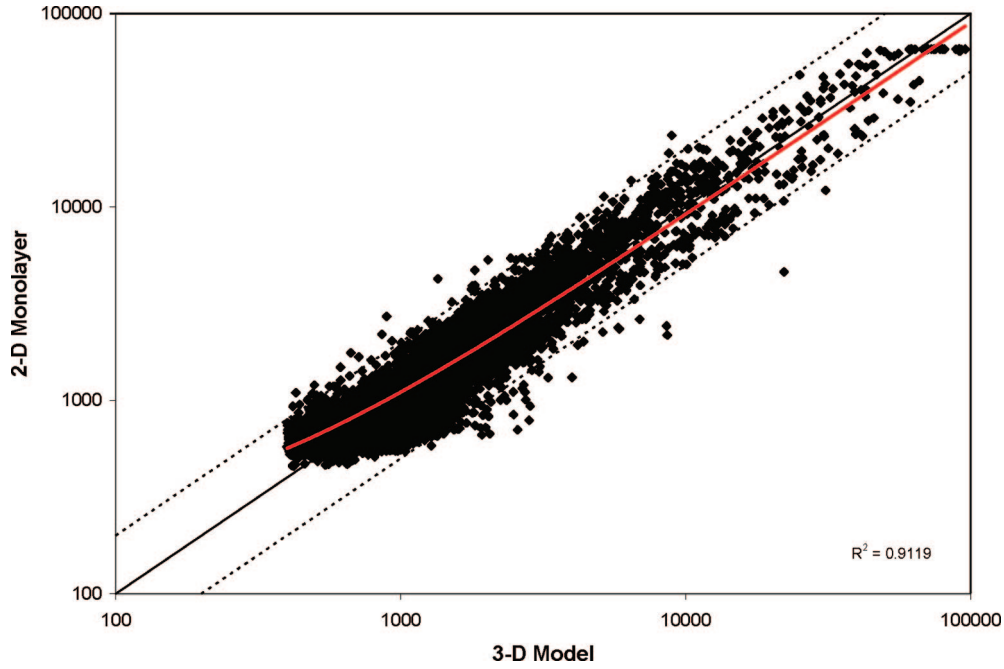

Figure 9. Microarray analysis of cells grown in 2-D monolayer vs 3-D model. The expressional profiles of more than 700 genes were altered in cells grown in 3-D model compared to that in the cells grown in 2-D monolayer. The figure represents one of three replicates.

3-D tumor models using different substrates such as Matrigel,[42] laminin-rich extracellular matrix,[6] irradiated HeLa cells[43] or Teflon membrane[44,45] have been developed to study the various aspects of tumor biology, phenotypic alterations, invasive and migratory behavior of cells, etc. Our study also reports significant genetic and structural variations in the cells grown in 3-D model vs in 2-D monolayer. It would be important, and is the focus of our future study, to compare the microarray data from cells grown in 3-D model to the cells from an actual tumor grown *in vivo* to further understand the significance of the changes in the gene expression profile seen in 3-D model. There have been only few attempts toward developing 3-D tumor model for *in vitro* evaluation of anticancer drugs. Spheroids as a 3-D model of tumor have been most commonly used to test drug effect.[46] However, recently, Fischbach et al. reported that carcinoma cells grown on polymeric scaffolds recreate the microenvironmental characteristics representative of tumors *in vivo* but not the spheroids.[47] Tumor histocultures from patients have been also used to study the drug response and to develop customized therapy, but the difficulty lies in acquiring tissue samples and maintaining the viability of tissues in culture, as well as their usefulness for the screening of a large number of therapeutics.[48] Our model uses biodegradable microparticles as a scaffold matrix, which are simple to produce in large quantity using a conventional emulsification method with consistent properties and using less complicated methodologies for cell seeding, maintaining in culture and for drug efficacy testing. Further, our 3-D model can be frozen and rescued so that one can prepare these scaffolds and store for future use. Although not yet tested, we believe that our scaffold structure could grow other cancer cells but may require optimization of conditions such as cell seeding density, media composition, etc. Further, the growth curve could depend on cell line as different cells have different proliferative rates.

Despite these advantages, there are certain limitations to our tumor model. For example, tumors are composed of heterotypic cell types and blood capillaries. To reduce the gap between the actual tumor and the tumor-like structure developed in our studies, there is a possibility of co-culturing our 3-D model with other cells. This could include cells that

(42) Debnath, J.; Brugge, J. S. Modelling glandular epithelial cancers in three-dimensional cultures. *Nat. Rev. Cancer* **2005**, *5*, 675–88.

(43) Sasaki, T.; Yamamoto, M.; Yamaguchi, T.; Sugiyama, S. Development of multicellular spheroids of HeLa cells cocultured with fibroblasts and their response to X-irradiation. *Cancer Res.* **1984**, *44*, 345–51.

(44) Hicks, K. O.; Ohms, S. J.; van Zijl, P. L.; Denny, W. A.; Hunter, P. J.; Wilson, W. R. An experimental and mathematical model for the extravascular transport of a DNA intercalator in tumours. *Br. J. Cancer* **1997**, *76*, 894–903.

(45) Wilson, W. R.; Hicks, K. O. Measurement of extravascular drug diffusion in multicellular layers. *Br. J. Cancer* **1999**, *79*, 1623–6.

(46) Monazzam, A.; Josephsson, R.; Blomqvist, C.; Carlsson, J.; Langstrom, B.; Bergstrom, M. Application of the multicellular tumour spheroid model to screen PET tracers for analysis of early response of chemotherapy in breast cancer. *Breast Cancer Res.* **2007**, *9*, R45.

(47) Fischbach, C.; Chen, R.; Matsumoto, T.; Schmelzle, T.; Brugge, J. S.; Polverini, P. J.; Mooney, D. J. Engineering tumors with 3D scaffolds. *Nat. Methods* **2007**, *4*, 855–60.

(48) Kubota, T.; Sasano, N.; Abe, O.; Nakao, I.; Kawamura, E.; Saito, T.; Endo, M.; Kimura, K.; Demura, H.; Sasano, H.; et al. Potential of the histoculture drug-response assay to contribute to cancer patient survival. *Clin. Cancer Res.* **1995**, *1*, 1537–43.

would support angiogenesis, such as human endothelial or endothelial progenitor cells, along with other cells that are typically present in tumors, such as fibroblasts and immunocompetent cells.

Conclusions

In this study, we have fabricated porous microparticles with composite interface containing PVA and chitosan that develop cancer cells into a tumorlike structure *in vitro*. The drug efficacy was significantly lower in 3-D model than in 2-D monolayer, suggesting the role of cellular architecture on drug uptake, distribution and efficacy. Our 3-D tumor model could be potentially used in developing effective drugs based on a better understanding of the role of chemical, biological and physical parameters in the process of drug diffusion through the tumor mass, drug retention and therapeutic outcome. A correlation between the drug effects seen in 3-D model to the *in vivo* efficacy would further establish the usefulness of our model in drug discovery.

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Supporting Information Available: Details of microarray analysis and three tables of genes, accession numbers, and fold decreases. This material is available free of charge via the Internet at http://pubs.acs.org.

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