



Polyelectrolyte microcapsule interactions with cells in two- and three-dimensional culture

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ABSTRACT

Microcapsules fabricated by layer-by-layer self-assembly have unique physicochemical properties that make them attractive for drug delivery applications. This study chiefly investigated the biocompatibility of one of the most stable types of microcapsules, those composed of poly-(sodium 4-styrene sulfonate) [PSS] and poly-(allylamine hydrochloride) [PAH], with cells cultured on two-dimensional (2D) substrates and in three-dimensional (3D) matrices. C6 glioma and 3T3 fibroblast cell morphology was observed after 24 h of co-culture with PSS/PAH microcapsules on a 2D substrate. Cells were also cultured with four other types of microcapsules, each composed of at least one naturally occurring polyelectrolyte. At microcapsule to cell ratios up to 100:1, it was found that PSS/PAH microcapsules do not affect number of viable cells more substantially than do the other microcapsules investigated. However, differences in number of viable cells were found as a function of microcapsule composition, and our results suggest particular biochemical interactions between cells and internalized microcapsules, rather than mechanical effects, are responsible for these differences. We then investigated the effects of PSS/PAH microcapsules on cells embedded in 3D collagen matrices, which more closely approximate the tumor environments in which microcapsules may be useful drug delivery agents. Matrix structure, cell invasion, and volumetric spheroid growth were investigated, and we show that these microcapsules have a negligible effect on cell invasion and tumor spheroid growth even at high concentration. Taken together, this work suggests that PSS/PAH microcapsules have sufficiently high biocompatibility with at least some cell lines for use as proof of principle drug delivery agents in *in vitro* studies.

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1. Introduction

Layer-by-layer (LbL) self-assembly is a technique in which electrostatic forces are exploited to alternately assemble oppositely charged polyelectrolytes on a substrate to form multilayer films [1]. The technique has been extended to allow deposition of multiple layers of oppositely charged polyelectrolytes on spherical colloidal templates. These templates can be decomposed after they are coated with the desired number of layers, resulting in hollow shells [2,3]. These hollow capsules, known as polyelectrolyte multilayer microcapsules (PMMs), may be composed of various materials including synthetic polyelectrolytes or natural polyelectrolytes such as polysaccharides [4], proteins [5,6] and lipids [7–9]. PMMs can be fabricated to have specific size, wall thickness, and permeability, among other properties.

The permeability of PMMs first attracted interest for temporally controlled drug release. The sustained release properties of PMMs were investigated using fluorescent dye as a release agent [10]. It was subsequently shown that PMM permeability can be tuned by altering environmental factors including pH [5], charge [11], salt concentration [12], temperature [13,14] and ionic strength [15]. Sealing PMMs with supplemental layers has also been shown to alter PMM permeability [5,16]. The tunable permeability of PMMs also allows facile loading of drugs into the hollow capsules. Another option made available by the LbL technique is direct coating of the polyelectrolytes on the surface of a drug crystal. In this case, the temporal drug release behavior and lipid/protein multilayer microcapsules can be controlled by tuning the number of layers [7,17].

In addition to potentially allowing a high degree of temporally controlled drug release, PMMs also potentially offer a high degree of distributionally controlled (or targeted) drug release [18]. To this end, various biomaterials have been used to fabricate, coat, and/or functionalize PMMs. Poly(ethylene glycol) has been used to modify the surface of microcapsules so as to make these potential drug carriers resistant to nonspecific protein adsorption and cellular uptake [19–22]. The high affinity of biotin terminated microcapsules for streptavidin surfaces has illustrated the potential of PMMs as targeted drug delivery carriers [23]. Other proteins and peptides

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have also been used to modify microcapsule surfaces, thus affording them adhesive specificity for certain cells, tissues, and organs [24–27].

While PMMs hold promise as ideal systems for both time and targeted release drug delivery, their biocompatibility and interactions with cells and the extracellular matrix (ECM) surrounding cells have not yet been extensively studied. A number of polyelectrolytes that have been used to fabricate PMMs have also been prepared as multilayer films. The effects of these films on cell adhesion and viability have been studied previously [28–32]. Additionally, while no studies have systematically compared cell proliferation and viability as a function of (unloaded) microcapsule concentration, size, and composition, several studies have investigated the effects of specific PMMs on cell viability and/or microcapsule uptake [21,22,33–39]. While the studies above have interrogated aspects of cell interaction with PMMs and the polyelectrolytes they are composed of, much remains to be learned about the potential effects of microcapsules on the viability, proliferation, morphology, and migratory capacity of cells. Because eventual *in vivo* application of microcapsules for drug delivery will occur in the three-dimensional (3D) environment of tissue, it is important to investigate the effect of unloaded microcapsules not only on cells cultured on two-dimensional (2D) substrates, but also on cells cultured in 3D environments and on the extracellular 3D environment itself. In this work, we systematically interrogated the effects of PSS/PAH microcapsules on viability, proliferation, and morphology of fibroblast cells cultured in 2D, glioma cells cultured in 2D, and glioma cells cultured in a 3D collagen matrix. While PSS/PAH microcapsules are not expected to be heavily used *in vivo* owing to their limited biodegradability, the ease with which they can be prepared and their exceptional stability would make them ideal for proof-of-principle drug delivery studies if their biocompatibility is suitably high in preparations and concentrations at which such studies would be performed. We show that only at very high concentrations do PSS/PAH microcapsules affect viability and proliferation of cells cultured in 2D and that these effects are even less substantial in 3D. We also show that at microcapsule to cell ratios of up to 100, PSS/PAH microcapsules have no more effect on cell viability and proliferation than do microcapsules made of a variety of fully biocompatible polyelectrolytes. Our study confirms that PSS/PAH microcapsules are suitable for proof-of-principle work focusing on microcapsules as potential drug delivery agents for cells cultured in typical conditions both in 2D and 3D preparations.

2. Materials and methods

2.1. Materials

Poly-(sodium 4-styrenesulfonate) [PSS], poly-(allylamine hydrochloride) [PAH], bovine serum albumin [BSA], alginate sodium salt [ALG], poly-L-lysine [PLL], dextran sulfate [DEXS], 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide (MTT) and fluorescein isothiocyanate (FITC) isomer I were purchased from Sigma–Aldrich. Chitosan chloride (CHI) was kindly provided by Frank Rauh at FMC Corporation. FITC-PAH was prepared by covalently binding FITC to PAH [40]. Manganese carbonate (MnCO_3) template particles were prepared by a simple mixing method [41]. Templates were prepared in three sizes: 1–2, 3–6, and 8–10 μm diameter. Size was controlled by adjusting the intensity of sonication during crystal formation, with increased intensity producing smaller templates. Particle diameters were confirmed by microscopy. 3T3 fibroblast cells were purchased from the American Type Culture Collection, and C6 glioma cells were provided by Professor Peter Canoll at Columbia University Medical School. Dulbecco's Modified Eagle Media (DMEM), Dulbecco's phosphate

buffered saline (D-PBS), trypsin–ethylenediaminetetraacetic acid (trypsin–EDTA), fetal bovine serum (FBS), calf serum, an antibiotic–antimycotic containing penicillin, streptomycin, and amphotericin, and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen. Formaldehyde solution (37%) was purchased from VWR. Collagen I was purchased from Inamed Biomaterials.

2.2. Preparation of microcapsules

Microcapsules were fabricated using the LbL self-assembly technique described previously [1–3]. We prepared PSS/PAH, PSS/FITC-PAH, BSA/PAH, DEXS/CHI, and ALG/PLL microcapsules. In all cases, six layers of each polyelectrolyte were used. PSS/PAH and ALG/PLL microcapsules were prepared in a variety of sizes (1–2, 3–6, and 8–10 μm diameter) while the remaining microcapsules were prepared only at 3–6 μm . For PSS/PAH microcapsules, 1 mg/ml negatively charged aqueous PSS solution (0.5 M NaCl) was added to a suspension of MnCO_3 particles. The PSS was allowed to adsorb onto the MnCO_3 particles for 10 min. Following three washings using centrifugation to remove non-adsorbed polyelectrolyte, 1 mg/ml positively charged aqueous PAH solution (0.5 M NaCl) was added to the suspension, and the entire procedure was repeated until the desired number of PSS and PAH layers was obtained. For PSS/FITC-PAH microcapsules, the fifth PAH layer was replaced with FITC-PAH. For BSA/PAH microcapsules, 1 mg/ml BSA in pH 7.4, 10 mM Tris–HCl buffer solution was used in place of PSS. BSA was allowed to adsorb for 30 min. For the preparation of DEXS/CHI and ALG/PLL microcapsules, each polyelectrolyte was dissolved in 0.5 M NaCl to give 1 mg/ml solution. The procedure for preparing DEXS/CHI and ALG/PLL microcapsules was identical to that for making PSS/PAH microcapsules, except these polyelectrolytes were allowed to adsorb for 1 h rather than the 10 min used for PSS and PAH. After deposition of the six double layers of each pair of polyelectrolytes around the MnCO_3 particles, hollow capsules were obtained by dissolving the cores of the coated MnCO_3 particles. Two techniques were used: for the PSS/PAH, ALG/PLL, and DEXS/CHI microcapsules, the microcapsules were placed in 0.1 M HCl for 5 min and were subsequently washed with EDTA and water. For the BSA/PAH microcapsules, 0.1 M EDTA was used to dissolve the MnCO_3 particles. Capsule concentration was quantified using a cytometer. Microcapsule surface charge was characterized by measuring ζ -potential on a Zetasizer (Malvern Instruments). All ζ -potential measurements were performed in de-ionized water.

2.3. Cell culture

2.3.1. Monolayer cell culture

C6 glioma cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 0.25 $\mu\text{g}/\text{ml}$ amphotericin B. 3T3 fibroblasts were cultured in identical culture medium except the FBS was replaced with 10% calf serum. Cells were kept at 37 °C in a humidified incubator with 5% CO_2 . For co-culturing cells with microcapsules, 1 ml cells at a concentration of $1 \times 10^5/\text{ml}$ were cultured in 24-well plate dish overnight, 100 μl of microcapsules in sterile water at the appropriate microcapsule concentration was added to each well, and the cell and microcapsules were placed in an incubator for 24 h in advance of MTT or imaging studies.

2.3.2. Preparation of glioma spheroids

C6 glioma cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 0.25 $\mu\text{g}/\text{ml}$ amphotericin B. MTSs of uniform size and shape were formed using the “hanging drop” procedure [42]. Briefly, 20 μl (~200 cells) of cell solution was dropped onto the inside cover of a 100 mm Petri

dish, and the Petri dish was filled with 10 ml culture medium. The dish was inverted and incubated for 7 days. The drops were held in place by surface tension, and the cells accumulated at the bottom of the droplet to form MTSSs, or spheroids. Spheroids of $\sim 500 \mu\text{m}$ in diameter were collected and deposited into collagen solutions. Spheroid containing solutions were then placed in an incubator to ensure cell health and to allow collagen gelation.

2.4. Collagen matrix preparation

Collagen matrices were prepared from the following ingredients: a stock collagen I solution at 3.1 mg/ml, $10\times$ DMEM solution, 7.5% (w/w) sodium bicarbonate, pre-prepared culture medium for cell culture, and NaOH (0.1N). Sodium bicarbonate (7.5%, w/w) in a volume equal to 2% of the total volume of the gel was added. NaOH was added to bring the pH to 7.4, and culture medium was added to ensure cell health during the experiment. The solution was well mixed with microcapsules and kept at 4°C before placement into homemade sample cells. The sample cells consisted of 2 cm diameter plexiglass cylinders of height up to 2 cm fully sealed with UV epoxy on glass coverslips. A nylon mesh was placed around the circumference of the cylinders to allow the collagen gels to anchor and prevent collapse of the gels under tension from migrating cells.

Six hundred microliters of collagen-microcapsule solution was added to the chambers. One spheroid was placed in each sample cell, and the sample cells are covered and incubated at 37°C and 5% CO_2 . This induced gelation of collagen while maintaining cell health. Full gelation occurred within 1 h, and a superlayer of culture medium was then added to maintain moisture and pH.

2.5. Viability assay

MTT assays were used to measure the viability of C6 and 3T3 cells cultured in 2D in the presence of microcapsules. Briefly, 1 ml aliquots of cell suspensions at a concentration of $1 \times 10^5/\text{ml}$ were incubated in the wells of a 24-well plate for 12 h to allow cells to attach onto the surface. Then, microcapsules were added serially at 1×10^7 , 1×10^6 , 1×10^5 , 1×10^4 , $1 \times 10^3/\text{ml}$. Four samples (in four wells on the same plate) were prepared at each microcapsule concentration. Four control wells with cells but no microcapsules were also prepared. The plate was placed in an incubator at 37°C for 24 h. Then, 1 mg/ml MTT solution was added into each well and incubated for 4 h. The medium was removed and replaced with MTT solvent (0.1 M HCl in absolute isopropanol). The MTT solvent solubilizes formazan crystals, which are only produced by live cells. Absorbance (A) determined at 570 nm using a microplate spectrophotometer (SpectraMax Plus³⁸⁴, Molecular Devices) is proportional to the number of live cells. Cell viability is expressed as

$$\text{viable cells (\% control)} = \frac{A_{\text{treated}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100$$

where A_{treated} is the average absorbance in wells containing cells cultured with a particular concentration of microcapsules, A_{blank} is the absorbance of the MTT solvent, and A_{control} is the average absorbance in wells containing cells but no microcapsules.

2.6. DAPI nuclear staining

Cells were cultured with microcapsules in a 1:100 or 1:10 ratio for 24 h and then washed with D-PBS three times to remove the culture medium. Fixation was performed by adding 37% formaldehyde solution, waiting 15 min and again washing the cells with D-PBS buffer. DAPI solution was added and incubated with the cells for 3–5 min. The sample was then rinsed several times and mounted using a mounting medium.

2.7. Optical microscopy

Bright field and DIC images were taken on either an Olympus Fluoview 300 in scanning mode using an Ar⁺ laser at 488 nm for excitation and a photomultiplier tube (PMT) for detection or on a Olympus IX 71 using a halogen lamp for excitation and a CCD camera for detection.

Fluorescence microscopy was used to visualize fluorescently labeled microcapsules in DAPI stained cells in detail. The samples were observed using an Olympus IX 71 microscope in brightfield configuration. A mercury lamp was the excitation source, filter sets appropriate for DAPI and FITC were used, and a CCD camera was used for detection.

To image the collagen matrix, confocal reflectance microscopy (CRM) was employed. The Olympus Fluoview 300 confocal laser scanning microscope with an Ar⁺ laser at 488 nm and a $60\times$ oil objective was used to perform these measurements. The confocal pinhole was set to measure slices $\sim 1 \mu\text{m}$ in depth along the optical axis. The reflectance signal returned through a beamsplitter and was directed by a mirror through the confocal pinhole to a PMT.

To investigate potential effects of microcapsules on the structure of collagen matrices, collagen solutions were mixed with microcapsules, gelled, and imaged using CRM. Two-dimensional slices of the 3D gels were taken in four samples $\sim 30 \mu\text{m}$ into each sample, though no significant differences in fiber density or isotropy were seen over $260 \mu\text{m}$ of the matrix (the working distance of the objective). The average mesh size of the matrix was determined as described in Ref. [43]. Briefly, pixels with intensity above a threshold (set so as to be above the intensity associated with background noise) were considered “on.” The distance between nearest neighbor on-pixels within a row or column defined a “mesh”; the distribution of mesh sizes was plotted for the rows and columns. This distribution was then fit to an exponential decay, $N(t) = N_0 e^{-t/\beta}$, with β representing the average mesh size.

2.8. Scanning electron microscopy (SEM)

SEM was used to characterize the size and morphology of MnCO_3 particles and microcapsules. A drop of sample suspension was placed on a silica wafer and dried at room temperature overnight. A thin film of gold ($\sim 2 \text{ nm}$) was sputtered onto the surface. A voltage of 5 kV was used during sample examination on a Hitachi 4700 SEM (Hitachi Ltd., Japan).

3. Results and discussion

3.1. Characterization of microcapsules

Fig. 1a shows $4 \mu\text{m}$ MnCO_3 particles before coating with polyelectrolytes. The particles are spherical and moderately monodisperse. After coating, MnCO_3 template cores were decomposed as described in Section 2. Fig. 1b shows an SEM image of PSS/PAH capsules after drying. The visible creases and folds show that the capsules collapse during the drying process, which is due to the evaporation of the aqueous content and provides evidence of the hollow nature of the capsules. The inset of Fig. 1b shows a confocal fluorescence image of PSS/FITC-PAH capsules suspended in water, demonstrating that the capsules are spherical before drying. All microcapsules fabricated were stable in water and cell culture medium, exhibiting neither collapse nor aggregation over weeks. ζ -potential measurements in water showed all microcapsules had a positive charge, as expected given that all outer layers used are cationic polyelectrolytes. Specifically, averaged over three batches, the four types of 3–6 μm microcapsules prepared had ζ -potentials of 28.5 ± 0.6 , 19 ± 2 , 25 ± 2 , and $8.4 \pm 0.5 \text{ mV}$ for the

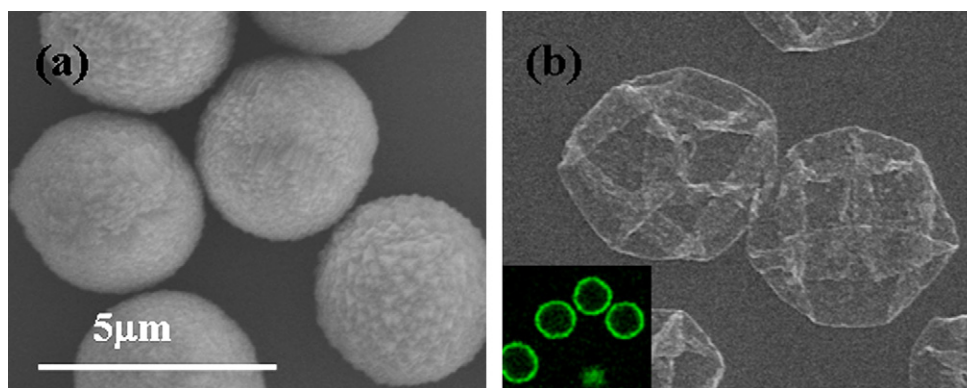


Fig. 1. SEM image of (a) MnCO_3 particles and (b) PSS/PAH microcapsules. The inset image in (b) shows hollow PSS/FITC-PAH microcapsules dispersed in water.

PSS/PAH, ALG/PLL, BSA/PAH, and DEXS/CHI microcapsules, respectively.

3.2. Morphology of cells cultured in monolayer with microcapsules

The morphology of C6 glioma and 3T3 fibroblast cells cultured in a monolayer with different concentrations of 3–6 μm PSS/PAH microcapsules was investigated via DIC microscopy. Fig. 2 shows the morphology of these cells after 24 h of co-culture. At a microcapsule to cell ratio $\leq 10:1$ (Fig. 2b–d), C6 and 3T3 cells cultured with microcapsules are extended, attached to the surface, and morphologically similar to cells in the same conditions in the absence of microcapsules (Fig. 2a). While cells are dense and approaching confluence at microcapsule concentrations up to $1 \times 10^5/\text{ml}$ (a 1:1 cell:microcapsule ratio), at a 10:1 microcapsule to cell ratio, when many cells contain multiple microcapsules (Fig. 2d), fewer cells are present. This, and the fact that the doubling time for both C6 and 3T3 cells cultured as described is <24 h, suggests that cell proliferation is affected at this microcapsule concentration. The fact that relatively few capsules are present on the substrate surface indicates that decrease in proliferation at this microcapsule concentration is not due to competition for surface adhesion between cells and microcapsules. Only when the PSS/PAH microcapsule to cell ratio reaches 100:1 (Fig. 2e), do cells become rounded and detach from the surface. This is also the only concentration at which there are obvious differences between C6 and 3T3 cells cultured with microcapsules. The microcapsules tend to aggregate around rounded 3T3 cells, while more microcapsules remain dispersed on the substrate surface in the presence of C6 cells. This may be due to differences in cell membrane composition and is consistent with other studies

finding cell line dependent differences in membrane aggregation and uptake of identical micro- or nano-particles [44,45].

3.3. Localization of microcapsules in cells

While Fig. 2 suggests cells readily take up the large PSS/PAH microcapsules used in this study, to confirm these capsules are indeed internalized and not residing atop the cells, higher magnification brightfield and fluorescent images were obtained (Fig. 3). In Fig. 3a, DIC measurements reveal microcapsules both within the cell and microcapsules adherent to the cell surface. The former are in focus in the same plane as the cell nucleus, while the latter cannot be focused in the same plane as the cell nucleus; instead, they are several microns above the central axial position of the cell nucleus. Some capsules taken up by the cells appear deformed from their spherical shape. The deformation may result from pressure present in the endocytotic vesicles in which the microcapsules are taken up [35]. To afford a more detailed view of microcapsules internalized by cells, fluorescently labeled PSS/FITC-PAH microcapsules were prepared, and dual imaging of cell nuclei and microcapsules was performed after DAPI staining (Fig. 3b). This provides further confirmation that large PSS/PAH microcapsules are readily internalized by glioma cells that continue to display normal morphology and intact nuclei.

3.4. Cell viability and apoptosis

While PSS/PAH microcapsules are inexpensive and straightforward to prepare as well as exceptionally stable, their use in *in vitro* studies has been limited by concerns about cytotoxicity, particularly due to the presence of the high concentration of sulfonic

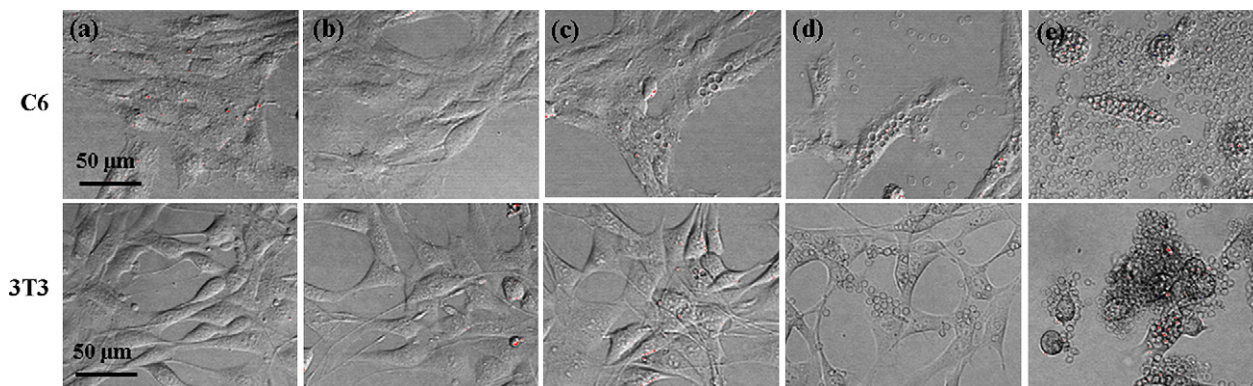


Fig. 2. DIC images of C6 glioma (upper panel) and 3T3 fibroblast (lower panel) cells cultured in monolayer with PSS/PAH microcapsules for 24 h. The concentration of microcapsules is (a) no microcapsules, (b) $1 \times 10^4/\text{ml}$, (c) $1 \times 10^5/\text{ml}$, (d) $1 \times 10^6/\text{ml}$, and (e) $1 \times 10^7/\text{ml}$. The cell concentration is $1 \times 10^5/\text{ml}$ in all cases.

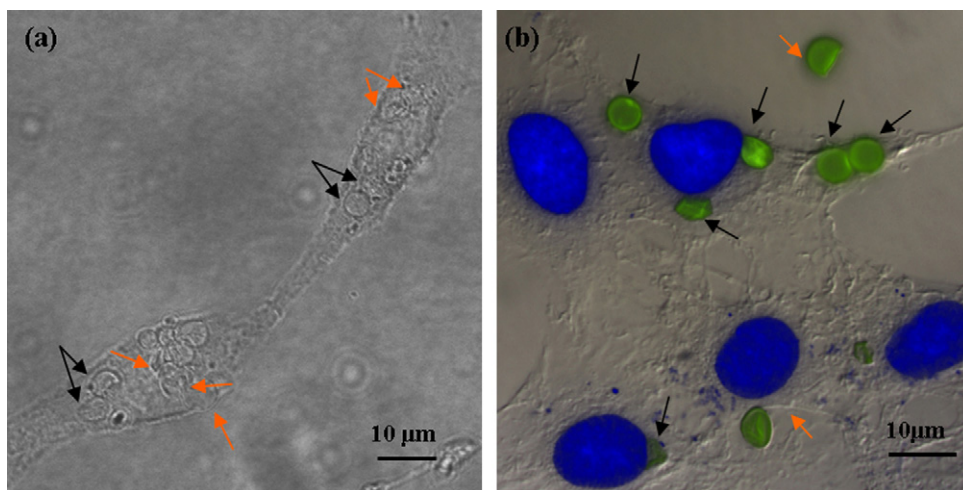


Fig. 3. Localization of microcapsules cultured with C6 glioma cells. (a) PSS/PAH microcapsules were imaged with bright field microscopy. (b) Cells are DAPI stained and capsules are PSS/FITC-PAH. The black arrows indicate capsules taken up by cells, and the orange arrows indicate non-internalized microcapsules. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

groups in PSS [46]. While Tryoen-Toth et al. found that PSS did not affect cell health when used as the outermost layer of a planar multilayer film [30], plating cells on a film of a particular material may not induce the same effects as culturing cells with microcapsules fabricated of identical material. Indeed, Wattendorf et al. and Liu et al. found that cell viability decreased slightly when cells were cultured with microcapsules containing PSS [22,33]. Liu et al. suggested this was due to competition for space on the substrate between cells and microcapsules [33]. Similarly, Kirchner et al. found cell attachment decreased at concentrations of 10^5 /ml 5 μm PSS/PAH microcapsules [32]. In this case, sedimentation of microcapsules onto cells was suggested as the probable cause. While these two studies implicated external mechanical factors in the decrease of viable attached cells in the presence of PSS/PAH microcapsules, chemical and mechanical effects of microcapsules *internal* to the cells could also contribute to differences in cell viability between cells cultured on multilayer films of a given material vs. those cultured with microcapsules composed of identical material.

The results presented in Fig. 2 suggest that a decrease in number of viable cells in the presence of high concentrations of PSS/PAH microcapsules can occur in the absence of competition for adhesion area on the substrate. The fact that we see decreased numbers of attached cells but few microcapsules on the substrate surface, few microcapsules resting atop cells, and very few cells rounding up and detaching from the surface at concentrations up to 10^6 /ml suggests that external mechanical effects are not the primary driver of decrease in cell number with increasing PSS/PAH microcapsule concentration. We note that at microcapsule concentrations of 10^5 /ml and higher, cell and microcapsule distribution on the substrate is inhomogeneous, and images such as that shown in Fig. 2 can only provide qualitative information on number of viable cells. To more quantitatively assess the biocompatibility of PSS/PAH microcapsules with C6 glioma and 3T3 fibroblast cells, we performed MTT assays.

To assess the importance of microcapsule composition independent of concentration, we compared the viability and proliferation of cells in the presence of microcapsules composed of PSS/PAH as well as other polyelectrolytes. In particular, we prepared microcapsules composed of either a mix of natural and synthetic polyelectrolytes [BSA/PAH microcapsules] or purely natural polyelectrolytes [DEXS/CHI and ALG/PLL microcapsules], each of which may be expected to have higher biocompatibility than PSS/PAH microcapsules. All microcapsules investigated have cationic outer layers, as reported in Section 3.1. However, BSA in the cell culture

medium employed is expected to adhere to the outer layers in all cases, resulting in an effective small negative charge on all microcapsules investigated [21]. Fig. 4a–d shows that C6 cell uptake of each type of 3–6 μm microcapsule type is similar when microcapsule to cell ratio is 10:1. Because the capsules are the same size and have similar outer layer composition and charge in the presence of cell culture medium, it is not surprising that cells take up similar quantities of each type of microcapsule.

C6 and 3T3 cells were cultured with 3–6 μm PSS/PAH and BSA/PAH microcapsules at concentrations from 10^3 /ml to 10^7 /ml. C6 cells were also cultured with ALG/PLL and DEXS/CHI capsules. Additionally, C6 cells were cultured with 1–2 and 8–10 μm PSS/PAH and ALG/PLL microcapsules. All results from these assays are presented in Fig. 4e. First, one notes that in all cases the number of viable cells decreases with increasing microcapsule concentration. Additionally, for both PSS/PAH and BSA/PAH 3–6 μm microcapsules, C6 cells are less affected than 3T3 cells by the presence of microcapsules, and the difference in viable cells relative to control is generally 5–10% higher in the C6 cells than the 3T3 cells. Of the four types of 3–6 μm microcapsules cultured with C6 cells, those cultured with BSA/PAH or ALG/PLL exhibit the highest viability at concentrations up to 10^6 /ml. However, for C6 cells up to a 1:1 microcapsule to cell concentration, the number of viable cells relative to control is >80% for all capsules investigated including those composed of PSS/PAH. At 10^6 and 10^7 /ml microcapsules, number of viable cells decreases substantially, and the difference is most prominent in C6 cells cultured with BSA/PAH microcapsules and least apparent in C6 cells cultured with DEXS/CHI microcapsules. Such differences as a function of microcapsule composition suggest decreased cell proliferation and viability as a function of microcapsule concentration is not driven solely by mechanical factors, which would be expected to be similar in all cases investigated. Instead, it suggests that specific biochemical effects of the materials employed are important. Despite evidence that material used clearly influences number of viable cells, we find no clear trend with respect to use of fully synthetic, partially synthetic, and fully natural polyelectrolyte microcapsules even at concentrations that on average result in several microcapsules taken up by each cell (see, for example, Fig. 4a–d).

Fig. 4e also reveals that while at concentrations up to 10^5 /ml C6 viability varies very little with microcapsule size from ~ 1 to ~ 10 μm , differences emerge at the highest concentrations investigated. At such concentrations, viability is significantly higher in the presence of 1–2 μm capsules than larger capsules. Indeed, even

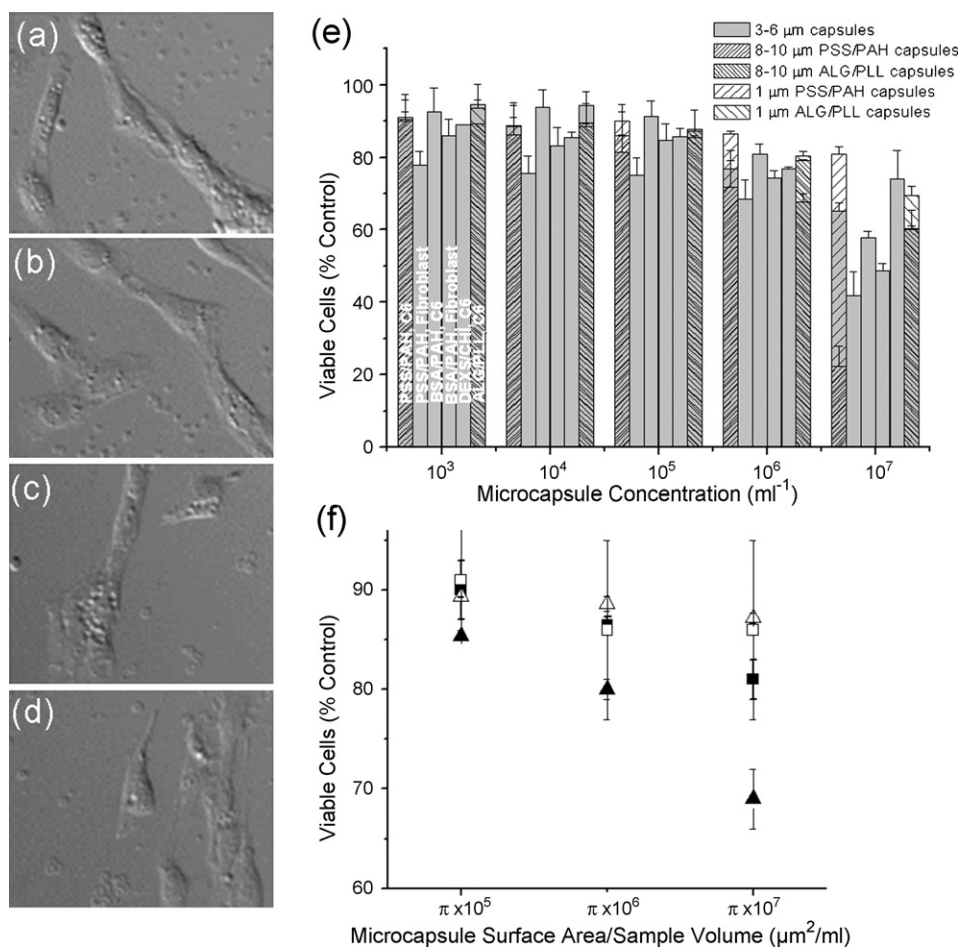


Fig. 4. DIC image of 10⁵/ml C6 cells with 10⁶/ml 3–6 μm (a) PSS/PAH, (b) BSA/PAH, (c) ALG/PLL, and (d) DEXS/CHI microcapsules. In all cases, microcapsule uptake and normal cell morphology are evident. (e) Number of viable cells relative to cells cultured in the absence of microcapsules as measured by MTT assay as a function of microcapsule concentration. Cell concentration is 1 × 10⁵/ml in all cases. Each bar represents an average of four samples, and the error bars represent one standard deviation. From left to right in each set of bars the systems are: C6 cells with PSS/PAH capsules, 3T3 cells with PSS/PAH capsules, C6 cells with BSA/PAH capsules, 3T3 cells with BSA/PAH capsules, C6 cells with DEXS/CHI capsules, and C6 cells with ALG/PLL capsules. 3–6 μm capsules are shown in gray, 8–10 μm microcapsules (PSS/PAH and ALG/PLL) are shown in densely striped pattern, and ~1 μm microcapsules (PSS/PAH and ALG/PLL) are shown in sparsely striped pattern. (f) Number of viable cells relative to control as a function of total microcapsule surface area per sample volume for 1 μm (filled symbols) and 10 μm (open symbols) PSS/PAH (squares) and ALG/PLL (triangles) microcapsules.

at 10⁷/ml microcapsules, C6 cells cultured with 1–2 μm PSS/PAH microcapsules have >80% the number of viable cells as the control. Of course, neither the total polyelectrolyte present nor the volume occupied by identical numbers of differently sized microcapsules is the same. If cell proliferation and viability is affected by the specific material of which the microcapsules are composed, viability should scale approximately with microcapsule surface area. If the effect of microcapsules in decreasing number of viable cells is chiefly caused by effects of microcapsules settling atop cells or competition for adhesion area, we would also expect viable cells to scale with microcapsule surface area. However, judging from visual observation and images such as that shown in Fig. 2, these two factors only appear relevant at 10⁷/ml 3–6 and 8–10 μm microcapsules. Fig. 4f shows cell viability as a function of surface area for the ~1 and ~10 μm PSS/PAH and ALG/PLL capsules investigated. The highest concentration of 1 μm capsules plotted is 10⁷/ml and that of 10 μm capsules is 10⁵/ml, concentrations at which little surface area of the substrate is covered with microcapsules. We note that the surface areas computed are approximations, as the “10 μm” microcapsules are actually 8–10 μm and the 1 μm microcapsules are actually 1–2 μm. Thus, the surface areas may be somewhat higher in the case of the “1 μm” than “10 μm” microcapsules. Even so, we find very similar results for number of viable cells in the presence of identical surface area of differently sized PSS/PAH microcap-

sules, but somewhat different results for ALG/PLL microcapsules at identical surface area. The poorer agreement seen for ALG/PLL capsules may be related to differences in total particle uptake as a function of microcapsule size and composition. We also note that when surface area is identical for 1 and 10 μm microcapsules, volume occupied will be approximately an order of magnitude higher in the case of the large microcapsules. Given that cells uptake all microcapsules investigated in substantial numbers and that at identical surface area the cells incubated with the larger microcapsules exhibit higher viability, it appears that the large volume occupied by these microcapsules inside the cells does not inhibit the proliferation or viability of these cells on the time scales investigated in this study. Taken together, our results indicate that the material that is present in the cell following microcapsule internalization, rather than mechanical effects of microcapsules either internal or external to the cells, is chiefly responsible for the decrease in number of viable cells as a function of microcapsule concentration.

To ascertain whether the decrease of MTT signal as a function of microcapsule concentration at high concentrations of large PSS/PAH microcapsules was due to cell death via apoptosis and not solely to a decrease in cell proliferation, DAPI staining was used to observe nuclei of cells cultured with microcapsules. Nuclei are considered to have the normal phenotype when the fluorescent signal is bright and homogeneously distributed, as in Fig. 2b. Apop-

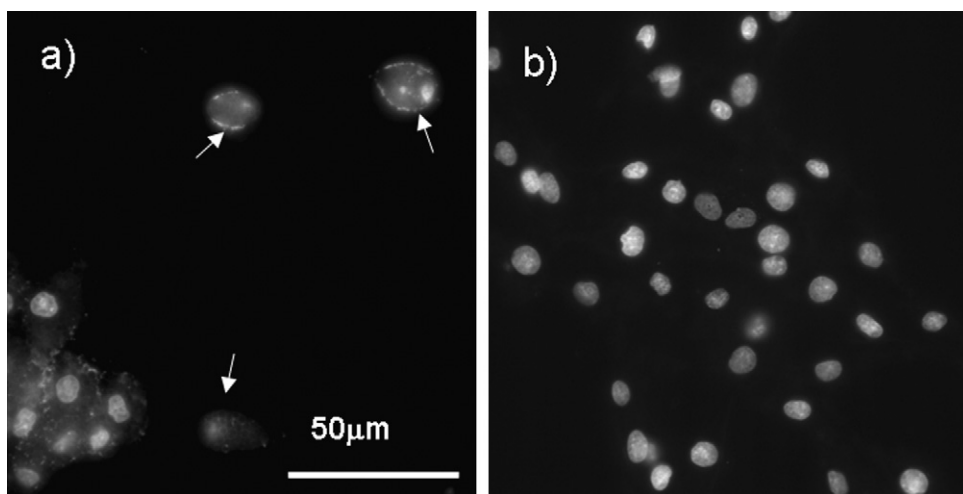


Fig. 5. DAPI stain of C6 glioma cells cultured with PSS/PAH microcapsules for 24 h at a (a) 100:1 and (b) 10:1 ratio of microcapsules to cells. Arrows point to cells that are in late apoptosis. Cell density is lower in (a) than (b) since many apoptotic rounded cells have fully detached from the surface and are washed away during the DAPI staining procedure.

otic nuclei can be identified by condensed chromatin gathered at the periphery of the nuclear membrane or fully fragmented nuclear bodies [30]. The result of DAPI nuclear staining of C6 cells cultured with large PSS/PAH microcapsules at the highest microcapsule to cell ratio (100:1) is shown in Fig. 5a. Some of the cells cultured are rounded and have fragments of DNA dispersed throughout the cell, indicating advanced apoptosis (Fig. 5a, arrows). Apoptotic condensation and inhomogeneous DAPI distribution was not observed in cells cultured with 3–6 μm PSS/PAH at lower microcapsule to cell ratios (Fig. 5b) or in cells cultured with any of the other types of microcapsules studied, even at the highest concentration investigated (data not shown). This result suggests that at $10^6/\text{ml}$ and lower microcapsule concentrations, decreased C6 cell proliferation is the chief cause of decrease of MTT signal for all microcapsule types. However, at the $10^7/\text{ml}$ microcapsule concentration, both lowered cell proliferation and apoptosis are responsible in the case of PSS/PAH microcapsules.

3.5. Invasion and growth of C6 spheroids in the presence of microcapsules

For use in drug delivery *in vivo*, microcapsules must be delivered through tissue filled with cells and ECM. Additionally, an increasing

number of studies *in vitro* use cells embedded in 3D environments since cell behavior and response to drug treatments may differ significantly when cells are embedded in 3D environments compared to when they are plated on 2D substrates [47]. Thus, in addition to studying the effect of microcapsules on cells in monolayer culture, we interrogate the effects of PSS/PAH microcapsules on C6 cells cultured as multicellular tumor spheroids (MTSs), 3D tumor approximations with cell–cell and cell–matrix contacts similar to those that exist *in vivo*. The MTSs are embedded in 3D biopolymer gels with composition, structure and mechanical properties similar to those of ECM *in vivo*. In order to evaluate whether 3–6 μm unfilled PSS/PAH microcapsules affect the growth and invasion of C6 glioma cells, potential effects of the microcapsules on the surrounding biopolymer matrix must first be identified. Such changes have been seen in the presence of polystyrene beads previously [48] and may be expected to affect the growth and invasion of such tumor systems [43]. We thus use CRM to compare the microscopic structure of pure 1.5 mg/ml collagen gels and 1.5 mg/ml collagen gels mixed with 3–6 μm PSS/PAH microcapsules in the absence of cells. Fig. 6 shows typical images of pure collagen and high concentration collagen–microcapsule matrices. Particular mesh size in these two images is 8.5 and 7 μm . No significant differences in collagen matrix mesh size is seen as a function of microcapsule

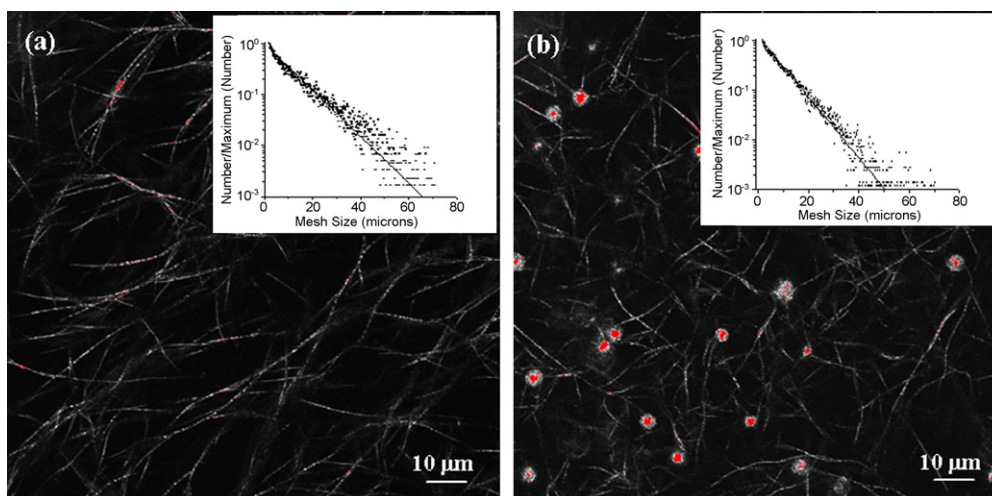


Fig. 6. Confocal reflectance images of two collagen matrices: (a) 1.5 mg/ml collagen and (b) 1.5 mg/ml collagen with $1 \times 10^7/\text{ml}$ microcapsules. Insets show the mesh size distribution as described in the text.

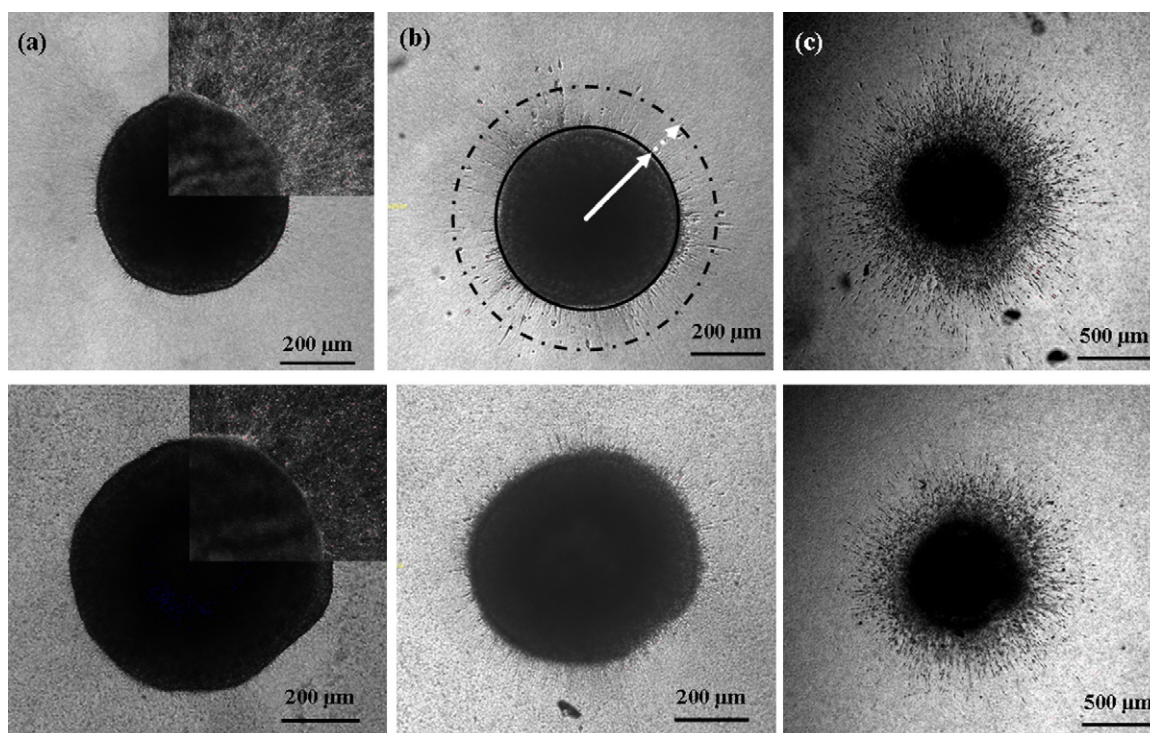


Fig. 7. Top panel: C6 MTS in a 1.5 mg/ml collagen gel; bottom panel: C6 MTS in a 1.5 mg/ml collagen gel with 10^7 /ml 3–6 μ m PSS/PAH microcapsules. (a) MTS 5 h after implantation; one quarter of the BF image is overlaid with a CRM image showing more substantially aligned (top) or less substantially aligned (bottom) collagen fibers surrounding the spheroid. (b) MTS 22 h after implantation. The MTS radius is depicted by the solid arrow and is defined by the extent of the dense cells in the center of the MTS. The invasive distance is depicted by the dashed arrow and is defined as the distance between the periphery of the MTS and a circle that circumscribes the invasive cells. (c) MTS 98 h after implantation.

presence, with average mesh size of $8 \pm 1 \mu\text{m}$ with no microcapsules and $7.1 \pm 0.1 \mu\text{m}$ with 1×10^7 microcapsules/ml. Thus, any change in MTS growth in environments with different numbers of microcapsules is unlikely to be caused by changes in matrix organization induced by the presence of microcapsules during gelation.

To identify the effect of microcapsules on the growth and invasion of C6 glioma MTSs, MTSs were placed in 1.5 mg/ml collagen gels mixed with 3–6 μm PSS/PAH microcapsules at the same microcapsule concentrations employed in the monolayer culture studies. Fig. 7 shows representative bright field images of MTSs in collagen gel matrices with and without microcapsules 5, 22, and 98 h after implantation. Invasive cells are observed to emerge from the central portion of the MTS, which is densely packed with cells, in both pure collagen and collagen-microcapsule matrices 5 h after implantation. On the same timescale, collagen fibers surrounding the MTS become aligned through tractional forces exerted by invasive cells around the MTS periphery in a sunburst pattern. There are more aligned fibers around the MTS in the pure collagen gel than around the MTS in the collagen gel mixed with microcapsules with a concentration of 10^7 /ml (Fig. 7). In those gels with lower microcapsule concentration, however, the degree of alignment is indistinguishable from that in the pure collagen gel (not shown). After 22 h, the number and density of invasive cells is slightly higher in pure collagen gels than in the collagen gels containing the highest concentration of microcapsules. After 98 h, more invasive cells are observed around the MTS body, and the density of invasive cells, invasive distance, and alignment of the surrounding collagen are somewhat greater in the pure collagen gels than in the collagen gels mixed with 10^7 /ml microcapsules (not shown). However, we find no obvious difference in the invasive cell density or collagen alignment between MTSs in pure collagen gels and in collagen gels with microcapsule concentration lower than 10^7 /ml.

Fig. 8a shows the growth of the MTS radius as a function of microcapsule concentration over the 98 h following implantation. Fig. 8b shows the invasive distance of cells as function of microcapsule concentration over the 98 h following implantation. Each trace in Fig. 8 is derived from an average over six MTSs. Fig. 8a indicates that PSS/PAH microcapsules do not affect the volumetric growth of the C6 MTSs over the 98 h following implantation, even at very high concentration. The invasive distance data shows that cells invade slightly faster in pure collagen gels than in collagen gels mixed with 10^7 /ml microcapsules (Fig. 8b), consistent with Fig. 7. However, there is no significant difference in the invasive behavior of cells in pure collagen gels and collagen gels mixed with microcapsules at lower concentrations. Only microcapsules at the highest concentration investigated affect the invasive behavior of cells. The limited invasive distance in these highest microcapsule concentration samples may be due to limited invasive cell proliferation or apoptosis (as in 2D culture) or decreased invasive cell motility.

These results show that C6 glioma MTSs are affected by the presence of high concentrations of large PSS/PAH microcapsules somewhat differently than are C6 cells in monolayer culture with the same concentration of PSS/PAH microcapsules. While the MTT assay results shown in Fig. 4e show that C6 viable cell number is $\sim 75\%$ for 3–6 μm PSS/PAH microcapsules at 10^6 /ml, the fact that invasive distance and MTS diameter are identical to within error at microcapsule concentrations up to 10^6 /ml indicates that cells cultured in 3D are less strongly affected by the presence of microcapsules than are cells in 2D. There are several possible reasons for the result. First, microcapsule to invasive cell ratio is not precisely known for the 3D systems and cells may take up fewer microcapsules in this environment than when plated on 2D substrates at identical concentrations. Despite this potential difference, the very robust growth of MTSs in the presence of very

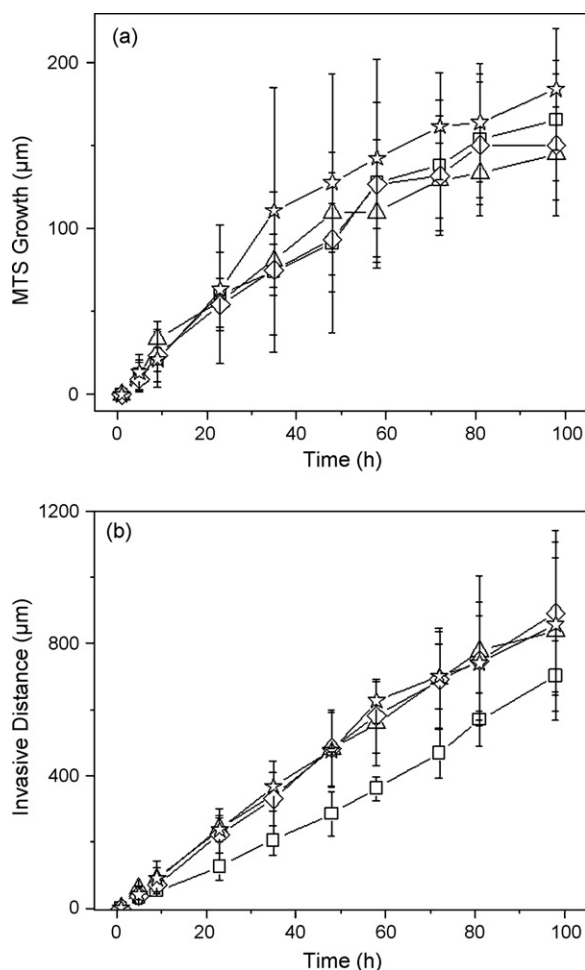


Fig. 8. (a) MTS growth in 1.5 mg/ml collagen gels with different microcapsule concentrations. (b) Invasive distance of MTS in 1.5 mg/ml collagen gels mixed with different concentrations of 3–6 μm PSS/PAH microcapsules. Control (stars), 10^3 /ml (diamonds), 10^5 /ml (triangles), and 10^7 (squares). Each trace is derived from an average over six MTSs. The error bars represent one standard deviation.

high concentrations of large PSS/PAH microcapsules suggests these microcapsules are useful drug delivery agents for proof-of-principle drug delivery studies using cells embedded in 3D tissue approximations.

4. Conclusion

In summary, this *in vitro* study of the interaction of microcapsules shows that for cells cultured in monolayer, number of viable cells relative to control is greater than 75% for microcapsule to cell ratios less than or equal to 1:1. This is true for both C6 and 3T3 cells and is independent of microcapsule size in the range of 1–10 μm and of microcapsule composition for the capsules used in this study. At a microcapsule to cell ratio of 10:1, number of viable cells starts to decrease more obviously, though almost all cells visualized appear extended and attached to the substrate, with no evidence that either competition for adhesion space or microcapsule sedimentation atop cells is substantially affecting number of viable cells. Our results thus suggest the decrease in viable cells is due chiefly to lower rates of proliferation induced by particular biochemical effects of internalized microcapsules. Over the full range of microcapsule composition and size studied, there is no clear trend in number of viable cells as a function of the type of polyelectrolytes used in the microcapsules. Indeed, while there is clear benefit to using biodegradable, natural polyelectrolyte-based microcapsules

for drug delivery applications *in vivo*, for proof of principle experiments consisting only of short term *in vitro* culture, cell viability and proliferation are not more adversely affected by the presence of PSS/PAH microcapsules than by that of microcapsules composed of natural polyelectrolytes. In addition to investigating the interaction of microcapsules with cells in monolayer culture, 3D C6 MTSs embedded in collagen matrices were also monitored in the presence of microcapsules. The microcapsules were shown to have minimal effect on matrix structure, cell invasion, and MTS growth. We thus have shown that the very easily prepared, stable, prototypical microcapsules composed of PSS/PAH are appropriate for use in proof of principle studies investigating various temporally or spatially controlled drug delivery in cells cultured on 2D substrates or in 3D environments.

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