

Corning® Ultra-Low Attachment Surface Promotes Spheroid Formation in MCF-7 Human Breast Cancer Cell Line

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SnAPPShots

A brief technical report
from the Corning
Applications Group

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Introduction

The generation of tumor spheroids *in vitro* is a useful model to examine malignant cells and tumorigenesis. Current techniques to generate tumor spheroids may involve using reconstituted basement membrane, agarose, or the hanging drop method. These techniques can be time consuming and may even require purchasing additional equipment. To address these issues, Corning offers a ready-to-use surface that allows for the formation of tumor spheroids. The Ultra-Low Attachment Surface product consists of a covalently bound hydrogel layer that is hydrophilic and neutrally charged. Since proteins and other biomolecules passively absorb to the polystyrene surfaces through either hydrophobic or ionic interactions, this hydrogel surface naturally inhibits nonspecific interactions of the cell to the surface eliminating unwanted cell attachment. This study evaluated the ability to generate functional tumor spheroids using tissue culture plastics coated with the Corning Ultra-Low Attachment Surface.

Previous reports in the literature have demonstrated that tumor spheroids have a slower growth rate than those cultured as a monolayer (1). Additionally, studies have demonstrated that in many tumor spheroids there is an increase in vascular endothelial growth factor (VEGF) secretion, a protein known to stimulate vasculogenesis and angiogenesis in tumor cells (1,2). One goal of this study was to examine these features in breast cancer tumor spheroids, MCF-7, cultured on the Corning Ultra-Low Attachment Surface compared to traditional tissue culture treated (TCT) surfaces. The results demonstrated that in addition to the formation of tumor spheroids on the Corning Ultra-Low Attachment Surface, these spheroids had a significant decrease in the growth rate and an increase in VEGF production compared to cells cultured on the TCT surfaces.

Evidence also suggests that cells cultured as a spheroid are more resistant to drug treatments compared to those cultured as a monolayer (on a TCT surface) (3). The next goal of this study was to examine whether (i) tumor spheroids could be generated and analyzed in a format ideal for High Throughput Screening and (ii) whether cells cultured on the Corning Ultra-Low Attachment Surface are more sensitive to drug treatments. The results demonstrated that functional cancer spheroids can be generated and analyzed in a format ideal for high-throughput screening and these spheroids may be more sensitive to drug treatment.

Method and Materials

Cell Culture

For cell growth assays MCF-7 (ATCC Cat. No. HTB-22) cells were cultured in IMDM (Corning cellgro®, Cat. No. 10-016-CM) supplemented with 10% FBS (PAA, Cat. No. A15-201). For high throughput screening assays MCF-7 cells were cultured in phenol red free IMDM (Life Technologies,™ Cat. No. 21056-023) supplemented with 1X B27 (Life Technologies, Cat. No. 17504044), 20 ng/mL epidermal growth factor (Sigma-Aldrich®, Cat. No. E9644), and 20 ng/mL basic fibroblast growth factor (Sigma-Aldrich, Cat. No. F0291). For cell sensitivity assays MCF-7 cells were cultured in phenol red free IMDM supplement with 2% FBS. Cells were incubated at 5% CO₂, 37°C and 98% relative humidity.

Cell Growth Assay

Cells were seeded onto Corning Ultra-Low Attachment Surface (Cat. No. 3815) and TCT (Cat. No. 430639) 25 cm² cell culture flasks at 20,000 cells/cm² (0.25 mL/cm²) and incubated for 4 days. Cell conditioned media was collected for the VEGF ELISA assay (see below). Cells cultured on the TCT vessel were harvested with Trypsin-EDTA (0.05%/0.5 mM) (Corning cellgro, Cat. No. 25-052-CV). Spheroids cultured on the Corning Ultra-Low Attachment Surface vessel were collected, washed with PBS, and incubated with Trypsin-EDTA (0.05%/0.5 mM) for four minutes at 37°C. Cell concentration was determined by trypan blue exclusion using the BioProfile® FLEX analyzer (Nova Biomedical).

VEGF ELISA

To detect VEGF levels, the Human VEGF Elisa kit was purchased from ThermoScientific (Cat. No. EHVEGF). The microplate provided in this kit was coated with anti-human VEGF165 antibody. The assay was performed following manufacturer's instructions.

High Throughput Screening Assay

MCF-7 cells were seeded on a 384 well black with clear bottom Corning® Ultra-Low Attachment Surface microplate (Cat. No. 3827) or a 384 well black with clear bottom TCT Surface microplate (Cat. No. 3764) at 80,000 cells/cm² (0.49 mL/cm²). After 48 hours, an NF-κB inhibitor, parthenolide, (Sigma-Aldrich, Cat. No. P0667) was added to cultures and incubated for 72 hours. Following incubation the CytoTox-One™ assay (Promega, Cat. No. G7890) was performed according to manufacturer's instructions and the microplates transferred to a Perkin EnVision® Multilabel Reader for detection of signal.

Cell Sensitivity Assay

MCF-7 cells were seeded on Corning Ultra-Low Attachment Surface 384 well black with clear bottom microplate (Cat. No. 3827) or Corning TCT 384 well black with clear bottom microplate (Cat. No. 3764) at 80,000 or 35,000 cells/cm² (0.49 mL/cm²), respectively. After 72 hours, parthenolide (Sigma-Aldrich, Cat. No. P0667) was added to MCF-7 cells and incubated for 24 hours. Following incubation the CytoTox-One™ assay (Promega, Cat. No. G7890) was performed as previously described.

Immunocytochemistry

MCF-7 cells were seeded on Corning Ultra-Low Attachment Surface 384 well black with clear bottom microplate at 80,000 cells/cm² (0.49 mL/cm²) and incubated for 96 hours. Immunostaining was performed as previously described (4). Briefly, spheroids were fixed and permeabilized for 3 hours at 4°C in 4% paraformaldehyde and 0.5% Triton X-100. Spheroids were then washed in PBS (3 x 5 minutes), dehydrated in an ascending series of methanol at 4°C in PBS (25%, 50%, 75%, and 95%, 20 minutes each and 100% for 1 hour), rehydrated in the same descending series and washed in PBS (3 x 5 minutes). After blocking in PBS containing 0.1% Triton X-100 and 3% Bovine Serum Albumin overnight at 4°C, spheroids were incubated with FITC-Mouse Anti-Human CD44 (1:50 dilution) (BD Bioscience) at 4°C for 48 hours. Spheroids were imaged using an AMG EVOS® F1 microscope.

Results

Cell Growth

The first goal of this study was to demonstrate that cells cultured on the Corning Ultra-Low Attachment surface have a

different morphology than those cultured on a TCT surface. After 72 hours, MCF-7 cells cultured on each surface have distinct morphological differences. Those cultured on the TCT surface proliferated as a flat monolayer (Fig. 1A), whereas cells expanded on the Ultra-Low Attachment Surface formed spheres (Fig. 1B). Based on previous work by Takagi et al. (1) which demonstrates that cells cultured as spheroids have a slower growth rate than those cultured as a monolayer, the next aim was to evaluate whether spheroids generated on the Ultra-Low Attachment Surface behaved in a similar manner. MCF-7 cells were seeded onto the 25 cm² TCT and Ultra-Low Attachment Surface vessels and harvested after four days in culture. Cells cultured on the Ultra-Low Attachment Surface vessel had a lower yield/cm² (Fig. 2A) and a slower growth rate (Fig. 2B). These data indicate that cells forming spheroids on the Corning Ultra Low Attachment Surface exhibit more *in-vivo*-like behavior than those cultured on a 2D TC-treated surface.

To evaluate whether the spheroids cultured on the Corning Ultra-Low Attachment Surface are functional, the cell conditioned media was collected and analyzed for VEGF secretion. VEGF has been previously shown to stimulate vasculogenesis and angiogenesis in tumor cells (1,2). The data presented in Fig. 2C show that spheroids cultured on the Corning Ultra-Low Attachment Surface have an increase in VEGF production compared to those grown on a TCT surface. Collectively, these studies demonstrate the Corning Ultra-Low Attachment Surface provides an environment where cells can behave in a more *in-vivo*-like manner, resulting in physiologically relevant tumor spheroids.

Toxicity Studies

The next goal of the study was to examine whether tumor spheroids could be generated and analyzed in a format ideal for high high throughput screening. MCF-7 cells were seeded onto a 384 well black clear bottom Corning Ultra-Low Attachment Surface microplate. After two days in culture, MCF-7 spheroids were treated with either vehicle control (0.01% DMSO) (Fig. 3A) or parthenolide, an NF-κB inhibitor, (Fig. 3B) for 72 hours. Evidence in the literature suggests that parthenolide induces cell death in MCF-7 spheroids (5). The representative images found in Fig. 3A and 3B indicate that parthenolide induces morphological changes of the spheroids. Additionally, cytotoxicity was analyzed in these spheroids using CytoTox-One (Fig. 3C). Prior to per-

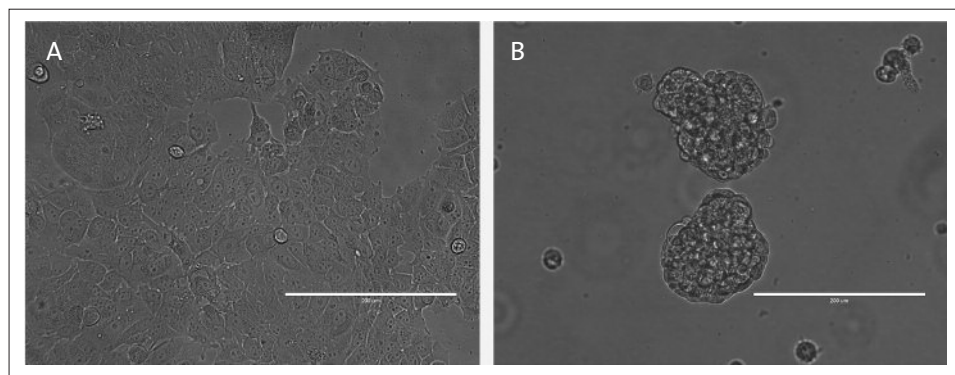


Figure 1. MCF-7 cells cultured on Corning Ultra-Low Attachment Surface forms tumor spheroids. MCF-7 were seeded onto the T-25 TCT (A) and Ultra-Low Attachment Surface (B) vessels. The cells were cultured for 72 hours and a representative image was taken using an AMG EVOS® F1 microscope. Scale bars represent 200 μm.

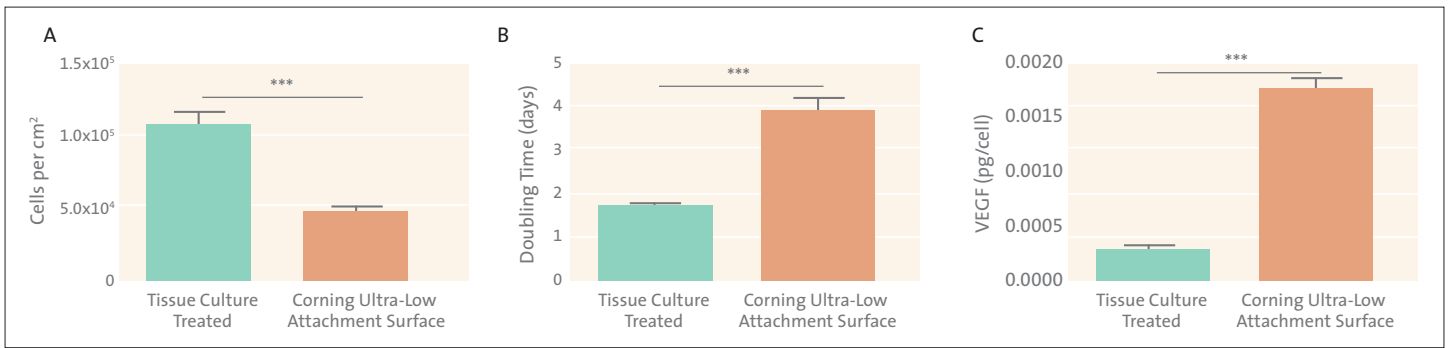


Figure 2. MCF-7 cells cultured on Corning® Ultra-Low Attachment Surface forms functional tumor spheroids. MCF-7 were seeded onto the 25 cm² TCT and Ultra-Low Attachment Surface flask at 20,000 cells/cm². The cells were harvested after 4 days in culture. Cells cultured on the TCT vessel had a higher yield/cm² (A), a shorter doubling time (B), and a decrease in VEGF production compared to those grown on Corning Ultra-Low Attachment Surface (C). Each experiment was performed in triplicate three independent times. These data are presented as the mean ± SEM, *** p<0.001, Paired t test.

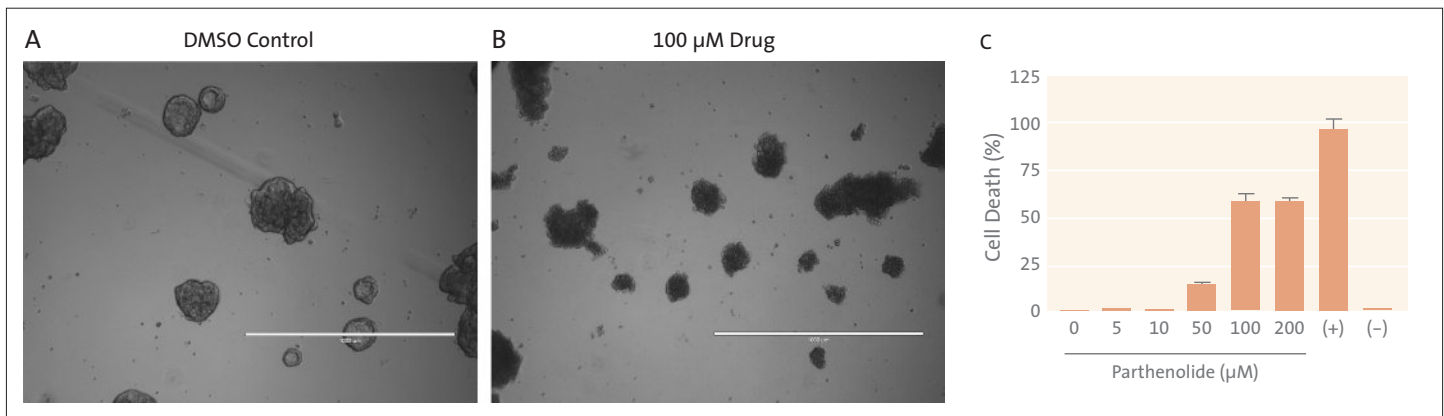


Figure 3. 384 well black clear bottom Corning Ultra-Low Attachment Surface microplate may be used for cancer and toxicity applications. MCF-7 spheroids were treated with either vehicle control (0.01% DMSO) (A) or 100 µM of the NF-κB inhibitor, parthenolide (B) for 72 hours. Additionally, cell death was analyzed in these spheroids using CytoTox-One (C). Prior to performing the assay, selected wells were incubated with a lysis buffer (+) for 15 minutes at room temperature. Non-treated wells (no DMSO added) are indicated by (-). Percent of cell death was evaluated compared to the lysed cells (+). Each sample was examined in 10 wells, three individual times. Scale bars represent 1000 µm.

forming the assay, selected wells were incubated with a lysis buffer, provided by Promega (+), for 15 minutes at room temperature. Non-treated wells (no DMSO added) are indicated by (-). The percent of cell death was evaluated compared to the lysed cells (+). These results demonstrate that parthenolide induces a dose-response increase in cell death compared to vehicle control.

Evidence also suggests that cells cultured as a spheroid are more resistant to drug treatments compared to those cultured as a monolayer (on a TCT surface) (3). To examine whether the MCF-7 spheroids cultured on the Ultra-Low Attachment Surface are more resistant to treatment, cells were cultured on both surfaces for 72 hours followed by 24 hour exposure to parthenolide. Cell treatment was shortened to 24 hours, because cells cultured on TCT demonstrated complete cell death when exposed to parthenolide for 72 hours. The results show that when cultured on the TCT surface, MCF-7 cells demonstrated greater than 65% cell death in the presence of 100 µM parthenolide, whereas cells cultured on the Corning Ultra-Low Attachment Surface did not yield greater than 50% cell death at a concentration of 100 µM (Fig. 4). Taken together, these data demonstrate that

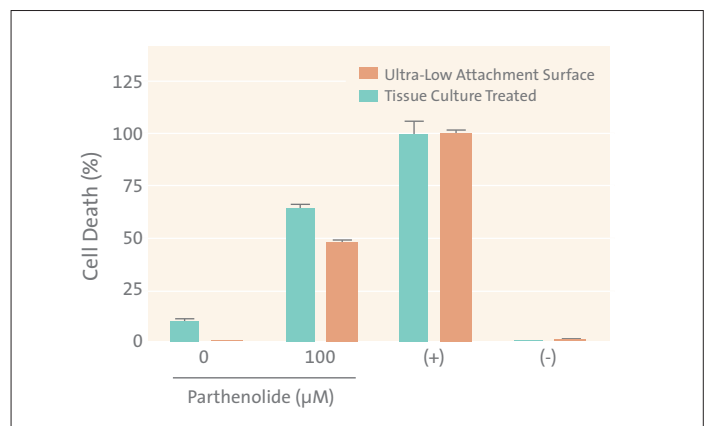


Figure 4. Trends suggest that MCF-7 cells cultured on a TCT surface are more sensitive to treatment compared to those cultured on the Corning Ultra-Low Attachment Surface. MCF-7 cells were cultured on either TCT or Corning Ultra-Low Attachment Surface for 72 hours followed by 24 hours exposure to parthenolide. Prior to performing the assay, selected wells were incubated with a lysis buffer, provided by Promega (+), for 15 minutes at room temperature. Non-treated wells (no DMSO added) are indicated by (-). Vehicle control (0 µM parthenolide) was 0.05% DMSO. Percent of cell death was evaluated compared to the lysed cells (+). Each sample was examined in 8 wells, at least two individual times.

the spheroids generated on the Corning® Ultra-Low Attachment Surface can be analyzed in a format ideal for high throughput screening.

Immunocytochemistry

Lastly, to evaluate whether spheroids cultured on the Corning Ultra-Low Attachment Surface are biologically relevant to tumors, spheroids were immunostained with a cell surface marker, CD-44, known to be present in most breast cancer cells (6). This representative image demonstrates the capabilities to culture and stain functional spheroids in the Corning Ultra-Low Attachment Surface 384 well microplate (Fig. 5).

Summary

- ▶ The Corning Ultra-Low Attachment products provide an environment for cells to behave in a more *in-vivo*-like manner. This surface is ideal for applications such as tumor spheroid production, stem cell embryoid body formation (data not shown) and several other applications.
- ▶ The new 384 well black clear bottom Ultra-Low Attachment Surface microplate has many applications in the area of cancer biology, including tumor spheroid development, drug screening studies and developing more *in-vivo*-like functional responses from model systems.
- ▶ The Corning Ultra-Low Attachment Surface generates functional cancer spheroids that may be analyzed in a format ideal for high-throughput screening.

References

1. Takagi, A., M. Watanabe, Y. Ishii, J. Morita, Y. Hirokawa, T. Matsuzaki, and T. Shiraishi. 2007. Three-dimensional cellular spheroid formation provides human prostate tumor cells with tissue-like features. *Anticancer Res.* 27:45-53.
2. Valcarcel, M., B. Arteta, A. Jaureguibeitia, A. Lopategi, I. Martinez, L. Mendoza, F.J. Muruzabal, C. Salado, and F. Vidal-Vanaclocha. 2008. Three-dimensional growth as multicellular spheroid activates the proangiogenic phenotype of colorectal carcinoma cells

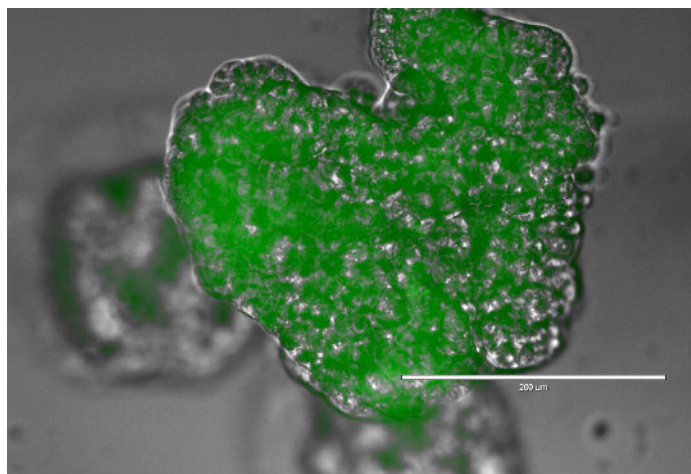


Figure 5. MCF-7 spheroids immunostained for a cell-surface marker, CD44, in the Corning Ultra-Low Attachment Surface 384 well black with clear bottom microplate. A representative image depicting MCF-7 spheroids immunostained with a cell surface marker, CD-44, found to be present in most breast cancer cells. Scale bar represents 200 μm.

- via LFA-1-dependent VEGF: implications on hepatic micro-metastasis. *J. Transl. Med.* 6:57.
3. Olive, P.L. and R.E. Durand. 1994. Drug and radiation resistance in spheroids: cell contact and kinetics. *Cancer Metastasis Rev.* 13:121-138.
4. Weiswald, L.B., J.M. Guinebreteire, S. Richon, D. Bellet, B. Saubamea, and V. Dangles-Marie. 2010. *In situ* protein expression in tumour spheres: development of an immunostaining protocol for confocal microscopy. *BMC Cancer* 10:106.
5. Nobert, G.S., M.M. Kraak, and S. Crawford. 2006. Estrogen dependent growth inhibitory effects of tamoxifen but not genistein in solid tumors derived from estrogen receptor positive (ER+) primary breast carcinoma MCF7: single agent and novel combined treatment approaches. *Bull Cancer* 93:E59-E66.
6. Engelmann, K., H. Shen, and O.J. Finn. 2008. MCF7 side population cells with characteristics of cancer stem/progenitor cells express the tumor antigen MUC1. *Cancer Res.* 68:2419-2426.

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