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Polymer surface functionalities that control human embryoid body cell adhesion revealed by high throughput surface characterization of combinatorial material microarrays

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Abstract

High throughput materials discovery using combinatorial polymer microarrays to screen for new biomaterials with new and improved function is established as a powerful strategy. Here we combine this screening approach with high throughput surface characterisation (HT-SC) to identify surface structure-function relationships. We explore how this combination can help to identify surface chemical moieties that control protein adsorption and subsequent cellular response. The adhesion of human embryoid body (hEB) cells to a large number (496) of different acrylate polymers synthesized in a microarray format is screened using a high throughput procedure. To determine the role of the polymer surface properties on hEB cell adhesion, detailed HT-SC of these acrylate polymers is carried out using time of flight secondary ion mass spectrometry (ToF SIMS), x-ray photoelectron spectroscopy (XPS), pico litre drop sessile water contact angle (WCA) measurement and atomic force microscopy (AFM). A structure-function relationship is identified between the ToF SIMS analysis of the surface chemistry after a fibronectin (Fn) pre-conditioning step and the cell adhesion to each spot using the multivariate analysis technique partial least squares (PLS) regression. Secondary ions indicative of the adsorbed Fn correlate with increased cell adhesion whereas glycol and other functionalities from the polymers are identified that reduce cell adhesion. Furthermore, a strong relationship between the ToF SIMS spectra of bare polymers and the cell adhesion to each spot is identified using PLS regression. This identifies a role for both the surface chemistry of the bare polymer and the pre-adsorbed Fn, as-represented in the ToF SIMS spectra, in controlling cellular adhesion. In contrast, no relationship is found between cell adhesion and wettability, surface roughness, elemental or functional surface composition. The correlation between ToF SIMS data of the surfaces and the cell adhesion demonstrates the ability of identifying surface moieties that control protein adsorption and subsequent cell adhesion using ToF SIMS and multivariate analysis.

1. Introduction

The relationship between the surface chemistry of materials and resulting cellular response has great importance for biomedical materials, regenerative medicine, tissue engineering and biosensors. Striking effects on the cellular behaviour of synthetic materials can be readily obtained by modification of material surface chemistry, an example being the significant improvement of cellular adhesion to polystyrene upon plasma treatment which is applied commercially to produce tissue culture ware [1,2]. Changes in cellular adhesion, morphology, motility, gene expression and differentiation have all been rationalised in terms of the surface properties of the materials on which cells have been cultured, including not only surface chemistry [3,4] but also surface wettability [5,6], topography [7,8] and mechanical properties [9]. It is widely recognised that proteins adsorbing onto material surfaces direct subsequent biological responses to the surface. These surface adsorbed protein may be from serum containing media only, or a pre-conditioning step with a cell adhesive protein such as Fn [10]. The identity, amount, orientation and conformation of proteins adsorbed to surfaces have been studied using surface characterisation techniques including X-ray photoelectron spectroscopy (XPS) [11] and time of flight secondary ion mass spectrometry (ToF SIMS) [12–14], as well as *in situ* techniques including surface plasmon resonance [15,16], quartz crystal micro balance [17,18], atomic force microscopy (AFM) [19]. Multivariate analysis has been used to analyse ToF SIMS data which contained information on protein conformation and identity within the complex spectra [12,13]. However, the relationship between the ToF SIMS spectrum of material surfaces with adsorbed proteins and subsequent biological responses, e.g. cell adhesion, has not yet been reported upon. It is hoped that the development of a method with which to identify relationships between cell response to materials and surface chemistry might aid in rational design of materials for biomedical applications.

Here, we report a surface structure-function relationship based on high throughput surface characterisation (*HT-SC*) approaches. The microarray platform employed here facilitates the automated high throughput characterisation of hundreds of materials [20,21]. The materials are acrylate polymers synthesised as 300 μm diameter spots on a cell resistant poly(hydroxyl methacrylate) (pHEMA) coated slide to form an array of 576 polymer spots in triplicate. Utilising 22 different acrylate monomers mixed pairwise in different proportions and UV photopolymerised, 496 unique homo- and co-polymers were formed. hEB cells from hESCs were cultured on these samples for 16 hrs to test their initial adhesion. To fully characterize the polymers we employed a suite of analysis techniques that we have adapted to *HT-SC*, including ToF SIMS, XPS, water contact angle (WCA) measurements, AFM and confocal Raman spectroscopy to analyse individual polymer spots in the microarray [22]. Using this analysis of the polymer spots, we are able to identify which are the important surface characteristics in determining hEB cell adhesion. In particular partial least squares (PLS) regression is applied to the challenge of analysing multiple ToF SIMS spectra from each spot since each spectrum contains hundreds of unique peaks. This method has been proven useful for micro-arrays in a study where it was determined that water contact angle could be predicted from the ToF SIMS spectra [23,24]. Hydrocarbon surface moieties were identified to correlate with high water contact angle and oxygen and nitrogen containing fragments were predicted to produce surfaces on which low water contact angles were measured. The physicochemical sense of this result gave us the confidence in the PLS approach to apply it to investigate the cellular response to the polymer microarray in this study.

2. Materials and methods

Microarray fabrication

16 major monomers were mixed pairwise with 6 minor monomers (Figure 1) in the following ratios: 100/0, 90/10, 85/15, 80/20, 75/25 and 70/30. The monomers were then spotted on 4 v/w % (in 95/5 vol% ethanol/water) pHEMA coated glass slides (epoxy monolayer-coated glass slides (Xenopore XENOSLIDE E, Hawthorne, NJ) using a robotic pin printer and photopolymerised for at least 10 min under 365 nm UV. Polymer arrays were dried at < 50 mTorr for at least seven days. The arrays were UV sterilized for 30 min before cell culturing, and then washed with PBS twice for 15 min to remove the residue monomer or solvent. Subsequently, the arrays were pre-conditioned with 25 $\mu\text{g mL}^{-1}$ Fn (Sigma) for 1 h, and then washed with Dulbecco's phosphate buffered saline (dPBS) and medium before cell seeding. Bare and pre-conditioned arrays washed with dPBS and deionised water were analysed using ToF SIMS.

Time of flight secondary ion mass spectrometry (ToF SIMS)

An ION-ToF IV instrument was operated using a Bi_3^+ primary ion source operated at 25 kV and in "bunched mode". A 1 pA primary ion beam was rastered, and both positive and negative secondary ions were collected from a 100 x100 μm area of each polymer spot in the microarray over a 10-second acquisition time. The primary ion dose was maintained below 10^{12} ions/ cm^2 to ensure static SIMS conditions. The acquisition of spectra was carried out in a high throughput manner and was completed within 3 hrs for all 576 polymer spots in a microarray. Ion masses were determined using a high resolution Time-of-Flight analyser allowing accurate mass assignment.

Raman Spectroscopy

Raman spectra were acquired using a 532 nm laser using a spectrometer (Horiba Jobin Yvon) with an automated sample stage. Spectra were acquired from each polymer in the microarray for 2 seconds. A x100 times objective lens was used to collect the signals. To test the degree of curing, triplicate samples of two monomers were manually spotted on a pHEMA coated slide using 3 μL of monomers by a micro pipette. Samples were then polymerised by UV (365 nm) for 10, 30, 60, 150, 300, 450, 600 seconds, respectively, under argon atmosphere. These were vacuumed (at < 50 mTorr) for one week to remove any volatile uncured monomer.

X-ray photoelectron spectroscopy

XPS was carried out on a Kratos Axis Ultra instrument using monochromated Al K α radiation (1486.6eV), 15mA emission current, 10 kV anode potential and a charge-compensating electron flood. High-resolution core levels were acquired at a pass energy of 20 eV. The takeoff angle of the photoelectron analyzer was 90°.

Water Contact Angle (WCA) Measurements

WCAs were measured using the sessile drop method on a fully automated Krüss DSA 100 instrument. A water drop with a volume of ~400 picolitre was used. Ultrapure water was used for the CA measurements (18.2M Ω -cm resistivity at 25°C). The side profiles of the drops were recorded for image analysis. WCA calculations were performed using a circle segment function intersecting with a straight baseline representing the surface.

AFM

AFM measurements were taken using a Nanoscope 3000A instrument in tapping mode. Silicon tips (radius < 10 nm) with a resonant frequency of approximately 300 kHz and a

force constant of 40 N/m were used (Tap300A1, Budget Sensors). A $5 \times 5 \mu\text{m}$ region of the each individual polymer was analysed in an automated manner and the root mean square (RMS) roughness was measured across this region.

Human embryonic body stem cell culture and counting

Undifferentiated hES cells (H13, WiCell, Wisconsin) were grown on an inactivated mouse embryonic fibroblast (MEF) feeder layer. To induce the formation of embryonic bodies (EBs), undifferentiated hES cells were treated with 1 mg/mL type-IV collagenase for 40 min, and then transferred from 6-well plates to low-adhesion petri dishes (10 cm, Ref:3262, Corning) containing 10mL of differentiation medium [80% knockout-DMEM, supplemented with 20% fetal bovine serum (FBS, Hyclone), 0.5% L-glutamine, 0.2% *b*-mercaptoethanol, and 1% nonessential amino acids (all from Invitrogen)]. hES cells in one 6-well plate was transferred to one petri dish. EBs were cultured for eight days at 37 °C and 5% CO₂, in a humidified atmosphere, with changes of media every two days. EBs were subsequently trypsinized and cultured (2 million cells per array) on Fn pre-conditioned polymer arrays for 16 hrs to test their initial adhesion. Polymer arrays were washed with PBS, fixed with Accustain (Sigma) solution for 30 min, permeabilized with 1% Triton X-100 in PBS for 10 min, and then stained with Cyto 24 (invitrogen) for 1 h. The arrays were gently washed with PBS and deionised water to remove buffer salts and air dried. Each spot was imaged with iCys laser-scanning cytometry (CompuCyte Corporation, MA), and cell numbers were quantified in a high throughput manner. Individual images of each spot were stitched together for display. Prior to cell culture polymer microarrays were incubated in 25 µg/ml Fn (Sigma) solution for 1 hour at 37 °C, then washed with dPBS (Invitrogen) once for 2 min and deionised water twice for 4 min.

Multivariate analysis

The ToF SIMS spectral data were analysed using principal component analysis (PCA), and the correlation between ToF SIMS spectra and hES cell adhesion was analysed using partial least squares (PLS) regression. 681 positive and 543 negative ions were selected from a group of polymers from the array containing all 22 monomers to form the peak lists. All peak intensities in a ToF SIMS spectrum were normalised to the total secondary ion counts to remove the influence of primary ion beam fluctuation. The positive and negative ion intensity data were arranged into one concatenated data matrix. Both multivariate analysis methods were carried out using the Eigenvector PLS_Toolbox 3.5. The SIMPLS algorithm was used for the PLS analysis. A “leave one out” cross validation method was used in the PLS analysis to find the number of latent variables to construct the PLS model. The ToF-SIMS and hES cell data were mean-centered before analysis. An R squared value is calculated to assess the degree of agreement between prediction and experiments.

$$R=1 - \frac{\sum (y_m - y_p)^2}{\sum (\bar{y} - y_m)^2}$$

y_m is the measured response variable, y_p is predicted response variable, $\bar{y}$ is mean of the measured response variable.

3. Results and Discussion

3.1. Fabrication of the microarray

The microarray was fabricated by UV photo initiated radical co-polymerization of each of the 16 *major* monomers (numbered 1 – 16, Figure 1) with each one of 6 *minor* monomers

(lettered A – F, Figure 1) in a pairwise manner on a pHEMA coated glass slide. Each microarray contains three replicate sub-arrays (Figure 1), which in turn includes 6 repeats of the 16 major monomers created as homopolymers and 480 copolymers.

3.2. Bulk chemical characterisation using Raman

To investigate the degree of curing of the polymers in the array, a confocal Raman spectrum was acquired from each spot under automation. The total acquisition time for 576 spots in a single array was approximately 3 hrs. Representative spectra are shown in Figure 2a. Large micro pipette spotted samples were used to assess the degree of curing as a function of UV exposure time. An estimate of the degree of polymerisation from the Raman data was made using the ratio between the C=C (shift = 1640 cm^{-1}) and C=O (shift = 1720 cm^{-1}) peak intensities. Since each acrylate group contains a carbon-carbon double bond and a carbonyl group, the ratio decreases with increasing degree of curing. The C=O/C=C ratio of two polymers photo polymerised for eight different UV exposure times are shown in Figure 2b. The ratio was found to decrease with increasing UV exposure duration from 0–60 s, after which it became constant suggesting that the uncured monomer content reached a minimum. The C=C/C=O ratio from the spots polymerised on the microarray was lower than the same micro pipette spotted material suggesting the polymerisation had progressed more effectively in this small volume. For all polymer spots, a UV exposure exceeding 10 min was applied in order to ensure maximum polymerisation.

3.3. Cell adhesion

The adhesion of human stem cells is critical for a range of cellular activities including proliferation and differentiation. To understand the influence of surface chemistry on stem cell adhesion, we have employed partially differentiated hEB cells rather than undifferentiated hESCs due to the fact that fully dissociated hESCs tend to undergo cell death during plating [21]. Furthermore, hEB cells are robust while still maintain enormous differentiation potential. The hEB cells obtained from trypsinized embryoid bodies (day 8) were seeded onto a Fn pre-conditioned array. Cell seeding procedure was carefully monitored to avoid the possible seeding inhomogeneity due to geometry or location of polymer spots. After culturing for 16 hrs, the sample was washed, stained with a DNA binding dye and then imaged; cells that had attached to each polymer were counted. The total cell number per polymer spot was used as a measure of the ability of each polymer to support initial cell adhesion. The individual images of polymer spots after cell culture are shown in Figure 3a. The cell numbers are arranged according to the polymer identity and presented as an intensity map in Figure 3b. Polymer spots exhibited a similar geometry as shown in Figure 3c. The standard deviation of cell number on the 6 replicas of each homopolymer was low (Supplementary Figure 1), which suggested a homogenous cell seeding density. Certain monomers stood out with a low cell adhesion, e.g. monomer 3 which contains an oligopropylene glycol segment, which is consistent with the view of this moiety as a cell adhesion resistant material. There is no strong trend between cell adhesion and substituent identity, however, homopolymers of monomers 15, 8, 14 and 13, which are all bi- or tri-acrylates, all correspond to relatively high cell numbers (Figure 3b), suggesting that there might be a relationship between the number of ester groups in the monomer and the observed cell adhesion. To investigate this further we plotted the measured cell number versus the concentration of ester groups in a monomer (number of ester group/total number of atoms) in Figure 4. It appears that cell adhesion increases with increasing ester group concentration until the ratio of 0.04 after which the cell number reaches a plateau. The ester group contains a carbonyl functionality, and it has been previously been proposed that the carbonyl group is beneficial for cell adhesion (e.g. bovine endothelial cells) [25,26]. Mono acrylates produce linear polymer whereas di and tri acrylates produce cross linked material. However, the increase of attached cell number with acrylate concentration is unlikely to be

due to crosslinking of di- and triacrylates since diacrylates 3, 6, 16 and 11 all showed relatively low cell number (Supplementary Figure 2). Therefore, the effect of the ester group concentration in the monomer structure on increasing cell number appears to be attributed to the chemical moiety.

3.4. Characterisation of polymer surface topography, wettability and elemental and functional composition

To fully characterise the surface chemical properties of the materials on the array, along with the wettability and the surface topography, we employed a HT-SC methodology which has been developed to acquire and process the large quantity of data [22]. Each polymer on the microarray was analysed by XPS, AFM, WCA and ToF SIMS prior to Fn pre-conditioning. We explored the correlation of total cell number with chemical or physical properties of the polymer spots in order to identify the underlying cell-material interactions.

No correlation was identified between cell number and the elemental and functional composition of carbon, oxygen and nitrogen measured for the polymer spots using XPS (Supplementary Figure 3). Most polymers had carbon composition between 60% and 80% and oxygen composition between 40% and 20%, a relatively small chemical compositional range which clearly supported a large range of cell adhesion. AFM revealed that the vast majority (94%) of the spots had a surface roughness of less than 5 nm, suggesting a relatively smooth surface for most polymers formed using this method (Supplementary Figure 4). The surface roughness was not found to correlate with cell adhesion, and there was a wide distribution of cell adhesion on materials with a roughness below 3 nm, suggesting that other surface parameters, such as surface chemistry, may play a more important role in controlling cell adhesion.

The plots of WCA versus cell adhesion for each major monomer are shown in Figure 5b. No correlation between the cell adhesion and WCA was seen when the entire library was considered (Figure 5a). However, when polymers containing tertiary amine groups (monomer E) were removed from the homopolymer comparison, polymers containing major monomers 2 and 9 showed a linear relationship between water contact angle and cell number when considered separately as shown in Figure 5c. This indicates that WCA only correlates with cell adhesion in some instances and only when considering a small sub set of polymers in the array of certain similar chemistries.

3.5 ToF SIMS analysis of Fn adsorbed polymer surface

ToF SIMS spectra generally contain many fragments (secondary ions) which result from the collision cascade of the primary ion with the surface. These hundreds of secondary ions contain information on the surface molecular structure of the sample. This information rich technique offers great opportunities for characterising the surface chemistry of the polymers and proteins adsorbed to them, but the complexity in interpreting the large number of fragments from all the polymer spots is an obstacle for the ready comparison of the data from each material in order to identify the major differences in surface chemistry. To overcome this difficulty, principal component analysis (PCA) has been used here to identify the major differences between ToF SIMS spectra of the large range of materials. PCA reduces the dimension of the ToF SIMS spectra to a small number of variables, called principal components (PCs) which are used to identify the differences between the samples. The major difference between the samples is contained in the first few PCs [27]. In particular, the PC analysis illustrates the different degrees of change observed for each polymer upon Fn adsorption. Comparing the ToF SIMS spectra of Fn pre-adsorbed homopolymers in the microarray, it is clear that some polymers showed similar surface chemistry to a sample of pure Fn adsorbed onto a glass coverslip, since they grouped with

pure Fn in the PCA graph (Figure 6a). This indicates that these surfaces are largely covered by Fn. Higher PC1 values are associated with higher intensities of characteristic ions from Fn that are assigned in Figure 6c. Most of these immonium ions have been reported previously as being characteristic of amino acids [12,28]. The differences in the ToF SIMS spectra seen between the various Fn adsorbed polymer samples demonstrate that Fn adsorption is influenced by the bare polymer chemistry. Polymer 8, 12 and 13 showed similar spectra compared to pure Fn which indicated more Fn coverage on them while polymer 3, 5, and 16 exhibited different ToF SIMS spectra (Figure 6a) which indicated less Fn coverage. Subsequent cell adhesion activity may be then mediated by the adsorbed Fn. Furthermore, all polymers adsorbed some Fn, even for those polymers resisting cell adhesion, e.g. polymer 3 (Figure 7). This observation is consistent with the general observation that none of the surfaces is completely protein resistant, and the fact that ToF SIMS has a very high sensitivity to protein adsorption [11].

The ability of ToF SIMS to probe the molecular structure of the surface and the differences in protein adsorption makes it possible to correlate surface chemistry of either the bare polymers or the protein adsorbed surfaces to cell adhesion. To identify correlations between the ToF SIMS data and cell adhesion, partial least squares (PLS) regression was employed. PLS regression can be used to identify the correlation between a multivariate (ToF SIMS spectra) and univariate data set (cell number) and is particularly useful for dealing with high dimensional data sets like ToF SIMS. PLS has previously been shown to be useful for correlating ToF SIMS spectra to cell growth and extracting information on the effect of chemical functionalities on cellular response for small sample numbers [26]. Most recently we have shown this to be applicable to identifying correlations between surface chemistry and cell response using polymer microarrays with dissociated human embryonic stem cells [29]. The PLS analysis produces a set of regression coefficients for each secondary ion, which is a measure of the degree of influence of a secondary ion on the univariate data set (cell number). The ToF SIMS spectra acquired from the Fn conditioned microarray were analysed using this method. A correlation between the SIMS spectra of the Fn adsorbed surfaces and cell adhesion was identified using PLS (Figure 8), which indicates that there is an interrelationship between cell adhesion and the chemistry of the protein adsorbed polymers. The regression coefficient reveals that the nitrogen containing ions from Fn are associated with high cell adhesion indicating that Fn encourages hEB cell adhesion, consistent with previous reports on Fn supporting cell adhesion [30]. Ions ($C_4H_9^+$) from the tertiary butyl group and oxygen containing ions from glycol functionalities were found to have negative regression coefficients, indicating that they correlate with lower cell adhesion. These ions were also responsible for the differences identified by PCA between the ToF SIMS spectra of polymer 3, 5 and 16 and pure Fn. Polymer 5, 3 and 16 contain tertiary butyl group and glycol functionality, respectively. This suggests a surface that resists cell adhesion from a combination of low Fn coverage on polymers containing these functionalities and the influence of these functionalities on the properties of the proteins in the subsequent adsorbed layer formed in the culture media. This PLS and PCA analysis indicates that surfaces supporting cell adhesion have a higher coverage of adsorbed Fn. It is well established that both pre-adsorbed and media adsorbed proteins affect cellular response through their identity, amount, orientation, conformation and distribution [31]. This is the first time a quantitative correlation has been established between the surface chemistry of a protein conditioned surface and cellular response which holds over such a large range of polymer chemistries. ToF SIMS analysis has been demonstrated to be able to probe the overall chemistry of the composite protein-polymer surface after conditioning with Fn including information on the amount, orientation and conformation.

Fn is commonly employed to encourage cell adhesion to materials and has previously been found to be useful in polymer microarray studies [10]. It is therefore important to note that

the adhesion of cells in this study is likely to be mediated by the pre-adsorption of Fn, with subsequent contributions from proteins adsorbed from the serum, and thus the cell-material interactions being studied will be heavily influenced by material-Fn interactions. Although there may be other proteins in the culture media that could also adsorb to the surfaces and play an important role on cellular response, the strong correlation identified between Fn adsorbed surfaces and cell adhesion indicates the vital role of Fn on encouraging hEB cell adhesion. However, this does not mean the exclusion of the possibility of contributions from other proteins in the culture media which could adsorb to the surface to promote or discourage cell adhesion. The chemistry of the polymers that modulate Fn adhesion after pre-conditioning in Fn and subsequently influence the cellular adhesion after exposure to the serum containing culture media is of great interest.

3.6 ToF SIMS analysis of the bare polymer surface

Multivariate analysis was carried out on the ToF SIMS data acquired from on the *bare* polymers before Fn pre-conditioning. PCA analysis was performed on the ToF SIMS data from the bare homopolymers, with the first four PCs and their respective loadings shown in Figure 9. The differences between spectra from the homopolymers are well represented using the first four PCs which capture 86.7% of the variance in the ToF SIMS spectra. The relative influence of each ion on the PC is presented in the loadings plot. Polymer 5 was differentiated from all of the other homopolymers due to higher intensities of ions $C_4H_9^+$ and $C_5H_9^+$ which are representative of the tertiary butyl group in monomer 5. Polymer 7 and 14 clustered as they showed higher intensity of $C_6H_5^+$ which originates from the benzene ring in the monomers. Polymer 3, 16 and 6 grouped due to higher intensities of ions $C_3H_7O^+$ and $C_2H_5O^+$ from glycol functionalities within their monomers. PCA analysis of ToF SIMS spectra differentiates not only polymers with different functionalities, but it can also reflect the variation of surface chemistry of polymers with the same functionality but slightly different composition. Taking PC4 as an example, a high positive loading of the characteristic ion ($C_2H_5O^+$) of ethylene glycol moieties and negative loadings of the hydrocarbon ions $C_3H_5^+$, $C_3H_6^+$ and $C_6H_5^+$, separates the four ethylene glycol moiety containing polymers (polymer 16, 2, 1, 9); the $C_2H_5O^+$ peak intensity decreased in the order of polymer 16, 2, 1, 9, which demonstrates the ability of ToF SIMS to probe the slight changes in surface molecular structures of polymers containing the same functionality. Interestingly, the cell number was found to decrease in the order of 9, 1, 2, 16, which corresponded to the increasing PC4 values of these polymers. Ethylene glycol functionalities have been widely reported to resist cell adhesion [32,33].

A strong relationship ($R^2=0.67$) between the cell number and the bare surface chemistries in the ToF SIMS spectra was identified (Figure 10a), and a set of regression coefficients capable of predicting the cell number on all spots was also generated by the PLS regression (Figure 10b). The most prominent 30 ToF SIMS ions which are positively and negatively correlated with cell number are listed in Figure 10b. Some of the secondary ions identified in the PLS regression coefficient are clearly associated with functionalities in the polymer, thus, the effect of secondary ions on cell adhesion can be interpreted in terms of the influence of surface functionalities. The association between secondary ion assignments and corresponding parent surface functionalities are presented in Table 1. Characteristic ToF SIMS ions of the ethylene ($C_2H_5O^+$) and propylene glycol ($C_3H_7O^+$) moieties showed high negative regression coefficients, indicating that surfaces containing glycol functionality correlate with low cell adhesion. This is consistent with the well known protein repellent nature of glycol moieties and their related tendency to resist cell adhesion [34, 35]. ToF SIMS ions indicative of the tertiary butyl moiety ($C_4H_9^+$) and phenyl group ($C_6H_5^+$) also showed relative high negative regression coefficients indicating that they correlate with low cell adhesion. These surface moieties have not previously been associated with minimising

cell adhesion. The amine functionality with the characteristic ion $C_3H_8N^+$ correlated strongly with high cell adhesion as seen from its positive regression coefficient. Certain hydrocarbon ions, such as $C_3H_7^+$ and $C_2H_3^+$, also correlated with high cell adhesion. These rather non specific ions are likely to arise from the polymer backbone, although other functionalities will also contribute to their intensity. The ToF SIMS ions correlating with lower cell numbers, e.g. $C_4H_9^+$, $C_3H_7O^+$, are found to be common in the PLS analysis of both the Fn conditioned and the bare polymer surfaces. For the Fn conditioned surfaces, ToF SIMS ions correlating with higher cell number are all from the protein structure, while mainly non-specific hydrocarbon ions correlated with higher cell adhesion on the bare polymer. This suggests that surfaces with low cell adhesion adsorb minimum Fn since ToF SIMS ions arising from surface chemical structures discouraging cell adhesion are from the bare surfaces. As the cell adhesion is mediated by Fn and proteins adsorbed from the media, the observed correlation between polymer surface chemistry and cell adhesion does not suggest a direct correlation but is indicative of a correlation between the material surface chemistry and protein adsorption.

A PLS regression model may suffer from over fitting, thus it is prudent to validate it using outside data. To validate the PLS model, a small proportion of the polymers were used to construct a model, and the rest of the polymer library were used to validate it. A subset of the array (128 polymers) was used to act as the training set to construct a PLS model which produced a set of regression coefficients; the cell adhesion on the remaining 448 polymers not included in the training set were calculated using the regression coefficients generated from the training set. This training set contained 2 repeats of 16 homopolymers and 96 copolymers containing 30% of 6 minor monomers. These 128 samples were chosen as they contain all the 22 monomer units and consequently include all ion species which could be generated from the microarray. The cell number predicted from the ToF SIMS data on the test series of polymers showed an R^2 value of 0.62 which validates the PLS model constructed from the training set (Figure 11). The ions with high regression coefficients in the training sample PLS model are consistent with those pulled out in the PLS model constructed from 576 samples (Supplementary Figure 5). This demonstrates that cell adhesion can be predicted based on the ToF SIMS spectra from the surface of a large number of chemically diverse polymer samples using a model constructed from a relatively small number of training samples.

A strong relationship between cell response and the information on the surface chemistry provided by ToF SIMS of the polymers before and after Fn conditioning has been identified using PLS. We have demonstrated that for a large number of chemically diverse polymeric materials it is not possible to find a correlation between cell response and any simple surface property, including WCA, roughness, elemental and functional composition. This is almost certainly in part because no single measurement can describe the complex surface structure fully, and moreover, SIMS is the only that provides an insight to the molecular structure. For example, in our studies, different polymer surface chemistries can have similar water contact angles on which very different cell numbers are observed. Polymers made up of monomers with different molecular structures also showed similar elemental compositions. In contrast, ToF SIMS analysis generates a spectrum which is very information rich, producing a fingerprint of the analysed surface containing compositional and structural information, although this information is relatively difficult to extract. The use of PLS to identify the important components in the SIMS spectra which correlate with cell response allows the important features to be identified and interpreted in isolation. Further work would be necessary to deconvolute the amount, orientation and conformation of the Fn, however, without this information we have shown that this approach can predict the cell response from the surface ToF SIMS spectra of protein adsorbed surfaces.

The identification of certain polymer chemical moieties which direct cellular response from the surface demonstrates that the information generated from the PLS regression methodology approach of the HT-SC is powerful. Amines and hydrocarbons we found to correlate with high adhesion and glycol, phenyl and tertiary butyl functionalities corresponding to lower cell numbers (Table 1). The identification of these functionalities could not have been achieved by considering the polymer structure and corresponding cellular response alone showing the need to utilise the molecular information contained in the ToF SIMS spectra to identify functional groups which influencing cell adhesion on the polymer microarray. Previous studies investigating the influence of surface chemistry on cell response have utilised alkanethiol self assembled monolayers (SAMs) with different terminal functional groups as model surfaces to achieve control of the density and orientation of the surface moieties [36–38]. In comparison, the surface structure of the copolymers studied here is much less ordered and well characterised, thereby requiring ToF SIMS to identify the functional groups at the surface and PLS to identify those which are important. While the information from the SAM approach is very useful in understanding the effect of various moieties on cell response, the use of polymers combined with analysis has more direct relevance to clinical applications.

Conclusions

We find a correlation between the hEB cell number on microarray polymer spots after 16 hours of culture and surface mass spectrometric data from the uppermost surface acquired using ToF SIMS using multivariate PLS regression. In contrast, no relationship was found which includes all materials using either WCA, surface roughness, or surface elemental or functional composition. Using PLS, chemical information contained in the ToF SIMS spectra of both Fn pre-adsorbed and bare polymer surfaces was found to correlation with cell adhesion in protein containing culture media. Polymer moieties included in the library such as glycol, tertiary butyl and tertiary amine were found to be the most prominent functionalities to influence cell adhesion. These findings indicate interrelationships between material surface chemistry, protein adsorption, and cell adhesion. Whilst such interrelationships have been widely reported in many systems, this is the first work to identify correlations across a very large number of diverse material chemistries in a combinatorial library using a surface analytical approach. These results highlight the importance of the molecular information contained in the ToF SIMS spectra in describing the surface chemistry that controls cell adhesion and will have wide application in the study of cell-material interactions. We believe that the application of this HT-SC approach to the high throughput materials discovery micro array format will accelerate the identification and development of new materials from large chemical libraries to achieve improved biomaterials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

An image of a microarray including 3 replicates on a microscope glass slide with the structures of the 22 monomers used. Each microarray contains 576 polymer spots which are made by pairwise mixing 16 major monomers denoted by numbers and 6 minor monomers denoted by letters in the following ratios: 100/0, 90/10, 85/15, 80/20, 75/25 and 70/30.

Figure 2.

(a) Raman spectra of manually spotted monomer 5 (top line), after 600 s polymerisation (middle line) and in situ polymerised monomer 5 in the microarray (bottom line). (b) The ratio of C=C to C=O peak versus polymerisation time. ●— monomer 5 (

); ○— monomer A (

)

Figure 3.

(a) Image of a microarray on which cells were cultured for 16 hrs, and from which cell number per polymer spot has been quantified. The white boxes are where spots at those positions fell off during cell culture. (b) A heat map of cell number per polymer spot arranged by the monomer identity and composition. Numbers denote major monomers while letters denote minor monomers. Each rectangular represents a polymer spot. (c) Representative images of polymer spots with low, medium and high cell adhesion (left to right). Cells were stained with Cyto 24. Scale bar 0.1 mm.

Figure 4.

The number of attached hES cells versus the ratio of number of ester groups to the number of total atoms per monomer molecule (including hydrogen) calculated from the monomer structure and mixed composition.

Figure 5.

Water contact angle versus cell number. (a) WCA versus cell number including all polymers. (b) Each graph represents polymers containing the same major monomer component denoted by numbers. Note the prominent effect of the monomer 3-dimethylamino propyl acrylate (denoted by letter E) on reducing water contact angle except for monomer 4-tert-butylcyclohexyl acrylate (denoted by number 5). (c) Linear relationship between water contact angle and cell number was found for polymers containing two major monomer components 2 and 9 respectively when copolymers containing monomer E were removed from the analysis.

Figure 6. (a) PC1 vs PC2 of 16 Fn adsorbed homopolymers showing homopolymer 8, 12 and 13 with similar chemistry compared to pure Fn adsorbed on a glass coverslip. (b) The 15 most positive loadings on PC1 showing higher PC1 values associated with higher intensities of characteristic ToF SIMS ions from Fn. (c) PCA analysis of Fn adsorbed polymer 8, 9, 12, 13, 14, 15 which shows polymer 8, 12 and 13 possessing the most similar surface chemistry compared to pure Fn.

Figure 7.

Comparison of the CN^- and CNO^- peaks of polymer 3 before and after Fn adsorption. The increase of these two peaks after Fn adsorption suggests even protein repellent polymers in the microarray adsorbed Fn. Polymer 3 with a protein repellent poly propylene glycol segment in the structure is among the polymers which showed the lowest CN^- and CNO^- peak intensities after Fn adsorption.

Figure 8.

Predicted versus measured hEB cells with ToF SIMS ions promoting and inhibiting cell adhesion listed. Ion formula are tentatively assigned from their masses. Positive and negative regression coefficients suggest promoting and inhibiting cell adhesion respectively. The greater the regression coefficient, the more influential the ion is on cell adhesion.

Figure 9.

PCA analysis on 16 homopolymers with the first four PCs shown. Each homopolymer has 6 repeats. We show (a) PC1 vs. PC2, (b) PC3 vs. PC4, (c) the loadings of the first four principle components with the most influential ToF SIMS ions labeled. The polymers containing glycol functionalities (labeled in red) are well separated in PC4. According to the scores and loadings of PC4, polymer 3, 16 and 6 present more glycol moieties on the surface compared with polymer 1, 2, 9 and 11.

Figure 10.

The PLS model constructed on 576 samples showing agreement between measured and predicted cell adhesion. The most influential 30 ToF SIMS ions on cell adhesion are listed. ToF SIMS ions with negative and positive regression coefficients inhibit and enhance cell adhesion, respectively.

Figure 11.

Validation of the PLS model constructed from 128 samples (5 latent variables) by using 448 samples. (a) a PLS model and a set of regression coefficients were generated from 128 training samples. (b) the generated regression coefficients were used to predict the cell adhesion on the rest 448 test samples.

Table 1

Functionalities and their associated ToF SIMS ion supporting or inhibiting adhesion of hES cells. Ions are identified by correlating ToF SIMS spectra to hES cells adhesion using PLS. Each ion is designated with a regression coefficient that determines the relative degree of influence on the adhesion of hES cells.

Effect on the adhesion of EB cells	Ions and corresponding regression coefficients	Functionality
Lower cell adhesion	$C_6H_5^+(-151)$, $C_6H_5O^-(-107)$	Aromatic
	$C_3H_7O^+(-124)$	Propylene glycol
	$C_4H_9^+(-205)$	Tertiary butyl
	$C_2H_5O^+(-101)$	Ethylene glycol
High cell adhesion	$C_3H_7^+(205)$, $C_2H_3^+(124)$, $C_4H_7^+(104)$, $C_2H_5^+(77)$, $C_4H_5^+(75)$, $C_5H_7^+(70)$, $C_5H_{11}^+(51)$, $CH_3^+(50)$	hydrocarbons
	$C_3H_8N^+(203)$, $C_2H_6N^+(69)$	Tertiary Amine