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Grafted thermo- and pH responsive co-polymers: Surface-properties and bacterial adsorption

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Abstract

A series of responsive polymers displaying pH and temperature-mediated phase changes were prepared from *N*-isopropylacrylamide and ω-carboxylic acid functionalised acrylamides. These polymers were grafted to surfaces and their characteristics probed by atomic force microscopy in aqueous solutions. The effects of pH and temperature induced phase transitions on the short-term adsorption of the bacteria *Salmonella typhimurium* and *Bacillus cereus* from pure cultures were assessed. Contact angle studies indicated that pH and temperature-dependent surface properties were exhibited by the graft polymer surfaces. Temperature-dependent surface morphology changes occurred through polymer graft phase transitions as observed in AFM and accompanying changes in adhesion forces underwater were found to correlate with surface properties obtained from contact angle measurements. Adsorption of *S. typhimurium* and *B. cereus* was not significantly altered as a function of pH, but attachment of both bacterial strains increased at temperatures above the polymer coil-globule transition indicating the importance of switching surface hydrophobicity in controlling short-term bacterial adsorption.

Keywords: Bacterial adsorption; Thermoresponsive polymers; Atomic force microscopy; *Salmonella typhimurium*; *Bacillus cereus*

1. Introduction

The possibility of generating synthetic polymers that can control bioadhesive processes such as cell attachment is of considerable industrial significance and clinical importance, as well as being a topic of much scientific interest (Blackwood, 2003). Potential applications for synthetic materials that are either bioadhesive or biocompatible are extremely widespread, ranging from surgical implants (Fournier et al., 2003; Hron, 2003), supports for in vitro cell culture and biotechnological screening (Hersel et al., 2003; Noiset et al., 2000; Neff et al., 1999), scaffolds for tissue engineering (Shin et al., 2003; Vats et al., 2003; Zacchi et al., 1998) through to anti-fouling surfaces or coatings in industrial processing and marine environments (Pasmore

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et al., 2002; Taton and Guire, 2002; Lewis et al., 2001; Brady, 1997).

In recent years, there has been a focus on preparing 'active' or responsive materials that exhibit different properties, such as a phase transition, under differing environmental conditions in order to manipulate or control bioadhesive interactions (Kazanci, 2003; Kwon et al., 2003; Yamato et al., 2002, 2000; Malmstadt et al., 2003; Lackey et al., 2002; Hoffman, 2000; Galaev and Mattiasson, 1999; Inoue et al., 1997) Potential uses for these classes of polymers include targeted delivery of drugs (Doorty et al., 2003) and anti-microbials or as 'smart' surfaces that can be switched from an adhesive to a non-adhesive state (Ebara et al., 2003; Rao et al., 2002). One area of particular interest is the utilisation of responsive polymers to control the attachment of prokaryotic cells to surfaces. Pioneering work by Lopez and co-workers demonstrated that attachment of *Halomonas* and *Staphyloccus* species to substrates could be controlled by surface-grafted poly(*N*-isopropylacrylamide) (PNIPAm), with greater numbers of cells adsorbing to the surface when it was of a wettability favourable for the individual bacterial species (Ista and Lopez, 1998; Ista et al., 1999). Further work by this group established that more complex organisms were able to detect substrates of different wettabilities, indicating the importance of surface physical chemistry on bioadhesion (Finlay et al., 2002).

We have been investigating whether the hydrophilic–hydrophobic switch of PNIPAm co-polymers at their lower critical solution temperature (LCST) can mediate the attachment of common pathogens and have reported the effects of neutral PNIPAm co-polymer switching on the adsorption of proteins as model conditioning films and on the attachment and release of representative microorganisms *Listeria monocytogenes*, *Salmonella typhimurium* and *Bacillus cereus*. Bioadhesion to these polymer grafts and brushes was found to be dependent on LCST-mediated changes in surface physico-chemistry and correlations were obtained between polymer conformations as observed in AFM and contact angle goniometry and overall extent of protein and cell attachment (Cunliffe et al., 2003, 2000).

However, the fact that most bacteria are negatively charged at ambient pH suggested that further control over bacterial adsorption might be achieved by utilising PNIPAm co-polymers containing anionic or acidic co-monomers as the surface-displayed brushes. We chose *S. typhimurium* and *B. cereus* as the test bacteria for these assays to afford a direct comparison with our earlier work and because these microorganisms are implicated in a variety of medical conditions (McCabe-Sellers and Beattie, 2004; Monack et al., 2004). In this paper, we report the first investigation into the effect of temperature and pH-mediated phase transition of these polymers on the short-term adsorption of *S. typhimurium* and *B. cereus*.

2. Materials and methods

High purity reagents, monomers and solvents for chemical synthesis were purchased from Aldrich, Acros or Fisher Scientific (UK) and used as received. Inhibitors were removed from *N*-isopropylacrylamide (NIPAm) and *N*-*t*-butylacrylamide by recrystallisation from hexane.

For contact angle goniometry, double distilled water (DDW, surface tension 72.8 mN m^{-1} at 20.0 °C) or phosphate buffered saline (150 mM NaCl at pH 5.6 or 7.4) were used. For these measurements a Kruss G10 contact angle measuring system equipped with a sealed humidity-controlled sample chamber, and automated image analysis system was used. Drops of liquid of known volume (1–4 μL) were applied from a micro-syringe to the surface of the polymer graft surfaces through a small port at the top of the cell: to avoid cross-contamination of liquids, a dedicated micro-syringe was used for each diagnostic liquid. Measurements of contact angles was conducted with a precision of ±0.5°.

2.1. Monomer and polymer synthesis

6-Acryloylaminohexanoic acid (6-AHA) was prepared by the method of Kuckling et al. (2000) and recrystallised before use: analytical data was consistent with the desired product.

Polymer **P1**: *N*-Isopropylacrylamide (4.52 g, 40 mmol) and *N*-*t*-butylacrylamide (1.27 g, 10 mmol) were dissolved in propan-2-ol (40 mL) in a thick walled Schlenk tube, and 3-mercaptopropanoic acid (0.018 g, 0.172 mmol) and 4,4′-azobis(4-cyanovaleric acid) (0.4 g, 1.42 mmol) were added. The solution was degassed by freeze-thaw cycles under vacuum at least three times and placed in a thermostatted oil bath at

65 °C for 24 h. After cooling to room temperature, the mixture was concentrated under reduced pressure and the residue added to diethyl ether (250 mL). The precipitated polymer was filtered and the residue was redissolved in THF and reprecipitated into diethyl ether (250 mL) three times. The purified polymer was then dried in vacuo at 20 °C overnight to leave a colourless powder (yield 4.97 g, 86%).

Polymers **P2** *and* **P3**: Monomers *N*-isopropylacrylamide (1.13 g, 10 mmol) and 6-acryloylaminohexanoic acid (0.366 g, 2 mmol or 0.184 g, 1 mmol) with 4,4′-azobis(4-cyanovaleric acid) (ACVA, 0.102 g, 0.4 mmol) as initiator and 3-mercaptopropanoic acid (0.004 g, 0.04 mmol) were dissolved in *n*-propanol (25 mL) in a Schlenk vessel. The resulting mixture was degassed by three freeze-thaw cycles under vacuum prior to polymerisation at 65–70 °C for 24 h. Once cooled to room temperature, the solution was concentrated under reduced pressure and purified by repeated precipitation/solvation cycles in the same manner as for **P1**. The yield of polymer after reprecipitation and drying was 0.95 g (73%).

2.2. Titration of polymer carboxyl groups

A solution of anionic polymer (1 mg mL^{-1}) in double distilled water was adjusted to pH 3 by addition of hydrochloric acid (10 mM) to protonate accessible carboxylic acid groups. The solutions were then titrated with sodium hydroxide (10 mM) and end-points determined by potentiometric methods taking into account the volume of acid added at the start-point.

2.3. Determination of lower critical solution temperatures

2.3.1. Turbidimetry

Polymers were dissolved in phosphate buffered saline solutions (150 mM NaCl at pH 7.4) to a concentration of 1–10 mg mL^{-1}. These solutions were first cooled to 5 °C and then heated to 50 °C at 0.5 °C min^{-1} in the thermostatted block of a Perkin-Elmer Lambda 40 instrument fitted with a temperature controlled heating and cooling facility (Haake K40). The cloud point was taken as the onset of a sharp increase in absorption at 500 nm or the range of temperature over which this occurred.

2.3.2. ^{1}H NMR

Polymer solutions (10 mg mL^{-1}) in D_2O were adjusted to pH 5.6 or 7.4 with DCl or NaOD (1 M, Aldrich). NMR spectra were recorded over a temperature range spanning 20–50 °C. The lower critical solution temperature transition was determined from line broadening and reductions in peak intensity of protons ($\delta = 3.98$ and 1.2 ppm) on the PNIPAm isopropyl side chains. Jeol EX-270 and Eclipse 400 spectrometers at 270 MHz and 399.8 MHz (^{1}H) and 67.6 and 100.5 MHz (^{13}C), respectively, were used for these measurements. Deuterated solvents and reagents used (D_2O, $CDCl_3$, NaOD and DCl) were purchased from Aldrich. All chemical shifts are reported in ppm relative to TMS or DSS.

2.4. Surface grafting of acid-functional polymers

Silica glass slides (Fisher, UK) were cleaned with sodium hydroxide (5 M), ammonium persulphate/sulphuric acid and water, dried and amine functionalised by treatment with 3-aminopropyltriethoxysilane (APTES) in aqueous acidified methanol solution according to previously reported methods (Cunliffe et al., 2003; Durfor et al., 1994).

Carboxylic acid terminated co-polymer **P1** and polycarboxyl polymers **P2** and **P3** were grafted to the surfaces by carbodiimide-mediated coupling. Amine-functional silica slides were placed in MOPS buffer (10 mM, pH 4.75, 10 mL) and the liquid supernatant stirred gently at 5 °C as polymers (0.1–1.0 g) were added. When all the polymer had dissolved, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (200 mg) was added with continued stirring. Further amounts of EDC (2× 200 mg) were added over a 2-h period. After addition was complete the surfaces were left at 5 °C for a further 24–72 h, washed thoroughly with deionized water and dried under a stream of nitrogen. The surfaces were stored in a dry nitrogen atmosphere prior to use.

2.5. Growth and labelling of microorganisms

Cultures of *S. typhimurium* NCTC 12023 and *B. cereus* (wild strain isolated at the Public Health Laboratory, Portsmouth UK) were grown, labelled and quantified according to our previous protocols (Cunliffe et al., 2003). Typical stock cultures used in bacterial

adsorption experiments were 10^6–10^7 cfu mL^{-1}: in all cases bacteria were used in stationary phase.

2.6. In vitro adsorption studies

Bacterial cultures (10^6–10^7 cfu mL^{-1}), either radiolabeled or stained with ethidium bromide were transferred to centrifuge tubes and centrifuged (8000 rpm, 10 min), washed twice in sterile buffer (PBS, pH 7.4, 6 mL) and resuspended in the assay solution (either double-distilled water (DDS) or phosphate buffered saline (PBS) at pH 5.6 or 7.4, 6 mL). Aliquots (200 μL) of the suspended bacteria were transferred to sterile assay solution (DDW, PBS at pH 5.6 or 7.4, 10 mL) in capped bottles. Functionalised silica glass test surfaces (1 cm^2), with and without polymer grafts, were then placed in the capped bottles and incubated, with gentle shaking (40 rpm), for 24 h at 10–12 or 37 °C. The slides were rinsed twice by immersion in sterile assay solution (10 mL) at the appropriate temperature and then examined by fluorescence microscopy (ethidum bromide stained bacteria) using a a Zeiss LSM 510 Meta instrument or transferred to scintillation vials containing Ultima Gold scintillant cocktail (Perkin Elmer 5 mL) in a Packard Tri-Carb 1900TR Liquid Scintillation Analyser was used (Packard Instrument Company, Meriden, CT, USA). All experiments were conducted on triplicate samples over five repeat experiments. The bacterial counts for the surfaces at the different temperatures were normalised against attachment of the same bacterium at underivatised glass substrates.

2.7. Instrumentation

2.7.1. Atomic force microscopy

Glass surfaces grafted with 3-aminopropyltriethoxysilane (APTES) and/or polymers were rinsed with water (18 MΩ), dried with nitrogen and mounted on nickel discs (mounting assemblies) prior to AFM investigation. AFM studies were performed using a TopoMetrix TM ×2000 Discoverer Scanning Probe Microscope (ThermoMicroscopes, UK) with a 70 μm × 70 μm × 12 μm tripod piezoelectric scanner. Topography measurements were conducted using "V"-shaped silicon nitride cantilevers bearing an integrated standard profile tip (length 200 μm, nominal spring constant (*K*) 0.032 N m^{-1}; Part No. 1520-00, ThermoMicroscopes, Santa Clara, CA, USA). Topographic imaging was performed in air and in aqueous buffer using a closed wet cell, modified to allow variable temperature adjustment. Contact mode imaging utilised an applied load and scan rate limited to ca. 1 nN and 3 Hz, respectively, to minimize compression and lateral damage to polymer grafts and underlying surfaces.

2.7.2. Infra-red spectroscopy

Infrared spectra were obtained employing a Perkin-Elmer Paragon 1000 FT-IR spectrophotometer, in transmittance mode, at a resolution of 4 cm^{-1}.

2.7.3. Gel permeation chromatography

GPC of polymer solutions was carried out at 33 °C using a LKB 2150 HPLC pump, fitted with Phenomenex Phenogel 5 linear column (30 cm × 5 μm bead size) with THF as the mobile phase at an elution rate of 1 mL min^{-1} using toluene as internal standard or on a Waters 150CV GPC instrument fitted with PLAquagel guard plus 2× mixed-OH, 30 cm, 10 μm columns using 0.01 M lithium bromide in *N*,*N*-dimethylformamide as the solvent system at 80 °C. Refractive index detection was used in both cases. Data capture and subsequent data handling was carried out using Viscotek 'Trisec' 3.0 software. Molecular weights were derived from comparison with polystyrene or poly(ethylene oxide) standards.

2.7.4. Microscopy

For confocal laser scanning microscopy a Zeiss LSM 510 Meta instrument was used, fitted with Argon and He/Ne lasers operating at excitation wavelengths of 453, 488 and 543 nm, using emission filters of 505–530 and 585–615 nm. Fields of view (6–10 per sample) were selected at random, and numbers of cells enumerated over triplicate fields.

3. Results

3.1. Properties of polymers in solution and grafted to surfaces

The polymers produced in this study readily formed solutions in phosphate buffered saline (PBS) at pH 7.4. The properties of these materials are given in Table 1.

Lower critical solution temperatures (LCST) at pH 5.6 and 7.4 were determined by turbidimetry. For the

Table 1
Polymer compositions and physical properties

Polymer	Monomer feed composition (mol%)	Monomer content	Molecular weight (kDa)	$\bar{M}_w/\bar{M}_n$	Cloud point pH 5.6 (per °C)	Cloud point pH 7.4 (per °C)
P1	NIPAm 80, *N*-*t*-butyl acrylamide 20	78:22[a]	5.8	1.71	23	24
P2	NIPAm 80/6-AHA 20	82:18[a]	2.7	1.67	29–31	33–44
P3	NIPAm 90/6-AHA 10	96:4[b]	3.8	1.77	32–33	34–37

[a] Determined by ^{1}H NMR.
[b] Potentiometric titration.

polymers with higher carboxyl content, the LCST values at higher pH were difficult to determine purely from turbidimetry and thus ^{1}H NMR spectroscopy was used to detect LCST via peak broadening and diminution of signal intensity of the *N*-isopropyl side chains (δ = 1.18 and 3.98 ppm). The increases in phase transition temperature with pH were consistent with the increase in ionisation of the pendant carboxyl groups, while the broadening of the LCST for **P2** and **P3**, especially at higher pH was indicative of the two component nature of the co-polymer backbones.

The neutral polymer **P1** and acidic co-polymers **P2** and **P3** were grafted to 3-aminopropyltriethoxysilane (APTES)-functionalised silica surfaces by carbodiimide mediated coupling, under conditions shown in previous studies to react more than 90% of accessible surface amine groups (Cunliffe et al., 1999). The primary properties of the polymer graft surfaces were characterised by contact angle goniometry. For the neutral surface (**P1**) the change in advancing contact angle exhibited across the polymer phase transition was typically 20°, in accord with prior reports of PNIPAm co-polymers attached to amine-functional glass surfaces, whereas the substrate amine surface varied in water contact angle by less than 6° over the same temperature range. Increases in aqueous contact angle were observed for PNIPAm-co-6-AHA polymers **P2** and **P3** grafted to surfaces over the different temperature ranges implying that the PNIPAm segments of these surfaces also experienced an LCST response. The responses of these acidic co-polymers to changes in ionisation state were probed at temperatures above and below LCST at pH 5.6 and 7.4 in PBS. These acidity regimes were chosen to reflect physiologically relevant conditions i.e. at skin surfaces and wounds (Dissemond et al., 2003) as well as those in the bloodstream.

The expected phase transitions with temperature at constant pH were evident in the increases in contact angles for **P1**–**P3** surfaces. Contact angle variations were not significant for the non-pH sensitive polymer **P1** but differences in contact angle across the pH range from 5.6 to pH 7.4 were apparent for **P2**. However, almost no change was exhibited by the **P3** surfaces over the different pH regimes, while the maximum contact angle difference at 45 °C ($\Delta\theta_{aq}$ = 14° for **P2**) was less than that due to temperature. In the case of **P3** surfaces the contact angles in PBS at the two pH values were little different to those in DDW although there was a very slight increase in θ_{aq} above the polymer LCST at pH 5.6 and a small decrease at pH 7.4. The contact angles were in all cases different from the underlying SiO_2-APTES substrate showing that the surfaces were well grafted and effectively covered by the polymer brushes.

3.2. Atomic force microscopy of polymer graft surfaces

Atomic force microscopy of the surfaces in aqueous solutions enabled polymer brush heights to be measured in their hydrated state and allowed changes in surface topography with temperature to be followed. These studies were initially carried out under buffer at the appropriate pH but the most reliable data for surface roughness were obtained under distilled water (solution pH range 7.0–6.4 over the course of the experiments), and under a narrower temperature range (22–35 °C) than the contact angle studies. This was due to the open nature of our AFM wet cell and the resulting evaporative loss of water leading to artefacts from buffer components appearing in the micrographs obtained at the upper end of the temperature range (45 °C). Qualitatively similar AFM images were obtained at pH 7.4 in PBS at 45 °C to those in DDW at pH 7.0 and 35 °C, while contact angle variations were very similar over these pH regions. We thus conducted

Table 2
Contact angles of polymer graft surfaces at varying pH

Surface	pH 5.6		pH 7.4	
	θ°_{aq} (12 °C)	θ°_{aq} (45 °C)	θ°_{aq} (12 °C)	θ°_{aq} (45 °C)
SiO_2-APTES-**P1**	35 ± 3	53 ± 5	36 ± 2	60 ± 1
SiO_2-APTES-**P2**	39 ± 1	58 ± 7	35 ± 4	44 ± 1
SiO_2-APTES-**P3**	34 ± 1	51 ± 2	34 ± 2	48 ± 2

quantitative AFM studies in DDW over regions above and below the LCST for **P1**, and over a range of temperatures (24–35 °C) around the phase transition for **P2** and **P3** in order to investigate surface changes over the broadened LCST span of these polymers.

Typical thicknesses of the brush layers were 2–4 nm for **P1**, 3–5 nm for **P2** and 2–3 nm for **P3**, although occasional isolated features of up to 30 nm in height were observed in individual fields indicating areas of multilayer deposition and/or glass substrate heterogeneity. For **P1** grafts qualitative changes in surface structure and roughness with variable temperature were apparent as noted in our earlier studies with polymers of this type attached to glass surfaces (Cunliffe et al., 2003) and the effects were small (ΔR_a = 0.3–0.7 nm). However, **P2** surfaces exhibited marked changes in topography but little change in overall roughness, whereas **P3** surfaces increased in both average roughness and roughness distribution (Table 2) over temperature ranges at which the polymers would be expected to be undergoing their phase transitions in solution Table 3.

Topography images obtained from the same area of the samples confirmed that the polymer phase transitions caused significant changes in surface structure for both polymers **P2** (Fig. 1) and **P3** (Fig. 2), over

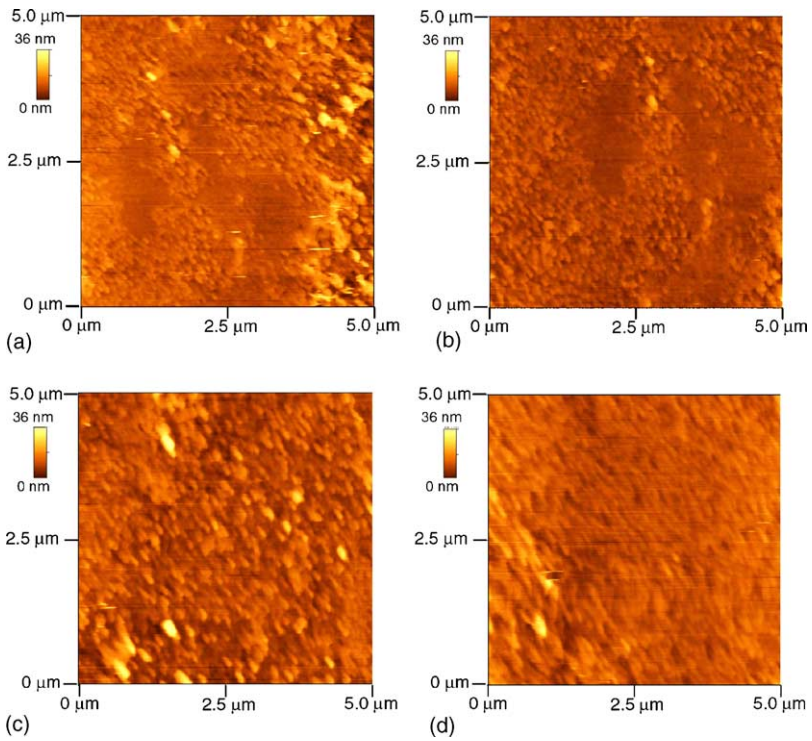

Fig. 1. AFM of **P2** graft surfaces in DDW. (a) 26.5 °C; (b) 27.9 °C; (c) 31.7 °C; (d) 34.4 °C.

Table 3
Surface properties of polymer brushes in DDW

Polymer	Temperature (°C)	R_a (nm)	RMS (nm)
P1	22	2.1	3.2
	37	2.9	3.9
P2	26.5	2.9	3.9
	34.4	2.9	2.8
P3	24.5	1.8	2.4
	34.1	2.5	3.3

temperature ranges (22–35 °C) that phase transitions were taking place.

To explore the changes in surface physical chemistry further we carried out adhesion force mapping in solution for the surfaces by measuring snap-to and pull-off forces as the tip was proximal to the surface. For **P1** surfaces low adhesion was exhibited by the hydrated polymer below LCST with a greater pull-off force being apparent above the polymer phase transition in close accord with previous results obtained for neutral thermoresponsive polymers (Jones et al., 2002). Force distance curves for **P2** and **P3** surfaces also demonstrated the changes in surface properties with temperature, with increases in pull-off forces as the polymers passed through phase transitions. The most pronounced effects were noted for polymer **P2** between 24 and 35 °C (Fig. 3), suggesting that surfaces grafted with this polymer were hydrophobic at lower temperatures than might be expected based on the phase transition temperature of this polymer in solution (33–44 °C).

For the **P2** surfaces the response was indicative of a heterogeneous surface either as a result of differences in graft density or as a result of different components of the co-polymer being exposed at different parts on the surface. Further analysis of force components (Fig. 4) confirmed the distributions of force patterns across the surface. In both temperature regimes a bimodal distribution was obtained, indicating that a combination of hydrophilic and hydrophobic regions were present on the polymer surface, but the frequency of strong (>6 nN) tip–surface interactions was much higher at the higher temperature, confirming the hydrophilic to hydrophobic switch of the overlying polymer brush.

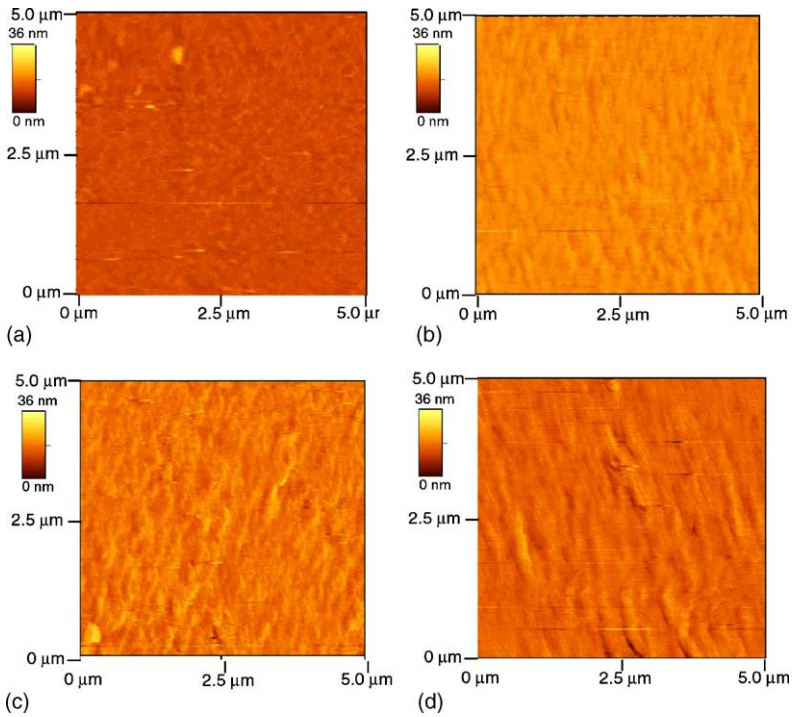

Fig. 2. AFM of **P3** graft surfaces in DDW. (a) 24.5 °C; (b) 27.2 °C; (c) 29.7 °C; (d) 34.1 °C.

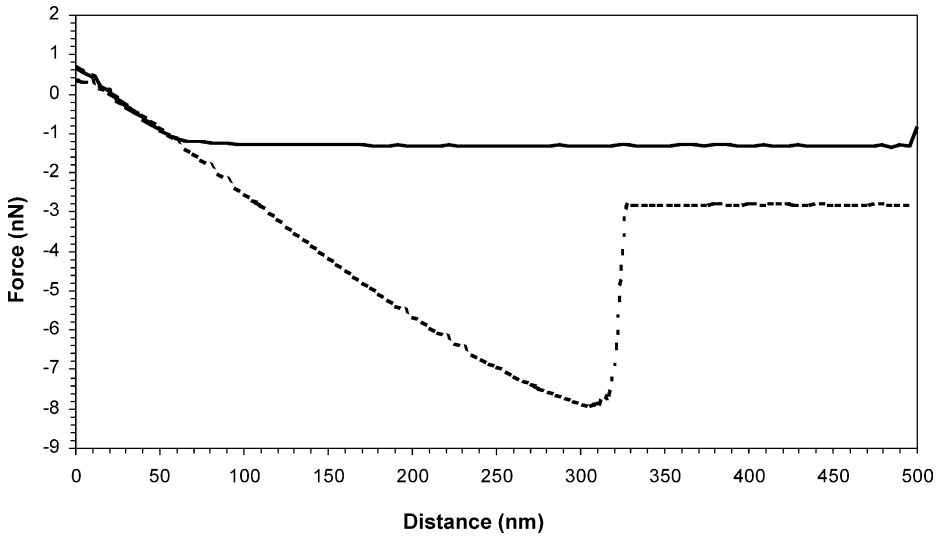

Fig. 3. Force distance curves for **P2** surfaces. Full line indicates forces measured at 24 °C while dotted line depicts forces measured at 34.4 °C.

Attempts to derive adhesion maps at the two different pH regimes were less conclusive, presumably because subtle changes in tip–surface interactions with pH at constant temperature were masked by the high ionic strength in both buffer solutions. Nevertheless, the fact that the co-polymer brushes displayed temperature driven responses in surface structure suggested that bacterial attachment might vary significantly above and below the LCST at constant pH, with a lesser variation in attachment with changing pH at constant temperature.

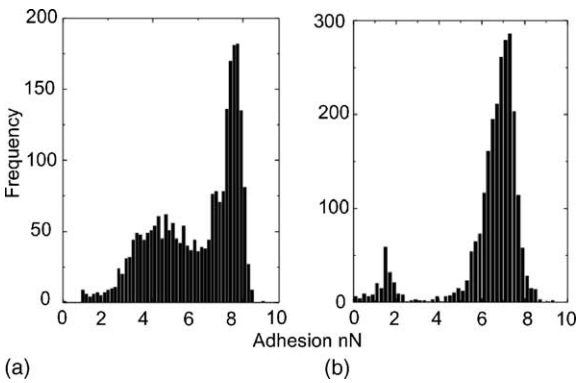

Fig. 4. Adhesion force mapping of surface-grafted **P2** at 24 °C (a) and 34.4 °C (b).

3.3. Bacterial adsorption assays

We carried out short-term bacterial attachment assays with two representative bacterial strains, a Gram-negative and motile bacterium (*S. typhimurium*) and Gram-positive, non-motile species (*B. cereus*). Incubation of the surfaces in bacterial suspensions was initially carried out in PBS at pH 7.0 at two temperatures (37 °C) and (10–12 °C) for 24 h. We reasoned from AFM data that the 37 °C assay would span the upper maxima of polymer LCST when **P1**–**P3** were grafted at the surfaces without unduly stressing the microorganisms, while 10–12 °C would be well below the temperature at which all the surface-grafted polymers would undergo the LCST transition. The results of these assays (data averaged from fluorescence micrographs of ethidium bromide stained bacteria and from scintillation counts of radio-labelled bacteria) are shown in Fig. 5.

For the neutral **P1** surfaces there was a significant increase in bacterial attachment at the higher assay temperature i.e. above the polymer LCST with *S. typhimurium* displaying a 2-fold increase in adsorption at 37 °C, while for *B. cereus* this increase was approximately 5-fold. Attachment of both bacterial strains to the anionic co-polymer surfaces showed rather less change across the temperature range although slight increases were observed in attachment to **P3** (40–45%)

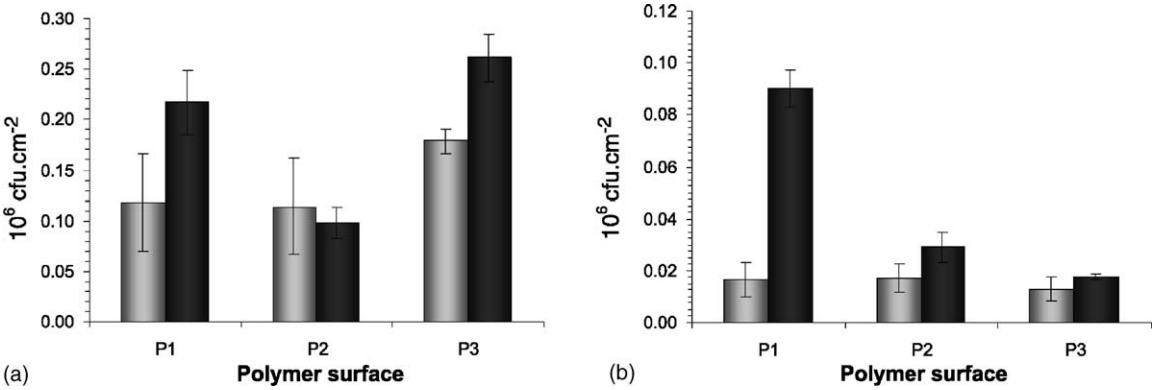

Fig. 5. Attachment of bacteria to polymer surfaces at pH 7.0. Light grey bars represent assay temperature of 10–12 °C, dark grey bars at 37 °C. Left hand side (a) shows attachment of *S. typhimurium* and (b) attachment of *B. cereus*.

at 37 °C. In all cases the numbers of cells present on **P2** were less than on the neutral surface as might be expected based on the increased number of ionisable carboxylic acid groups on this polymer compared to **P3**, and both the anionic co-polymer surfaces reduced the numbers of *B. cereus* compared to **P1**. The overall extent of bacterial adsorption correlated well with our previous study (Cunliffe et al., 2003) in that approximately 1–10% of the total number of cells in suspension (10^7 cfu mL^{-1} for *S. typhimurium*, 10^6 cfu mL^{-1} for *B. cereus*) attached, with the highest adsorption occurring to the neutral surface above the LCST.

We then probed the adsorption of the same bacteria strains under the two further pH regimes at which contact angles had been measured. Surfaces were again incubated at 10–12 and 37 °C for 24 h then rinsed twice at the assay temperature prior to microscopy and scintillation count analysis. The numbers of bacteria attached are shown in Fig. 6.

For *S. typhimurium* (Fig. 6) small increases in adsorption were apparent above polymer LCST, with the greatest increase (50%) to **P3** surfaces at pH 7.4. The **P2** surfaces adsorbed fewer bacteria than **P3** at the higher assay temperatures and the same or fewer bacteria at 10–12 °C though differences were less than 50% of total numbers of cells attached. Variation across the pH ranges was also small, with lower numbers of bacteria found on **P2** surfaces at pH 7.4 compared to pH 5.6, whereas for **P3** surfaces an increase in attached bacteria occurred at the higher pH.

A greater degree of variation was found for *B. cereus* over the same temperature ranges with proportionally

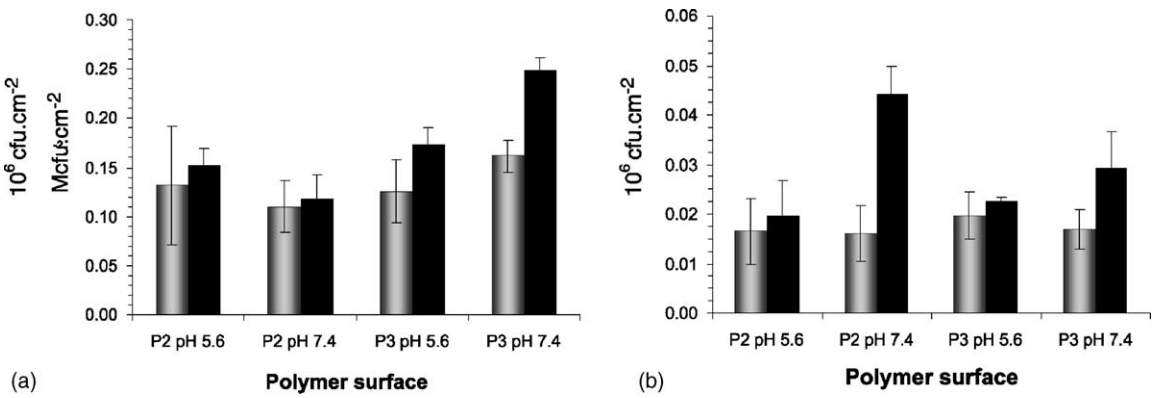

Fig. 6. Attachment of bacteria to co-polymer surfaces at pH 5.6 and 7.4. Light grey bars represent assay temperature of 10–12 °C, dark grey bars at 37 °C. Left hand side (a) shows attachment of *S. typhimurium* and (b) attachment of *B. cereus*.

larger increases in attachment for **P2** and **P3** surfaces at 37 °C at the higher pH value. Over twice as many bacteria attached to **P2** at 37 °C at pH 7.4 compared to 10–12 °C, and over both sets of surfaces adsorption of *B. cereus* was higher at temperatures above polymer LCST. However, these increases were less than one order of magnitude and there was almost no change in adsorption with pH, with very similar numbers of cells attaching to surfaces at pH 5.6 and 7.4 at 10–12 °C irrespective of the surface.

4. Discussion

Our hypothesis was that under normal physiological pH regimes (i.e. pH 7.4) co-polymers of PNIPAm with an acrylamidoalkanoic acid would be ionised and chain extended and thus repellent to bacterial cells, whereas at higher temperatures or lower pH values the surfaces would be hydrophobic and chain-collapsed, increasing attachment of these particular bacterial strains. In order to test the hypothesis, it was necessary to produce polymers grafted to surfaces that were as similar as possible to each other but varying only in the critical parameters of co-monomer content and phase transition.

Accordingly, polymers **P1**–**P3** were prepared with chain transfer agents to control molecular weight and **P1** was synthesised with an acidic initiator to provide appropriate functionality to attach to aminopropyl-derivatised surfaces. The presence of terminal (**P1**) and pendant (**P2** and **P3**) carboxyl functionality was verified by titration. All the polymers exhibited phase changes in solution over biologically relevant pH and temperature regimes: these transition temperatures were similar to those reported for analogous polymers by Kuckling et al. (2000) although the actual monomer contents and molecular weights of the polymers in this study were slightly different. Grafting of these polymers resulted in smooth surfaces under aqueous solutions at 10–20 °C as shown by AFM confirming the suitability of these materials for the bioadsorption assays.

The surfaces displayed changes in physical properties, as shown by contact angles of aqueous droplets, which corresponded well with their behaviour in solution. Thus, **P1** surfaces exhibited a contact angle change of 20° in water as the polymer brush dehydrated above the LCST. The magnitude of this change was slightly less than that reported by Ista et al. (1999), who used a plasma polymerisation method to generate multipoint attachment of PNIPAm to surfaces but similar to those reported by Kidoaki et al. (2001) who prepared PNIPAm grafts from iniferter-coated surfaces. The co-polymers **P2** and **P3** displayed broadly similar contact angles to **P1** as expected owing to their high PNIPAm content, however incorporation of the higher proportion of the acidic co-monomer raised the overall contact angles for **P2**, as expected for the non-ionised polycarboxylic acid component (Wang et al., 2002; Lee et al., 1996). Increases in contact angle with temperature due to the PNIPAm segments were also observed for **P2** and **P3**, although the magnitudes of these increases (12°–15°) were less than for **P1**.

Contact angle experiments in buffer solutions at pH 5.6 and 7.4 indicated that the **P2** surfaces were responsive to changes in acidity, while the **P3** surfaces exhibited almost no change across the pH range: these variations agreed well with the behaviour of the polymers in solution. However, neither surface became noticeably more hydrophilic than the neutral PNIPAm co-polymer grafted surface **P1** even though at pH 7.4 a substantial proportion of carboxyl groups on **P2** and **P3** would be expected to be ionised. This was most probably a result of the reduced number of accessible carboxyl groups on the surface-bound polymers following carbodiimide-mediated grafting. Although it is unlikely that all the side-chain carboxyls in **P2** and **P3** formed amide bonds with the APTES substrates, it was clear from the low extent of pH response that the concentration of ionisable acid groups at the graft surfaces was smaller than for the same polymers in solution. In general poly(acrylic acid) and poly(alkanoic acid)s display broad pK_a values of 4–5 and thus a proportion of side chain carboxyls are ionised around pH 5–6, with a sharp increase in ionic content above pH 6. Indeed Kuckling et al. (2000) noted a large change in LCST of poly(*N*-isopropylacrylamide-co-acrylamidohexanoic acid) co-polymers between pH 6–9 attributed to carboxylate ion formation. The fact that the structurally similar polymer **P2** in this study showed a broad 4–13 °C increase in LCST across the two pH values in solution but a relatively small change ($\Delta\theta_{aq} < 14°$) in contact angle supports the hypothesis that most of the carboxyl groups on the side chains had either reacted with the amine-tipped substrate or were rendered inaccessible most likely

via co-operative H-bonding or ionic interactions with the underlying surface. The contact angle differences between **P2** and **P3** surfaces compared to **P1**, and variations in θ_{aq} for **P2** and **P3** between pH 5.6 and 7.4, did nevertheless suggest that the carboxyl components of these polymers were exerting an effect on the brush conformations but that the dominant effect on the surface behaviour was due to the PNIPAm segments. These observations were confirmed by the adhesion force profiling of the surfaces, with force-distance curves for **P2** and **P3** being very similar (albeit different in overall magnitude) to those observed for PNIPAm homopolymer brushes (Jones et al., 2002). The slight residual adhesion observed at lower temperatures for the **P2** surfaces was indicative of a second component in the polymer brush being approached by the tip. It was not possible to determine by this technique whether the increased pull-off force to remove the tip was due to poly(6-acryloylaminohexanoic acid) segments collapsed at the surface through a phase transition proper or merely conformationally restricted through interaction with the APTES underlying layer.

For bacterial adsorption studies we were limited to a maximum temperature of 37 °C, which was above the LCST for all the polymers at pH 5.6–7.0 but potentially within the region at which the phase transition occurred for **P2** at pH 7.4. However, the broad range of LCST for **P2** at pH 7.4 and the fact that we observed measurable changes in both surface structure and adhesion force between 24 and 34 °C, coupled with the previously reported suppression (by 2–4 °C) of phase transition temperatures for surface-grafted PNIPAm (Takei et al., 1994) implied that this surface too exhibited a hydrophilic–hydrophobic switch over the two assay temperatures under all the pH and temperature regimes in the bacterial adsorption experiments.

For bacterial assays carried out in PBS at pH 7.0 followed by rinse cycles in DDW the attachment of *S. typhimurium* increased above polymer LCST for surfaces **P1** and **P3** but was not significantly different for **P2** even though all the surfaces displayed similar increases in water contact angle over the temperature range. For *B. cereus*, the numbers of cells present on all the surfaces at 37 °C was higher than those on the surfaces at 10–12 °C, with the greatest change apparent for the neutral **P1** surfaces. These results were in agreement with our earlier study on the attachment of *S. typhimurium* and *B. cereus* to neutral thermoresponsive polymers (Cunliffe et al., 2003) and suggested that, as detected in the contact angle measurements, the behaviour of the polymer brushes was most strongly influenced by the neutral PNIPAm components.

Across the pH ranges the variations in *S. typhimurium* adsorption were low, with only **P3** surfaces at higher pH showing marked differences at pH 7.4 ($\Delta_{adsorption} = 8.7 \times 10^4$ cfu cm^{-2}, $P = 0.15$) with expected polymer phase transitions. Minor differences in attachment of *B. cereus* were also observed with temperature, but only for **P2** at pH 7.4 were the numbers of cells present at the two temperatures close to statistically significant variations from the two sample means ($P = 0.1$, *z*-test two sample variance from mean values). There were no significant differences in adsorption with pH for this bacterium, suggesting that any changes in polymer conformation as a result of variations in ionic content were not sufficient to alter initial cell attachment. In addition, changes in surface roughness for the sets of polymer brush surfaces were small, implying that while topographic changes were visible by AFM, any variations in bacterial attachment over the temperature regimes were not a clear function of average surface roughness.

The overall pattern of bacterial attachment was thus rather complex. For the neutral surfaces the behaviour was as predicted owing to the known tendency for these bacterial strains to adhere in greater numbers to more hydrophobic rather than hydrophilic substrates (van Loosdrecht et al., 1987a). Increases in water contact angles were accordingly reflected in higher bacterial counts at the surfaces. For the carboxylic acid containing co-polymer surfaces, minor variations in cell adsorption with temperature were observed while increases in numbers of attached cells with temperature at constant pH were less at pH 5.6 and 7.4 than at pH 7.0 although the overall magnitudes were very similar. In addition, there was no strong correlation of numbers of attached cells with the assay suspension pH even though both the **P2** and **P3** surfaces exhibited changes in contact angle with temperature and pH, albeit a small change for **P3** between pH 5.6 and 7.4. Considering the low carboxyl content of **P3** the lack of pH response is not too surprising, as there may simply not have been enough carboxylate groups to effect biologically significant changes in conformation irrespective of solution pH. The fact that contact angle measurements

suggested that the PNIPAm components were the main contributors to the surface behaviour in this co-polymer reinforces the view that carboxylate content was low at all the pH values, not just at pH 5.6, and is a good indication as to why the **P3** surfaces did not respond more strongly to solution acidity. However, because bacterial cells carry a net negative charge it is possible that a cell repellent effect might have arisen from these co-polymer surfaces even when relatively low numbers of the pendant carboxylic acid groups were ionised, as would occur across the pH range under investigation. Charge–charge interactions are of longer range than H-bonding or steric factors, thus in order for bacteria to attach to a surface they must first overcome electric double layer repulsions so the presence of any additional charge, even 'buried' under a thin PNIPAm loop, might provide a rate-limiting barrier to cell adsorption. While surfaces **P3** behaved in contact angle studies essentially as if they were PNIPAm only, in buffers of high ionic strength a small, but possibly significant, ion–ion repulsive effect may have been a factor in the resulting adhesion profile. For the potentially more acidic and ionisable **P2** surfaces the low variation of bacterial attachment with pH was not expected and again points to the greater contribution of the PNIPAm segments compared to the poly(6-acryloylaminohexanoic acid) components. This also suggests, as noted in the contact angle measurements, that much of the carboxyl functionality in the **P2** surface also was 'locked' via covalent or non-covalent interactions with the surface, and was not a strong contributory factor in cell adsorption. In this respect, the co-polymers in this study were behaving in a rather different fashion to those reported by Larsson et al. (2001) who noted differences in behaviour of PNIPAm co-polymer anchored to colloidal silica surfaces compared to PNIPAm homopolymer, in this case attributed to repulsion interactions of the co-polymer with the anionic substrate. With our cationic amine-functional substrates the contribution of the anionic component was perhaps not surprisingly rather less than that observed by Larsson et al. (2001).

Comparison of the results across the two bacterial strains indicated that *B. cereus* cells attached in lower numbers than *S. typhimurium* to all the surfaces under the various assay conditions. *B. cereus* cells appeared to be more sensitive to changes in contact angle than *S. typhimurium*, as in all cases the numbers of cells attached were higher at 37 °C than at 10–12 °C. This behaviour was shown most clearly for *B. cereus* attachment to **P2** co-polymer at pH 7.4 and 37 °C: the number of *B. cereus* cells adsorbed was much higher (by a factor of >2) than was the case for *S. typhimurium*, which actually showed a slight decrease in attachment under the same conditions. This was most likely a consequence of the cell surface structures present on *B. cereus*, which consist of lipoteichoic acids and a variety of protein residues: collapse of the carboxyl-containing co-polymers above LCST may thus have prevented unfavourable interactions of the polymer anionic side chains with the bacterial lipoteichoates. The fact that there was little change in adsorption with pH suggests that these interactions were of lesser magnitude than those due to non-specific hydrophobic interactions with collapsed PNIPAm blocks. *B. cereus* cells have been noted to be most hydrophobic at late stationary phase (Peng et al., 2001) and thus under the conditions of the assays (cells were grown to stationary phase then incubated with the surfaces) it is not therefore surprising that the bacteria were found to attach in higher numbers to the more hydrophobic substrates. The origin of the cell surface hydrophobicity is partially due to protein residues on *Bacillus* spp. that include a sub-type dependent S-layer protein: the overall hydrophilicity/hydrophobicity of the cells varies with the presence and crystallinity of this S-layer (Miyamoto et al., 1997) which in turn is dependent on the storage and growth conditions of the microorganism. *B. cereus* is also capable of spore formation, which leads to rather variable surface characteristics, and is itself temperature-dependent (Gonzalez et al., 1999; Mazas et al., 1997). While the cells in these experiments were not in optimum survival media, over the timescales of the experiments (24 h) it is unlikely that significant sporulation had occurred and no evidence for spore formation was found in microscopy images selected at random during the bacterial adsorption experiments. Thus, the overall pattern of adhesion of this bacterial strain was in accord with prior studies (van Loosdrecht et al., 1987b, 1990) with a small but reproducible increase in numbers of attached cells as the surfaces were 'switched' via temperature, but not by pH.

The other bacterial strain in these experiments, *S. typhimurium* showed a similar pattern of attachment to *B. cereus*. *Salmonellae* species are able to use a range of surface structures including fimbriae or pili to promote attachment and also are highly

motile, employing flagellae to reach a surface, but their 'colloidal' properties are largely dependent on strain-specific lipopolysaccharides, and/or capsule and slime layers at their surface (Austin et al., 1998). In our assays attachment of *S. typhimurium* varied according to the switch of the polymers via temperature rather than pH, suggesting a non-specific mode of adsorption over the timescales of the experiments. Minor variations in attachment across the sets of surfaces were found according to pH, but these were probably not of statistical significance compared to mean variations, suggesting that these cells too were more sensitive to changes in surface layer hydrophobicity than to any other factor in our assay (van Loosdrecht et al., 1989).

Overall, it was apparent that surface switches in hydrophilicity and hydrophobicity as a result of polymer brush phase changes with temperature were factors in mediating bacterial attachment. These findings were in accord with our earlier observations that *S. typhimurium* and *B. cereus* attached in similar numbers to non-responsive surfaces over the two assay temperatures but varied in adsorption to neutral responsive polymer grafted materials (Cunliffe et al., 2003), however it was also evident that the effects of pH-dependent polymer ionisation over the acidity ranges in this study were of less significance. This may have been due to the low degree of surface change by the polymers over the pH ranges but may also reflect the ability of cells to adopt different attachment mechanisms in response to variations in surface environment even though these are not normally manifest over short time periods. Our consideration of the bacteria as 'living colloids' is of course an approximation, albeit reasonable over the timescales of the adsorption experiments, since longer term bacterial attachment relies on the ability of the cells to initiate extra-cellular polymeric substance (EPS) production leading to irreversible adhesion and biofilm formation. It should also be noted that only 1–10% of the total cell populations actually adhered to the surfaces, suggesting that we were not observing biofilm formation per se but rather were detecting sub-sections of the population that were able to adsorb in a non-specific manner to the polymer brushes: the fact that this adsorption was related to overall polymer surface hydrophobicity supports this assertion. Nevertheless, the results are of significance in that the mediation of short-term attachment via hydrophilic–hydrophobic switching within relatively simple random co-polymers implies that further control of cell attachment might be possible via more regulated polymer architectures capable of more selective switching. For example, the growth of block co-polymer brushes from surfaces via living polymerisation routes would enable individual components of the brushes, such as charged or thermoresponsive blocks, to be placed at specific points within the polymer chain. This should allow particular factors such as pH and temperature response to be decoupled from each other and their effect on cell adsorption to be quantified. Ultimately it might be possible to control whether a bacterial cell can attach to a surface using these polymer brushes or indeed to direct a polymer itself to attach to a specific cell via a pH or temperature trigger. We are currently investigating block co-polymers prepared via living polymerisation routes in order to test the above hypotheses.

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