Correlation of the Adhesive Properties of Cells to *N*-Isopropylacrylamide/*N*-*tert*-Butylacrylamide Copolymer Surfaces with Changes in Surface Structure Using Contact Angle Measurements, Molecular Simulations, and Raman Spectroscopy

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A series of copolymers of *N*-isopropylacrylamide (NIPAM) and the more hydrophobic comonomer *N*-*tert*-butylacrylamide (NTBAM), with increasing NTBAM content (i.e., increasing hydrophobicity) were prepared. The adhesion of human epithelial cells on polymer films prepared from copolymers of NIPAM: NTBAM was observed to increase with increasing polymer hydrophobicity. However, in the absence of serum, cell adhesion to the different surfaces was statistically indistinguishable. Thus, it appears that the copolymer films differentially support cell adhesion due to selective adsorption of proteins from the physiological environment (the serum). Using contact angle measurements, molecular simulations, and Raman spectroscopy to characterize the different surfaces, we show evidence that the different behavior of cells on the films of increasing hydrophobicity is actually due to the different chemical properties of the surfaces with increasing content of NTBAM in the copolymers. As the NTBAM content is increased, the number of NH residues at the surface decreases, due to the additional steric hindrance of the bulkier NTBAM group, which results in decreased hydrogen bonding and thus decreased adsorption of proteins such as albumin. However, in some cases, the adsorption is driven by hydrophobic interactions, and proteins such as fibronectin were found to adsorb more to the films with a higher content of NTBAM. There appears, thus, to be a direct correlation between surface composition, i.e., the functional groups exposed at the surface, and protein binding and subsequent cell adhesion.

Introduction

The response of biological tissue to polymer materials in vivo is one of the most fundamental and complex challenges of modern restorative medicine. Whether a material is biocompatible or not is determined partly by the properties of the material surface that interact with the surrounding cell or tissue. To promote tissue formation and integration, a biocompatible material provides a surface which enables cell adhesion, spreading, proliferation, and migration. Understanding and controlling cellular responses to interactions with materials is one of the key issues in the development of biomaterials.[1]

The properties of a polymeric material which influence cell interactions include surface charge, surface hydrophobicity, surface texture, and surface energy.[2] Along with these surface physicochemical characteristics, individual functional groups have also been demonstrated to effect protein adsorption and cellular response.[3−6] Tengvall et al. demonstrated that surfaces displaying CH_3 groups bound proteins more firmly in comparison to surfaces displaying OH groups.[5] This study also found differences with respect to the amounts of individual proteins adsorbed to the respective surfaces, with fibrinogen adsorbing more to CH_3 surfaces. Gessner et al. hypothesized that differences with respect to protein adsorption on surfaces with similar hydrophobicities were due to the presence of different surface chemical groups.[7] Surface functional groups have also been documented as having an effect on cell−biomaterial interaction.

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For example, tissue culture polystyrene (TCP) displays greater cell adhesive and spreading properties than would be expected on the basis of its surface hydrophobicity alone. These improved cellular interactions are believed to occur due to the increased concentration of hydroxyl groups on the surface of the treated polystyrene.[3] The relative importance of many other functional groups has been investigated with relation to cellular interaction and biocompatibility. Lindblad et al. compared the influence of methyl and hydroxyl groups in vitro and in vivo and demonstrated that hydroxyl groups promoted cellular interaction in vitro in comparison to methyl groups.[6] In vivo, however, methyl groups were found to cause a heightened immune response through the increased adhesion of inflammatory cells. McClary and Grainger investigated cellular responses including adhesion, growth, and cytoskeletal organization, using surface adhesion molecules (SAMs) with differing terminal groups (CH_3 and COOH).[8] This study revealed that cells on CH_3 surfaces displayed reduced cell adhesion, growth, and spreading in comparison to cells on COOH surfaces. Morphological differences were attributed to differences in cytoskeletal organization and the reduced activation of Rho A, which is important for the formation of stress fibers within cells. Tang et al. investigated the effect of a number of other functional groups on immune response and also found that surface functionalities do have an effect on cellular response but argued that functionalities alone might not be sufficient to control the response.[9]

Recently, thermoresponsive polymers such as poly(*N*-isopropylacrylamide) (poly(NIPAM)) have been shown to have reversible interactions with cells. This polymer exhibits a lower critical solution temperature of 32 °C and is insoluble in water at temperatures in excess of this value. This temperature responsiveness leads to an interesting application whereby cells adhere to the polymer surface at temperatures in excess of 32 °C and are spontaneously released at temperatures below this value,[10] providing an elegant and mild method to harvest cells.[11,12] Much work has been done to increase the rate of detachment of cell sheets from the polymer surfaces, with the most efficient methods being to prepare the polymer layers on porous substrates,[13] or to add hydrophilic comonomers, both of which increase the rate of rehydration of the polymer films. Lowering the temperature at which the rehydration occurred was also considered as a possibility, via the addition of hydrophobic comonomers.[14] However, lower rehydration (transition) temperatures were found to result in slower release of the cell sheets. Additionally, it was found that holding the cells for the long times required for detachment at lower temperatures led to deterioration of cellular metabolic functions, making such copolymers unsuitable for release of cell sheets.[14] Thus, hydrophobic copolymers of PNIPAM are not suitable for use in tissue cell-sheet engineering.

N-isopropylacrylamide N-*tert*-butylacrylamide

Figure 1. Structures of the NIPAM and NTBAM monomers.

On the other hand, there have been many studies which show that there is a sigmoidal relationship between hydrophobicity and cell adhesion, with the amount of cell adhesion increasing with increasing surface hydrophobicity.[15] Since cells are known to adhere to poly(NIPAM) films,[16] the development of a series of copolymers of NIPAM and the more hydrophobic comonomer *N-tert*-butylacrylamide (NTBAM) was seen as a logical avenue to acquire more systematic information about the effect of increasing surface hydrophobicity on polymer surface/cell interaction.[17,18] To this end, three copolymers were prepared, containing 15, 35, and 50% of the NTBAM comonomer respectively, thus increasing the hydrophobicity somewhat without becoming too hydrophobic. The hydrophobicity of the copolymer surfaces was estimated from measurements of the contact angle with water and the results showed a small but significant increase of contact angle with increasing amounts of NTBAM in the polymer,[18] due to the extra CH_3 group present in the NTBAM monomer compared to the NIPAM monomer, the structures of which are shown in Figure 1.

This modest change in hydrophobicity resulted in a significant increase in the amount of cells adhered to the surfaces with increasing NTBAM content.[18] However, in the absence of serum, the amount of cell adhesion to the different surfaces was statistically indistinguishable.[19] This is significant, as it implies that the different cell adhesion profiles in the presence of serum result from differential adsorption of proteins by the copolymer films, leading to the establishment of altered cell-signaling pathways and ultimately modified numbers of adhered cells. This leads to the question as to whether the different behavior is a result simply of the increased hydrophobicity or is a more complex response to the change in chemical composition of the surface in the presence of NTBAM.

In view of these results, the logical next step would appear to be to obtain information about the specific surface properties of these copolymers, and how the surface properties change upon increasing the NTBAM content in the films. In this article, we present the results of the investigation of

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the components of the surface free energy of the surfaces with increasing NTBAM content, a Raman spectroscopic investigation of the film surfaces, and molecular simulations of the effect of increasing NTBAM content on the solvent-accessible surface area. Cell and protein adhesion to the different surfaces are correlated to the changes in the surface properties (free energy or composition) with increasing NTBAM content to obtain information about the specificity of cell−polymer interactions.

Experimental Section

Copolymer Preparation and Molecular Weight Determination. *N*-isopropylacrylamide (NIPAM) monomer (purity >99%) from Phase Separations Ltd. (Clwyd, U.K.) and *N-tert*-Butylacrylamide (NTBAM) from Fluka (Dorset, England) were recrystallized twice from hexane before use. The NIPAM/NTBAM copolymer series (100% poly(NIPAM), 85:15 NIPAM/NTBAM, 65:35 NIPAM/NTBAM, 50:50 NIPAM/NTBAM, and 100% poly(NTBAM)) were prepared by free radical polymerization in benzene using AIBN (*N*,*N*-azobisisobutyronitrile) as initiator, as described previously.[17]

The molecular weight of the polymers (number average and weight average) was determined using combined refractive index and light scattering. The setup consisted of four styragel columns (10^5, 10^4, 10^3, and 500 Å) connected to a Wyatt Optilab 903 Interferometric Refractometer and to a Wyatt Dawn DSP laser photometer. The mobile phase was THF, the flow rate was 0.45 mL/min, and 200 μL of 1 wt % polymer in THF was injected. The average molecular weights were in the range 2.1×10^5 to 1.4×10^6 and the polydispersities were approximately 2.

Preparation of Polymer and Copolymer Films. The polymer films were prepared from 5 wt % polymer solution in dry ethanol. Controlled amounts of the polymer solution were spread on the surfaces to result in 5-μm-thick polymer layers. For the contact angle and Raman measurements, the films were cast on microscope slides. For the cell and protein adhesion studies, the films were cast onto 96-well TCP plates. The ethanol was allowed to evaporate in a laminar-flow hood (or in covered Petri dishes for the contact angle and Raman studies), creating 5-μm-thick polymer films.

Contact Angle Measurements. Static contact angle measurements on the polymer (copolymer) surfaces were performed using a contact angle goniometer (A-100 Ramé-Hart, Mountain Lakes, NJ) at 37 °C using a temperature control cell. Between 9 and 36 contact angle measurements were performed for each liquid and polymer (copolymer) film. Three standard reference liquids were chosen: water (Milli-Q PLUS unit, Millipore Corp., Bedford, MA), ethylene glycol (99.5%, AnalaR from BDH), and diiodomethane (synthesis grade, Merck Schuchardt) were used as supplied. Water is bipolar (both a Lewis acid and Lewis base), ethylene glycol is monopolar basic (the acidic property is negligible and the basic property is appreciable), and diiodomethane is apolar (exhibits neither acidic nor basic properties). Thus, the liquids have a spread of properties, enabling separation of the individual components contributing to the surface free energy of the copolymer surfaces.

The surface free energy can be represented as a sum of contributions from each of the various types of specific interactions, such as van der Waals forces, polar interactions, induction forces, and hydrogen bonding.[20] A number of attempts have been made to distinguish the relative importance of the different contributions to the interactions between surfaces with significant polar constituents, some of which ended in a blind alley,[21] or were vehemently contested.[22,23] Fowkes[24] suggested that all the terms attributable to apolar interactions (i.e., dispersion, or van der Waals interactions) be replaced by a single dispersion term, γ^d, and that all the terms attributable to the different kinds of polar interactions should be replaced by a single term, γ^{AB}, referring to Lewis acid−base interactions

$$\gamma = \gamma^d + \gamma^{AB} \tag{1}$$

where the latter can be further divided into the acid and base parts, γ^+ and γ^-

$$\gamma^{AB} = \sqrt{2(\gamma^+\gamma^-)} \tag{2}$$

One important limitation of eq 1 is that the terms on the right side are always interdependent. The individual components of the surface energy of a solid can be estimated by measuring the contact angles with several reference liquids which differ in terms of their interaction type (i.e., dipolar, monopolar, apolar, as described above)

$$(1 + \cos\Theta)\gamma_L = 2[(\gamma_S^d\gamma_L^d)^{1/2} + (\gamma_S^+\gamma_L^-)^{1/2} + (\gamma_S^-\gamma_L^+)^{1/2}] \tag{3}$$

for which the values of the corresponding surface energy components are known.[25] Here γ_L and γ_S correspond to the liquid and solid surface energies, respectively. Obviously, the result of such determinations depends explicitly upon the reference parameter set assigned to the test liquids.

The surface energy components of the reference liquids used in this study are shown in Table 1. Further complications arise when test liquids interact with the substrate in a way that differs from ideal wetting, causing, for instance, its swelling or dissolution.

Table 1. Surface Energy Components (mJ/m^2) of the Test Liquids

test liquid	γ_L	γ_L^d	γ_L^p	γ_L^+	γ_L^-
water	72.8	21.8	51.0	25.5	25.5
ethylene glycol	48.0	29.0	19.0	1.92	47.0
diiodomethane	50.8	50.8	0	0	0

Raman Spectra. Raman spectra were measured in backscattering geometry using an Instruments SA Labram 1B Raman microspectrometer, equipped with an argon laser with an output of 514.5 nm and a cooled CCD detector. An Olympus BX microscope with 100× lens were used. A total of 15 accumulations of 20-s scans were conducted for each spectrum between 400 and 4000 cm^{-1}.

Cell Adhesion. Human epithelial (HeLa) cells were seeded onto either TCP or copolymer films of NIPAM/NTBAM and subsequently allowed to adhere. Medium was removed from individual wells at each time-point and the numbers of nonadherent cells were determined by cell counting using a haemocytometer. Adherent cells were washed with pre-warmed (37 °C) phosphate buffer saline (PBS) to remove any excess medium. The number of adherent cells was also determined by cell counting.

Protein Adsorption. To analyze protein adsorption on the surface of the various substrata, TCP dishes and NIPAM/NTBAM copolymer films were exposed to DMEM (Dulbecco's modified Eagle's medium) supplemented with either 10% FCS (fetal calf serum), albumin (500 mg/mL), or fibronectin (50 mg/mL). After 6 h protein-containing solutions were removed and surfaces were gently rinsed with pre-warmed (37 °C), double-distilled water. The adsorbed proteins were desorbed by protein solubilizing solution

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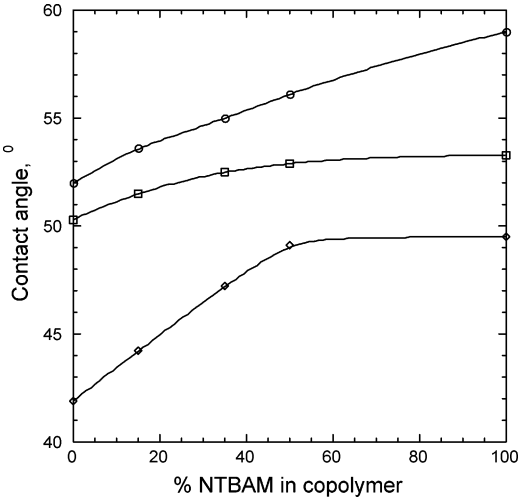

Figure 2. Contact angles of three liquids on pure polymer and copolymer films of NIPAM/NTBAM at 37 °C as a function of increasing NTBAM content: (○) water; (□) ethylene glycol; (◇) diidomethane.

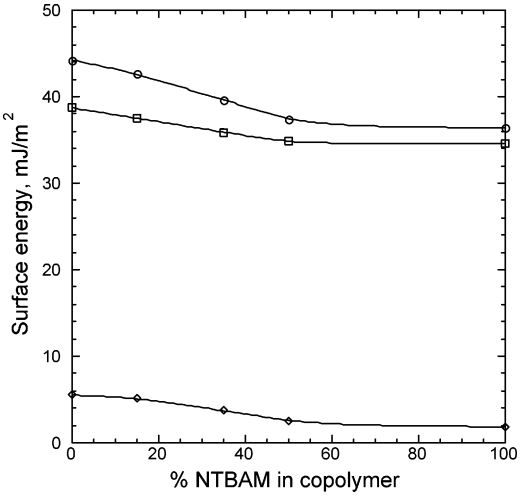

Figure 3. Surface energy contributions for pure polymer and copolymer films of NIPAM/NTBAM at 37 °C plotted as a function of increasing NTBAM content: (○) total surface energy; (□) van der Waals part of surface energy; (◇) acid−base part of surface energy.

(3% (w/v) SDS, 1 mg/mL DTT in 8 M urea), overnight at 37 °C. Subsequently, desorption solution was removed and the desorbed proteins were concentrated using Centricon YM-30 filters (Millipore, Malborough, MA). Protein was resuspended in 500 mL of 1.0 M Tris pH 7.4. Protein amounts were determined using a micro BCA assay. A 25-μL portion of each sample was added to a 96-well microplate (in duplicate), followed by 200 μL of BCA reagent, and was incubated for 60 min at 37 °C. Absorbance was measured at 590 nm in a Dynatech MRX5000 multiwell plate reader (Dynatech Laboratories, Chantilly, VA). A calibration curve was made with the provided BSA standards. The unknown protein concentration in each sample was extrapolated from the standard curve.

Results

Contact Angle and Surface Energy Measurements. The contact angle values measured in each of the three reference liquids are plotted as a function of increasing NTBAM content in Figure 2. As expected, the contact angle in water increases gradually with the gradual change from 100% poly(NIPAM) to 100% poly(NTBAM), in accordance with earlier results. A similar trend, of increasing contact angle with increasing NTBAM content, was also observed with EG and DIM. From the measurements shown in Figure 2 it can be seen that the apolar contribution to the contact angle is the most affected by the presence of higher amounts of NTBAM, since the contact angles in DIM, the apolar solvent, are most affected by the increase in NTBAM content. This suggests that van der Waals type interactions become stronger on the more hydrophobic surfaces.

As described above, the components of the free energy can be calculated using the two-component method of Good et al. The van der Waals and acid−base components of the free energy were calculated, and are plotted in Figure 3 along with the total free energy, as a function of the NTBAM content. As can be seen, it is the acid/base contribution that displays the most significant decrease with increasing amounts of NTBAM in the film, showing a 3-fold decrease from 100% PNIPAM to 100% NTBAM (see also Table 5 for the actual numbers). This suggests that the increasing amounts of NTBAM in the copolymers result in a decrease in the importance of hydrogen bonding type interactions.

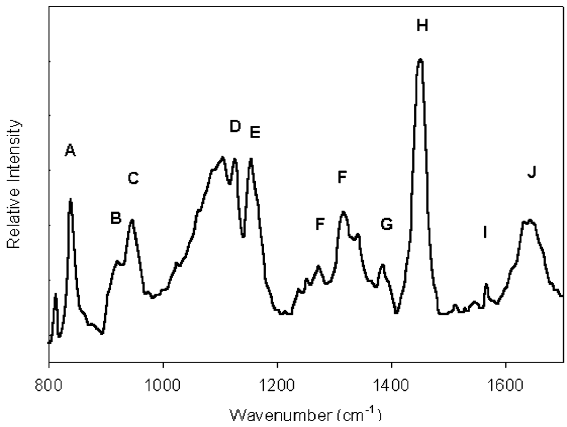

Figure 4. Raman spectrum of 100% poly(NIPAM) films at 25 °C.

Table 2. Assignments of Raman Peaks of Poly(NIPAM) Films Shown in Figure 4

indicator	wavenumber (cm^{-1})	assignment
A	836	CH_3 twist[26]
B	920	CH_3 rock,[27] (*tert*-butyl)
C	945	CH_3 rock,[27] (*iso*-propyl)
D	1105	C−C skeletal in-phase stretch[26,28]
E	1145	N−C bond stretch[27]
F	1273, 1314	doublet of overlapping C−N and C−H[27]
G	1383	CH_3 bend[28]
H	1450	CH_3 bend (scissors deformation)[29]
I	1540	amide 2 N−H deformation
J	1642	amide 1 C═O stretch[29]

Raman Spectra. *800 to 1600 cm^{-1} Region.* The Raman spectrum of 100% poly(NIPAM) in the frequency range 800−1700 cm^{-1} is presented in Figure 4. The peak assignments are detailed in Table 2.

Figure 5 shows the Raman spectrum of 100% poly(NTBAM). The peak assignments of the major peaks in the poly(NTBAM) film are given in Table 3.

There are some noticeable differences between the spectra of the two polymers, and it can be seen that there are some

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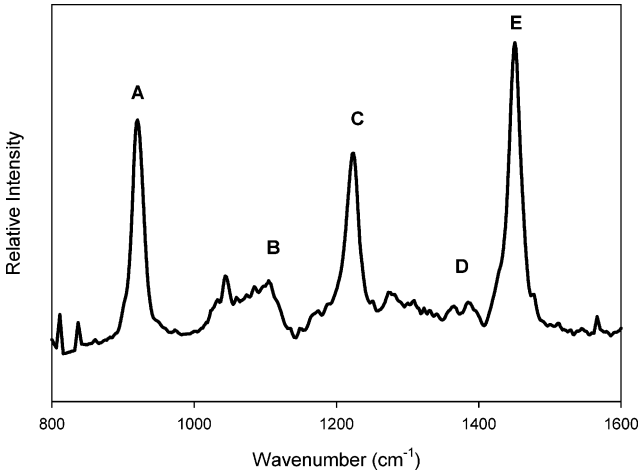

Figure 5. Raman spectrum of 100% poly(NTBAM) film at 25 °C.

Table 3. Assignments of Poly(NTBAM) Polymer Peaks Found in Figure 5

indicator	wavenumber (cm^{-1})	assignment
A	920	*tert*-butyl CH_3 rock
B	1105	C−C skeletal in-phase stretch[26,28]
C	1250	N−C bond opening stretch[27]
D	1368, 1390	*tert*-butyl interaction between CH_3 groups[27]
E	1450	CH_3 bend (scissors deformation)[28]

identifying peaks for each polymer type. The presence and intensity of the 920 cm^{-1} peak is indicative of the concentration of NTBAM in the copolymer. This is associated with the CH_3 rock vibration and is particularly marked at this wavenumber in the *tert*-butyl group. There is a corresponding CH_3 rock resulting in a peak at 945 cm^{-1} for the isopropyl group in the pure NIPAM system. These peaks are clear and identifiable and can be of use in a mixed polymer system. Figure 6 shows the Raman intensities for pure poly(NIPAM), pure poly(NTBAM), and the copolymers of 85:15, 65:35, and 50:50 in the region 800−1600 cm^{-1} where these differences are clearly visible.

The changing copolymer nature is also reflected in the comparison of the intensities of the peaks at 920 cm^{-1} and at 945 cm^{-1} with respect to the peak at 1450 cm^{-1}. The 1450 cm^{-1} peak is used as a reference peak, as it appears in all spectra with good intensity. The 920 cm^{-1} peak is related to the amount of NTBAM present, and the 945 cm^{-1} peak is related to the amount of NIPAM present in the copolymers, as described above. To clarify the relationships between these changing peaks, the intensities of the 1450, 920, and 945 cm^{-1} are fitted to a Gaussian−Lorentzian function and the amplitude of the fitted peaks at 920 cm^{-1} and 945 cm^{-1} are expressed as a ratio of the 1450 cm^{-1} peak. Figure 7 shows a graph of the intensities expressed as a ratio with the intensity of the 1450 cm^{-1} peak as the NTBAM content is increased. It can be seen that there is a large increase in the 920 cm^{-1} peak corresponding to increasing NTBAM content, and to a lesser extent, a decrease in the intensity of the 945 cm^{-1} peak corresponding to the decreasing amount of NIPAM. These observations show good agreement with a linear dependence of the peak ratios on the NTBAM content. A more detailed analysis using, for example, 2-D correlation

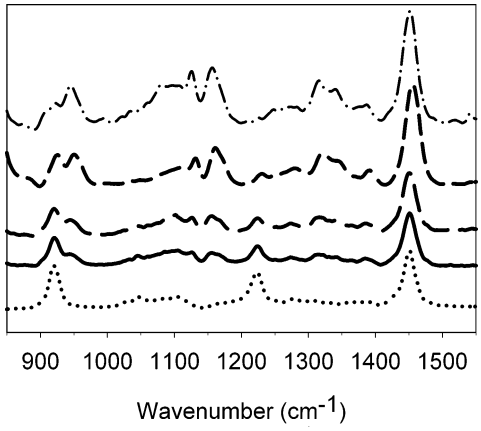

Figure 6. Raman intensities in 800−1600 cm^{-1} region at 25 °C for (from top to bottom): poly(NIPAM), 85:15, 65:35, 50:50, and poly(NTBAM) films, respectively. Note change in peaks at 900−950 cm^{-1}. Spectra are separated for illustrative purposes.

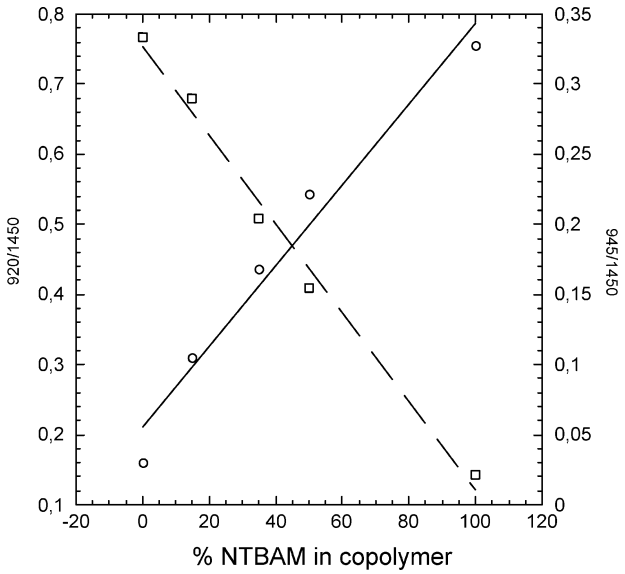

Figure 7. Ratio of intensity of peaks at 920 and 1450 cm^{-1} (○) corresponding to NTBAM; and 945 and 1450 cm^{-1} (□) corresponding to NIPAM, as a function of NTBAM content in the copolymer film.

spectroscopy could be employed on a larger data set for further elucidation.

2800−3200 cm^{-1} Region. Figure 8 shows the scattering intensities of the peaks at 2875 and 2924 cm^{-1}. As described previously[28,29] the peak at 2924 cm^{-1} is attributed to CH_2 asymmetric stretching and is a backbone vibration of the polymer. This is verified by showing that the intensity of this peak does not vary with the side chain type in Figure 8. However, there is a decrease in the 2875 cm^{-1} stretch on increasing the amount of NTBAM. Assigned to the single C−H stretch it is normally weak in IR spectra but shows well in Raman and is indicative of the decreasing amount of isopropyl groups present at the surface of the polymer with increasing NTBAM content.

In expressing the ratios of the intensities of these two peaks (at 2924 and 2875 cm^{-1}), a correlation is found as shown in Figure 9. This indicates that the intensity of the 2875 cm^{-1}

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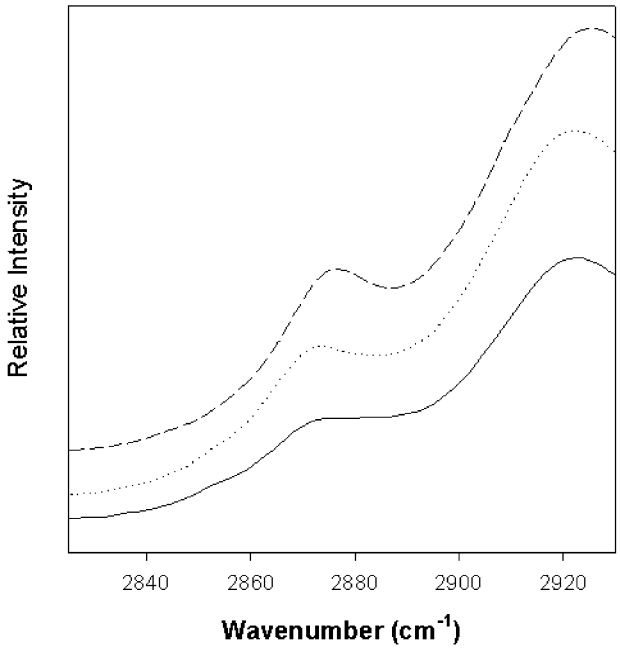

Figure 8. Decreasing intensity of 2875 cm^{-1} peak on increasing proportion of NTBAM in (upper spectrum to lower) 85:15, 65:35, and 50:50 NIPAM:NTBAM copolymers. Spectra are separated for illustrative purposes.

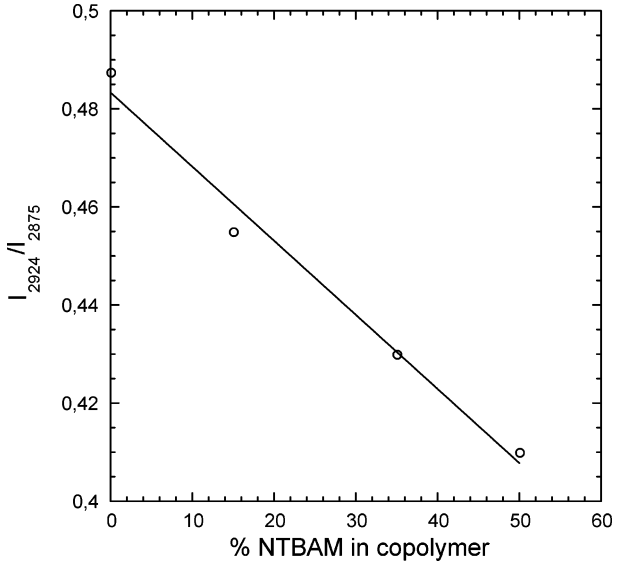

Figure 9. Plot of the ratios of the peaks at 2924 and 2875 cm^{-1} versus percentage NTBAM in the copolymer films.

peak is directly proportional to the percentage of NTBAM present at the surface of these films. This method can directly assess the proportion of C−H groups at the polymer surface, and it can be seen that the amount of isopropyl groups at the surface decreases with increasing NTBAM content. This may be of advantage in biomedical applications where the nature of the functional group at the surface is of importance in assessing cellular adhesion and growth.

Molecular Simulations. Initial chain conformations were generated using the random flight generator whereby the polymer chain is grown in a stepwise manner by adding links to one end.[30] For each new link added, bond length and valent angle restrictions apply, but the torsional angle defining the link orientation relative to the rest of the chain is randomized.

Table 4. Solvent-Accessible Surface Areas and Solvation Free Energies of Polymer Chains Calculated Using the OMNISOL Program[37]

chain composition (12 link chain)	solvent-accessible surface area, nm^2	solvation free energy, kcal/mol	
		water	octane
100/0 NIPAM/NTBAM	17.8	−88.1	−85.2
50/50 NIPAM/NTBAM	18.4	−71.5	−77.6
0/100 NIPAM/NTBAM	19.3	−68.3	−77.3

Only self-avoiding conformations are permitted. Although no conformational energy searching was attempted, a sufficient number of initial conformations was generated in order to afford a meaningful statistical average.

Each chain has been placed in close proximity to a $Si_{400}O_{800}H_{336}$ cluster (with Si and O atoms arranged in the lattice of cristobalite) used to simulate the silica surface, and the chain configuration has been optimized using the MM2 method of molecular mechanics.[31] The cluster geometry was fixed. It should be recognized, however, that the optimized chain conformations were essentially static, whereas in reality, the polymer chains are continuously moving around. To take into account this effect, molecular dynamics simulations are normally carried out. However, since the MM2 field fails to accurately simulate the molecular environment (solvation or intermolecular interactions) whereas the use of more realistic interaction potentials is prohibited by enormous computational demands, only static chain conformations have been considered in this study.

The free energy of hydration of the polymer chains has been calculated using the method of geometry-dependent atomic surface tensions with implicit electrostatics developed and implemented in the OMNISOL program by Hawkins, et al.,[32] and later rationalized and ported on a PC platform by one of us.[30]

The molecular simulations seem to indicate that the poly(NIPAM) packing is able to accommodate NTBAM up to 50% before a modification of the solid structure takes place. Second, the addition of the NTBAM leads to decreased surface exposure of the N−H groups, even at small additions of NTBAM, despite the unmodified NIPAM polymer structure. Furthermore, the results suggest that the modified structure at NTBAM content in excess of 50% does not change the exposure of the N−H groups in a sudden manner. As the content of NTBAM groups exceeds 50%, a noticeable decrease in the solvation free energy of the polymer chain is observed even though the overall solvent-accessible surface area steadily increases on moving from poly(NIPAM) to poly(NTBAM) surfaces. The fact that the surface energy of polymer-coated surfaces decreases with increasing NTBAM fraction (as shown in Table 4) can be explained by better shielding of the amide groups (N−H groups) by *tert*-butyl groups than by isopropyl groups.

The molecular simulations also show that the sterical repulsion between *tert*-butyl groups is slightly higher than that between isopropyl groups, resulting in a looser backbone conformation in *tert*-butyl substituted chains at the surface,

(30) Zhmud, B.; Poptoshev, E.; Pugh, R. J. *Langmuir* **1998**, *14* (13), 3620.
(31) Lipkowitz, K. B. *QCPE Bull.* **1992**, 6.
(32) Hawkins, G. D.; Cramer, C. J.; Truhlar, D. G. *J. Phys. Chem.* **1997**, *101*, 7147.

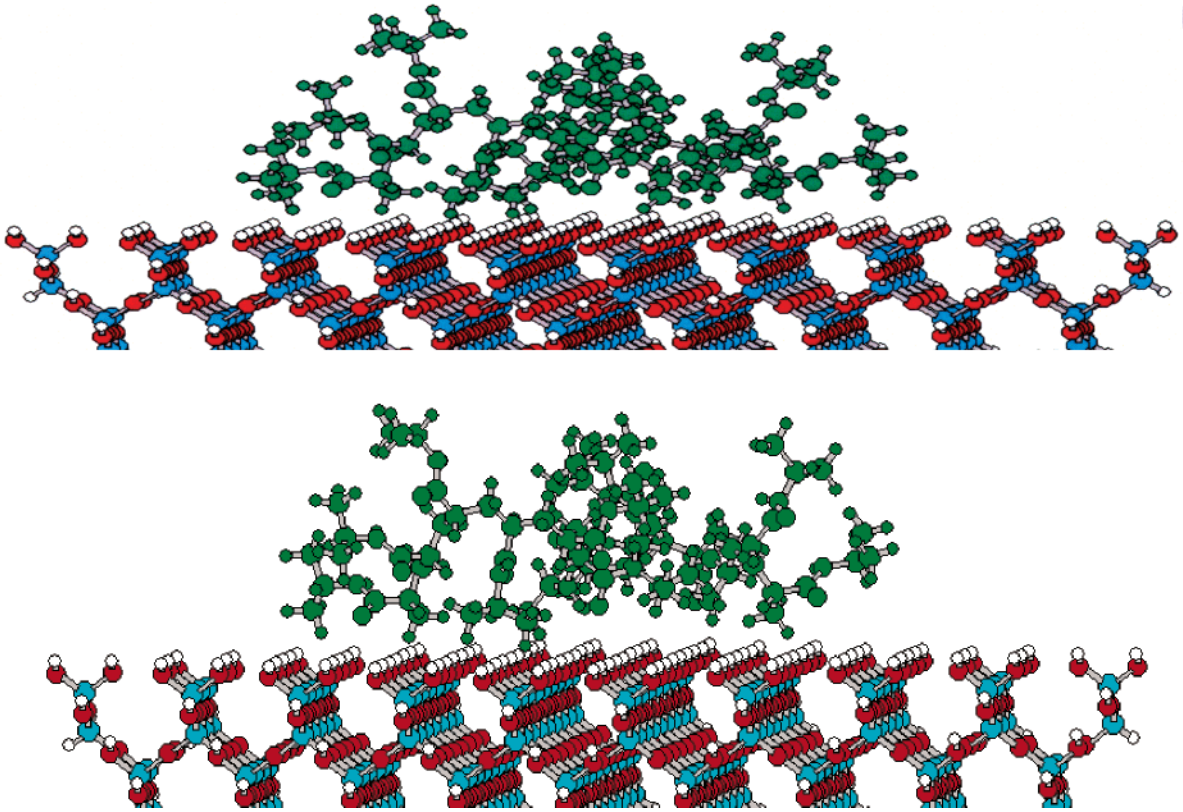

Figure 10. Molecular model of 12-link poly(NIPAM) (top) and poly(NTBAM) (bottom) chains adsorbed to the surface of silica. The surface of silica has been represented by fully hydroxylated $Si_{400}O_{800}H_{336}$ cluster with Si and O atoms arranged in the lattice of β-cristobalite.

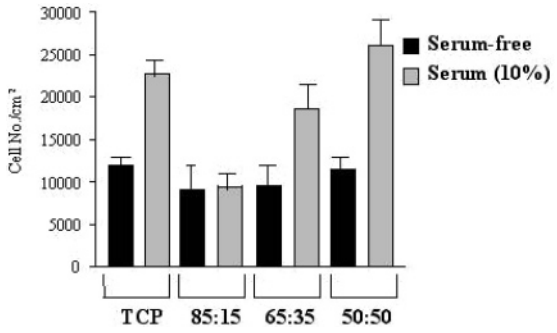

Figure 11. Effect of serum on the adhesion of HeLa cells to either TCP or copolymer films of NIPAM/NTBAM as a function of the NTBAM content. Data taken from Allen et al.[19] Error bars refer to the SEM ($n = 3$), and their meaning is discussed further in Allen et al.[19] TCP refers to tissue culture polystyrene and is used as a control or reference surface.

as shown in Figure 10. Besides that, the inductive effect of the *tert*-butyl group is slightly higher than that of the isopropyl group, resulting in higher polarizability of the amide moiety. These factors may contribute to higher affinity between NTBAM-coated surfaces and biological membranes.

Cell Adhesion. The number of cells that had adhered to TCP and copolymer films 6 h post-seeding in the presence and absence of 10% serum is shown in Figure 11. In the complete absence of serum, there was no discernible difference in the adhesion of HeLa cells to the various copolymer films of NIPAM/NTBAM irrespective of surface hydrophobicity. Furthermore, we found no significant difference in the ability of HeLa cells to adhere to TCP and NIPAM/NTBAM copolymer films under these conditions. In contrast, upon the introduction of serum into the cell culture medium, the ability of cells to adhere to TCP and NIPAM/NTBAM copolymer films improved. A direct comparison of each surface revealed that the presence of serum in the medium significantly promoted cell adhesion to TCP. Interestingly, an inter-comparison of cell adhesion on NIPAM/NTBAM copolymer films of varying ratios revealed that the ability of serum to promote cell adhesion was modulated according to NTBAM content. In this respect, the presence of serum in the medium significantly promoted cell adhesion to the most hydrophobic NIPAM/NTBAM copolymer films of ratios 50:50 and 65:35, but had no effect on the most hydrophilic NIPAM/NTBAM copolymer 85:15 (Figure 11).

Protein Adsorption. Incorporation of NTBAM had strong effects on protein adsorption to copolymer films, as shown in Figure 12. Using a combination of qualitative and quantitative methods, it was determined that total serum protein adsorption from cell culture medium decreased with increasing NTBAM content. Further quantitative analysis of two important serum proteins, albumin and fibronectin, demonstrated that surface hydrophobicity can differentially affect individual protein adsorption profiles. Albumin adsorption displayed a trend similar to that of total protein adsorption and decreased with increasing surface hydrophobicity. In contrast, fibronectin adsorption was found to increase with corresponding increases in NTBAM content, as shown in Figure 12.

Discussion

The importance of these results for the main issue of interest, namely cell adhesion, lies with the fact that the adhesion process is dependent on the preadsorption of

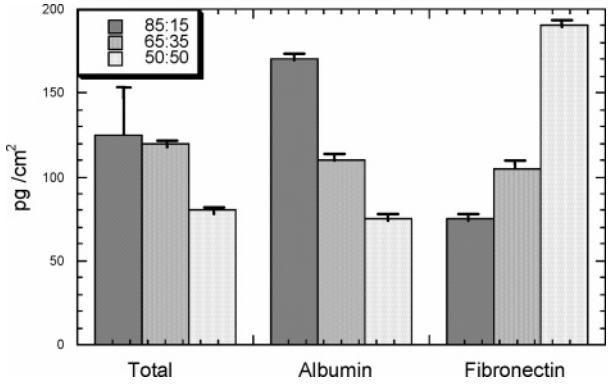

Figure 12. Extent of total protein adsorption, and adsorption of albumin or fibronectin to copolymer surfaces of increasing NTBAM content. Data taken from Allen et al.,[19] where a more detailed discussion of the separation and identification of the bound proteins is given. Error bars referr to the SEM ($n = 3$) and are discussed in detail in Allen et al.[19]

Table 5. Surface Energy and Number of Cells Adhered to the Surfaces[a]

% NTBAM in copolymer	surface free energy (mJ/m^2)					cells adhered (cells/cm^2)
	γ	γ_S^d	γ_S^{AB}	γ_S^+	γ_S^-	
0	44.2	38.7	5.52	0.19	40.2	-
15	42.6	37.5	5.09	0.17	38.9	9512.7
35	39.6	35.9	3.70	0.09	37.6	18565.5
50	37.4	34.8	2.56	0.05	35.3	26106.1
100	36.4	34.6	1.85	0.03	31.7	-

[a] (−) Implies that the number of cells adhered to this surface was not determined. Cell studies were not performed on pure NTBAM or copolymers with NTBAM>NIPAM as it was considered that these may be too hydrophobic for good cell growth, based on the sigmoidal relationship between cell adhesion and surface hydrophobicity reported by others.[15]

proteins from serum.[19] Considering earlier results and conclusions, the subsequent question to be answered appears to be whether the adsorption of proteins to the polymer surface is preferentially determined by the general hydrophobicity of the polymer surface or by changes in the chemical groups exposed at the surface, such as the exposure or lack thereof of the N−H groups at the surface.

A direct comparison of the surface energy data and the cell adhesion data is given in Table 5, from which it is clear that the number of cells adhering to the surfaces increased considerably with the decrease in surface energy that occurred with increasing NTBAM content. To gain more information about the type of acid−base interactions occurring, the γ^+ and γ^- contributions were determined (see Table 5). While there is a clear trend for a decrease in the surface energy of all the components (dispersion, acid−base, and acidic (γ^+) and basic (γ^-) components shown separately) with increasing NTBAM content in the copolymer, the effect of NTBAM content is most significant on the acid component of the free energy, γ^+. All of the surfaces are found to be relatively strongly electron donating (i.e., basic) and weakly electron accepting (i.e., acidic). There is an obvious correlation between the cell adhesion and γ^+, with cell adhesion occurring much more effectively on surfaces with $\gamma^+ < 0.1$ than on surfaces with $\gamma^+ > 0.1$. Good et al. observed a similar trend with HEMA/EMA copolymers, and found that having $\gamma^+ \approx 0$ was a necessary condition for cell growth on their polymer films.[33] It would appear that a value of γ^+ close to 0 is also a condition for cell attachment on NIPAM/NTBAM copolymer films.

Since this series of copolymers differs only in the ratio of isopropyl to *tert*-butyl groups, very little variation in the apolar component of the free energy with composition is expected. Indeed, there is very little variation, as shown in Table 5, just a slight decrease with increasing NTBAM content. Good et al. showed for their series of HEMA/EMA copolymers that the average γ^d was 39.5 mJ/m^2 and that the departure from this average was not significant.[33] The mean γ^d for the series of copolymers used here is 35.5 mJ/m^2, and as can be seen in Table 5, there is not much departure from this value, which is also close to the value reported by Good et al.

It has recently been observed that there are some problems associated with measuring the contact angles of polymer films, due to a distinct stick/slip pattern on several of the polymers, due to film deformation along the line where the drop is pinned to the surface.[34] However, through careful selection of organic liquids, the authors of that work were able to measure contact angles without this stick/slip behavior, and from these they calculated surface energies from 38.9 mJ/m^2 for poly(NIPAM) to 31 mJ/m^2 for poly(NTBAM). Although their values are a little lower than those reported here, the difference is not so large, and so we can assume that the components of the surface free energy calculated here are also in the correct range, and thus that it is reasonable to make correlations between the cell adhesion behavior and the changes in components of the surface free energy.

The gradual decrease in the γ^- component of the free energy with increasing NTBAM content, as well as the abrupt change at a NTBAM content of 50% shown in Table 5, provides further evidence for the reduction in the concentration of N−H groups at the polymer surface with increasing NTBAM content, due to the additional shielding provided by the *tert*-butyl groups. *tert*-Butyl groups have a higher tendency to protrude outside than isopropyl groups, rendering the coated surface more paraffin-like, and hence of lower surface energy at higher NTBAM contents. However, when the surface is exposed to a solvent (as is the case during the contact angle measurements, and during cell and protein adhesion studies), significant changes in the packing density take place. This is due to the changes in the hydration/solvation energy (which are big compared to the heat energy $\sim kT$), resulting in easier self-association (compaction) of chains in solution and at the interface. Paralleling this with surfactants, adding one hydrocarbon unit causes a significant reduction in cmc and csac (critical surfactant association concentration). In contrast, the chain configuration in a vacuum tends (i.e., in dry films) to become looser (more expanded) after the substitution, as the sterical repulsion between *tert*-butyl moieties is stronger than that between isopropyl moieties.

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(34) Gilcreest, V. P.; Carroll, W. M.; Rochev, Y. A.; Blute, I.; Dawson, K. A.; Gorelov, A. V. *Langmuir* **2004**, *20*, 10138.

Raman spectroscopy has been used previously to map surfaces of poly(NIPAM) to determine surface topography under different solvent and temperature environments, but has not yet been applied to the specific identification of groups expressed at the surface.[28,35] This type of spectroscopy is ideally suited to these systems as there is little interference from water in the resulting spectra. Additional specificity is obtained as the Raman instrument is focused on a specific small region of the surface allowing detailed study of that region.

From previous work[29] it has been suggested that the side chain group of NIPAM is restrained in the trans conformation in situations where there is no phase separation and that the cis isomer is found in the phase-separated and dehydrated system. In these systems, which are precipitated from ethanol, it appears that the side chains are in the trans position as the CH_2 and CH_3 asymmetric stretches all appear at less than 3000 cm^{-1}. This hypothesis is borne out by studying the signal at 1250−1270 cm^{-1}. In this region a signature H−N−C═O bond opening stretch of the N−C bond is observed which shows up very weakly in poly(NIPAM) at 1266 cm^{-1}. The retention of the side chain in the trans position would encourage hydrogen bonding between adjacent N−H and C═O groups, restricting the H−N−C═O opening motion. Poly(NTBAM) does not have such a restricted motion and as a result there is a strong signal at 1250 cm^{-1} linked to this H−N−C═O bond opening motion (Figure 5). Thus, it would appear that increasing the NTBAM content in the copolymer films reduces the ability of the surface to form hydrogen bonds via the N−H groups by suppressing the N−H groups at the surface, in good agreement with the reduction in the acid−base component of the surface free energy (as shown in Table 5) as the NTBAM content increases.

In general within the literature increasing surface hydrophobicity is accompanied by increasing protein adsorption; however, this does not appear to be the case with this series of NIPAM/NTBAM copolymers. The increased presence of NTBAM, and the subsequent reduction of N−H groups available for hydrogen bonding, may account for the reduction in protein adsorption. Recently, Gessner et al. used 2D gel electrophoresis to study the effect of surface hydrophobicity on the adsorption of proteins from serum.[7] These investigators found that increasing surface hydrophobicity promoted protein adsorption to the surface of a material over a wide range of hydrophobicities. However, these investigators also found one exception to this general rule. They found that despite the increased surface hydrophobicity of N-*tert*-butylstyrene in comparison to methylstyrene, there was an apparent reduction in the total amount of serum protein adsorbed. These investigators hypothesized that this reduction was due to the presence of *tert*-butyl groups sterically hindering the adsorption of serum proteins to the surface.[7] Thus, the increased presence of *tert*-butyl groups gained through the addition of NTBAM may contribute to the reduction in overall protein adsorption (i.e., from the 10% serum solution) observed in our system.

Comparison of adsorption profiles of two well-known serum proteins from single protein solutions, albumin and fibronectin, revealed contrasting adsorption profiles (decreasing and increasing, respectively). Adsorption of albumin followed a pattern similar to that of total serum; this result was not surprising as albumin comprises 58−74% of total serum proteins. In contrast, fibronectin adsorption correlated with increasing surface hydrophobicity. Due to its relatively large molecular weight, fibronectin may not be as susceptible to steric hindrance effects of *N-tert*-butyl groups, and thus its adsorption is not inhibited by the increased presence of NTBAM. Additionally, the increased binding of fibronectin to the surfaces with a higher NTBAM content could be due to the increased presence of CH_3 groups at the surface—Tengvall et al. also reported that CH_3 groups were more favorable than OH groups for adsoption of fibronectin.[5] Thus it is probable that the binding of fibronectin is driven by apolar interactions and is suppressed on surfaces which are hydrogen binding. It has been argued that fibronectin and vitronectin play a major role in determining cellular response, in particular adhesion to the surface of biomaterials. There appears to be a correlation between fibronectin adsorption to the surface of NIPAM/NTBAM copolymer films and the cell adhesion profile, as the number of adherent cells also increases with the NTBAM content, indicating the importance of the surface composition on the protein binding and thus the cell-signaling and cell adhesion.

Finally, it should be remembered that the chain conformation in water and in, say, diiodomethane, is not going to be identical (as can be demonstrated by sum frequency generation spectroscopy (SFG) or ellipsometry), which somewhat undermines the usefulness of the Good approach to calculate the surface free energy from the contact angle data. However, taken in combination with the molecular simulation and Ramen spectroscopy data, there is convincing evidence that it is not simply the increase in hydrophobicity with increasing NTBAM content that is responsible for the increased cell adhesion at higher NTBAM contents, but rather it is the decrease in the number of N−H groups exposed at the surface, as well as the increase of CH_3 groups at the surface, which determines the degree to which hydrogen bonding or van der Waals interactions can take place, and the degree to which protein adsorption is affected by steric hindrance. These two effects determine which proteins are preferentially adsorbed from the serum, and thus which cell-signaling pathways are established, and ultimately the extent to which cell adhesion occurs.

Conclusion

From this study it is clear that cell- and protein-adhesion on surfaces of increasing hydrophobicity is actually due to subtle changes in the underlying chemical nature of the surfaces changes—that is, to changes in the proportion of N−H groups at the surface, and the affect of this on the type of interactions that occur at the surface. Increasing the NTBAM content in the copolymers appears to reduce the amount of N−H groups exposed at the surface, due to the additional steric hindering of the larger *tert*-butyl group relative to the isopropyl group. This means that at high

(35) Maeda, Y.; Yamamoto, H.; Ikeda, I. *Macromolecules* **2003**, *36*, 5055.

NTBAM contents, the surface resembles a paraffin surface, with a subsequent reduction of the acidic and basic characteristics of the surface free energy. This alters the hydrogen bonding capabilities of the surfaces and results in altered patterns of protein adsorption, and leads ultimately to increased cell adhesion at higher NTBAM contents.

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