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Rationalising the design of polymeric thermoresponsive biomaterials

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We investigated the cell adhesion and growth of a series of thermoresponsive copolymers of *N*-isopropylacrylamide (NIPA) and N-*tert*-butylacrylamide (NtBA) above their lower critical solubility temperatures (LCST). It was found that cell adhesion and growth on the solvent cast films improved with increasing the NtBA content in the copolymers. The improvement was dependent on cell line. The surfaces of copolymers were analysed by atomic force microscopy. The topography of polymer films was not dependent on composition. The differences in the cell attachment and growth were attributed to the variation of surface energy with composition. The surface energy of copolymers decreased with the increase in the NtBA content. We conclude that poly(*N*-isopropylacrylamide) (poly(NIPA)) is a relatively poor substrate for cell growth and proliferation. However, its ability to support cell growth can be significantly improved by suitable modification.

Introduction

Surface characteristics such as surface topography, surface free energy, electrical charge and chemical composition are all known to play significant roles in cell adhesion. Influence of surface free energy has been extensively studied [1, 2] and there appears to be an optimum range of surface energy (as determined by contact angle measurements), which promotes mammalian cell adhesion. To date, the study of free surface energy has involved the use of different class of biomaterials such as polymers, metals, ceramic etc., with different values of surface energy. However, such materials vary greatly in surface chemistry making the study on the influence of surface energy alone difficult. We synthesised a series of co-polymers on the basis of *N*-isopropylacrylamide (NIPA) and N-*tert* -butylacrylamide (NtBA) (Fig. 1) which should display similar surface chemistry with different free surface energy at 37 °C [3]. Poly(*N*-isopropylacrylamide) (poly(NIPA)) is a well-known temperature sensitive polymer, exhibiting a lower critical solution temperature (LCST) at 32 °C in water. This unique thermoresponsive property of poly(NIPA) and its copolymers makes it particularly relevant as a novel method for drug delivery and tissue engineering technology. Thus, in our previous work it was demonstrated that increasing amount of the monomer NtBA results in a reduction of the LCST [4]. Moreover, it was shown that the drug release from NIPA/NtBA co-polymer films can be manipulated by changes in co-polymer composition [5]. The main objectives of this work are to determine the surface characteristics of poly(NIPA-co-NtBA) films and to find the correlation between these characteristics and cell behaviour.

Materials and methods

Materials

The monomers, namely NIPA (97%, Aldrich) and NtBA, (purum, Fluka Chemie, Switzerland) were recrystallised from hexane and acetone, correspondingly. 2,2′-Azobis(2-methylpropionitrile) (AIBN), (Phase Separation LTD, Queensferry, Clwyd, UK) was recrystallised from methanol. All other solvents were reagent grade and were dried and distilled before use.

Copolymers synthesis

Poly(NIPA), poly(NtBA) and poly(NIPA-co-NtBA)(15, 20, 35, 40, 50 mol % NtBA) were prepared by free radical polymerisation, using AIBN (0.5 mol % of AIBN) as an initiator in benzene (10%, w/w) under argon. After polymerisation at 60 °C for 24 h, the mixture was precipitated in hexane. Precipitation was repeated three times using acetone as a solvent and hexane as a non-solvent, and the product was dried at room temperature in vacuum. Increasing the amount of the hydrophobic

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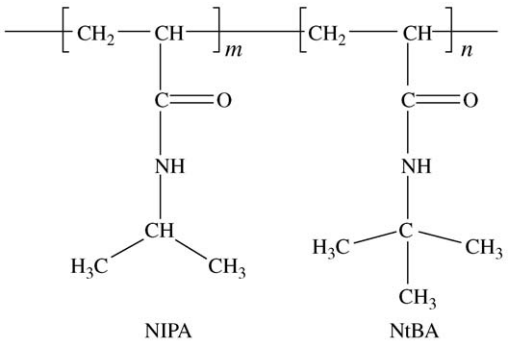


Figure 1 Chemical structure of NIPA/NtBA copolymers.

monomer NtBA in the co-polymer lowers the LCST as demonstrated in Fig. 2.

Poly(NIPA-co-NtBA) films for cell culture experiments were cast in six-well plate (tissue culture grade polystyrene – TCPS) from a 5% (w/w) solution of polymer/dry ethanol (100%). The ethanol was allowed to evaporate for 24 h in a laminar flow cabinet creating polymers films 5 μm thick. $22 \times 22\,mm^2$ glass coverslips were used as substrates for the purpose of AFM investigations. Thickness ranged between 5 and 10 μm, as determined by micrometry. Analysis of surface roughness elucidated that films cast on tissue culture grade polystyrene had similar roughness values to films cast on glass coverslips. All poly(NIPA-co-NtBA) films were transparent.

Surface roughness characterisation

The topography of PNIPAM/PNIPA films was observed using a Digital Instruments Dimension 3100 in air, at ambient temperature and humidity conditions. A triangular silicon-nitride tip mounted on a cantilever (stiffness constant 0.57 N/m) was operated in contact mode. In total, a matrix of 256 × 256 data points along an *x*–*y* plane were analysed for a single scan. A scan size of 50 μm × 50 μm with a low scan rate of 1.39 Hz was employed. First order flattening was carried out on all height images to eradicate any bowing phenomenon present. Each film was randomly scanned in five different locations. The roughness of the films was reported as root mean square roughness (RMS) values, where RMS denotes the standard deviation of the *Z*-values within a given area.

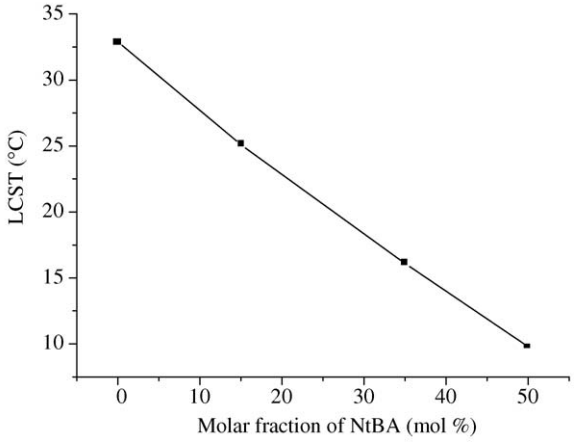


Figure 2 Lower critical solution temperature of poly(NIPA-co-NtBA) copolymers as a function of NtBA contents.

Surface energy

Advancing contact angle measurements were performed using sessile drop method and a home built goniometer. The goniometer was assembled on the optical rail from Newport Optics with optomechanical components from Newport Optics and Edmund Optics. The imaging part of the goniometer and DROPimage analysis software was acquired from Ramé-Hart Inc. For the contact angle measurements we used polymer films prepared by solvent casting on microscopy cover glasses. Polymer samples were placed on temperature controlled tilt stage. All contact angle measurements were obtained at $20 \pm 0.05\,^{\circ}C$. In a typical experiment a drop was deposited on the surface with an initial radius about 3 mm. For advancing contact angle experiment a thin stainless steel needle was inserted in the centre of the drop from above. The volume of a drop was increased by pumping liquid into the drop using a syringe pump.

Pumping speed was adjusted to maintain the rates of advancing below 0.5 mm/min. Drop images were acquired every 3 s. Drop profiles were extracted and contact angles were calculated by numerical derivation of the profile at the contact point.

Test liquids used included benzyl benzoate, 1-iodonaphthalene, dibenzylamine, 1,3-diiodopropane, 1-bromonaphthalene, ethanolamine, glycerol and 1,2-dibromoethane (all from Aldrich). Some liquid–copolymer combinations exhibited time dependent contact angles or so-called slip/stick behaviour [6]. Such contact angles were excluded from any further analysis.

Cell culture

Human epithelial larynx carcinoma cells (Hep2) and mouse fibroblast-like cells (L929) were used for cell growth and cell adhesion studies. Relevant cell culture media and associated supplements including 10% fetal bovine serum were used, as detailed by conventional protocols. All cell types were maintained at 37 °C under 95% air/5% CO_2. Cells were cultivated on the precoated six-well plates. Wells were seeded with 3 ml cells suspension $(20\,000\,cells/sm^2)$. All experiments were carried out in the presence of 10% fetal bovine serum. Cells on the surface of the polymer films after 1 and 48 h were harvested by trypsinisation (0.25% trypsin, for 5 min. Trypsin–EDTA, Gibco). The number of viable cells was counted using a haemocytometer counting chamber.

Results and discussion

Cells adhesion and growth

Previously, our work demonstrated that increasing NtBA contents in poly(NIPA-co-NtBA) copolymers substrates improves cell adhesion and growth [4, 7]. Also, relatively poor cell growth and cell adhesion was observed on poly(NIPA) film. The results were obtained with epithelial HEp2 cell lines confirm these conclusions (Figs. 3 and 4). However, there was no

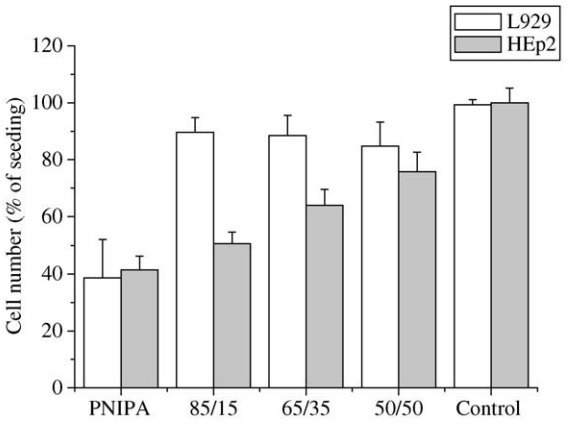


Figure 3 Adhesion of L929 and Hep2 cells on copolymers (NIPA mol%/NtBAmol%) 1 h after seeding. Control is tissue culture-grade polystyrene.

significant difference between Hep2 cell growth on copolymer with composition 50%/50% and cell growth on tissue culture grade polystyrene after 48 h. In contrast, the adhesion and growth of L929 fibroblast-like cells demonstrate very similar characteristics on different copolymers and TCPS except for poly(NIPA).

Surface roughness

There was no significant difference observed in surface roughness between any of the films varying in copolymer ratio – 85 : 15, 65 : 35 and 50 : 50. Table I divulges the RMS roughness values attained for poly(NIPA-co-NtBA) films.

It is evident from the height images obtained from AFM analysis that the film surfaces are relatively smooth and flat, with no apparent structures visible on them. Fig. 5 evinces how uniform the surface roughness is for the film with monomer ratio 85/15.

TABLE I RMS roughness values. Values listed are mean ± SD

Copolymer ratio	85 : 15	65 : 35	50 : 50
RMS (nm)	15.8 ± 1.2	16.3 ± 1.5	15.3 ± 6.5

Surface energy

It is customary to correlate cell adhesion and growth with the surface properties of biomaterials. Contact angle of water is the most popular surface characteristic due to its relative ease of measurement. However, the determination of water contact angles was not possible for the polymers employed in the present work. Contact angle measurements were attempted for all NIPA/NtBA composition (including poly(NIPA) and poly(NtBA)) above their corresponding LCST. Pronounced slip/stick behaviour was observed in all cases. The reason for such behaviour could be attributed due to a dual hydrophilic/hydrophobic nature of thermoresponsive copolymers. Although thermoresponsive polymers are not soluble above their LCST, water can penetrate a polymer matrix and cause time-dependent contact angles and slip/stick behaviour. As a result, we could not obtain unique and meaningful water contact angles for the thermoresponsive polymers and copolymers. Nevertheless, other test liquids produced time-independent and reproducible contact angles that enabled us to calculate surface energies. Surface energy could be calculated from contact angles using either a surface tension component approach or an equation of state approach. The approach of surface tension components usually requires the measurements of contact angles with dispersive/non-dispersive i.e. non-polar/polar liquid pair. Unfortunately, PNIPA and its copolymers investigated here (with the exception of poly(NtBA)) are soluble or swell in virtually all polar organic solvents available to us. Consequently, equation of state approach was used in the form proposed by Li and Neumann [8]. In their approach contact angle, θ is associated with liquid surface tension, γ_{lv} and solid surface tension (or surface energy), γ_{sv}, by the equation

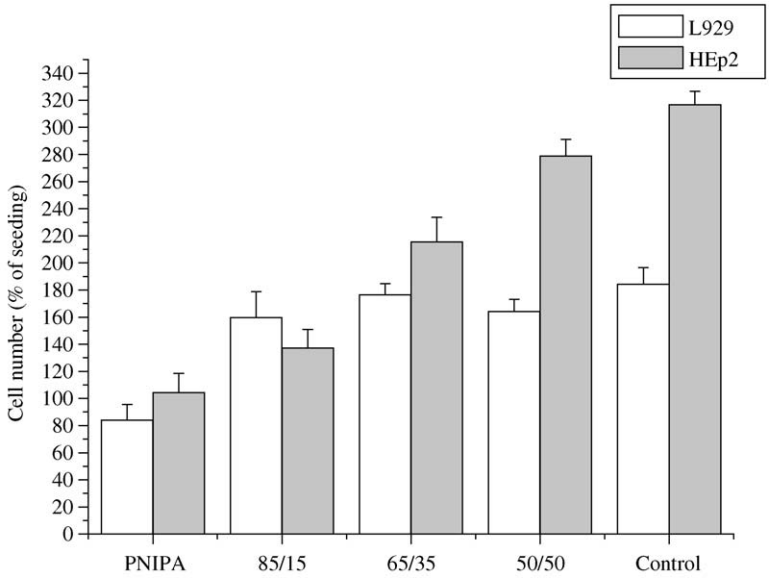
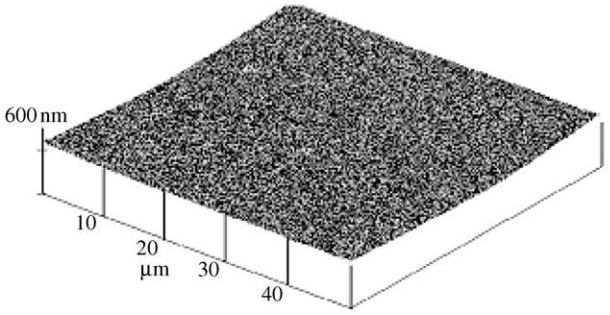


Figure 4 Growth of L929 and Hep2 cells on copolymers (NIPA mol%/NtBAmol%) 48 h after seeding. Control is tissue culture-grade polystyrene.



Digital instruments NaroScope	
Scan size	50 μm
Scan rate	1.387 Hz
Image data	Height
Data scale	250.0 nm
Image Statistics	
Img. Z range	67.263 nm
Img. mean	0.129 nm
Img. RMA (Rq)	15.315 nm
Img. Ra	13.110 nm
Img. Rmax	66.025 nm

Figure 5 AFM height images of an 85 : 15 film. AFM parameters employed during film characterisation included.

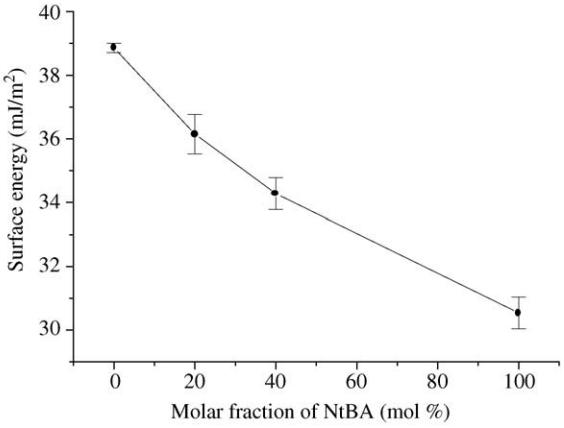


Figure 6 Surface energy of copolymers plotted as a function of their chemical composition.

$$\cos\theta = -1 + 2\sqrt{\frac{\gamma_{sv}}{\gamma_{lv}}}\,\mathbf{e}^{-\beta(\gamma_{lv}-\gamma_{sv})^2} \qquad (1)$$

The value of parameter β was determined as $0.0001247\,m^2/mJ^2$ from the measurements on the extended range of polymer surfaces and test liquids.

We used Equation 1 to obtain the surface energy by non-linear least-square analysis with β as constant and γ_{sv} as an adjustable parameter. The dependence of surface energy on polymer composition is presented in Fig. 6. Within experimental error, surface energy decreased linearly with increasing hydrophobic monomer content.

The resulting surface energies could be compared with other polymer systems. For example, surface energy of poly(NIPA) is close to that of poly(methyl methacrylate) ($\gamma_{sv} = 38.5 \pm 0.5\,mJ/m^2$, water contact angle, $\theta_w = 73.72°$). Poly(NtBA) is close in its surface properties to polystyrene ($\gamma_{sv} = 29.9 \pm 0.5$, $\theta_w = 88.42°$) or poly(*t*-butyl methacrylate) ($\gamma_{sv} = 28.8\,mJ/m^2$, $\theta_w = 90.73°$). The surface energies of the above polymers were obtained by the same equation of state approach (Equation 1) and cited from [6]. It is clear that, from the point of view of surface thermodynamics, poly(NIPA) and its copolymers with NtBA belong to a class of polymers which are considered hydrophobic (θ_w is in the range 70–90°) and are not adequate substrates for cell adhesion. For example, polystyrene is usually surface modified for tissue culture application with the resulting reduction of water contact angle from 88° to about 50°.

The relationship between surface physical chemistry and cell adhesion and growth is yet not completely clear. Nevertheless, a general propensity for improved cell adhesion and spreading was observed with the increase of substrate hydrophilicity (as deduced from the contact angle of water). It was demonstrated that cell spreading and substratum surface free energy showed a characteristic sigmoid relationship [1]; good spreading only occurred when free energy was higher than approximately $60\,mJ/m^2$. However, the opposite tendencies have also been observed when 2-hydroxyethyl methacrylate (HEMA) was copolymerised with more hydrophobic monomer ethyl methacrylate (EMA) [9]. Cell adhesion was suppressed when the content of hydrophilic monomer in the copolymers was increased. The series of poly(NIPA-co-NtBA) polymers follow a similar trend. Failed attempts to obtain a meaningful contact angle of water on poly(NIPA-co-NtBA) polymers indicate that water can be taken by the polymer matrices, thus changing their properties and ultimately affecting cell adhesion.

Conclusions

It was found that poly(NIPA) is a relatively poor substrate to support cell adhesion and growth. So we proposed a strategy to improve poly(NIPA) biocompatibility. The introduction of more hydrophobic monomer NtBA into poly(NIPA) decreases the surface energy of the resulting copolymer. However, the cell adhesion and growth is significantly improved with the increase of NtBA content. The magnitude of this improvement is cell line dependent. The copolymers with higher NtBA content can emulate tissue culture grade polystyrene in their ability to support cell growth.

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