Why the dish makes a difference: Quantitative comparison of polystyrene culture surfaces

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ABSTRACT

There is wide anecdotal recognition that biological cell viability and behavior can vary significantly as a function of the source of commercial tissue culture polystyrene (TCPS) culture vessels to which those cells adhere. However, this marked material dependency is typically resolved by selecting and then consistently using the same manufacturer's product – following protocol – rather than by investigating the material properties that may be responsible for such experimental variation. Here, we quantified several physical properties of TCPS surfaces obtained from a wide range of commercial sources and processing steps, through the use of atomic force microscopy (AFM)-based imaging and analysis, goniometry and protein adsorption quantification. We identify qualitative differences in surface features, as well as quantitative differences in surface roughness and wettability that cannot be attributed solely to differences in surface chemistry. We also find significant differences in cell morphology and proliferation among cells cultured on different TCPS surfaces, and resolve a correlation between nanoscale surface roughness and cell proliferation rate for both cell types considered. Interestingly, AFM images of living adherent cells on these nanotextured surfaces demonstrate direct interactions between cellular protrusions and topographically distinct features. These results illustrate and quantify the significant differences in material surface properties among these ubiquitous materials, allowing us to better understand why the dish can make a difference in biological experiments.

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1. Introduction

Researchers studying biological systems attempt to mimic physiological conditions by systematically controlling specific aspects of *in vitro* culture. These parameters include solution pH, culture media nutrient composition, oxygen tension, frequency of media exchange and the number of cells per volume of liquid media. It is widely appreciated from practical experience, and strict adherence to established cell culture protocols, that each individual feature can significantly influence cell behavior [1–3]. Even the choice of modern culture surfaces, including the source of tissue culture polystyrene (TCPS), is known anecdotally to play a key role in repeatability of cell culture observations though the reasons remain unclear. Here, we explore and quantify variations in physical properties among such culture surfaces, as well as the corresponding effects on protein adsorption and cell behaviors.

Through the 1950s, borosilicate glass was the culture surface of choice [4]. So-called tissue culture polystyrene was accidently discovered when researchers at Falcon Plastics Company were attempting to coat plastic with glass to create cultureware. Instead, the oxygen plasma treatment of the polystyrene produced an optimal surface without the glass [5]. Today, TCPS is one of the most commonly used surfaces in mammalian cell biology [5]. Numerous commercial sources of TCPS are available, creating a wide variety of manufacturers, surface chemistries and culture formats [6]. Polystyrene manufactured for tissue culture uses a special resin formulation with tightly controlled molecular weight distribution. The tissue culture formats (flasks, dishes, multi-well plates, etc.) are created using high speed, hot runner injection molding [5]. After molding, polystyrene plates and flasks are placed in a vacuum chamber and undergo reactive gas plasma and secondary treatments to render the surfaces hydrophilic, enhancing cell–substrate adhesion [5]. Many manufacturers offer special proprietary techniques and qualification standards, the details of which are seldom published [7], such as the Nunclon® Delta or Greiner Bio-One Advanced TC™ brands, to certify the consistency of their manufacturing processes and repeatable surface characteristics. Despite these

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assurances by various manufacturers, inconsistent or irreproducible results within a given set of *in vitro* experiments are often blamed on the culture dish. A common "solution" is to then change the format or brand of dish [8,9]. However, given that TCPS is a material that is a means to an end in most biological and biomaterials investigations, the source(s) of variation among cell culture responses to different types and sources of TCPS typically remain uninvestigated.

We considered whether the largely overlooked differences in material surface features and properties among commercial TCPS sources contribute strongly to such discrepancies among *in vitro* culture results for ostensibly identical experiments. Researchers increasingly appreciate that surface features such as roughness and mechanical stiffness, independent of surface chemistry and media conditions, can play a significant role in guiding cell behavior [10–12]. Substratum topography can alter organization of the cytoskeleton, and influence attendant properties such as adhesion, proliferation, migration and differentiation potential [13–16]. Measurable effects of surface roughness on cell behavior have been demonstrated for polystyrene, as well as for hydroxyapatite, polydimethylsiloxane, polymethyl methacrylate and even titanium [17–20]. The size of these features can also play a significant role in cellular response [21,22]. In this study, we considered numerous samples of commonly used TCPS culture vessels to quantify variations in surface topography and other physical features as a function of commercial source and of culture vessel formats. We then investigated whether these differences affected cell morphology and behavior. The aim of this study was not to identify or claim that one TCPS source is "better" than others, but rather to understand how the material processing and surface properties varied and correlated with protein adsorption and cell responses.

2. Methods

2.1. Atomic force microscopy

Samples were prepared from tissue culture surfaces found in Table 1 and characterized via atomic force microscopy (AFM; MFP-3D Asylum Research, Santa Barbara, CA) within an inverted optical microscope (IX51, Olympus America, Inc.) and imaged in air using AFM cantilevers of nominal spring constant $k = 0.035$ N m^{-1} and probe radius $R = 25$ nm (MLCT, Veeco, Malvern, PA). At least five surfaces were analyzed for each sample type and manufacturer. Root mean squared (RMS) roughness values were extracted from height trace images using the scientific computing software Igor Pro (Wavemetrics Portland, OR) and reported as reported as mean ± standard error of measurement.

2.2. Atomic force microscopy of cells on dishes

NIH 3T3 fibroblasts were cultured on Falcon Petri dishes of 35 mm diameter (P35) at a density of ~15,000 cells cm^{-2} in 10% bovine calf serum (BCS) in Dulbecco's modified Eagle's medium (DMEM). After 1 day of incubation, media was aspirated and cells were incubated for 15 min at room temperature in a 4% paraformaldehyde (AlfaAesar 43368 Ward Hill, MA) solution in phosphate

buffered saline (PBS). Five rapid washes in PBS + 0.05% Tween-20 (Teknova P1176 Hollister, CA) were performed before imaging via AFM under contact mode, as described above, in 1× PBS.

2.3. Contact angle measurements

Approaching contact angle measurements were taken with a VCA 2000 Video Contact Angle System (AST Inc.) goniometer. Contact angles were measured by dropping a single droplet of double deionized water (ddH$_2$O), DMEM or DMEM + 10% BCS onto samples prepared from the tissue culture side of Celltreat®, Corning®, Cyto One®, Falcon™, Greiner Bio One Cellstar™, Nunclon™, Sarstedt, 75 cm^2 TCPS culture flasks (see Table 1) and compared to a 100 mm diameter VWR non-tissue culture Petri dish (#25384-088). VCA OptimaXE (AST Inc.) software was used to estimate angles on the left and right sides of contact.

2.4. Immunocytochemistry

To assay orientation of vinculin and F-actin, NIH 3T3 murine fibroblasts were fixed using 4% paraformaldehyde (AlfaAesar 43368 Ward Hill, MA) in PBS for 15 min at room temperature after 24 h. Following fixation, cells were washed briefly with PBS containing 0.05% Tween-20 and permeabilized for 3 min at room temperature with 0.1% Triton X-100 (Fluka 93443, Switzerland). To minimize non-specific binding, cells were treated with 3% bovine serum albumin (BSA; Sigma, A7906) in PBS for 30 min before staining. Cells were incubated at room temperature with relevant primary antibodies in 3% BSA for monoclonal anti-mouse vinculin (Sigma V4505, 1:200). Cells were then incubated with secondary antibody goat anti-mouse IgG (Abcam6785, 1:400). Cells were also double labeled with Alexa Fluor 555 Phalloidin (Molecular Probes, A34055, 1:1000) for 60 min. Cells were rinsed three times (10 min each) with PBS and imaged by fluorescence microscopy (IX-81, Olympus America, Inc.) and captured using Slidebook 5.0 (Intelligent Imaging Innovations, Inc., Denver, CO). Cell nuclei were also counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Millipore 90229, 1:2000).

2.5. Cell area

Human bone-marrow-derived mesenchymal stromal or "stem" cells (hMSCs; ReachBio Seattle, WA) were expanded until passages 4–6, and plated onto P35 TCPS dishes at low density (~5000 cells cm^{-2}) to maintain subconfluent culture conditions. NIH 3T3 murine fibroblasts were seeded in P35 dishes from each manufacturer at a density of ~15,000 cells cm^{-2}. hMSCs were cultured in typical basal MSC culture media consisting of complete MesenCult medium (MesenCult basal plus with 20% MesenCult Supplemental; StemCell Technologies, Vancouver, BC) and 2 μM L-glutamine, 100 units ml^{-1} penicillin and 100 μl ml^{-1} streptomycin (Invitrogen, 15140-163, Carlsbad, CA). hMSCs and NIH 3T3 fibroblasts were fixed and immunocytochemistry was conducted, as described above. Cells were imaged by fluorescence microscopy (IX-81, Olympus America, Inc.) and captured using Slidebook 5.0 (Intelligent Imaging Innovations, Inc., Denver, CO). Cell areas were

Table 1
Manufacturer-specific catalog numbers for TCPS samples analyzed in this study.

	Celltreat®	Corning®	CytoOne®	Falcon™	Cellstar™	Nunclon™	Sarstedt
75 cm^2 flask	229341	430641	CC7682-4875	353135	658175	178905	83.1813.302
35 mm Petri dish	229635	430165	CC7682-3340	353001	627160	153066	83.1800
60 mm Petri dish	229660	430166	CC7682-3359	353002	628160	150228	83.1801
Six-well plate	229106	3516	CC7682-7506	353046	657165	140675	83.1839
96-well plate	229196	3598	CC7682-7596	353072	655162	160004	83.1835

quantified using Cell Profiler Software 2.0 (http://www.cellprofil-er.org/, Cambridge, MA) [23,24].

2.6. Population doubling

NIH 3T3 fibroblasts and hMSCs were seeded in P35 dishes from each manufacturer at a density of ∼15,000 cells cm^{-2} and 5000 cells cm^{-2}, respectively. After 24 h and 48 h post seeding, cells were incubated at room temperature in 1 ml of a 4% paraformaldehyde in PBS. Samples were then washed quickly three times with 1 ml of PBS + 0.05% Tween-20. Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Millipore 90229, 1:2000). Samples were imaged by fluorescence microscopy (IX-81, Olympus America, Inc.) and captured using Slidebook 5.0 (Intelligent Imaging Innovations, Inc. Denver, CO). Cell nuclei were counted using ImageJ analysis software (http://imagej.nih.gov/ij).

2.7. Protein adsorption assay

100 µg ml^{-1} FITC-conjugated bovine albumin (Sigma, A9771) was dissolved in ddH$_2$O and deposited on P35 Petri dishes (Table 1) Plates were incubated at 37 °C for 1 h. The solution was carefully aspirated and analyzed using a Cary 50 UV–visible spectrophotometer. Absorbance was measured at 459 nm and reported as percentage compared to nominal concentration of 100 µg ml^{-1}.

2.8. Collagen adsorption

5 µg cm^{-2} rat tail collagen type-I (Sigma, 40236) in 0.02 M acetic acid was dried overnight at 37 °C onto 60 mm diameter Petri dishes from Table 1. Samples were imaged in 1× PBS in contact mode AFM, as described above, and compared to collagen deposited on a MatTek (#P50G-0-30-F) glass bottom dish. RMS roughness values were calculated as described above.

2.9. Quantification of focal adhesion orientation

To quantify the orientation of focal adhesions, images were analyzed via a customized MATLAB script that quantified the orientation vector magnitudes for each adhesion, as described previously [25]. The average angular standard deviation was calculated from immunocytochemical staining of 3T3 fibroblasts adhered to stated TCPS sources. A lower average angular standard deviation <α$_{sd}$> indicates a higher order of alignment among the feature of interest (here, the many vinculin-stained regions indicative of adhesion complexes within a given cell). At least nine cells comprising many adhesion complexes were analyzed for each condition, and all values of average angular standard deviation were reported as mean ± standard error of measurement.

3. Results

Characterization of surface topography of TCPS cultureware revealed significant differences among commercial sources (Fig. 1). Samples were obtained from Celltreat®, Corning®, Cyto One®, Falcon™, Greiner Bio One Cellstar™, Nunclon™, Sarstedt (see Table 1) and imaged using contact mode AFM (see Section 2). Height images obtained in AFM contact mode imaging demonstrate pronounced qualitative variations across manufacturers in surface features, when viewed at equal image dimensions and resolution. Surprisingly, few such images of bare TCPS exist in the literature, despite their prolific use in in vitro cell and tissue culture. Thus, we imaged numerous formats from each manufacturer, including 75 cm^2 TCPS flasks (T75), 60 mm diameter TCPS Petri dishes (P60), as well as six-well TCPS and 96-well TCPS plates to deter-

mine if such variation existed across a single brand. First, we will discuss these topographic differences evident in Fig. 1 qualitatively, and then quantify properties such as surface roughness and wettability.

3.1. Overall image features

Celltreat®, Corning® T75 and Falcon™ TCPS T75 exhibited randomly oriented, raised features (appearing similar to and hereafter referred to as "fibers") on the material surface, while Greiner Bio One Cellstar™, Nunclon™, Sarstedt surfaces exhibited a greater degree of fiber alignment (n = 5 for each format). Cyto One® exhibited the most unique surface features, with a qualitatively smoother topography with the exception of various "pits" confirmed by topographic line traces of the height images. These "pits" were also prominent in Corning®, Greiner Bio One Cellstar™ and Nunclon™ dishes. The cross-sectional area of these pits varied greatly within a given culture vessel, among manufacturers, and among formats for a given manufacturer, ranging from <1 µm^2 to >100 µm^2. These fiber-like and pit-like features are signatures of the manufacturing process, which varies among manufacturers and typically includes injection into machined metallic molds and that can include trapped bubbles.

Note that the corresponding pit diameters are comparable to that of TCPS-adhered cells such as human umbilical vein endothelial cells [25].

Interestingly, there was little variation among vessel formats for a given commercial source: no significant differences in topography or degree of fiber alignment were observed among T75, P60 and six-well plates for a given manufacturer, with the exception of 96-well plates. For example, pitting in Cyto One® TCPS 96-well plates was more prominent and of smaller pit diameter than in other formats. Nunclon™ does not use a molded 96-well plate, but instead adheres a proprietary thin sheet of TCPS, optimized for optical imaging. This TCPS surface was, in comparison with the other formats that exhibited either prominent fibers or pits, featureless when viewed at the same height scale. Previous studies have shown that such features can have significant effects on cell adhesion, cytoskeletal organization and cell morphology [21]. Note that Celltreat® 96-well TCPS plates are not produced as flat-bottomed wells, resulting in the apparent arc in image contrast in Fig. 1.

3.2. Surface roughness

Closer inspection of these fiber features revealed a typical lateral width of ∼250 nm and height of ∼5 nm for Falcon™ P60 dishes (Fig. 2A) and of ∼500 nm and ∼12 nm, respectively, for Nunclon™ P60 dishes (Fig. 2B). We did not deconvolute the AFM probe shape from these images, but used cantilevered probes of diameter ∼20 nm such that these features of 10 s and 100 s nm are reasonable estimates of the actual surface feature dimensions. TCPS fibers typically range between 50 and 500 nm in diameter, depending on the manufacturer, which is the same range and order of magnitude as the diameter of the native extracellular matrix fiber diameters to which tissue cells adhere in vivo [26,28,29].

Further characterization of TCPS surfaces revealed a statistically significant difference (P < 0.001) in RMS surface roughness values across all manufacturers' P60 TCPS dishes (Fig. 2C), as determined by analysis of AFM contact mode images (n = 5 for each manufacturer). As observed in Fig. 1, Cyto One® appeared to have the smoothest surface of all manufacturers with a RMS surface roughness of ∼1 nm, while Nunclon™ had the largest surface features and a RMS surface roughness of ∼6 nm.
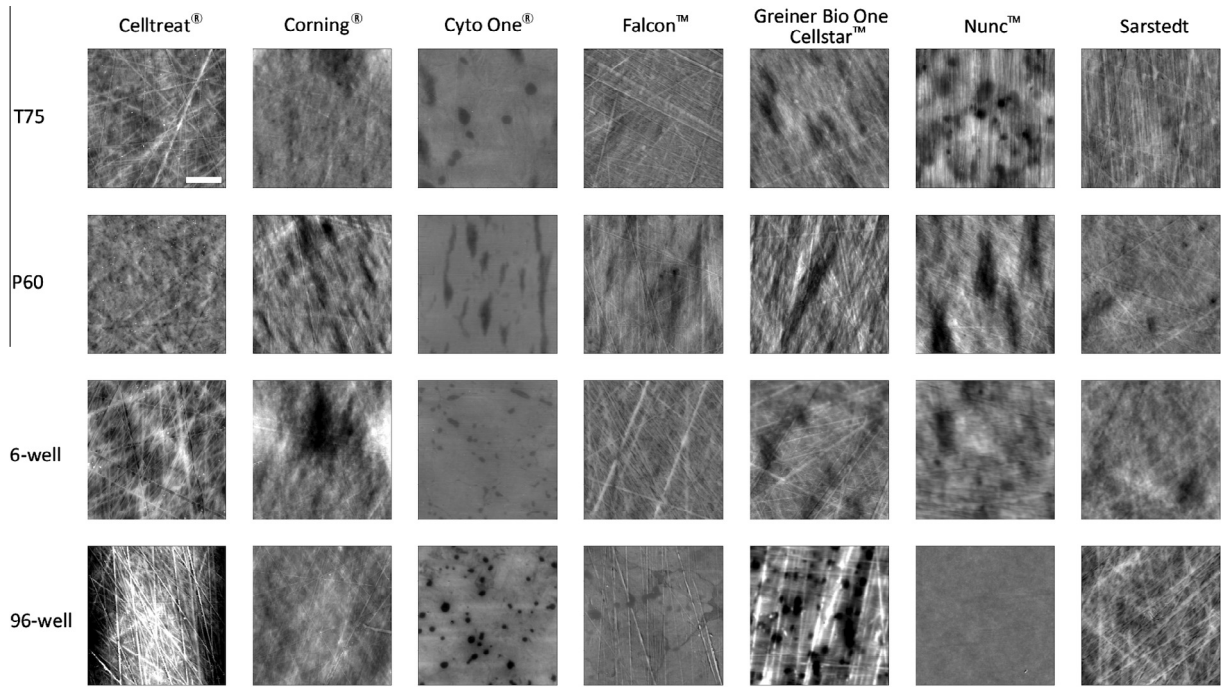
Fig. 1. AFM contact mode images of surface topographies for various tissue culture polystyrene samples. AFM contact mode height trace images were collected over 20×20 µm areas on tissue culture polystyrene 75 cm² TCPS flasks (T75), 60 mm diameter TCPS Petri dishes (P60), 6-well TCPS multiwell culture plates and 96-well TCPS multiwell culture plates manufactured by Celltreat®, Corning®, Cyto One®, Falcon™, Greiner Bio One Cellstar™, Nunc™ and Sarstedt. Imaging demonstrates that manufacturers largely maintain similar topographical features among their dish formats while surface topography differ significantly from between companies. All AFM height trace images are displayed at equivalent scales. Scale bar = 2 µm.
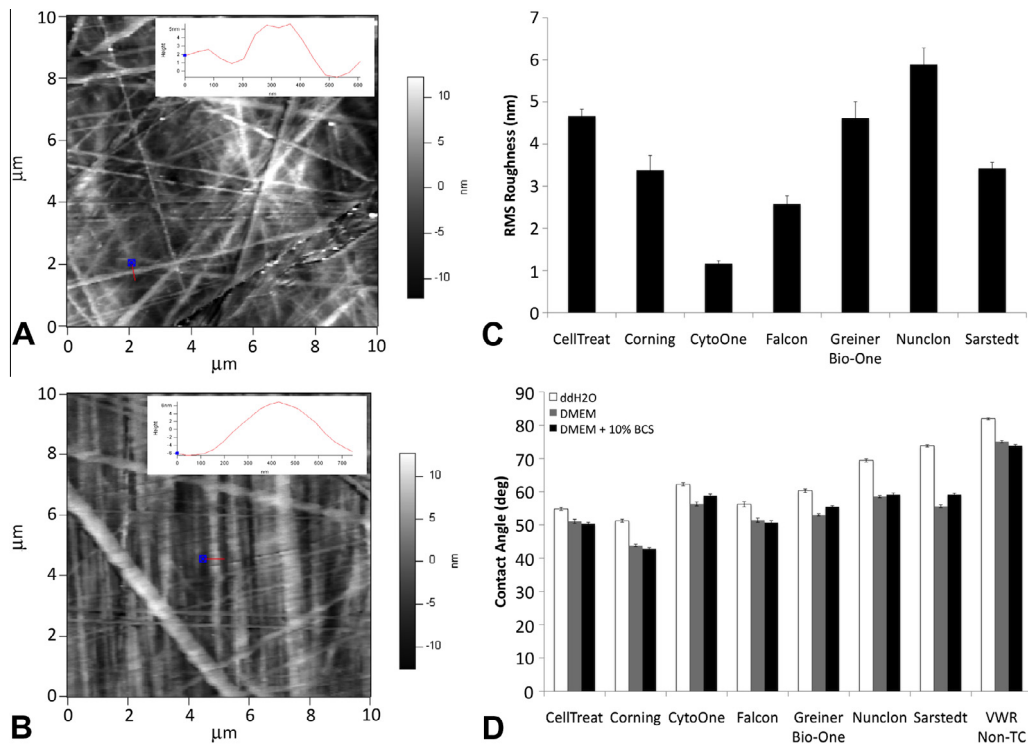
Fig. 2. Surface characterization of TCPS surfaces. (A) 10×10 µm AFM height trace image of Falcon™ 60 mm tissue culture polystyrene dish. Representative cross-section of a single fiber (red line in C) demonstrates fiber diameter of ∼250 nm (inset). (B) 10×10 µm AFM height trace image of Nunc™ 60 mm tissue culture polystyrene dish. Representative cross section of a single fiber (red line in D) demonstrates fiber diameter of ∼500 nm (inset). (C) RMS roughness (n = 5) values as measured by AFM height trace images (AFM) for 60 mm diameter (P60) TCPS surfaces. Values reported as mean ± standard error of measurement. One-way ANOVA demonstrates a statistically significant difference for all roughness measurements ($P < 0.001$). (D) Advancing contact angle measurements as measured via computed goniometry for ddH$_2$O, DMEM and DMEM + 10% BCS on Celltreat®, Corning®, Cyto One®, Falcon™, Greiner Bio One Cellstar™, Nunclon™, Sarstedt and VWR non-tissue culture (non-TC) 75 cm² TCPS culture flasks. n = 10 measurements for each surface. Values reported as mean ± standard error of measurement. One-way ANOVA demonstrates a statistically significant difference for all contact angles ($P < 0.001$) with or without the VWR non-TC surface.

3.3. Contact angle

In addition to surface roughness, we measured advancing contact angle measurements to compare surface wettability (see Section 2). Advancing contact angle measurements across all TCPS sources (Fig. 2D) demonstrated a statistically significant difference ($P < 0.001$) in wettability with ddH$_2$O, typical cell culture medium such as DMEM and DMEM containing an additional 10% BCS. Importantly, the presence of ions in DMEM as well as the additional presence of proteins in DMEM + BCS did not abrogate the manufacturer-specific differences in contact angle. Thus, potential differences among manufacturers in final surface functionality (e.g. the number of hydroxyls as a function of plasma treatment details) was not ameliorated by ions or proteins in the media. This suggests that surface topography played a stronger role than apparent surface charge in the wettability among these TCPS surfaces, as has been noted previously by Busscher et al. for other polymers [30], although we identified no direct, strong correlation of surface wettability with any specific surface feature (e.g., pit dimensions, fiber dimensions or fiber orientation).

3.4. Protein adsorption

We assessed protein adsorption via visible light spectrophotometry of adsorbed FITC-conjugated albumin (Fig. 3A) and collagen (Fig. S.1); see Section 2. Although the high repeatability of albumin adsorption measurements resulted in statistically significant differences among TCPS sources ($P < 0.001$), the overall range of these measurements was 27–29% adsorption of FITC-conjugated albumin among all samples.

This small variation in the amount of protein adsorbed (ranging only from 27% to 29%) is insignificant in terms of in vitro cell culture response as a function of the amount of protein adsorbed. Concordantly, Clinchy et al. found no statistically significant difference in adsorption of another protein, IgG antibodies, to 96-well TCPS plates across multiple manufacturers [31]. We also did not observe statistically significant differences among the TCPS sources we considered herein for another extracellular matrix protein, collagen type-I (Fig. S.1). It is important to note that deposition of 5 µg cm^{-2} rat tail collagen type-I thin films onto these TCPS samples produced surfaces of almost equivalent roughness ($P > 0.05$) after collagen adsorption.

It remains possible that other characteristics of the adsorbed proteins (e.g. protein conformation or configuration to expose binding epitopes) may differ appreciably; such conformation differences can play a significant role in guiding cell adhesion to adhesive ligands [32]. However, as noted below, in the present case we found no significant difference in the initial number of cells adhered for either human mesenchymal stem cells or fibroblasts on various TCPS manufacturers.

3.5. Cell responses

To explore the effects of these TCPS formats – which differed significantly among sources in terms of several physical properties – on in vitro cell culture, we considered two responses: the area over which attached cells spread on the TCPS and the rate at which attached cells proliferated (see Section 2). Cell spread area was quantified optically via actin (phalloidin) staining of primary human mesenchymal stromal or "stem" cells (hMSCs), after 24 h culture in P35 TCPS dishes. A statistically significant difference ($P < 0.001$, $n > 390$ cells) in average cell area was observed among culture on TCPS from different manufacturers (Fig. 3B). Interestingly, no statistically significant difference in cell area was
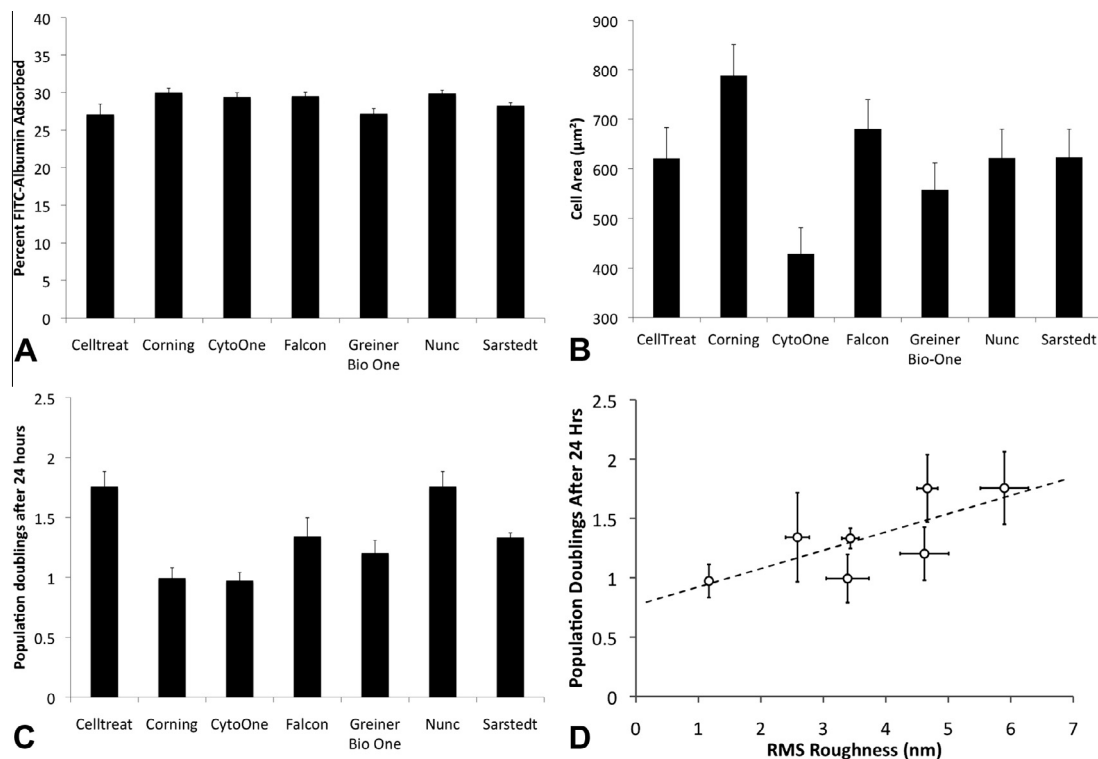

Fig. 3. Characterization of relevant in vitro cell culture properties. (A) Percent FITC-conjugated bovine albumin (Sigma, A9771) adsorption as measured by visible light spectrophotometry at 459 nm wavelength on 35 mm diameter (P35) TCPS surfaces. n = 6 measurements for each surface. Values reported as mean ± standard deviation. One-way ANOVA demonstrates a statistically significant difference for adsorbed FITC-albumin ($P < 0.001$). (B) Cell spreading area of human mesenchymal stem cells cultured on 60 mm diameter (P60) TCPS surfaces. (C) Population doublings after 24 h for NIH 3T3 fibroblasts cultured on P35 TCPS surfaces (n = 3). Values reported as mean ± standard error of measurement. One-way ANOVA demonstrates a statistically significant difference ($P < 0.001$). (D) Linear regression analysis demonstrates a weak but non-trivial correlation between RMS roughness and population doublings (R^2 = 0.56). Values reported as mean ± standard error of measurement.

observed for NIH 3T3 murine fibroblasts (data not shown). Cell proliferation in P35 TCPS dishes was also quantified for hMSCs (Fig. S.2) and the more rapidly proliferating cell type, NIH 3T3 murine fibroblasts; fibroblasts exhibited a statistically significant difference in population doublings after 24 h among various TCPS sources (Fig. 3C, $P < 0.001$). Consistent with our protein adsorption study (Fig. 3A), the initial number of attached hMSCs or of attached fibroblasts did not differ significantly as a function of TCPS source (4 h post-seeding, where $P = 0.416$ and $P = 0.356$, respectively).

These findings for cell spread area and proliferation rate are consistent with previous, separate observations in the wider literature. Direct comparison of easter oyster (Crassostrea virginica) cells cultured on Corning and Falcon dishes also demonstrated a significant difference in cell spreading behavior of these cells as a function of TCPS source [33]. Further, others [3] have noted significant variation in population growth of hMSCs as a function of TCPS supplier. Finally, one previous study of three TCPS brands (Falcon, Wisent and Sarstedt) obtained similar results regarding contact angle and protein adsorption, while noting significant differences in monocyte adhesion and retention over 7 days [34].

Researchers have not previously correlated these noted differences in cell response with differences in quantifiable physical properties of the culture surface, instead attributing these differences to proprietary processes used by each manufacturer [5,34]. Interestingly, we observed a strong correlation between population doublings of NIH 3T3 murine fibroblasts over 24 h with the TCPS surface roughness ($r = 0.75$, $P = 0.0272$, where r is the Pearson product-moment correlation coefficient between means, and values of 0.5 are considered to indicate strong correlations [35]). We note that causality for this correlative relationship between surface roughness and cell proliferation rate is beyond the scope of the current study.

Finally, Fig. 4 highlights the potential for direct interaction of NIH 3T3 murine fibroblasts with Falcon™ P35 TCPS surface features. Immunostaining of vinculin – a protein found in the focal adhesion complex linking the intracellular cytoskeleton to integrin and thus to the extracellular matrix proteins – suggests a potential correlation between cellular adhesion patterns and underlying topography (Fig. 4A and B). One-way ANOVA demonstrated a statistically significant difference among manufacturers in the average angular standard deviation of vinculin-stained regions indicating adhesion complexes ($P < 0.001$). Average angular standard deviation $\langle \alpha_{sd} \rangle$ is an objective measure of relative alignment of features [27], and lower $\langle \alpha_{sd} \rangle$ indicates a higher degree of alignment among focal adhesions within a given cell. AFM contact mode height and deflection images of fixed 3T3 murine fibroblasts (Fig. 4C–F) further illustrate this direct interaction and patterning between fibroblast filopodia and TCPS fibers. Here, we observed single and multiple cellular protrusions directly corresponding to underlying polystyrene fibers (green highlights, Fig. 4C and D). As integrin signaling locally activates small Rho GTPases, the organization of TCPS fibers may affect the formation of lamellipodia and filopodia, which are linked to signaling molecules involved in cytoskeletal response and ultimately affect cell mechanical stress and cell motility [36]. Orientation of filopodia can be mediated by both surface topography and soluble factors, although the mechanism linking the two remains unknown [37].

4. Discussion

A researcher will often find empirically that a particular brand of tissue culture dishes work well for his/her cell type of interest and observations of interest, while another manufacturer's cultureware results in poorer cell viability or altered behavior [8,9]. Through the use of AFM contact mode imaging and additional measurements of contact angle and protein adsorption, we have observed significant variation in physical features of TCPS vessels among a wide range of commercial sources. To our knowledge, this is the first study that systematically characterizes the topography of a large variety of TCPS brands and formats, and the impact of these features on cell culture for two adherent cell types.

Differences in TCPS physical properties from a given commercial source remain largely consistent across formats (i.e. 60 mm diameter dishes vs. 75 cm^2 flasks from the same manufacturer), suggesting that the results are attributable to proprietary polystyrene vessel manufacturing processes. The details of the manufacturing processes and quality control methods used are rarely published, and few studies exist directly comparing one TCPS brand to another for in vitro cell culture use [7]. Clearly, manufacturing process steps differ among TCPS commercial sources. This variation results in qualitative differences in surface features, as well as quantitative differences in surface roughness and wettability that cannot be attributed simply to differences in surface chemistry. (Fig. 2 shows that addition of ions or serum proteins did not mitigate manufacturer-dependent differences in contact angle.) Such differences in manufacturing could include the age, lot or mixture of the resin used, or various injection molding parameters such as flow rates and mold surface topography [14]. In fact, a recent study demonstrated that use of the same injection mold across different material types (e.g., polystyrene, polycarbonate and polyethylene imine) did not significantly alter cell morphology and lactate dehydrogenase activity, despite altered surface chemistries [38], suggesting that final surface topography most strongly dictated cell response. Importantly, the fiber-like features observed on the TCPS surfaces herein are on the same order of magnitude as typical extracellular matrix fibers, and Fig. 4 demonstrates the potential for direct interaction between cells and these features.

Numerous cell types have exhibited altered behavior in response to variations in surface topography such as feature width and depth, orientation and roughness [14,39,40]. In fact, recent studies have shown that cell responses to given chemical or physical cues are often cell-type-specific. McGrail et al. have identified differing responses of fibroblasts and mesenchymal stem cells in response to the same tumor-secreted soluble factors, in terms of distinct changes in cellular morphology, stress fiber density and adhesion [41]. Furthermore, numerous studies have demonstrated cell-type-specific responses to substrate groove patterns. For example, the groove depth required to induce significant morphological changes in fibroblasts was twice as shallow as that required for human endothelial cells and smooth muscle cells [42]. Nerve cells selectively align to nanoscale fibers [43]. Adhesion and motility of fibroblasts [21] and smooth muscle cells [44], cytokine production in epithelial cells [45] and differentiation potential of mesenchymal stem cells [46–48] and myoblasts [49] are also reported to depend markedly on surface topography. Feature size and spacing have the ability to impact cell signaling, integrin mediated adhesion, DNA transcription, cell motility and extracellular matrix organization. However, the detailed mechanisms that mediate these responses to physical cues remain incompletely understood. Indeed, our study found statistically significant differences in variations of cell area for hMSCs, but not for fibroblasts, adhered to the same range of TCPS sources (Fig. 3B). Conversely, we observed significant changes in the cell doubling response of fibroblasts, which demonstrated a strong correlation between TCPS surface roughness and cell proliferation rate, but not for hMSCs (Fig. 3C and D). Given that nanotopography serves as one of several cues to cell behavior, it has been proposed that nanoscale topographic features similar to those found in basement membrane structures should be an integral part of the design of biomedical implants and in vitro microenvironments where controlled cellular responses are desired [36]. Our findings are one of several that demonstrate the response of such topographic cues to be
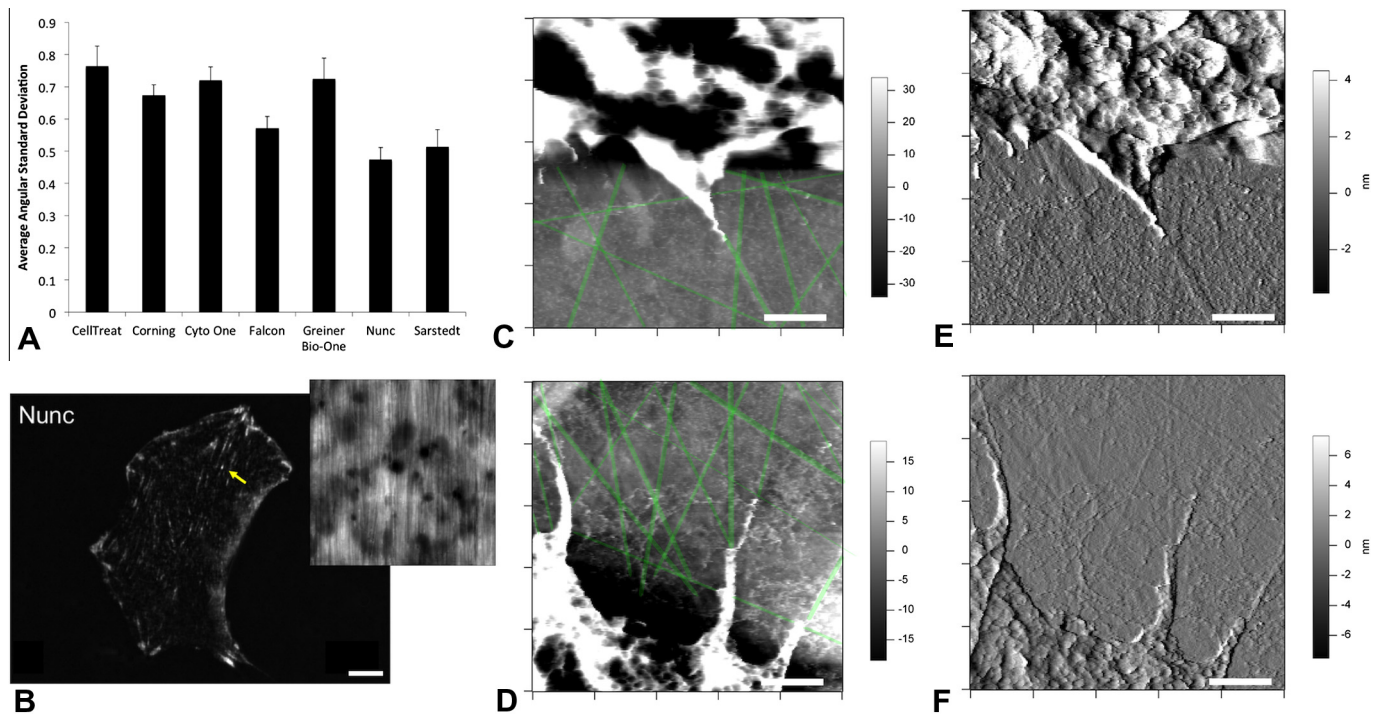
Fig. 4. Focal adhesion contacts of NIH 3T3 fibroblasts interacting with Falcon™ TCPS features. (A) Average angular standard deviation for focal adhesions, as determined by vinculin staining (white features, with one such indicated by a yellow arrow in (B) of NIH 3T3 fibroblasts 16 h post-seeding on P35 TCPS dishes of 35 mm diameter. (B) Nunc (P35) TCPS representative vinculin image with AFM 20 × 20 μm contact mode deflection images from Fig. 1 (inset). Scale bar represents 20 μm. (C, D) 5 × 5 μm (AFM) height image of fixed NIH 3T3 murine fibroblasts 16 h post-seeding on Falcon™ 60 mm tissue culture polystyrene dishes. Distinct correspondence with TCPS fibers (highlighted in green) are observed with extended cell filopodia, suggesting dish topography may significantly influence cell interactions with underlying substrata. (E, F) Corresponding AFM deflection (error) image for C and D, respectively. Scale bars = 1 μm.

cell-type-specific, ostensibly due to the diversity of ligand–receptor interactions and downstream metabolic signaling pathways among different cell types.

The cell culture substratum must be carefully chosen as a delicate balance among the chemistry, mechanics and even surface topography at the cell–biomaterial interface [50].

However, surface topography of ostensibly smooth, featureless culture surfaces is often overlooked, with the tacit assumption that TCPS sources are either equivalent or indescribably but importantly different for a given *in vitro* outcome. Published data are also often presented without citing the particular manufacturer of TCPS used [51]. In fact, a recent study by Lavenus et al. claimed that TCPS and glass were of similar roughness, although the reported measurements in that work indicated TCPS to exhibit RMS roughness that was an order of magnitude greater than that of glass; for the specific case of hMSCs, those authors also noted significant differences in morphology and number of cells initially adhered to TCPS and glass and suspected but did not quantify that this was attributable to differences in protein adsorption [52]. Furthermore, published protocols and methods state specifically that sequential steps should use one TCPS source vs. another (e.g., Corning for step one, and Falcon for step two [49]); others suggest interchangeable use of two specified brands [50]. It is unclear whether this specificity is due to historical convenience or to prior success of the protocol. Anecdotally, many researchers have found that cell behavior depends strongly on the choice of TCPS source, and a few studies have shown this explicitly without further characterization of the TCPS itself and often with uncorroborated assertion that differences in surface treatment among manufacturers is responsible for differences in experimental outcomes [27].

It is important to note that we do not intend to conclude (nor can we conclude) that one particular brand of TCPS is superior to another. Results for other cell types and for specific features of pro-

tein adsorption or cell responses may differ greatly than results presented here. Additionally, the desired response of protein–substratum and cell–substratum interactions can be unique to a given experimental aim. Instead, we argue that it is important to recognize the significant, quantifiable differences in physical properties that exist across seemingly similar material types and chemistries (i.e., all so-called TCPS), which may in turn play significant roles in cueing cell behavior and in influencing experimental results.

Appendix A. Figures with essential color discrimination

Certain figures in this article, particularly Figs. 2 and 4, are difficult to interpret in black and white. The full color images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2013.02.035

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2013.02.035.

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