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Graphical analysis of mammalian cell adhesion *in vitro*

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ABSTRACT

Short-term (< 2 h) cell adhesion kinetics of 3 different mammalian cell types: MDCK (epithelioid), MC3T3-E1 (osteoblastic), and MDA-MB-231 (cancerous) on 7 different substratum surface chemistries spanning the experimentally-observable range of water wettability (surface energy) are graphically analyzed to qualitatively elucidate commonalities and differences among cell/surface/suspending media combinations. We find that short-term mammalian cell attachment/adhesion *in vitro* correlates with substratum surface energy as measured by water adhesion tension, $\tau \equiv \gamma_{lv}\cos\theta$, where γ_{lv} is water liquid-vapor interfacial energy (72.8 mJ/m^2) and $\cos\theta$ is the cosine of the advancing contact angle subtended by a water droplet on the substratum surface. No definitive functional relationships among cell-adhesion kinetic parameters and τ were observed as in previous work, but previously-observed general trends were reproduced, especially including a sharp transition in the magnitude of kinetic parameters from relatively low-to-high near $\tau = 0\ mJ/m^2$, although the exact adhesion tension at which this transition occurs is difficult to accurately estimate from the current data set. We note, however, that the transition is within the hydrophobic range based on the $\tau = 30\ mJ/m^2$ surface-energetic dividing line that has been proposed to differentiate "hydrophobic" surfaces from "hydrophilic". Thus, a rather sharp hydrophobic/hydrophilic contrast is observed for cell adhesion for disparate cell/surface combinations.

1. Introduction

Bioadhesion is a topic of perennial interest in diverse fields of endeavor such as biomaterials and civil engineering [1–4]. In the former, mammalian cell adhesion is a controlling factor in tissue integration of materials used in various medical devices and prostheses [5,6]. Bioadhesion is also important in the technology of *in vitro* cell/tissue culture for which a large quantity of tissue-culture-grade polystyrene (TCPS) is used in an array of sterile-disposable containers in the form of bottles, flasks, and micro-well plates that are now ubiquitous in biotechnology laboratories world-wide. TCPS is commercially surface-treated to render inherently hydrophobic polystyrene more hydrophilic to improve bioadhesivity and thus useful as a cell-substratum surface for *in vitro* cell culture.

Perhaps the most widely observed instance of cellular bioadhesion is *in vitro* cell culture. A typical experimental protocol is to create a mono-disperse suspension of animal cells derived from a tissue of interest in a growth-medium formulation. Growth medium is typically comprised of aqueous isotonic saline, balanced salts, optionally supplemented with blood-serum proteins and/or amino acids. There are many such commercially-available formulations for this purpose, as well as viable cell lines delivered in the form of frozen ampoules from organizations such as American Type Culture Collection (ATCC). Together with commercial sterile-disposable cultureware (typically TCPS and glass), commercially-available cells and media have transformed *in vitro* cell culture from a rather specialized tool of cell biology and cell biologists into a widely-used biotechnology that nearly every serious biology/biomaterials laboratory can apply. In fact, the tech-

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nique of *in vitro* cell culture is now an integral part of biology and biomedical engineering curricula in many, if not all, colleges and universities focusing on these skills to satisfy strong interest among aspiring biologists and biomedical engineers.

Despite the ubiquity and long scientific history of mammalian cell culture, there remain significant outstanding questions regarding the specific mechanisms involved in the cell attachment and adhesion process, even though broad descriptive mechanisms have been available since the early 1970′s [7] that are widely published in various text books and review articles [8–11]. It is widely promulgated within such sources that cell adhesion to material surfaces such as TCPS (surface-treated or tissue-culture polystyrene, PS) from suspension in serum-supplemented media is mediated by surface-adsorbed protein ligands (adhesins) specific for cell-membrane-bound receptors [3,12,13]. Mating of receptors with ligands purportedly binds anchorage-dependent cells to the substratum surface and initiates cell-signaling cascades that ultimately lead to cell spreading and replication [2,10,14,15]. However, relatively less attention to ubiquitous surface/interfacial forces is given in these descriptive biological adhesion mechanisms [9,16]. As a result, more physicochemical theories have arisen that attempt to quantitatively link bioadhesion with surface/interfacial energetics [16–21]. Not surprisingly, perhaps, given the complexity of bioadhesion processes, these theories are quite complex and difficult to experimentally validate. For example, the Dupré work of adhesion applied in Ref. [18] for cell adhesion apparently can explain interfacial aspects of cell adhesion phenomena. However, a number of parameters used in this theory (such as interfacial tensions between cell and liquid, γ_{cl} or between cell and surface, γ_{cs}) are not generally possible to directly measure because cells in the initial stage of adhesion are small and deformable hydrogel-like objects that are in an inappropriate form for application of tensiometric tools of investigation. As a consequence, indirect and complex theoretical arguments are made to estimate these unknown/immeasurable parameters, greatly complicating direct elucidation of cause-and-effect in cell adhesion. Perhaps not surprisingly, therefore, general consensus regarding the role of interfacial energetics in bioadhesion has not been reached, especially with respect to a pronounced hydrophilic/hydrophobic contrast in bioadhesion; with cell adhesion favoring hydrophilic surfaces that are not as efficient protein adsorbents as hydrophobic surfaces to which cells do not efficiently adhere in comparison to hydrophilic surfaces. For examples, Lim et al. demonstrated that cell adhesion efficiency exhibited a striking hydrophilic/hydrophobic contrast that pivots around a nominal contact angle $\theta = 65^\circ$, with higher cell attachment rates to hydrophilic surfaces ($\theta < 65^\circ$) and lower attachment rates to hydrophobic surfaces ($\theta > 65^\circ$) [9,17,22]. Parhi et al. showed that protein-adsorbent hydrophobic surfaces were not conducive to cell attachment [9]. But these reports do not end in consistency in the literature that appear, from time to time, reporting higher cell attachment to hydrophobic surfaces [23,24].

This work attempts to clarify this inconsistency by applying a graphical approach to comparing cell-attachment rates of three separate cell types to seven surfaces incrementally spanning the full range of water wetttability in a way that circumvents previously-applied complex mathematical approaches that seem to only to obscure cause-and-effect relationships rather than highlight qualitative comparisons of different cells to different surfaces. We apply a recently-developed cell adhesion kinetics method termed “suspension depletion” that is rapid to apply and has well-defined statistics. This approach also circumvents a bias inherent in frequently-applied “surface–rinse-and-count-adherent cells” methods of adherent-cell enumeration. Taken together, we are able to confirm a hydrophilic/hydrophobic contrast in cell adhesion in vitro, thus illuminating a basic aspect of the bioadhesion of mammalian cells from protienaceous growth media to a variety of surfaces spanning the full range of surface energy.

2. Materials and methods

2.1. Cell-adhesion substrata

Tissue-culture-grade polystyrene (TCPS, Corning, 50.74 ± 1.76 mm, internal diameter, N = 4) and glass (VWR) 60×15 mm Petri dishes (50.09 ± 0.20 mm, internal diameter, N = 3) (19.7 and 20.2 cm^2 inner surface area, respectively) were used in this work as test cell-adhesion substrata. Internal diameters were measured with a digital caliper.

Glass petri dishes were re-used repeatedly during the course of this study. Glass dishes were first washed in aqueous bleach solution, air dried, wrapped in Al foil, and annealed at approximately 400 °C to eliminate all traces of biological debris from prior use. Glass coverslips (VWR, 0.13×0.17 mm) were processed along with dishes in the cleaning and silanization procedures (below) as witness samples suitable for contact angle measurements. Glass was first cleaned and activated by piranha solution (30% H_2O_2 in concentrated H_2SO_4) at approximately 80 °C for 60 min, followed by 3 x sequential washes in each of 18 MΩ deionized water and 100% ethanol. Piranha-solution-treated glass was then air dried at 110 °C and subsequently treated for 20 min by air-plasma discharge at 13.6 MHz at 100 W (Harrick Plasma of Ithaca, NY). Glass cover slips were held in Pyrex glass dishes whereas glass Petri dishes were placed directly into the barrel of the discharge unit. So-treated glass surfaces were found to be fully-water-wettable (with advancing water contact angle <5°) and designated “clean glass”.

2.2. Silanization of clean-glass substrata

Silanes (used as received from vendor) applied in this work were 3-aminopropyltriethoxysilane (APTES, Sigma-Aldrich), *n*-propyltriethoxysilane (PTES, Sigma-Aldrich), vinyltriethoxysilane (VTES, Sigma-Aldrich), and octadecyltricholorosilane (OTS, Gelest Inc.). APTES silanization was carried out in 95:5 v/v ethanol–water solutions by 20 min reaction of clean glass with 5% v/v APTES solution that had been hydrolyzed for 6 h in the ethanol–water mixture before use.

APTES-treated glass was washed with ethanol and dried overnight in a vacuum oven at 110 °C. PTES and VTES silanization followed the APTES procedure except that 90:10 ethanol–water containing 0.5% v/v glacial acetic acid was used.

OTS silanization was carried out by 1.5 h reaction of glass with 5% v/v OTS in chloroform in a glove box (Plas-Labs, 815-PGB, La Petite). OTS-silanized samples were rinsed 3 x with chloroform before curing at 60 °C on a hotplate housed in the glove box. OTS-treated glass dishes were optionally dip-coated in a 0.2% solution of 1,1-pentadecafluorooctylmethacrylate in tricholorotrifloroethane (Nyebar, Nye Lubricants) to render silanized surfaces slightly more hydrophobic [28].

2.3. Surface analysis

Advancing water contact angles ($N \geq 5$) measurements on glass coverslip witness samples were carried out by an automatic contact-angle goniometer (First Ten Angstroms Inc.) that employed the captive-drop method of measuring advancing/receding contact angles. θ values were converted to adhesion tension $\tau \equiv \gamma_{lv}\cos\theta$ that is a thermodynamic measure of the strength of water (in this case) adsorption to the test surface [21,26,27]; where γ_{lv} is the water liquid-vapor (lv) interfacial tension and θ is the advancing contact angle subtended by water droplets on the test surface.