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Experimental and Computational Analysis of Cellular Interactions with Nylon-3-Bearing Substrates

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Abstract

The ability to design biomaterials that interact with biological environments in a predictable manner necessitates an improved understanding of how surface chemistry influences events such as protein adsorption and cell adhesion. In this work, we examined mechanisms governing the interactions between 3T3 fibroblasts and nylon-3 polymers, which have a protein-like polyamide backbone and are highly amenable to tuning of chemical and physical properties. Protein adsorption and cell adhesion to a library of nylon-3 polymers were characterized and analyzed by partial least squares regression. This analysis revealed that specific chemical features of the nylon-3 polymers correlated with the extent of protein adsorption, which, in turn, correlated with cell adhesion in a serum-containing environment. In contrast, in a serum-free environment, cell adhesion could be predicted solely from chemical properties. Enzymatic treatments of 3T3 cells prior to plating indicated that proteins bound to the cell surface mediated cell-nylon-3 polymer interactions under serum-free conditions, with additional analysis suggesting that cell-associated fibronectin played a dominant role in adhesion in the absence of serum. The mechanistic insight gained from these studies can be used to inform the design of new polymer structures in addition to providing a basis for continued development of nylon-3 copolymers for tissue engineering applications.

Keywords

Cell adhesion; Protein adsorption; Cell-material interactions; Nylon-3; PLSR

1. Introduction

Substrates and scaffolds that support cell attachment, growth and differentiation are crucial components in the construction of engineered tissues. Although the intrinsic bioactivity of natural materials enables the construction of scaffolds that can actively direct cell function, there are also many disadvantages that accompany the use of biologically produced substances as biomaterials. It can be challenging to create consistent, controlled, and tailored

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environments using natural materials¹; moreover, natural materials can be antigenic and can harbor pathogens².

The chemist's ability to vary the molecular and nanoscale structure of engineered synthetic materials should, in principle, enable control over the signals these materials convey to cells (either directly or via adsorbed proteins)^{3–5}. Construction of two- and three-dimensional cell niches has been achieved using many types of degradable and non-degradable synthetic materials such as polystyrene⁶, polyethylene glycol^{7–11}, polyurethane^{9,12}, poly(methyl methacrylate)¹³, polyglycolide-co-lactide¹⁴, poly(*N*-isopropylacrylamide)¹⁵, polysaccharides^{7,12}, and self-assembling peptides¹⁶. Direct cellular recognition of these materials can be enabled by incorporation of short cell-adhesive peptides (e.g., RGD, YIGSR, REDV)^{9,17–19} or cell-binding domains of serum proteins (e.g., fibronectin, laminin)^{20,21}. The interactions between cells and synthetic materials may also be manipulated by tuning the chemical and physical properties of the materials (e.g., hydrophobicity, wettability, topology, charge density^{6,22,23}) to alter the adsorption of cell-adhesive proteins. However, because of limitations in preparative capabilities and an incomplete understanding of signaling between cells and their natural substrates, the full promise of synthetic materials with respect to predictably controlling cell-material interactions has yet to be realized.

Our pursuit of nylon-3 copolymers as potential biomaterials is motivated by the hypothesis that the protein-like backbone of these under-explored substances (β -amino acid residues instead of α -amino acid residues) should render them protein-mimetic without being susceptible to enzymatic degradation. In a preliminary study, we screened a small library of nylon-3 copolymers and discovered not only that some were capable of supporting improved cell adhesion and spreading when compared to positive control adhesive substrates (i.e., collagen), but also that significant differences in cell adhesion could be achieved via relatively subtle changes in copolymer composition^{24,25}. Interestingly, some of the nylon-3 copolymers could support the adhesion and spreading of fibroblasts in a serum-free environment.

The properties of nylon-3 materials can be altered by varying the identity, proportion and stereochemistry of the β -lactam precursors employed for the ring-opening polymerization process (Figure 1). The ease with which these alterations can be made synthetically enables research intended to identify polymer characteristics that are most important in terms of cell adhesion. In the present study we take advantage of these features to examine factors that influence fibroblast adhesion to surfaces that bear nylon-3 copolymers. Understanding how these materials achieve the range of adhesive behavior identified in our previous screening studies is important not only for further development of this specific class of polymers, but also for elucidating generalizable mechanisms by which variations in materials properties influence cell adhesion.

2. Materials and methods

Fibronectin (F1141), anti-collagen type I antibody (C2456), and anti-mouse IgG-FITC conjugate (F5262) were from Sigma-Aldrich (St. Louis, MO); PureCol™ type I collagen (5005-B) was from Advanced Biomatrix (San Diego, CA); NHS ester-functionalized glass slides (CodeLink activated slides, DN01-0025) were from SurModics (Eden Prairie, MN); Multi-channel silicone coverslips containing 50 wells (103350) were from Grace Biolabs (Bend, OR); Dulbecco's Modified Eagle Medium (DMEM), cell culture supplies, mammalian cell LIVE/DEAD Viability/Cytotoxicity Kit (L3224), NanoOrange Protein Quantitation Kit (N6666), and vitronectin (PHE0011) were from Invitrogen (Carlsbad, CA); Anti-fibronectin antibody (sc-80559) and anti-vitronectin antibody (sc-80558) were from

Santa Cruz Biotechnology (Santa Cruz, CA); Human plasmin (527621) was from CalBiochem (San Diego, CA); Collagenase type 1 (LS004197) and type 2 (LS004177) were from Worthington Biochemical (Lakewood, NJ); NIH 3T3 fibroblast cells were from the American Type Tissue Collection (ATCC, Manassas, VA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification.

2.1. Nylon-3 polymer synthesis

Monomer synthesis, polymerization and characterization were conducted by following previously reported methods^{24,26–29}. Each random copolymer was composed of two subunits, termed 'A' and 'B', in varied combination (Figure 1a). Polymer synthesis was conducted in a glove box maintained at low humidity. Briefly, a mixture of monomer A, monomer B, and the co-initiator in tetrahydrofuran (THF) was treated with a solution of lithium bis(trimethylsilyl)amide in THF. The polymerization was allowed to continue for 6 h to consume all monomer, and the reaction was then terminated by adding methanol to the reaction vessel. Pentane was added to the reaction solution to precipitate the polymer, which was collected as a pellet after centrifugation and dried under nitrogen. The solid was redissolved in THF, precipitated, and dried twice more as described above. At this stage, the side chain amino groups bear protecting groups; the polymer was deprotected by treating with neat trifluoroacetic acid at room temperature for 2 h. Diethyl ether was then added to precipitate the polymer, which was collected as a pellet after centrifugation and dried under nitrogen. The solid was redissolved in methanol, precipitated, and dried twice more as described above to yield the final polymer as a TFA salt. Polymers at the protected stage were analyzed by gel-permeation chromatography (GPC); the instrument was equipped with a Wyatt multi-angle light scattering detector and a refractive index detector. Number-averaged molecular weight (M_n) and polydispersity index (PDI) were calculated using ASTRA 5.3.3.15 software with a dn/dc value of 0.1 mL/g for all polymers.

2.2. Preparation of nylon-3 polymer-functionalized glass slides

N-Hydroxysuccinimide ester-functionalized CodeLink activated microarray slides were covered with a 50-well silicone coverslip and incubated with either control solution C1 (40 mM NaHCO₃ containing 15% glycerol), control solution C2 (50 mM aminoethanol in 100 mM NaHCO₃ containing 15% glycerol), or a solution containing an individual nylon-3 polymer (2 mg/ml in 40 mM NaHCO₃ containing 15% glycerol). The glass slide was incubated in a water-loaded humidifying chamber for 12 h at room temperature, rinsed thoroughly with Milli-Q water, and dried with N₂.

2.3. Cell adhesion and protein adsorption in the presence of serum

NIH 3T3 fibroblasts were cultured in tissue culture polystyrene petri dishes in DMEM containing 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine at 37°C in a 5% CO₂ environment. Cells at 80%–90% confluency were removed from dishes via treatment with 4 mL of 0.05% trypsin and 0.02% EDTA for 3–4 min at 37°C, after which 5 mL of the aforementioned growth medium was added to quench the trypsin. The detached cells were transferred to a 15 mL centrifuge tube and centrifuged at 1500 rpm for 3 min. After the supernatant was removed under vacuum, the cell pellet was resuspended in culture medium to yield a final cell concentration of 1.9×10^5 cell/mL. This cell suspension was added to each well of the nylon-3 polymer-functionalized glass slide to reach a cell concentration of 21,700 cells/cm²; the slide was placed in a petri dish and incubated for 2 h at 37 °C, followed by addition of 30 mL medium into the petri dish to immerse the entire slide. At one day post-seeding, excess medium was removed, and the slide was incubated with 850 µL of a LIVE/DEAD staining solution containing calcein AM (2 µM) and ethidium homodimer-1 (4 µM). Cell adhesion was assessed quantitatively by scanning the whole slide on a GeneTAC UC 4 × 4 scanner (Genomic Solutions) at 512 nm

after 20 minutes of staining and by analyzing data using NIH MacBiophotonics ImageJ (version 1.43m) software.

The adsorption of individual proteins from a complex protein mixture (i.e., FBS) onto nylon-3-modified substrates was measured. For this analysis, 8 μL of DMEM containing 10% FBS was added to each well of the polymer-functionalized glass slide, and the slide was incubated in a humidified chamber at room temperature for 2 h. Each well was washed with 10 μL PBS ($\times 3$), 10 μL Milli-Q water ($\times 1$), and dried with N_2 . The amount of total protein adsorbed following incubation with 10% FBS was assessed via a NanoOrange assay, while the adsorption of individual proteins (collagen, fibronectin, and vitronectin) was assessed via standard immunodetection methods. To quantify total adsorbed protein, 8 μL of NanoOrange working solution was added to each well, and the slide was incubated in a humidified chamber at 37°C for 1 h, cooled to room temperature, and scanned on a GeneTAC UC 4×4 scanner at 595 nm. To quantify the adsorption of collagen from the FBS onto the polymer substrates, samples were incubated with 8 μL mouse anti-collagen primary antibody (1:100 dilution of original solution, 52 $\mu\text{g/mL}$) followed by reaction with 8 μL goat anti-mouse IgG-FITC conjugate secondary antibody (1:50 dilution of original solution, 60 $\mu\text{g/mL}$). Fibronectin and vitronectin were detected in separate wells using mouse anti-fibronectin (1:50 dilution of original solution, 0.29 $\mu\text{g/mL}$) and mouse anti-vitronectin (1:50 dilution of original solution, 0.29 $\mu\text{g/mL}$) primary antibodies, respectively. Each well was washed with 10 μL PBS containing 0.05% Tween 20 (v/v) ($\times 3$), 10 μL Milli-Q water ($\times 1$), dried with N_2 , and scanned on a GeneTAC UC 4×4 scanner at 512 nm. In all cell adhesion and protein adsorption experiments, all polymer samples were evaluated simultaneously on a single slide. Two controls (C1 and C2, described in Section 2.2) were also present on every slide in order to detect experiment-to-experiment variations in fluorescence intensity readings.

2.4. Cell adhesion in a serum-free environment

Cell adhesion experiments were performed in a serum-free environment using the procedures described for the serum-containing environment (Section 2.3), except that the culture medium did not contain FBS and a higher cell seeding density (28,600 cells/ cm^2) was used. In order to examine whether serum-free cell adhesion to the polymers was mediated by cell-surface proteins, 3T3 cells were treated with specific proteases prior to seeding upon the polymer substrates. Cells were detached from culture substrates and centrifuged as described in Section 2.3, and the resulting cell pellet was resuspended in 3 mL serum-free DMEM containing 0.25 μM plasmin or 10 U/mL collagenase (types I and II), and incubated for 15 min at 37°C . The protease-treated cell suspensions were re-centrifuged at 1500 rpm for 3 min to remove the supernatant. The cell pellet was then resuspended in serum-free DMEM to a final concentration of 2.5×10^5 cells/mL. This cell suspension was added into each individual well of the polymer-functionalized glass slide to reach a concentration of 28,600 cells/ cm^2 , incubated, and assayed for adhesion and viability using methods described in Section 2.3.

The adsorption of individual adhesive proteins to the polymers was measured following incubation of the polymer substrates with defined protein solutions. A fresh working solution of each protein was prepared by diluting the original protein solution (as purchased) directly in serum-free DMEM to yield pH 7.4 solutions of fibronectin (44.2 $\mu\text{g/mL}$), collagen type I (17.7 $\mu\text{g/mL}$), and vitronectin (17.7 $\mu\text{g/mL}$). These solutions were then used directly in protein adsorption experiments. Eight microliters of fibronectin solution in serum-free DMEM was added to each individual well of the polymer-functionalized glass slide at a density of 5.05 $\mu\text{g}/\text{cm}^2$, and the slide was incubated in a humidified chamber at room temperature for 2 h. The amount of protein adsorbed in each well was determined via the NanoOrange assay, as described in Section 2.3. This process was repeated with collagen

type I and vitronectin at a coating density of 2.02 $\mu\text{g}/\text{cm}^2$; the higher adsorption efficiencies of these latter two proteins led to approximately equivalent final coating densities for the three conditions.

2.5. PLSR modeling

Polymer chemical composition, physical properties, and cellular adhesion were analyzed by partial least squares regression (PLSR) in SimcaP+ v.12.0.1 (Umetrics; San Jose, CA)³⁰. Chemical properties examined included the number of methyl groups in subunit A, number of carbons in subunit B, the presence or absence of a ring structure in subunit B, the ratio of subunit A to the total number of subunits [A:(A+B)], the total number of subunits, and the polymer molecular weight (M_n). Physical properties included the levels of total protein adsorption and individual protein adsorption for vitronectin (VN), fibronectin (FN) and collagen (Coll) under serum-free and serum-containing conditions (Supplementary Table 1).

In PLSR, the X matrix of independent observations is linearly regressed against the Y matrix of dependent observations. PLSR projects variables in the X and Y matrices onto new dimensions called principal components by using weighted linear combinations of variables in their corresponding matrices. The first principal component captures the strongest variation in the original data matrix, and each succeeding principal component captures remaining variation. The number of principal components that results in the minimum error is used to build the PLSR model. Essentially, the PLSR algorithm will try to find a multi-parameter function in the X-matrix that quantitatively predicts the data observed in the Y-matrix. Two primary metrics were used to determine the relative strength of each model: R^2Y and Q^2Y . R^2Y is the regression coefficient for the model and indicates how well the model fits the data in the Y matrix. Q^2Y is the cross-validated regression coefficient describing the fraction of variation in the Y matrix that is captured by the model and is determined from a leave-one-out cross-validation³¹. By maximizing R^2Y and Q^2Y (to at most 1), the selected model form has the best fit for the data in the training set.

2.6. Statistical methods

Data are presented as mean $\pm$ standard deviation. Statistical analysis of the data was performed using ANOVA with Tukey's HSD post-hoc test with p values less than or equal to 0.05 considered to be statistically significant. Statistical analyses of linear regression fits were performed using ANOVA with p values less than 0.05 considered to be statistically significant.

3. Results and Discussion

3.1 Protein adsorption and cell attachment to nylon-3 polymer-bearing surfaces in a serum-containing environment

The ability of materials to regulate protein adsorption in a serum-containing environment is highly relevant to both in vitro and in vivo studies; in vitro studies are conducted most commonly in the presence of serum-containing media, and in vivo studies necessarily involve a highly complex environment containing a vast number of proteins. Initial studies with 3T3 cells in serum-containing medium involved three groups of nylon-3 copolymers with different subunit identities and proportions: P1 – P4 (MM + CH), P5 – P8 (MM + CO), and P9 – P12 (DM + CH) (Figure 1). Several of these copolymers were previously identified as supportive of 3T3 cell adhesion³². Copolymer P13 (MM + DH), which does not support 3T3 cell adhesion, was included for comparison.

To gain insight on how nylon-3 polymer chemistry influences protein adsorption, we monitored the level of total protein, Coll, FN, or VN that adsorbed to these surfaces from a

serum-containing medium. Our results in Figure 2a–d indicate that the extent of protein adsorption in the presence of serum varied with polymer composition. However, given the numerous variables involved (e.g., monomer identity, ratio of A and B, molecular weight), it is difficult to directly identify the effects of each variable and determine which are the most critical. Indeed, no single chemical property demonstrated a perfect correlation with protein adsorption or cell adhesion outcomes (data not shown). Thus, we elected to examine the potential multivariate relationship among polymer properties using PLSR³³. We built a model with an X matrix consisting of the chemical properties of the nylon-3 polymer compositions and a Y matrix consisting of protein adsorption measurements from the serum-containing experiments (Supplementary Table 2). The model describing the relationship between chemical properties and total serum protein adsorption yielded a good fit and was predictive by cross-validation ($R^2Y = 0.814$, $Q^2Y = 0.652$, Figure 3a). Analysis of the two components demonstrated that the number of methyl groups in subunit A (cationic), number of carbons in subunit B (hydrophobic), and subunit A ratio (defined as $A:(A+B)$) were positively correlated with total protein adsorption (Figure 3b). The number of methyl groups in subunit A was the highest ranked variable in our model, followed by the number of carbons in the subunit B side chain (Supplementary Table 3); when we analyzed the two sub-groups containing one or two methyl groups (i.e., MM or DM), we found that polymers with two methyl groups had significantly more total protein adsorption (175.8 ± 5.1 vs. 153.1 ± 18.5 , $p=0.0066$). Likewise, when we examined the impact of number of side chain carbons in subunit B for polymers with one methyl group in subunit A, we found that six side-chain carbons in subunit B (i.e., CO) resulted in significantly higher protein adsorption than four side-chain carbons (i.e., CH; 170.1 ± 7.7 vs. 141.5 ± 11.6 , $p=0.0084$). Both of these subunit changes increase overall polymer hydrophobicity because both add CH_2 units to the respective subunits. The other chemical properties (the presence or absence of a ring structure in the subunit B side chain, number of subunits, and polymer molecular weight (M_n)) had conflicting contributions in the two components.

We next analyzed the relationship between chemical properties and the adsorption of individual proteins from complete serum onto the nylon-3 surfaces (Supplementary Table 2). As in the model developed to predict only total protein adsorption, the number of methyl groups in subunit A was the most highly ranked variable and projected positively in component 1 (Supplementary Table 3). Therefore, we conclude that the level of protein adsorption is influenced by multiple chemical properties, with increased methyl groups in subunit A (i.e., DM in place of MM) being the most dominant factor to increase adsorption, and other properties fine-tuning the level.

As shown in Figure 2, polymer composition was able to regulate not only protein adsorption, but also cell adhesion. We therefore examined whether the chemical properties of the nylon-3 polymers could be used to directly predict the extent of cell adhesion (Supplementary Table 2). We found that, while models could be fit from this data set ($R^2Y = 0.402$), these models had limited predictive ability ($Q^2Y = 0.293$) and were weaker than our models predicting protein adsorption. Cell adhesion to synthetic materials is typically mediated by adsorbed serum-derived proteins such as collagen, fibronectin, and vitronectin^{34–37}; therefore, we next analyzed whether the protein adsorption patterns that resulted from the nylon 3-copolymer could predict cell adhesion (Supplementary Table 2). Our results indicate that cell adhesion in a serum-containing environment could indeed be more accurately predicted from protein adsorption than from chemical properties ($R^2Y = 0.651$, $Q^2Y = 0.561$).

To further evaluate the relationship between the adsorption of proteins from serum and cell adhesion, cell adhesion was plotted against the adsorption of total and individual proteins derived from the FBS mixture on all thirteen nylon-3 copolymers (Figure 4a–d). Controls

C1 and C2 (see Materials and Methods for details) were not used in this analysis but are shown in Figure 4a–d as unfilled diamonds to enable comparison to nylon-3 polymers. A regression analysis showed that cell adhesion was linearly proportional to total protein adsorption with an R^2 value of 0.64 and a p value of 0.00099 (Figure 4a); in contrast, none of the cell-single protein plots supported a statistically significant linear regression fitting (Figure 4b–d). Alternative fitting approaches were also used (e.g., logarithmic), but did not yield a significant improvement over the linear regression. These findings suggest that different serum proteins may collaborate to mediate cell-polymer interactions either by way of interacting with target cells directly or by indirectly promoting the adsorption and optimizing the conformation of cell-adhesive proteins³⁸.

3.2 Protein adsorption and cell attachment to nylon-3 polymer-bearing surfaces in a serum-free environment

We previously observed that NIH 3T3 cells in serum-free media can adhere to surfaces bearing nylon-3 copolymers³², and confirmed that result in the library of polymers presented here (Figure 5a). Since cell adhesion is typically mediated through adsorbed proteins, the ability of some nylon-3 copolymer compositions to support fibroblast adhesion in the absence of serum was unexpected. It seems unlikely that the sequence- and stereo-random nylon-3 copolymers could interact directly with cell-surface receptors, and we therefore set out to determine the source of cell adhesion under serum-free conditions. As a first step, we developed a PLSR model predicting cell adhesion from polymer chemical properties under serum-free conditions (Supplementary Table 2). This model fit the data ($R^2Y = 0.619$) and was moderately predictive ($Q^2Y = 0.402$). Analysis of the loadings in principal component one showed that adhesion correlated with an increase in methyl groups in subunit A, an increase in the number of carbons in subunit B, the presence of a ring structure, and a higher percentage of subunit A (Figure 5b). Since these chemical properties were also positively correlated with protein adsorption in the serum-containing environment tested earlier (Figure 3b), we hypothesized that proteins were also acting as cell adhesion intermediates in this serum-free environment.

Thus, we investigated whether 3T3 cell adhesion to surfaces bearing nylon-3 copolymers in the absence of serum could be mediated by proteins initially borne by the cells themselves. Adhesive proteins known to reside on cell surfaces include collagen, fibronectin, and vitronectin³⁹. A short protease treatment of cells was used to explore possible roles of these proteins in 3T3 cell adhesion: collagenase selectively degrades collagen, while plasmin degrades both fibronectin and vitronectin but not collagen. NIH 3T3 cells were treated with either collagenase or plasmin immediately prior to being seeded on nylon-3-modified surfaces. As shown in Figure 6, pretreatment with plasmin caused a substantial decrease in cell adhesion, relative to no pretreatment of the cells, for most of the polymer-modified surfaces. The decrease is particularly noticeable for polymers that strongly encourage adhesion (i.e., the majority of polymers used in this study). In many of these cases, plasmin treatment caused 3T3 cell adhesion to decline to the level seen with a relatively non-adhesive polymer (P13). These results suggest that a crucial role is played by fibronectin and/or vitronectin associated with the surface of 3T3 cells in their adhesion to nylon-3-bearing surfaces.

Collagenase pretreatment caused a decrease in 3T3 cell adhesion to many but not all of the polymer-modified surfaces; adhesion on P3, P7 and P12, for example, did not seem to be affected by this pretreatment (Figure 6). Even when collagenase led to a decline in adhesion, this effect was often smaller than the effect exerted by plasmin pretreatment. These results raise the possibility that collagen associated with 3T3 cells can facilitate adhesion to the modified surfaces, but that collagen is generally less effective in this regard than fibronectin and/or vitronectin.

To gain further insight into which individual cell-surface proteins were mediating cell adhesion to nylon-3-modified surfaces, we independently monitored the amount of Coll, FN, or VN that adsorbed to these surfaces from solutions that contained only the protein in question. In contrast to our analysis in Figure 2, these experiments examined individual protein adsorption in the absence of serum competition. From these results in Figure 7a–c, we confirm that the regulation of protein adsorption by chemical features of the polymer followed the same trend identified in the serum-containing environment. Comparing (MM + CH) to (DM + CH) shows that the addition of a single CH₂ group to the cationic subunit of the polymer increased the adsorption of individual proteins, as well as cell adhesion. Similarly, increasing the size of the ring structure in the hydrophobic subunit – going from (MM + CH) to (MM + CO) – increased the adsorption of individual proteins.

To further evaluate the relationship between individual cell-surface proteins and cell adhesion in a serum-free environment, we plotted cell adhesion against the adsorption of Coll, FN, and VN, respectively, on all thirteen polymers (Figure 8a–c). Controls C1 and C2 (see Materials and Methods) were not used in this analysis but these data are shown in Figure 8a–c as unfilled diamonds to provide a comparison to nylon-3 polymers. Cell adhesion was linearly proportional to Coll adsorption ($R^2=0.44$, $p=0.014$, Figure 8a), FN adsorption ($R^2=0.59$, $p=0.0022$, Figure 8b), and VN adsorption ($R^2=0.40$, $p=0.02$, Figure 8c), with FN exhibiting the best correlation.

Combining the results of the cell surface protein degradation experiments (Figure 6) and linear regression fitting of cell adhesion–protein adsorption (Figure 8), we conclude that the interactions between 3T3 cells and surface-immobilized nylon-3 copolymers under serum-free conditions are mediated by cell-surface proteins, with FN exerting a somewhat stronger effect than either Coll or VN³⁹.

4. Conclusions

The peptide-mimetic backbone structure of nylon-3 polymers has motivated us to explore these biomaterials as substrates for cell culture and tissue engineering applications. Through a combined experimental/computational approach, we found that specific chemical properties of cationic/hydrophobic nylon-3 copolymers exhibited a significant correlation with protein adsorption, which, in turn, was correlated with 3T3 fibroblast adhesion. While the proteins in the serum-containing environment originated from the serum, as expected, the proteins that enabled cell adhesion under serum-free conditions most likely originated on the surfaces of the cells themselves.

Nylon-3 copolymers are highly tunable, based on variation of the structures, stereochemistries, and proportions of subunits, average chain length and other characteristics. Thus, these materials should be useful as a platform for quantitatively examining how specific chemical features regulate the interactions between synthetic materials and their biological environments. Moreover, the cooperative relationship between substrate chemistry and protein adsorption identified by our analysis suggests that cell-material interactions can be further refined in a quantitatively predictable manner via manipulation of the protein environment. The importance of cell-surface proteins in serum-free cell adhesion demonstrated by our findings also supports the use of mild cell detachment or trypsinization conditions in future studies. Although the set of nylon-3 copolymers used for these studies is relatively modest in size, the approach we have employed may be expanded by evaluating a broader diversity of nylon-3 copolymers, enabling further development of the computational model that correlates polymer chemistry with biological outcomes. A robust model should ultimately enable the rational development of new polymers with optimized or diversified biological functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(a) β -Lactam monomers used to construct nylon-3 polymers. (b) Nylon-3 copolymers used in this study. All polymers are random in subunit sequence and stereochemistry, composed of a cationic subunit (monomethyl (MM) or dimethyl (DM)) and a hydrophobic subunit (cyclohexyl (CH) or cyclooctyl (CO)), or a hydrophilic subunit (dihydroxyl (DH)). Polymers P1 to P12 correspond to previously reported Pol-1 to Pol-12, but P13 corresponds to previously reported Pol-15²⁴.

Figure 2.

Amount of (a) total serum protein, (b) collagen, (c) fibronectin, and (d) vitronectin adsorption, as well as (e) cell adhesion upon nylon-3 polymers with different subunit pairings ((MM + CH, black), (MM + CO, white), and (DM + CH, striped)) and different cationic subunit content (MM or DM) in a serum-containing environment (n=3 per condition). In (a), the fluorescence intensity is based upon NanoOrange staining; in (b)–(d), the fluorescence intensity is based upon immunological staining for individual adsorbed proteins; in (e), the fluorescence intensity is based on staining for live cells. (**) p<0.05 compared to polymers with same composition containing 60% and 70% MM or DM; (*) p<0.05 compared to polymer with same composition containing 60% MM or DM; (^^) p<0.05 compared to the other two polymers within each % MM/DM subgroup; (^) p<0.05 compared to the lowest protein or cell attractive polymer within each % MM/DM subgroup.

Figure 3.

(a) Results of PLSR model using chemical properties to predict total protein adsorption. (b) Loadings plot of the PLSR model. In (b), “# mers” refers to the number of subunits in each polymer, “Sub A # methyl” refers to the number of methyl groups in subunit A, “Sub B #C” refers to the number of carbons in the subunit B side chain; “Sub B ring” refers to the presence or absence of a ring structure in subunit B, and “Sub A ratio” is the ratio of subunit A to the sum of subunits A+B.

Figure 4.

Relationship between protein adsorption and cell adhesion upon nylon-3 polymer-modified surfaces in a serum-containing environment for (a) total serum protein, (b) collagen, (c) fibronectin, and (d) vitronectin, all derived from treatment of polymer-bearing surfaces with a solution of FBS. Each data point represents one polymer; $n=3$ samples per condition. The X axis in (a) represents fluorescence intensity of NanoOrange staining for adsorbed total serum protein; in parts (b), (c), and (d), the X axis represents fluorescence intensity of immunological staining for Coll, FN, and VN adsorption, respectively. The Y axis in all panels represents the fluorescence intensity of cell viability staining. $p = 0.00099$, 0.11 , 0.14 and 0.073 respectively for (a), (b), (c) and (d) via ANOVA analysis of regression. C1 and C2 (unfilled diamonds) are controls that were not used in the linear regression.

Figure 5.

(a) Cell adhesion upon nylon-3 polymers in a serum-free environment; fluorescence intensity is based on staining for live cells ($n=3$ per condition). (b) Loadings plot for PLSR model composed of a X matrix of chemical properties regressed against a Y matrix of cell adhesion in a serum-free environment. (**) $p<0.05$ compared to polymers with same composition containing 60% and 70% MM or DM; (*) $p<0.05$ compared to polymer with same composition containing 60% MM or DM.

Figure 6.

Adhesion of cells to nylon-3 polymers in a serum-free environment following targeted degradation of cell-surface proteins via treatment with plasmin or collagenase immediately prior to adding the cell suspension to polymer-functionalized glass surfaces (n=3 per condition). (**) $p < 0.05$ compared to other two conditions within the same grouping; (*) $p < 0.05$ compared to the lowest value within the same grouping.

Figure 7.

Amount of (a) collagen, (b), fibronectin, and (c) vitronectin adsorption upon nylon-3 copolymers pairs of subunits ((MM + CH, black), (MM + CO, white), and (DM + CH, striped)) and different cationic subunit content in a serum-free environment. Fluorescence is based upon NanoOrange staining; n=3 per condition. (**) p<0.05 compared to polymers with same composition containing 60% and 70% MM or DM; (*) p<0.05 compared to polymer with same composition containing 60% MM or DM; (^^) p<0.05 compared to the other two polymers within each % MM/DM subgroup; (^) p<0.05 compared to the lowest protein polymer within each % MM/DM subgroup.

Figure 8.

Relationship between protein adsorption and cell adhesion for 3T3 cells cultured upon nylon-3 polymer-modified surfaces in a serum-free environment. Each data point represents one polymer; $n=3$ samples per condition. The X axes in (a), (b), and (c) represent the fluorescence intensity of NanoOrange staining for adsorbed Coll, FN, and VN respectively. The Y axis in all panels represents the fluorescence intensity of cell viability staining. $p = 0.014$ (a), 0.0022 (b) and 0.020 (c) according to ANOVA statistical analysis of regression. C1 and C2 (unfilled diamonds) are controls that were not used in the linear regression.