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Controlling the adhesion of the diatom *Navicula perminuta* using poly(*N*-isopropylacrylamide-*co*-*N*-(1-phenylethyl) acrylamide) films

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Keywords: adhesion, diatoms, Lower Critical Solution Temperature, Navicula perminuta, stimuli, thermo-responsive surfaces

Abstract: Poly(*N*-isopropylacrylamide)-*co*-*N*-(1-phenylethyl) acrylamide [P(NIPAAm-*co*-PEAAm)] thermo-responsive thin films with a lower critical solution temperature (LCST) adjusted to fit marine applications were used to investigate the effect of changes in the wetting properties of a surface on the adhesion of the diatom *Navicula perminuta*, an organism which forms slime films on surfaces immersed in aquatic environment. Although the strength of attachment of cells was affected by whether the film was

collapsed or expanded, no significant decrease in adhesion strength occurred upon temperature decrease. The effects were attributed to possible strong interactions between the hydrophobic segments of the responsive film when collapsed with components in the adhesive complex.

Introduction

The unique dynamic and controllable properties of *stimuli*-responsive materials are of major interest for the control of biomolecular adsorption, cell adhesion and function, having found diverse applications including in biosensing (Ding *et al.* 2001), and cell culture (Yang *et al.* 2005). Since the adhesion of cells and organisms to surfaces is mediated by the deposition of extracellular substances, changes of the interaction of these substances with the surface (as induced by the phase transition of the responsive polymer) may affect the anchorage strength of cells and organisms, and cause detachment. The release of mammalian cells from thermo-responsive surfaces based on poly(*N*-isopropylacrylamide) (PNIPAAm) and derived copolymers by decreasing temperature to below the LCST has been widely investigated (Schmaljohann *et al*. 2003, Yang *et al.* 2005). Of particular interest are the investigations on the potential of responsive surfaces for biofouling control (Ista *et al*. 1999, 2010, Cunliffe *et al.* 2000, 2003, Alarcon *et al.* 2005). It has been shown that bacterial attachment to surfaces can be controlled by the temperature-dependent properties of thermo-responsive surfaces, depending on the surface preferences of the bacterial species (Ista *et al.* 1999, 2010).

Diatoms dominate the slime films that develop on all illuminated surfaces immersed in the aquatic environment. Raphid diatoms are unicellular algae that adhere to surfaces through the production of sticky extracellular polymeric substances secreted through an elongate slit, the raphe, in one or both valves (Molino & Wetherbee 2008). Diatom slimes significantly enhance drag (Schultz 2007) and are responsible for impaired function of underwater equipment, especially optical sensors, in both freshwater and marine environments.

To investigate the ability of diatoms to respond to the properties of the surface on which they attach, the authors recently developed poly(*N*-isopropylacrylamide)-*co*-*N*-(1-phenylethyl) acrylamide [P(NIPAAm-*co*-PEAAm)] films with a transition temperature suited to applications in marine environment (Cordeiro *et al.* 2009). The temperature-dependent properties of the hydrophobically-modified PNIPAAm films were extensively characterized using a variety of surface sensitive techniques, providing insights on the interfacial properties of the generated

films (Cordeiro *et al.* 2009, Duval *et al.* 2009). In the present paper, the efficacy of P(NIPAAm-*co*-PEAAm) copolymer films to modulate initial attachment and adhesion strength of the diatom *Navicula perminuta* was investigated.

Materials and methods

Thermo-responsive films

The synthesis and characterization of poly(*N*-isopropylacrylamide-*co*-*N*-(1-phenylethyl) acrylamide) (P(NIPAAm-*co*-PEEAm)) copolymers are described elsewhere (Cordeiro *et al.* 2009). Thermo-responsive P(NIPAAm-*co*-PEEAm) thin films (copolymer ratio NIPAAm : PEEAm = 0.9 : 0.1) were immobilized onto Teflon AF coated Nextrion® glass slides or silicon wafers using a low pressure argon plasma treatment (Schmaljohann *et al.* 2004, Cordeiro *et al*. 2009). After plasma immobilization, the dry film thickness of the thermo-responsive layer was measured by ellipsometry (M-44, J. A. Woolam Co., Inc., USA) and found to be about 12 nm. Temperature-dependent swelling/deswelling experiments were performed using a home-built cell with an angle of incidence of 74.8° (Werner *et al.* 1999). The sample was immersed in a substitute for commercial artificial seawater prepared using the five main components of natural sea water as described in (ASTM D1141-98 2003) and stored overnight at 5 °C. The sample was then placed in the flow-cell, heated at a rate of 1 °C/min and ellipsometry data acquired continuously. A rate of -1 °C/min was used for the cooling curves (Cordeiro *et al.* 2009).

Experiments with diatoms

Surfaces to test the attachment of diatoms at 5 °C were incubated overnight in 9 ml of sterile distilled water in a Quadriperm dish (Fisher Ltd, UK). Three hours prior to assay distilled water was replaced with 0.2 µm filtered artificial seawater (ASW) ('Tropic Marin', Aquarientechnik GmbH, Germany). Surfaces to test settlement at room temperature were similarly equilibrated in 9 ml of ASW in an environmental cabinet at 25 °C for 3 hours prior to assay. Cultures of the diatom *Navicula perminuta* were grown in F/2 medium (Guillard &

Ryther 1962). One milliliter of a concentrated suspension of *N. perminuta* cells was added to each well of the Quadriperm dishes and mixed with the 9 ml of ASW in which slides had been equilibrated. Final working concentration of cells was equivalent to 0.181 µg chl*a* ml^{-1}. Dishes were placed in environmental cabinets at 5 °C or 25 °C and cells were allowed 2 hours in which to settle and attach to surfaces.

Settled, non-attached cells were removed by gentle washing at the settlement temperature. Slides were washed *in situ* in their dishes and did not pass through the air: water interface. This was particularly important to avoid any re-organization of the film upon contacting air (Cordeiro *et al.* 2009). Replicates for the quantification of attached cells were fixed in 2.5 vol% glutaraldehyde (Sigma-Aldrich, UK) in ASW; to provide the initial settlement density of cells on each coating.

The volume of each well of the remaining samples was adjusted to 10 ml. Where a temperature switch was not required, dishes were returned to the appropriate environmental cabinet. To effect the 25 °C to 5 °C temperature change, Quadriperm dishes were placed over ice. For the reverse temperature switch, dishes were placed on a slide-drying hotplate. The rate of temperature change was monitored using a Digitron 3208 I. S. probe and fifteen minutes was allowed for the switch to be completed before the temperature of each well was checked. The slides were exposed to flow at the final temperature of each sample using a novel apparatus (see Detachment Studies), and then fixed as described above. The difference in cell numbers between unexposed slides and those subjected to flow was used to calculate the mean cell removal as a percentage of the initial settled population.

Cells were visualized by virtue of autofluorescence of chlorophyll using a Zeiss Axioplan epifluorescence microscope. Semi-automated counts were made using Zeiss Kontron 3000 image analysis software with 30 random fields of view being quantified for each replicate.

Detachment apparatus

A novel method was devised to test the adhesion strength of cells without the need for slides to be exposed to air. Washed slides, still in 10 ml of ASW in their Quadriperm dishes,

were transferred to a 5 l tank of ASW (at either 5 °C or 25 °C), gently submerged, and placed horizontally on a stand. A recirculation pump (Fluval Ltd, UK) in the same vessel circulated the seawater through 13 mm tubing at a rate of 680 l h^{-1}. Tubing was rigged to a retort stand that had been placed on an oscillating platform (Luckham Ltd, UK) with a travel distance of 10 cm. The central 1 cm of three replicate slides was then exposed to flow for 5 min.

Data Analysis

Detachment data was analyzed to separate the effect of the P(NIPAAm-*co*-PEAAm) copolymer film passing through the LCST on the adhesion strength of *Navicula* cells, from the effects of varying temperature on cell physiology. The contribution of the different variables (Table 1) to the total variation in the dataset was made explicit.

INSERT TABLE 1

Detachment data were arcsine-transformed and subjected to the Anderson-Darling test for conformity before parametric statistics were employed. An initial 1-way ANOVA on the influence of surface type revealed that removal from the glass standard and the P(NIPAAm-*co*-PEAAm) polymer film was significantly different (p = 0.01), and subsequent analyses were therefore conducted separately for the two surface types. A 2-way ANOVA with post-hoc, pairwise Tukey test was used to determine the significance of the attachment and detachment temperatures. A separate 1-way ANOVA was then used to determine the significance of transition through the LCST.

Results and discussion

Thermo-responsive thin films

To investigate the ability of marine organisms to recognize changes in the properties of the surface to which they settle, the authors recently developed and characterized P(NIPAAm-*co*-PEAAm) copolymer films with a transition temperature fitting applications in marine environment (Cordeiro *et al.* 2009). The films reversibly swelled and collapsed in artificial

seawater in dependence of temperature (Figure 1), with a transition temperature of 15 °C and a swelling degree of ca. 9 below the LCST (Cordeiro *et al.* 2009). The curves represented in Figure 1 correspond to the dynamic swelling and deswelling of the thermo-responsive film. The kinetics and equilibrium of swelling can be found described elsewhere (Schmaljohann *et al.* 2004, Cordeiro *et al.* 2009).

INSERT FIGURE 1

The imaging of the expanded and collapsed film by atomic force microcopy revealed very smooth surfaces (roughness ca. 1 nm), with no morphological changes occurring with the transition temperature (Cordeiro *et al.* 2009). Inverse contact angles measurements performed in artificial seawater showed that the receding contact angle decreased from 35° at 25 °C to 20-25° at 5 °C, confirming the increased hydrophilicity of the film below the LCST (Cordeiro *et al.* 2009).

Attachment studies

Diatom cells are not motile in the water column, and in nature reach a surface through transport in currents and gravity. In laboratory assays, the cells sink rapidly and form an even covering on the test surfaces. For a given suspension density, the number of cells per unit area settled on a surface should therefore be constant, but the proportion of that population which *attach* is influenced by a range of environmental factors, not least the physicochemical properties of the substratum. To investigate the influence of the temperature-dependent surface properties of P(NIPAAm-*co*-PEAAm) copolymer thin films on the attachment of *Navicula*, cells were settled for 2 h at either 25 °C (i.e. above the LCST) or at 5 °C (i.e. below the LCST). The number of attached cells on the thermo-responsive surface and on Nextrion® glass controls at 5 °C and 25 °C are shown in Figure 2A.

A two-way ANOVA using the surface types (Nexterion® glass, P(NIPAAm-*co*-PEAAm) copolymer film) and settlement temperatures (25 °C, 5 °C) as the treatment variables confirmed that both have a significant influence on the density of attached cells (p = 0.001). Temperature may influence the number of attached cells on the P(NIPAAm-*co*-PEAAm)

copolymer film in two ways. Firstly, the physiology of cellular attachment may be influenced, which is confirmed by the differential densities of cells on the Nexterion® glass controls at 5 °C and 25 °C. Secondly, the physicochemical characteristics of the thermo-responsive polymer film depend on the environmental temperature (Cordeiro *et al.* 2009). Given that temperature over this range should have no effect on the glass substrate, standardizing the P(NIPAAm-*co*-PEAAm) copolymer film data to the glass control should exclude the effect of temperature on cell physiology. Standardized data (Figure 2B) therefore make explicit the effect of temperature on diatom attachment as mediated by the physicochemical differences of the thermo-responsive surface depending on whether the temperature is below (5 °C) or above (25 °C) the LCST. A Student's t-test on standardized attachment data confirmed what can be seen graphically in Figure 2B: the density of *N. perminuta* cells on the thermo-responsive film does not differ significantly from the settlement temperature ($p = 0.05$), suggesting that initial cell attachment is not influenced by the differing physicochemical characteristics of the surface above and below the LCST.

INSERT FIGURE 2

Detachment studies

To determine whether the temperature-dependent physicochemical properties of the thermo-responsive layer have an effect on cell adhesion strength, samples settled at either 5 °C or 25 °C were exposed to flow at the respective temperature using the apparatus described in the experimental section. The removal of adhered diatom cells from the glass control and from P(NIPAAm-*co*-PEAAm) copolymer surfaces are expressed in Figure 3 as percentage removal of the attached cell population.

INSERT FIGURE 3

The attachment strength of cells of *Navicula* to Nexterion® glass was not notably influenced by the attachment temperature (Figure 3). Results from a 2-way ANOVA confirm that the strength of attachment of cells on the glass control does not differ significantly ($p = 0.05$) with varying settlement temperature. This suggests that, once sufficient EPS has been

produced to attach the cell, the strength of attachment is independent of the ambient temperature (for the 2 h duration of the experiment). It is not therefore necessary to normalize detachment data to negate the effect of temperature on cell physiology. In the parallel analysis on the P(NIPAAm-*co*-PEAAm) copolymer films, the temperature at which settlement occurs is found to significantly influence the attachment strength (p = 0.001). Cells settled at 25 °C, when the polymer surface is collapsed and more hydrophobic, are more strongly attached than those settled at the lower temperature (i.e. onto a more hydrophilic surface). In general, diatom adhesion is weaker on hydrophilic surfaces when compared to hydrophobic surfaces (e.g. Finlay *et al*. 2002), which is in good agreement with the results obtained.

Temperature-triggered effects on cell adhesion

To determine whether the dynamic nature of the polymer film could moderate cell adhesion strength, the adhesion strength of cells attached to surfaces maintained at static temperatures below and above the LCST were compared with the adhesion strength of cells attached to surfaces heated or cooled passing trough the LCST after settlement (Figure 4).

INSERT FIGURE 4

A one-way ANOVA comparing the removal of cells from Nexterion® glass controls shows that there are no significant differences (p = 0.05) in adhesion strength irrespective of whether cells pass through the LCST of the polymer layer, in either direction, or are maintained at their starting temperatures (Figure 4A). Therefore, the quick temperature variations tested do not appear to have an effect on cell physiology nor on the interaction of the adhesion complex with the surface.

The comparison of removal of cells from P(NIPAAm-*co*-PEAAm) copolymer films when maintained at constant temperature with the removal of cells after temperature switch is shown in Figure 4B. One-way ANOVA revealed no significant differences (p = 0.05) in cell adhesion strength whether cells were settled and maintained at 25 °C or if these were cooled down to below the temperature transition of the responsive polymer surface after cell settlement. Since the adhesion strength of *diatom* cells generally decreases with increasing hydrophilicity,

which is in agreement with the fact that cells adhered more strongly to the collapsed film (i.e. when settled and maintained at 25 °C) than to the expanded film (i.e. when settled and maintained at 5 °C) (Figure 3), it was hypothesized that the adhesion strength of cells may decrease with decreasing temperature after initial cell attachment, due to changes in surface properties and as a result in surface-adhesive complex interactions. It is however possible that the adhesive complex secreted by the adhering cells strongly interacts with the exposed hydrophobic segments of the collapsed thermo-responsive film, and under the experimental conditions tested these interactions may not significantly be affected by the transition temperature, possibly even hindering film hydration and expansion (i.e. re-organization of polymer segments) upon temperature decrease.

Higher removal of cells from P(NIPAAm-*co*-PEAAm) films was observed when the surfaces were settled and maintained at 5 °C as compared with surfaces heated to above the LCST after cell settlement. Results from a 1-way ANOVA confirm that the strength of attachment of the cells onto the film kept in the expanded state during the whole experiment is weaker than onto films submitted to temperature increase (p = 0.001). Upon temperature increase, the polymer film undergoes dehydration and adopts a compact conformation, favoring the interaction of the polymer hydrophobic (phenyl and isopropyl) groups with the components of the adhesive complex and with the attached cells, thereby increasing cell adhesion strength.

Conclusions

Stimuli-responsive surfaces open new avenues to mediate non-specific bioadhesion and probe biological adhesion mechanisms. However, isolating the influence of a thermo-responsive polymer on cell attachment and adhesion requires careful analysis. Although altering the temperature will change the physicochemical characteristics of thermo-responsive films, the biochemistry and physiology of the cell will also be altered, as may the molecular interaction between the adhesive complex and the substratum. In such studies, it is therefore paramount to include appropriate controls to quantify the ancillary effects of assay temperature, and for the experimental design to allow variation in the dataset to be correctly partitioned between the

temperature effect on the properties of the surface and the direct effects on cellular processes. The work presented here is a first attempt towards gaining new insights on the nature of interactions of diatoms with dynamic surfaces. The results show that although cell adhesion strength can be tuned by controlling surface properties, with surface hydrophobicity playing an important role, the pre-deposition of biopolymers and the interactions of the components of the adhesive complex secreted by the attached cells with surface exposed polymer segments may influence the molecular rearrangements of polymer chains triggered by temperature switch (Cheng *et al* 2005), possibly delaying or impeding the transition of the polymer. Future investigations of the interactions of relevant adhesive components with tailored responsive surfaces will enable a further understanding of the molecular processes involved in the adhesion of diatoms.

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spectroscopic ellipsometry (part I). *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **156**: 3–17.

Tables

Table 1: Description of the variables considered in the statistical analysis of data.

Variables	**Description**
Surface type	Nexterion® glass, P(NIPAAm-*co*-PEAAm) film
Attachment temperature	5 °C; 25 °C
Detachment temperature	5° C; 25 °C
Temperature switch	5 °C to 25 °C; 25 °C to 5 °C

Figures caption

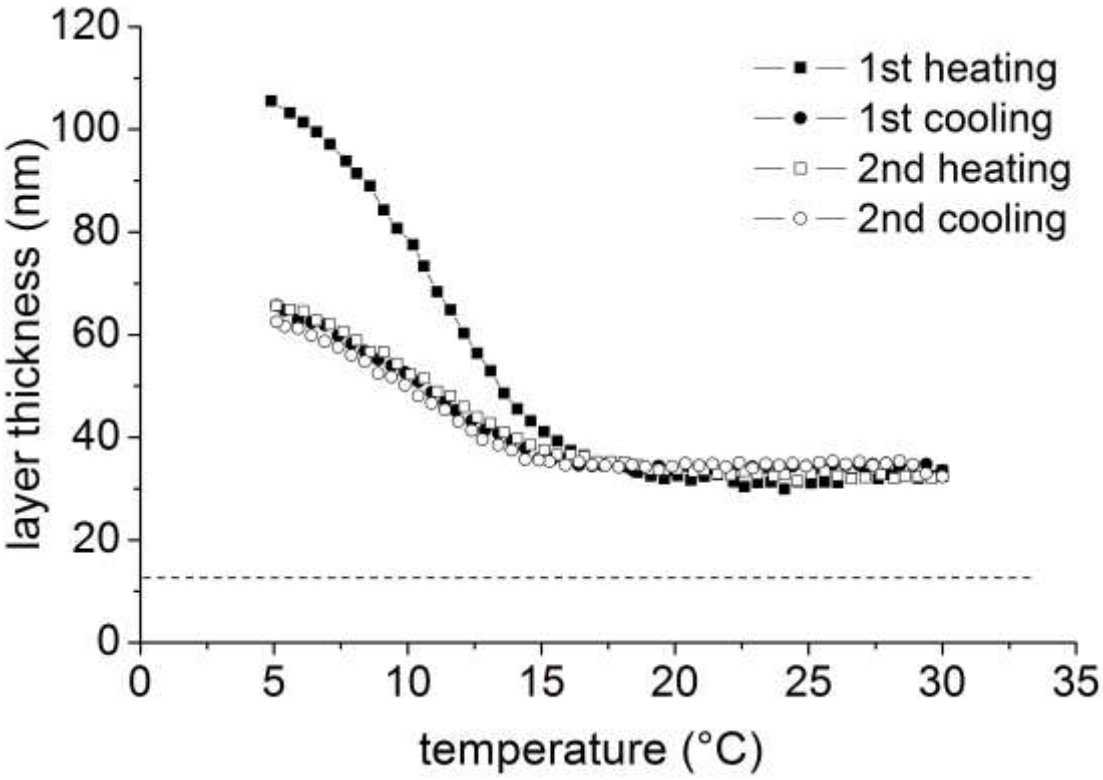
Figure 1: P(NIPAAm-co-PEAAm) film thickness versus temperature during first and second heating /cooling cycles in artificial seawater (heating/cooling rate = 1 °C). The dashed line represents the dry film thickness.

Figure 2: (A) Density (mm^2) of attached *N. perminuta* cells on P(NIPAAm-*co*-PEAAm) copolymer surface and Nexterion® glass control after 2 h settlement at either 5 °C or 25 °C. **(B)** Cells of *N. perminuta* attached to P(NIPAAm-*co*-PEAAm) surface, expressed as a percentage of attachment on the control, after 2 h settlement at either 5 °C or 25 °C. N = 90, error bars = ± 2 x Standard Error derived from arcsine transformed data.

Figure 3: Removal of attached cells of *N. perminuta* from Nexterion® glass control and from P(NIPAAm-*co*-PEAAm) surfaces by a standard normal flow (Attachment temperature °C > Detachment temperature °C). N = 90, error bars = ± 2 x Standard Error derived from arcsine transformed data.

Figure 4: Removal of attached cells of *N. perminuta* from **(A)** Nexterion® glass, and **(B)** from P(NIPAAm-*co*-PEAAm) copolymer surfaces by a standard normal flow (Attachment temperature °C > Detachment temperature °C). N = 90, error bars = ± 2 x Standard Error derived from arcsine transformed data.

FIGURE 1


1st heating
1st cooling
2nd heating
2nd cooling
layer thickness (nm)
temperature (°C)
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20
40
60
80
100
120
0
5
10
15
20
25
30
35

FIGURE 2 A

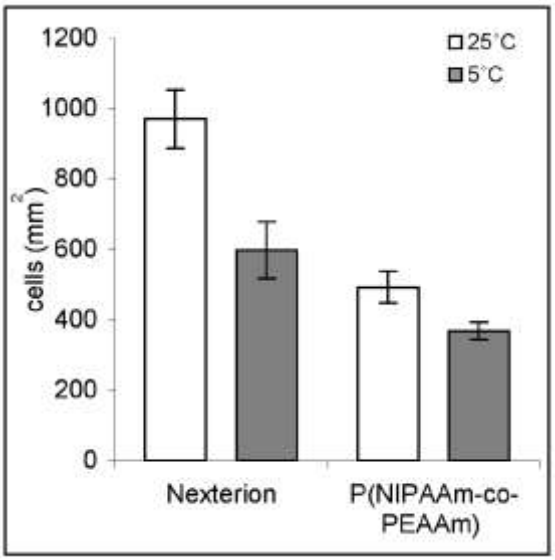

FIGURE 2 B

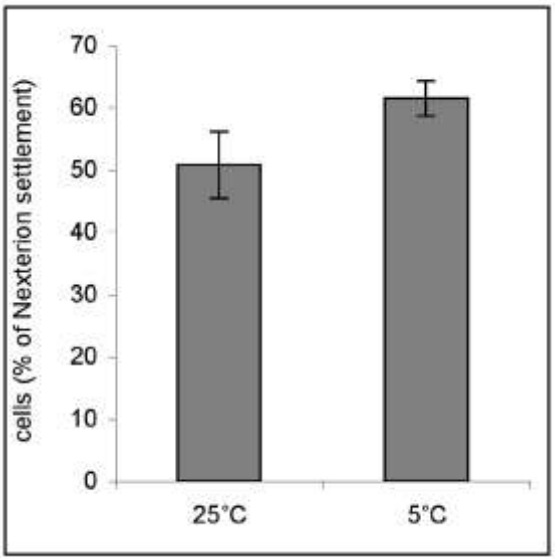

FIGURE 3

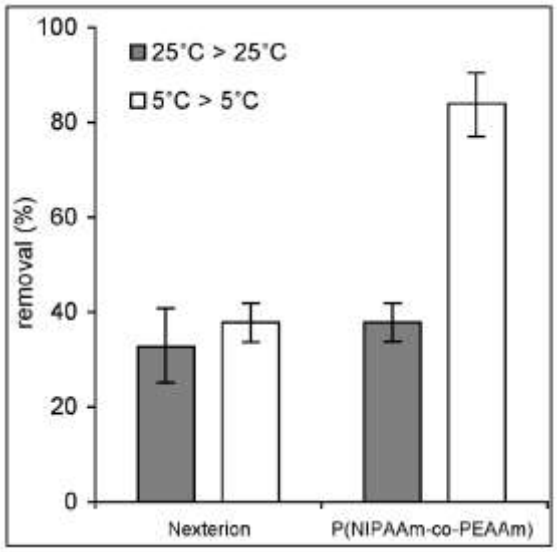
100
80
60
40
20
0
removal (%)
25°C > 25°C
5°C > 5°C
Nexterion
P(NIPAAm-co-PEAAm)

FIGURE 4 A

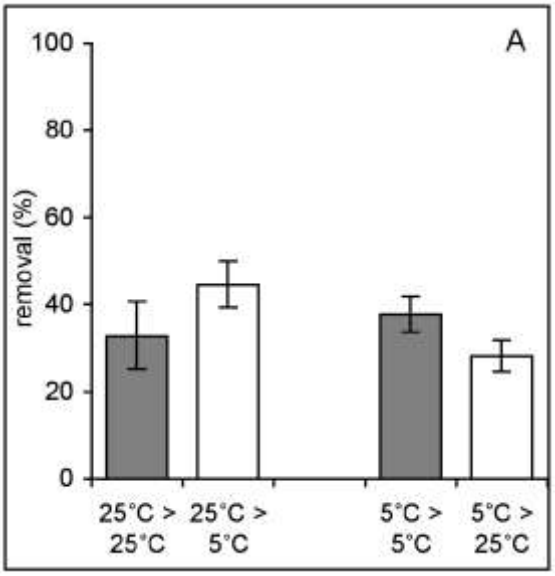
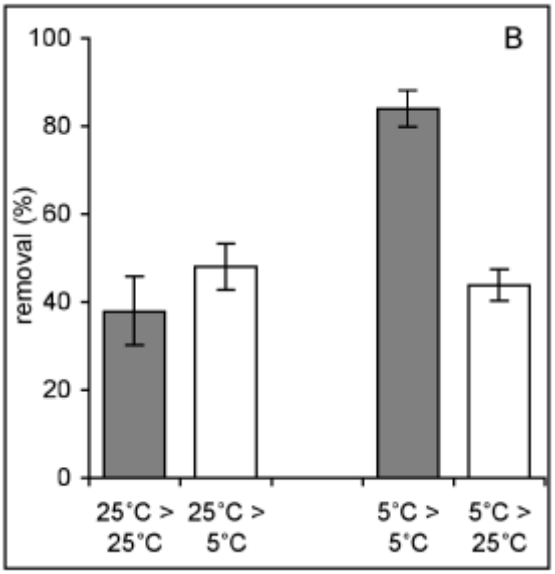
FIGURE 4 B