Characterization of Porous PLGA/PLA Microparticles as a Scaffold for Three Dimensional Growth of Breast Cancer Cells

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We have designed and evaluated biodegradable porous polymeric microparticles as a scaffold for cell growth. The hypothesis was that microparticles with optimized composition and properties would have better cell adhesion and hence cell growth into a tissue-like structure. Solvent-evaporation method was modified using sucrose as an additive to form large porous microparticles of poly(D,L-lactic-*co*-glycolic) (PLGA) and polylactide (PLA) polymers. Microparticles containing hydrophilic polymers (poly(vinyl alcohol) and chitosan) incorporated in their internal matrix structure were also formulated. Different formulations of microparticles were evaluated for physical properties, cell adhesion, and cell growth in culture. PLA microparticles containing poly(vinyl alcohol) (PVA) in the matrix structure (PLA-PVA) and treated with serum prior to cell seeding demonstrated better cell adhesion and cell growth than other formulations of microparticles. Cells were seen to grow into clumps, engulfing microparticles completely with time, and forming a 3-D tissue-like structure. Cell density of 1.5×10^6 cells per mg of microparticles was achieved in 9 days of culture, which was a 7-fold increase from the initial seeding cell density. The mechanism of better cell growth on PLA-PVA microparticles appears to be due to the PVA associated with the internal matrix structure of microparticles. These microparticles demonstrated better wetting in culture and also cell adhesion. In addition to tissue engineering applications, microparticles with cancer cells grown into a tissue-like structure in vitro can be potentially used as a model system for preclinical evaluation of the cytotoxic effect of anticancer agents.

Introduction

Cells grown in vitro in three dimensions (3-D) on a polymeric scaffold have been extensively investigated in tissue engineering applications. Recently, 3-D cultures are suggested as a better model to study the complex biological processes than cells grown in monolayers.[1,2] In cancer research, cancer cells grown into a tissue-like structure using a scaffold may closely mimic the biology of tumor development in vivo and hence can serve a better model system for preclinical evaluation of the cytotoxic effect of anticancer agents than cells grown in monolayers.[3,4]

Growth of cells in 3-D typically involves cell seeding onto a scaffold which, following their adhesion, spreading, proliferation, and differentiation, can develop into a tissue-like structure.[5] One of the essential requirements for promoting 3-D growth of cells in vitro is to design a suitable polymeric scaffold that provides a structural template for cell adhesion and growth. Ideally, a scaffold should biodegrade at a rate matching the rate of extracellular matrix deposition so that no residual polymer is left.[6] It is also necessary that scaffolds do not form any product(s) which may interfere in cell growth. Toward this goal, many synthetic polymers such as polylactide (PLA), polyglycolide (PGA) and their copolymers, poly(D,L-lactic-*co*-glycolic) (PLGA),[7] as well as natural polymers, such collagen,[8] alginate,[9] and chitosan[10] have been investigated as a scaffold matrix.

PLA and PLGA are the most widely used biocompatible and biodegradable polymers for fabrication into different forms of scaffolds such as fibers, mesh, membranes, and sponge.[5,11,12] Most synthetic biodegradable polymers such as PLA and PLGA polymers have better mechanical strength than natural polymers such as collagen or chitosan, but natural polymers exhibit better cell adhesion than synthetic polymers. Hence, one way to address this problem is to modify the polymer surface to improve cell adhesion. Surface modification of PLGA/PLA polymers has been reported either by blending them with hydrophilic polymers or by coating scaffolds with an extra cellular matrix (ECM).[13,14] Recent studies have demonstrated the significance of polymer characteristics used in the fabrication of scaffolds such as their hydrophilicity and surface charge, and the effect of different treatments on cell adhesion and proliferation.[15−17]

We have designed large porous microparticles using a modified solvent evaporation method with different surface and physical properties and evaluated them as a scaffold using breast cancer (MCF-7) cells as a model cancer cell line. It was hypothesized that microparticles with optimized composition and properties would have better cell adhesion and hence cell growth into a tissue-like structure. Although different types of matrixes have been investigated for cell

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Table 1. Formulation Parameters for Preparation of PLA Microparticles (Part A) and Physical Characterization of Different Formulations of PLA Microparticles (mean ± s.e.m, $n = 3$) (Part B)

A

PLGA/PLA conc.	aqueous phase volume (W_1)	ratio of primary emulsion (W_1/O) to external aqueous phase volume (W_2)	size (μm)[a] s.e.m.
200 mg/4 mL	800 μL (1:5)	1:50 (2−100 mL)	108 ± 4
200 mg/4 mL	800 μL (1:5)	1:100 (2−200 mL)	160 ±12
200 mg/4 mL	800 μL (1:5)	1:200 (1−200 mL)	182 ± 9
200 mg/4 mL	800 μL (1:5)	1:300 (1.5−450 mL)	267 ± 8

B

PLGA/PLA polymer conc. (O)	aqueous phase volume (W_1)	primary emulsion (W_1/O) to external aqueous phase volume (W_2)	size[b] (μm) ± s.e.m	pore size by volume (μm)	intrusion volume (cc/g)
200 mg/4 mL	800 μL (1:5) (without sucrose)	1:100 (2−200 mL)	167 ± 8	20.4 ± 0.02	2.6 ± 0.13
200 mg/4 mL	200 μL (1:20)	1:100 (2−200 mL)	145 ± 6	18.5 ± 0.03	5.4 ± 0.23
200 mg/4 mL	400 μL (1:10)	1:100 (2−200 mL)	156 ± 9	22.8 ± 0.04	7.8 ± 0.34
200 mg/4 mL	**800 μL (1:5)**	**1:100 (2−200 mL)**	**162 ± 13**	**30.1 ± 0.13**	**13.6 ± 0.42**
200 mg/4 mL	2000 μL (1:2)	1:100 (2−200 mL)	155 ± 8	ND	ND

[a] Mean particle size was determined from several scanning electron microscopic fields. [b] Mean particle size was determined from several scanning electron microscopic fields. Shaded parameters were used to formulate microparticles for cell growth study. ND = not determined because a major fraction of microparticles was broken.

growth, there are no reports of developing microparticles of PLGA and PLA as a scaffold. Therefore, it would be interesting to determine how different microparticle parameters and their composition influence cell adhesion and cell growth.

Material and Methods

Materials. PLGA (intrinsic viscosity 0.65 dL/g copolymer ratio 50:50) and PLA (intrinsic viscosity 0.17 dL/g) were purchased from Birmingham Polymers, Inc. (Birmingham, AL). Poly(vinyl alcohol) (PVA, average MW 30 000−70 000 Da), bovine serum albumin (Fraction V), sucrose, fibronectin, and bovine collagen type I were purchased from Sigma Chemical Co. (St. Louis, MO). RPMI-1640, trypsin EDTA, crystal violet, and fetal calf serum were purchased from Gibco BRL (Grand Island, NY). All other chemicals were of analytical grade.

Formulation of Microparticles. Porous PLGA/PLA microparticles were prepared by modifying the solvent-evaporation method. Briefly, 800 μL of aqueous phase containing 2.5% w/v bovine serum albumin (BSA) and 10% w/v sucrose (W_1) was emulsified into the polymer solution (O) (200 mg of PLGA or PLA polymer in 4 mL of dichloromethane) using a homogenizer (Biospacte Product Inc, Bartlesville, OK) at 1000 rpm to form a primary (W_1/O) emulsion. BSA was added into the aqueous phase to enhance stability of the primary emulsion. Selection of the molecular weight of PLGA and PLA polymers for microparticle formulations was based on their estimated identical degradation behavior. PLA polymers have relatively slower degradation rates than PLGA polymers of similar molecular weight. Hence, PLA polymers of lower molecular weight than PLGA polymers were used in the formulation of microparticles. The primary emulsion was added dropwise into a 1% w/v aqueous solution of PVA containing 10% w/v sucrose (W_2) with stirring on a magnetic stir plate to form a multiple emulsion (W_1/O/W_2). The emulsion was stirred overnight on a magnetic stir plate to evaporate the organic solvent. Microparticles were recovered by centrifugation at 15 000 g, washed three times with distilled water, and then lyophilized for 48 h. Different size microparticles were prepared by changing the primary emulsion (W_1/O) to PVA phase (W_2) volume ratio, whereas microparticles with different porosity were prepared by increasing the internal aqueous phase volume (W_1) in the primary (W_1/O) emulsion (Table 1). To formulate PLA and PLGA microparticles with PVA or chitosan incorporated in their internal matrix structure, these agents were added in the internal aqueous phase (W_1) of the primary emulsion. For this purpose, a chitosan solution (1.25%) was prepared in 5% v/v lactic acid, whereas PVA was used as a 5% w/v aqueous solution, and

Table 2. MCF-7 Cell Growth on Different Formulation of Microparticles and with Different Treatments at 7 Days Post-Seeding[a]

			dry microparticles		microparticles treated with alcohol	
treatments formulations (2 mg)	PVA or chitosan content per mg microparticles	initial cell seeding (1×10^6)	serum final cell density (1×10^6)	serum + collagen final cell density (1×10^6)	serum final cell density (1×10^6)	serum + collagen final cell density (1×10^6)
PLGA	2.4 ± 0.05	0.45	0.5 ± 0.05	1.25 ± 0.03	0.9 ± 0.03	1.2 ± 0.09
PLGA-PVA	3.2 ± 0.23	0.45	0.8 ± 0.16^b	1.5 ± 0.08	1.0 ± 0.08	2.2 ± 0.10
PLGA-chitosan	2.1 ± 0.19	0.45	1.2 ± 0.13	1.75 ± 0.05	0.75 ± 0.05	2.3 ± 0.11
PLA	3.3 ± 0.06	0.45	1.2 ± 0.06^c	1.5 ± 0.09	1.5 ± 0.09	1.5 ± 0.10
PLA-PVA	4.8 ± 0.12	0.45	3.5 ± 0.17^b	2.95 ± 0.13	3.0 ± 0.07	2.8 ± 0.09
PLA-chitosan	2.3 ± 0.23	0.45	1.75 ± 0.04	2.95 ± 0.11	2.75 ± 0.09	2.2 ± 0.12

[a] Data as mean ± s.e.m. ($n = 3$). [b] $P < 0.05$. [c] $P < 0.05$.

the required amount of BSA and sucrose were dissolved into these solutions prior to emulsification into the polymer phase (O).

Characterization of Microparticles. Surface morphology of microparticles was characterized by scanning electron microscopy (SEM) operating at an accelerating voltage of 10−30 kV (JEOL JSM-T220A scanning electron microscopy, MA). Microparticles were sputter-coated with gold−palladium at a thickness of about 70 nm prior to the acquisition of SEM images. The average particle diameter was determined from the SEM pictures of different formulations of microparticles. Microparticles were also characterized for porosity using Mercury Porosimetry at Porous Materials Inc., Ithaca, NY.

Determination of PVA and Chitosan Associated with Microparticles. The amount of PVA associated with the microparticles was determined by a colorimetric method based on the formation of a colored complex between two adjacent hydroxyl groups of PVA and an iodine molecule.[18] 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. Samples were neutralized with 900 μL of 1 N HCl, and the volume was adjusted to 5 mL with distilled water. To each sample were added 3 mL of a 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. The absorbance of the samples was measured at 690 nm following incubation for 15 min at room temperature. A standard plot of PVA was prepared under identical conditions.

The amount of chitosan associated with microparticles was determined using a colorimetric method described by Muzzarelli with some minor modifications.[19] Briefly, each formulation of microparticles (~2 mg) was treated with 1 mL of 0.01 v/v lactic acid solution and incubated overnight at 37 °C. Samples were centrifuged at 1000 rpm, and the supernatant was used to determine chitosan content. A stock solution of Cibacron brilliant red 3B-A dye (Aldrich) was prepared (1.5 mg/mL) in water. An aliquot of the dye solution was diluted with 0.1 M glycine hydrochloride buffer of pH 3.2 so that the dye concentration is 75 μg/mL. To each sample (500 μL) was added 3 mL of the dye solution, and the sample was mixed and incubated for 30 min at room temperature. The absorbance of the samples was measured at 575 nm using a spectrophotometer (model UV-10601PC, Shimadzu Scientific Instruments, Columbia, MD). Microparticles without chitosan were used as a control. A standard plot of chitosan was prepared under similar conditions.

Effect of Different Treatments to Microparticles on Cell Adhesion. The following protocol was used to systematically determine the effect of a single or combination of treatments to microparticles on cell adhesion. Typically, each formulation of microparticles in an eppendorf tube was soaked for 3 h at 37 °C either in 70% alcohol (2 mg/mL), collagen type I (50 μg/mg of polymer particles in 500 μL of 10 mM acetic acid), fibronectin (10 μg/mg microparticles in 500 μL of PBS), or 50% fetal calf serum in RPMI-1460 medium (2 mg/mL). Microparticles were centrifuged (Eppendorf microcentrifuge 5417R Brinkmann Instruments, Westbury, NY) at 10 000 g for 10 min at 4 °C, supernatant was discarded from each sample, and microparticles from each tube were transferred to different wells of six-well plates using 1 mL of RPMI-1640 medium. Medium from each well was aspirated carefully, leaving microparticles in wells for cell seeding as described below. The preliminary assessment of cell adhesion to different formulations of microparticles was determined by observing each plate after a 24 h incubation period under an inverted microscope (Nicon, Osaka, Japan). The estimate of cell adhesion to microparticles was based on the area occupied by cells in wells in the presence or absence of microparticles. Thus, the smaller the area occupied by cells in wells, the greater the cell adhesion to the microparticles would be. Based on the preliminary assessment, the effect of the combination of treatments on cell growth was determined over one week (Table 2). Photomicrographs of selected plates with cells attached to microparticles were taken with an inverted microscope fitted with a digital camera.

Cell Culture and Cell Seeding. MCF-7 cells were maintained in RPMI-1640 medium supplemented with 10% FCS in T-150 flasks in an incubator at 37 °C and 5% CO_2. Microparticles (~2 mg) treated as above were taken in each well of six-well plates, and a cell suspension (1×10^6 cell/mL) in RPMI medium (500 μL) was added onto microparticles in each well. After 3 h of incubation, 1.5 mL of complete RPMI medium was added and plates were incubated in a CO_2 incubator, replacing medium on every alternate day. To study the effect of cell seeding density on cell growth, different cell seeding densities (0.1×10^6 cell/mg to 1×10^6 cells/mg of microparticles) were used. Microparticles were aspirated from culture plates at different time points to determine cell growth kinetics. Cells were

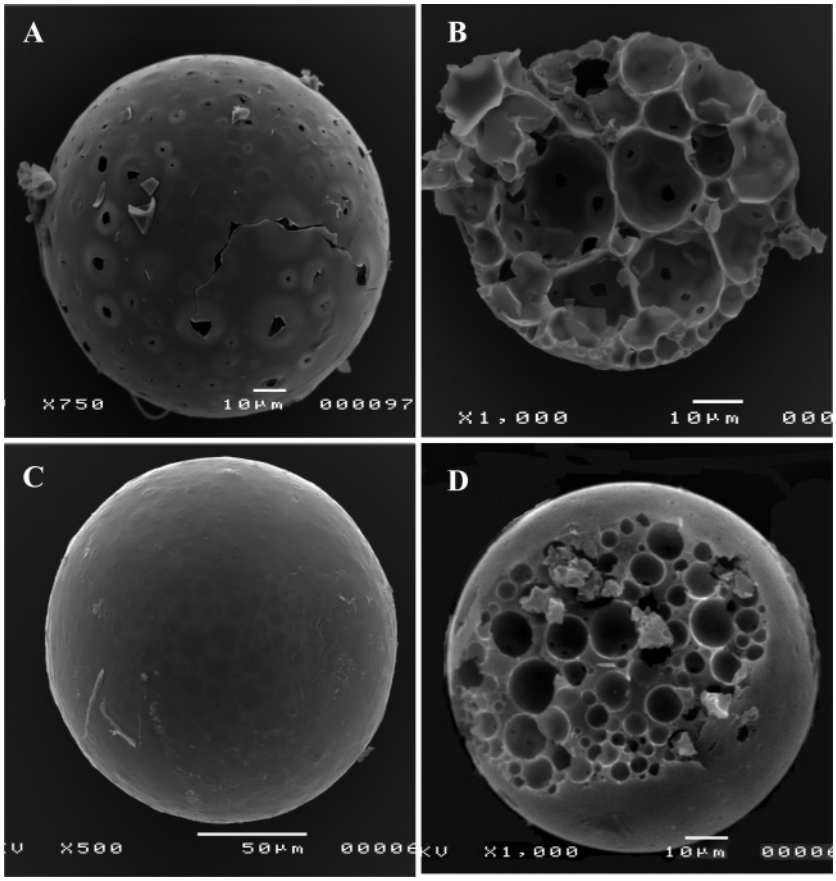

Figure 1. Scanning electron micrograph of an outside and inside of a typical PLA/PLGA microparticle formulated with (A and B) and without (C and D) sucrose in the internal phase (W_1) of the primary emulsion (W_1/O).

detached by treating microparticles with 1 mL of 0.1 M citrate buffer containing 0.1% crystal violet for 1 h at 37 °C. Detached cells were counted using a haemocytometer.[20]

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

Results

Formulation and Characterization of Microparticles. Protocol to formulate large and porous microparticles was developed in two steps. First, we studied the effect of primary emulsion (W_1/O) volume to external phase (W_2) volume ratio on particle size. It was found that the increase in primary emulsion volume to external phase volume ratio increased the particle size (Table 1A). In the second set of experiments, aqueous phase (W_1) volume in the primary emulsion (W_1/O) was varied, keeping the primary emulsion volume to the external phase volume (W_2) ratio same. It was found that the increase in volume of the aqueous phase (W_1) in the primary emulsion (W_1/O) increased the porosity of microparticles (Table 1B). However, the microparticles formulated with a higher aqueous phase volume ratio (1:2 aqueous to polymer volume ratio) in the primary emulsion were more fragile, and a large fraction of it contained fragmented microparticles. Therefore, microparticles with an aqueous phase volume ratio of 1:5 v/v in the primary emulsion were used for further studies (bold values in Table 1B).

It was also determined that sucrose used in the internal aqueous phase (W_1) of the primary emulsion played a role in the formation of microparticles with an interconnected porous internal matrix structure. Although a similar formulation without sucrose formed microparticles with pores in the matrix, the pores were smaller and not interconnected (Figure 1). Furthermore, the microparticles formulated without sucrose had no surface pores. Porosity measurements demonstrated a significant difference in the average pore diameter and intrusion volume of microparticles formulated with and without sucrose (Table 1B).

In all of the formulations of microparticles, PVA was used in the external aqueous phase (W_2) as an emulsion stabilizing agent, but in some of the formulations, it was also used in the internal aqueous phase (W_1) of the primary emulsion (W_1/O). The objective was to formulate microparticles with PVA incorporated in the internal matrix structure of microparticles. In a similar way, microparticles with chitosan were also prepared. These microparticles were in the same size range as those formulated without PVA or chitosan in the internal matrix structure, but the microparticles formulated with chitosan demonstrated a rougher surface than those formulated with PVA (Figure 2). This could be due to deposition of cationic chitosan on anionic microparticle surface. The wetting property of the microparticles was determined by measuring the time required for them to settle down in water at room temperature. Microparticles with PVA or chitosan in their internal matrix structure had a better wetting property

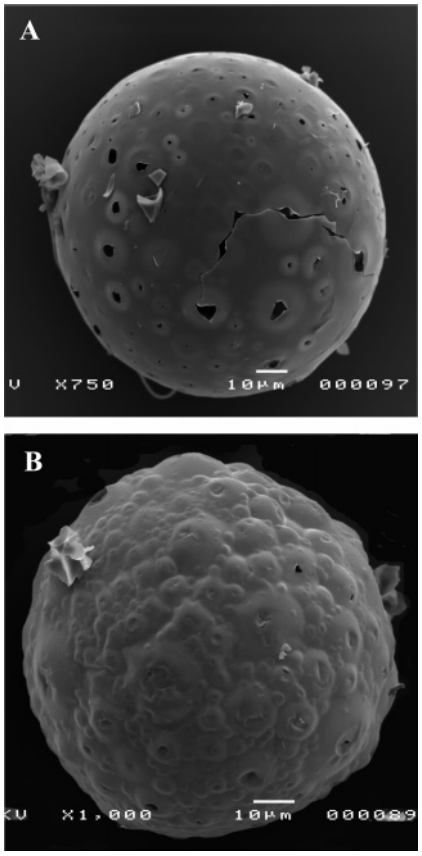

Figure 2. Scanning electron micrograph of a typical PLA/PLGA microparticle (A) and microparticles formulated with chitosan (B).

(settle within 5 min) than microparticles without these agents (required 6−12 h to settle down).

Effect of Composition of Microparticles and Different Treatments to Microparticles on Cell Adhesion and Growth. We further determined if the polymer used (PLGA or PLA) for microparticle formulations affects cell adhesion properties. In general, it was observed that cell adhesion to microparticles formulated with PLA polymer was better than to the corresponding formulations of PLGA polymer. PLGA microparticles with or without PVA chitosan in the internal matrix structure did not show significant cell adhesion at 24 h, even after treatment either with fibronectin, collagen, serum, or ethanol (0−10% cell adhesion). However, cell adhesion increased marginally when PLGA microparticles were treated first with alcohol and then with collagen (∼20%). The corresponding formulations of PLA microparticles demonstrated significantly better cell adhesion (10−50% cell adhesion; Figure 3). Microparticles with chitosan incorporated in the internal matrix structure demonstrated better cell adhesion than those formulated without it, but there was no significant difference in cell adhesion properties of microparticles formulated with PLA and PLGA polymers. Of all of the formulations and treatments, PLA microparticles with PVA incorporated in the internal matrix structure (PLA-PVA) and soaked in serum prior to cell seeding demonstrated better cell adhesion (40−50% cell adhesion) than the other formulations of microparticles or treatments tested. Based on the above preliminary assessment of cell adhesion, selected treatments were further tested alone and in combination for cell growth over one week. In these studies also

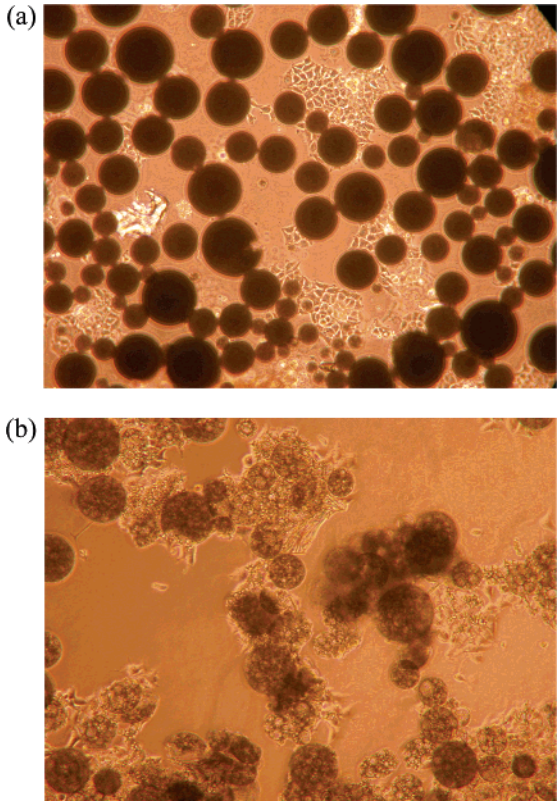

Figure 3. Photographs showing adhesion of MCF-7 cells to (a) PLGA-PVA and (b) PLA-PVA at 24 h following incubation. There was no adhesion of cells to PLGA-PVA microparticles and were mostly seen floating in the medium but significantly greater cell adhesion was seen to PLA-PVA microparticles. Magnification 200×.

PLA microparticles demonstrated better cell growth than the corresponding formulations of PLGA microparticles (Table 2). The results of all of the studies demonstrated that PLA-PVA microparticles treated with serum prior to cell seeding had better cell adhesion and cell growth (Table 2). Hence, these microparticles were used in further studies to determine the effect of cell seeding density on cell growth as described below.

Analysis of PVA and Chitosan Content of PLA and PLGA Microparticles. To understand the reason for better cell adhesion and growth to PLA-PVA microparticles than the corresponding PLGA microparticles, or those formulated without PVA in the internal matrix structure, different formulations were analyzed for the PVA content. PVA content of PLA microparticles in which PVA was incorporated in the matrix structure was greater than that of the corresponding formulation of PLGA microparticles or those formulated without PVA in the matrix structure (Table 2). Thus, it seems that the PVA associated with microparticles plays an important role in cell adhesion and cell growth. However, similar analysis of PLA and PLGA microparticles for chitosan content did not show a significant difference between them.

Influence of Cell Seeding Density on Cell Growth. It was seen that lower cell seeding density demonstrated a lag phase in cell growth, whereas higher cell seeding density demonstrated a lower growth rate (Figure 4). The microparticle surface might have been covered to its saturation capacity at a higher cell seeding density, and hence further

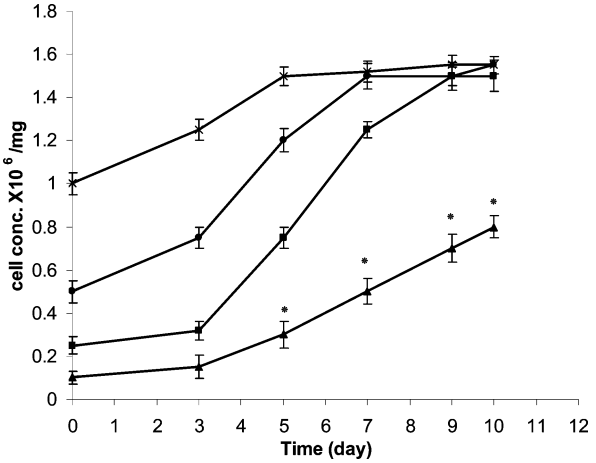

Figure 4. Kinetics of growth of MCF-7 cells on PLA-PVA microparticles with different initial cell seeding density. (▲, 0.1 × 10^6 cell/mg), (■, 0.25 × 10^6 cell/mg), (●, 0.5 × 10^6 cell/mg), and (×, 1 × 10^6 cell/mg). Data as mean ± sem, $n = 3$ *$p < 0.05$ vs other groups.

cell growth may have been limited. At a lower cell seeding density, cells grew slowly, probably because of less cell−cell contact, resulting in a lag phase. A seeding density of 0.25 × 10^6 cells per mg microparticles demonstrated a better cell growth rate. Microparticles were seen almost completly covered with cells within 5 days post-seeding; cells later grew in smaller clumps around microparticles and then formed clusters (Figure 5).

Discussion

Polymeric microparticles are commonly used for drug delivery applications, but their fabrication specifically as a scaffold for cell growth has not been reported. This could be because the microparticles formulated using conversional methods do not provide a support matrix structure needed for cell anchoring and for their subsequent proliferation and growth into a tissue-like structure. In this study, we have demonstrated that microparticles formulated under appropriate formulation conditions and with composition can provide a matrix structure that is suitable for cell adhesion and growth. Sucrose used in the aqueous phase of the primary emulsion which, upon leaching from microparticles, formed an internal matrix structure with interconnected large voids (Figure 1). Microparticles formulated without sucrose had smaller pores and these were not interconnected. Sucrose is known to increase the surface tension of water,[21] and hence, when it was used in the internal phase of the primary emulsion, the water droplets formed could have been larger than those formed when it was not used. Thus, as sucrose leached out from microparticles during washing, it left large void structures inside the microparticle matrix structure (Figure 1). The porous matrix structure provides a greater surface area to promote cell adhesion, migration, and spreading, and subsequent growth into a tissue-like structure.[22]

PLGA and PLA polymers are commonly used polymers for tissue engineering applications because of their biocompatibility as well as high mechanical strength that maintains the matrix porous structure for cell migration and growth.

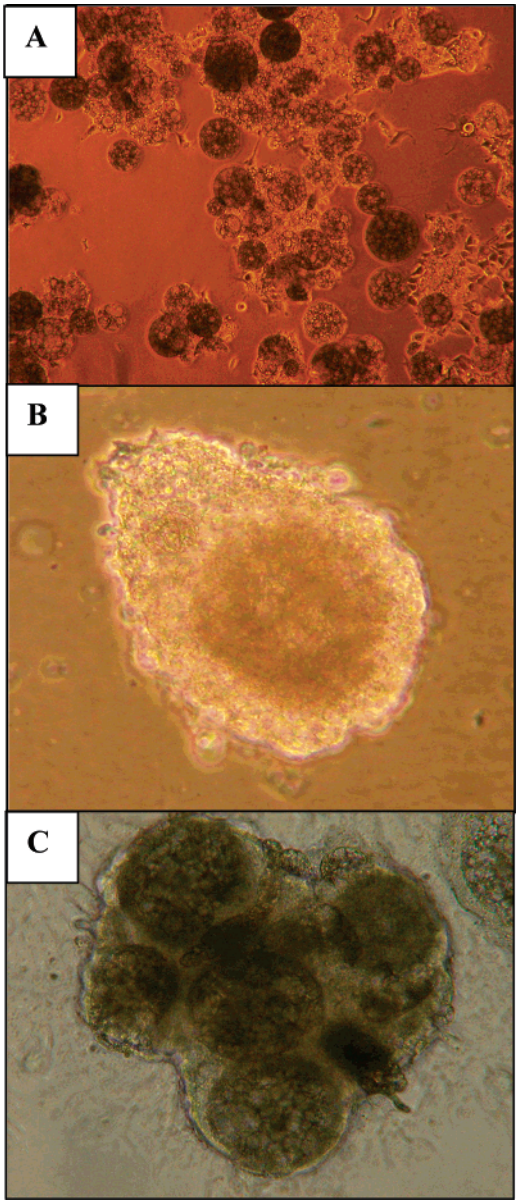

Figure 5. Representative photographs of MCF-7 cells grown on PLA-PVA microparticles at 72 (A), 120 (B), and 168 h (C) post-seeding. Magnification 200X. Panels B and C were zoomed with a digital camera attached to the microscope.

However, since PLA/PLGA polymers are hydrophobic, matrixes formulated using these polymers are difficult to wet in cell culture medium, and hence have limited cell adhesion. Therefore, these matrixes are either treated with solvents (e.g., ethanol) and/or coated or blended with other hydrophilic polymers to promote cell adhesion.[23,24] Coating the surface after wetting with ethanol has been demonstrated to improve cell adhesion to PLA/PGA matrixes.[11] We also observed improvement in cell adhesion to microparticles following their treatment with ethanol, however the effect was marginal and also particles aggregated following the treatment.

PLA/PLGA microparticles formulated with PVA in the internal matrix structure demonstrated better cell adhesion and cell growth than those formulated without it. This could be because of formation of an internal matrix structure with an interface coated with hydrophilic PVA, thus enhancing

their wetting due to reduction in contact angle.[25] Although the enhancement in cell adhesion and growth was seen when chitosan was added in the internal matrix structure, the effect was significantly lower than that observed with PVA. The hydrophilic nature of these microparticles due to associate PVA or chitosan is evident from their relatively rapid sedimentation than that of those formulated without it.[26] An interesting observation was the better cell adhesion to PLA microparticles than to the corresponding formulation of PLGA microparticles and more to the microparticles in which PVA was incorporated in the internal matrix structure than to those without it. PVA analysis demonstrated that PLA microparticles had a greater amount of associated PVA than to the corresponding formulations of PLGA microparticles, suggesting that the amount of PVA associated with microparticles plays a role in cell adhesion and growth. It is known that PVA interpenetrates the PLGA/PLA polymer at the interface during particle formulation. This occurs because partially hydrolyzed PVA, which is a copolymer of poly(vinyl acetate) and poly(vinyl alcohol), has considerable block copolymer characteristics. Therefore, the hydrophobic vinyl acetate part of PVA anchors at the oil−water interface and interpenetrate the PLA/PLGA polymer chains, thus forming an interface.[18] The anchoring of the hydrophobic portion of the PVA molecule may be better to hydrophobic PLA than to the relatively hydrophilic PLGA polymer. This explains the greater amount of PVA that remains associated with PLA microparticles than to PLGA microparticles.[25] Since the PVA content is greater, PLA−PVA microparticles have a more hydrophilic interface than the corresponding formulation of PLGA microparticles.[26] Microphotographs of PLGA−PVA microparticles in wells following cell seeding demonstrated that these microparticles are floating in culture medium and are not in contact with cells. A similar observation was made when PLA or PLGA microparticles without incorporated PVA or chitosan in the internal matrix structure were used. On the other hand, cells are seen adhered to PLA−PVA microparticles under the same experimental condition (Figure 3).

It has been reported that hydroxyl groups on the polymer surface play a positive role in cell adhesion and growth because of the interactions involving hydrogen bonding between hydroxyl groups of the polymer surface and cell surface.[26] In this case, the hydroxyl functional groups of the PVA anchored on microparticle matrix structure at the interface probably have better interactions with polar groups on the cell surface, thus promoting cell adhesion and their spreading into the matrix structure of microparticles.[27−29] Cells were seen to attach to PLA microparticles within 24 h of incubation, whereas the corresponding formulation of PLGA microparticles required 48 h or more for the same degree of cell adhesion. Since PVA is integrated with the polymer structure of microparticles, it could be a better approach to modify polymer interface than just simply coating the preformed particles with hydrophilic polymers. Physically adsorbed polymers could desorb quickly, and therefore may not provide their long-term surface effect on cell adhesion and growth. Association of chitosan to microparticles could be ionic because chitosan is cationic in nature, and hence, there was no difference in the associated chitosan between PLA and PLGA microparticles. This may also explain the similar cell growth seen with PLA−chitosan and PLGA−chitosan microparticles (Table 2).

A cell concentration of 1.5×10^6 per mg microparticles was achieved with the PLA−PVA formulation, which is a 7-fold increase in cell density from the seeding cell density in about 9 days (Figure 4). This cell concentration is about 2-fold greater than that which has been reported previously for MCF-7 cells with a chitosan scaffold.[20] The development of a 3-D dimensional architecture of cancer cells has been associated with reduced apotosis,[30] a higher level of expression of receptors,[31] and partial differentiation, particularly of breast cancer cells, as compared to cells grown in monolayers.[32] Cells growth in 3-D culture also promotes extracellular matrix synthesis to form a tissue-like structure.[33,34] Clumping of microparticles with incubation suggests that cells are probably secreting extracellular matrix components such as collagen (Figure 5). Thus, a three dimensionally grown tissue-like structure can be used as a model to understand cell metabolism and the basic biology of tumor development as well as a tool to study the effect of anticancer agents in preclinical evaluation. It has been recently shown that the tissue microenvironment is a major regulator in tumor induction as well as its suppression and involves complex cell−cell signaling pathways.[35] Therefore, it will be interesting to determine how closely the tissue-like structure developed in vitro resembles the tumor induced in vivo so that one can understand their potential applications as well as limitations.

Conclusions

We report a simple method of fabrication of large porous microparticles using PLGA/PLA polymers which can be used as a scaffold for cell growth. Our results demonstrated that PVA incorporated in the internal matrix structure of microparticles plays an important role in cell adhesion and cell growth. Cells were shown to grow into a tissue-like structure on microparticles in about 5 days post-seeding. Further studies could involve further optimization of composition of microparticles to enhance cell adhesion and growth and feasibility of growing normal cells on microparticles in a bioreactor for large scale production for their use in cell therapy. Microparticles with cells can be easily implanted at the target site using an injector or a catheter because of their micron size and spherical shape. In summary, microparticles developed in our studies can be used as a scaffold for various biomedical applications.

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References and Notes

(1) Abbott, A. *Nature* **2003**, *424*, 870−872.
(2) Cukierman, E.; Pankov, R.; Stevens, D. R.; Yamada, K. M. *Science* **2001**, *294*, 1708−1712.
(3) Schmeichel, K. L.; Bissell, M. J. *J Cell Sci.* **2003**, *116*, 2377−2388.
(4) Jacks, T.; Weinberg, R. A. *Cell* **2002**, *111*, 923−925.
(5) Sachlos, E.; Czernuszka, J. T. *Eur. Cell Mater.* **2003**, *5*, 29−39;.
(6) Vunjak-Novakovic, G.; Freed, L. E. *Adv. Drug Delivery Rev.* **1998**, *33*, 15−30.
(7) Mikos, A. G.; Bao, Y.; Cima, L. G.; Ingber, D. E.; Vacanti, J. P.; Langer, R. *J. Biomed. Mater. Res.* **1993**, *27*, 183−189.
(8) Liao, S. S.; Cui, F. Z. *Tissue Eng.* **2004**, *10*, 73−80.
(9) Alsberg, E.; Kong, H. J.; Hirano, Y.; Smith, M. K.; Albeiruti, A.; Mooney, D. J. *J. Dent. Res.* **2003**, *82*, 903−908.
(10) Madihally, S. V.; Matthew, H. W. *Biomaterials* **1999**, *20*, 1133−1142.
(11) Mikos, A. G.; Lyman, M. D.; Freed, L. E.; Langer, R. *Biomaterials* **1994**, *15*, 55−58.
(12) Murphy, W. L.; Dennis, R. G.; Kileny, J. L.; Mooney, D. J. *Tissue Eng.* **2002**, *8*, 43−52.
(13) Chen, G.; Sato, T.; Ushida, T.; Hirochika, R.; Shirasaki, Y.; Ochiai, N.; Tateishi, T. *J. Biomed. Mater. Res.* **2003**, *67A*, 1170−1180.
(14) Chen, G.; Sato, T.; Ushida, T.; Hirochika, R.; Tateishi, T. *FEBS Lett.* **2003**, *542*, 95−99.
(15) Zhu, H.; Ji, J.; Lin, R.; Gao, C.; Feng, L.; Shen, J. *J. Biomed. Mater. Res.* **2002**, *62*, 532−539.
(16) Yang, J.; Shi, G.; Bei, J.; Wang, S.; Cao, Y.; Shang, Q.; Yang, G.; Wang, W. *J. Biomed. Mater. Res.* **2002**, *62*, 438−446.
(17) Cui, Y. L.; Qi, A. D.; Liu, W. G.; Wang, X. H.; Wang, H.; Ma, D. M.; Yao, K. D. *Biomaterials* **2003**, *24*, 3859−3868.
(18) Sahoo, S. K.; Panyam, J.; Prabha, S.; Labhasetwar, V. *J. Controlled Release* **2002**, *82*, 105−114.
(19) Muzzarelli, R. A. *Anal. Biochem.* **1998**, *260*, 255−257.
(20) Dhiman, H. K.; Ray, A. R.; Panda, A. K. *Biomaterials* **2004**, *25*, 5147−5154.
(21) Lee, J. C.; Timasheff, S. N. *J. Biol. Chem.* **1981**, *256*, 7193−7201.
(22) Hou, Q.; Grijpma, D. W.; Feijen, J. *J. Biomed. Mater. Res.* **2003**, *67B*, 732−740.
(23) Lavik, E.; Teng, Y. D.; Snyder, E.; Langer, R. *Methods Mol. Biol.* **2002**, *198*, 89−97.
(24) Gao, J.; Niklason, L.; Langer, R. *J. Biomed. Mater. Res.* **1998**, *42*, 417−424.
(25) Shakesheff, K. M.; Evora, C.; Soriano, I. I.; Langer, R. *J. Colloid Interface Sci.* **1997**, *185*, 538−547.
(26) Oh, S. H.; Kang, S. G.; Kim, E. S.; Cho, S. H.; Lee, J. H. *Biomaterials* **2003**, *24*, 4011−4021.
(27) Mooney, D. J.; Park, S.; Kaufmann, P. M.; Sano, K.; McNamara, K.; Vacanti, J. P.; Langer, R. *J. Biomed. Mater. Res.* **1995**, *29*, 959−965.
(28) Lee, J. H.; Park, J. W.; Lee, H. B. *Biomaterials* **1991**, *12*, 443−448.
(29) Lee, J. H.; Jung, H. W.; Kang, I. K.; Lee, H. B. *Biomaterials* **1994**, *15*, 705−711.
(30) Weaver, V. M.; Lelievre, S.; Lakins, J. N.; Chrenek, M. A.; Jones, J. C.; Giancotti, F.; Werb, Z.; Bissell, M. J. *Cancer Cell* **2002**, *2*, 205−216.
(31) Anders, M.; Hansen, R.; Ding, R. X.; Rauen, K. A.; Bissell, M. J.; Korn, W. M. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 1943−1948.
(32) Spancake, K. M.; Anderson, C. B.; Weaver, V. M.; Matsunami, N.; Bissell, M. J.; White, R. L. *Cancer Res.* **1999**, *59*, 6042−6045.
(33) Overstreet, M.; Sohrabi, A.; Polotsky, A.; Hungerford, D. S.; Frondoza, C. G. *In Vitro Cell Dev. Biol. Anim.* **2003**, *39*, 228−234.
(34) Fishman, J. H.; Gonzalez, A.; Osborne, M. P. *Biochem. Biophys. Res. Commun.* **1996**, *220*, 467−471.
(35) Radisky, D. C.; Bissell, M. J. *Science* **2004**, *303*, 775−777.

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