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Assessing embryonic stem cell response to surface chemistry using plasma polymer gradients

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ABSTRACT

The control of cell–material interactions is the key to a broad range of biomedical interactions. Gradient surfaces have recently been established as tools allowing the high-throughput screening and optimization of these interactions. In this paper, we show that plasma polymer gradients can reveal the subtle influence of surface chemistry on embryonic stem cell behavior and probe the mechanisms by which this occurs. Lateral gradients of surface chemistry were generated by plasma polymerization of diethylene glycol dimethyl ether on top of a substrate coated with an acrylic acid plasma polymer using a tilted slide as a mask. Gradient surfaces were characterized by X-ray photoelectron spectroscopy, infrared microscopy mapping and profilometry. By changing the plasma polymerization time, the gradient profile could be easily manipulated. To demonstrate the utility of these surfaces for the screening of cell–material interactions, we studied the response of mouse embryonic stem (ES) cells to these gradients and compared the performance of different plasma polymerization times during gradient fabrication. We observed a strong correlation between surface chemistry and cell attachment, colony size and retention of stem cell markers. Cell adhesion and colony formation showed striking differences on gradients with different plasma polymer deposition times. Deposition time influenced the depth of the plasma film deposited and the relative position of surface functional group density on the substrate, but not the range of plasma-generated species.

1. Introduction

The cellular response to biomaterial surfaces, such as attachment and proliferation, is known to be mediated by the material's surface chemistry. Indeed, surface chemistry is also able to instruct cell function and direct cell differentiation [1–10]. However, it has proven difficult to predict cell fate outcomes based on the molecular structure of the polymer substrate [11,12]. The advent of high-throughput screening techniques including cell microarrays and surface-bound gradients has precipitated a paradigm shift in the field [13–17]. Surface chemistry gradients are a format that is particularly well suited to study subtleties in cell response to surface chemistry. The format permits one variable, such as functional group density, to be continuously changed with respect to the position on a test surface whilst other parameters are held constant [18–22]. These platforms also require significantly lower cell numbers, lower quantities of culture medium and sample materials than ad hoc testing of surface chemical properties with discrete samples.

Plasma polymerization lends itself to surface gradient formation, since it supports the introduction of a wide range of surface chemistries and forms well-adherent layers on a range of substrates [23–25]. The technique also allows surfaces to be modified independently of the underlying substrate, with little change to surface topography. Several methods using plasma polymerization to create gradients of surface functionality have been described, such as changing the input feed stock while a mask occluding part of the substrate is moved concurrently, or using a knife edge electrode, resulting in non-uniform plasma glow discharge [26–29]. Whilst these techniques are effective in depositing plasma polymer gradients, sophisticated and expensive reactors or permanent modifications are required. The diffusion of monomer underneath a solid mask can also be exploited to create plasma polymer gradients [30,31]. The distance of the mask from the substrate can control the depth of the plasma film and the slope of the gradient [31,32]. Furthermore, plasma polymerization time can be varied to influence the depth of the plasma film deposited. Such a diffusion-based gradient deposition process can be implemented in almost any reactor setup for negligible cost.

Recent work on carboxylic acid plasma polymer (octadiene-acrylic acid) gradient surfaces demonstrated a relationship between retention of "stemness" in mouse embryonic stem (ES) cells and

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the degree of cell-surface adhesion [28]. Increased cell spreading and the absence of three-dimensional (3-D) colony organization were observed to coincide with loss of a stem cell marker expression. However, others have suggested that a comparatively larger colony size is optimal for maintaining cell pluripotency [33]. Here, we use a different chemical moiety known to limit cell adhesion and cell spreading in order to generalize the results. Polyethylene oxide (PEO) is well recognized in the biomaterials field for its ability to provide surface coatings which result in low protein adsorption and low cell adhesion [34–36]. Plasma polymerization can be used to deposit PEO-like coatings onto a wide range of surfaces, through the use of monomers such as diethylene glycol dimethyl ether (diglyme, DG) [27]. PEO-like coatings produced by plasma polymerization are known to swell in aqueous media [37–40]. Grafted poly(acrylic acid) and acrylic acid (AA) plasma polymers have been shown to promote protein and cell adhesion [28,41,42]. In this study, we have combined the use of cell adhesive AA plasma polymer with low fouling DG plasma polymer to create chemical gradients suitable to assess ES cell response as measured by cell attachment, colony formation and the expression of stem cell markers. This work sheds light on the important relationship between inhibition of cell spreading and of stem cell differentiation.

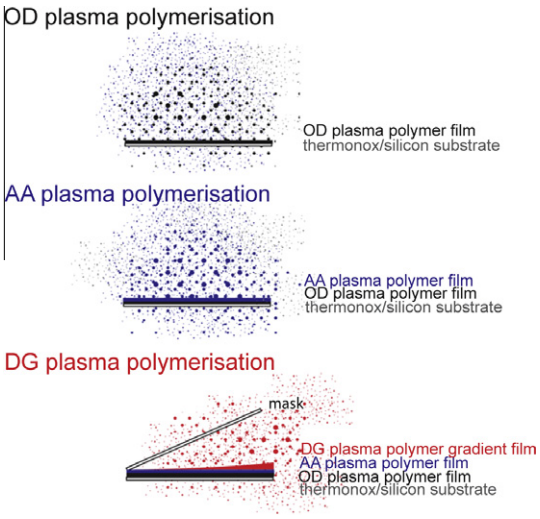

Fig. 1. Schematic of plasma polymer layers deposition on silicon (Si) or Thermanox: octadiene (OD) plasma polymer, acrylic acid (AA) plasma polymer and the final diethylene glycol dimethyl ether (DG) plasma polymer gradient layer.

2. Materials and methods

2.1. Gradient generation via plasma polymerization

Thermanox plastic coverslips (cell culture treated, Nunc, Roskilde, Denmark) and p type silicon wafer (Si) (110, boron doped, Virginia Semiconductors, Fredericksburg, VA, USA) were cut to size, rinsed in ethanol and dried under a gentle stream of nitrogen. Plasma polymerization was performed using a custom-built reactor as previously described [43]. Briefly, the apparatus consists of a cylindrical glass chamber, 10 cm in diameter and 50 cm in length. A copper wire wound around the glass plate was utilized to generate the RF power (RF operating at 13.56 MHz and amplifier inductively coupled to the sample chamber). Samples were placed in the center of the chamber on a glass plate. The chamber was evacuated to a base pressure of 1.2×10^{-3} mbar. All monomers were freeze–pump–thaw degassed three times to remove traces of oxygen. For all monomers, a constant flow rate of the monomer was set to 2 standard cubic centimeters per min (SCCM). A base coat of 1,7-octadiene (OD) (97%, Alfa Aeser, Ward Hill, MA, USA) was applied to the substrates for 10 min at generator output power measured at 2 W. Deposition of an AA plasma polymer layer directly onto the Thermanox or silicon substrates resulted in film dissolution when immersed in water, regardless of the plasma deposition power (data not shown). For this reason, an adhesion layer of OD plasma polymer was required between the substrate and the AA plasma polymer coating. Subsequently, an AA plasma polymer layer ($\geqslant$99%, Sigma Aldrich, St Louis, MO, USA) was deposited on top of the OD plasma polymer layer. Optimal conditions for AA plasma polymer layer deposition were determined by X-ray photoelectron spectroscopy (XPS) measurement of COOH functional group retention and stability in neutral aqueous medium. Deposition at 4 W power retained the highest number of COOH groups, whilst still maintaining stability in aqueous medium (Supplementary material, Fig. S1). In order to achieve a thickness gradient, a glass slide, tilted 12° to the substrate surface with the sides enclosed, was placed over the silicon wafer or Thermanox coverslips as a mask (Fig. 1). The outer edge of the silicon wafer protruded 5 mm from the opening of the glass slide. A gradient of DG plasma polymer layer ($\geqslant$ 98%, Sigma Aldrich) was then deposited for 5 or 10 min at an output power of 4 W. The resulting gradients had a length of 3 cm with the 0 cm position corresponding to the point where the glass slide mask contacted the silicon or Thermanox substrate.

2.2. Surface characterization

2.2.1. Infrared microscopy (IRm)

For IRm studies, plasma polymer gradients described above were deposited onto p-type silicon wafers (Virginia Semiconductors) in order to permit transmission IRm. IRm studies were performed at the Australian Synchrotron. The IR beamline is equipped with a Bruker Hyperion 2000 IR microscope (Bruker Optics, Ettlingen, Germany) and a Bruker Vertex 80 IR spectrometer. A 20×20 μm^2 aperture was utilized for all experiments, with a spacing of 200 μm between spectrum collections. All spectra were background corrected with an unetched silicon wafer of the same type.

2.2.2. X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy experiments were carried out using an AXIS HSi spectrometer (Kratos Analytical Ltd, Shimadzu Corporation, Kyoto, Japan) equipped with a monochromatized Al Kα source. The pressure during analysis was typically 5×10^{-8} mbar. The elemental composition of surfaces was determined from survey spectra, collected at a pass energy of 320 eV at intervals of 2 mm along the length of the gradient (corresponding to the *x*-axis). High-resolution spectra were obtained at a pass energy of 40 eV. Binding energies were referenced to the aliphatic carbon peak at 285.0 eV. For high resolution C 1s spectra, curve fitting of the various components was performed using Casa XPS (Casa Software Ltd, Knutsford, Cheshire, UK). Component peak shapes were fitted using a Gaussian–Lorentzian model.

To quantify carboxylic acid (COOH) group density, surfaces were reacted with trifluoroethylamine (TFEA) (125 mM) (Sigma-Aldrich) for 2 h in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (50 mM) (Sigma-Aldrich) followed by extensive washing in MilliQ water prior to XPS analysis. The fluorine contained within the TFEA provided an easily identifiable XPS label and allowed the quantification of available COOH groups on the surface.

2.2.3. Profilometry

Plasma film thickness along the gradient was measured using a Dektak 6M stylus profilometer (Veeco, Plainview, NY, USA). Using a

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