Journal of Industrial Microbiology & Biotechnology (1998) 20, 121–125

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Lower critical solubility temperature materials as biofouling release agents

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Poly(*N*-isopropylacrylamide) (PNIPAAM) exhibits a lower critical solubility temperature (LCST) of 32°C. Using thin films of this compound as a model system, the potential of 'smart polymers' as biofouling-release agents was examined. PNIPAAM-coated glass slides were incubated in artificial sea water containing the marine bacterium *Halomonas marina* or in natural bay water at a temperature above the LCST. Upon rinsing of the biofouled samples with artificial sea water below the LCST, the dissolution of the coating released over 90% of the attached fouling material, a significant increase over the release obtained for glass controls. These experiments demonstrate the potential of PNIPAAM and similar polymers as possible fouling-release agents, and suggest that tethered PNIPAAM (or similar polymers) may be useful as regenerable fouling-release surfaces.

Keywords: Poly(*N*-isopropylacrylamide); *Halomonas marina*; fouling-release; LCST; smart polymers

Introduction

Most materials, whether natural or artificial, become quickly covered with a collection of organic compounds, microorganisms and their byproducts when submerged in the natural aqueous environment [4]. This accumulated layer is commonly referred to as a biofilm, and represents a mode of growth that is the preferred way of life for most bacteria studied to date [7]. Biofilm formation gives an advantage to microorganisms, due to a combination of increased concentrations of nutrients at the water/solid interface, possibilities of interactions with other bacteria within a consortium, and protection from desiccation, predation, and antimicrobial agents [7]. When the colonized surfaces are those in which human beings have a vested interest in keeping clean—diverse examples include catheters, heat exchange columns and hulls of ships—the accumulation of microbes, sometimes associated microorganisms, and their byproducts is referred to as biofouling [4].

Until recently, the strategies for controlling biofouling have been generally limited to killing of the attached micro- and macro-organisms with toxins, which usually contain heavy metals or organo-metallic complexes, or to mechanical removal of the attached material [6]. Both of these techniques are problematic in terms of human health and environmental pollution and are not feasible for those materials which are in intimate contact with human tissues, such as implants, contact lenses, or catheters. An understanding of the processes involved in biofouling should enable the development of more environmentally friendly anti- and non-fouling compounds. Much of the emphasis on biofilm research in recent years has been to examine the complex interactions between the components of the biofilm and the surface supporting it [1,16], and to use this information to produce materials that reduce the net amount of microbial and organic accumulation [5]. There are generally two classes of materials that reduce biofouling, those that reduce attachment of bacteria in the first place (fouling-resistant materials, eg oligo(ethylene glycol) [12]) and those that easily release attached microbes (fouling-release surfaces, eg polydimethylsiloxane [2]).

Within the last decade, interest has grown in the use of 'smart polymers' for a variety of biotechnological uses [9]. Examples of smart polymers are those that undergo rapid, reversible phase changes in response to small changes in environmental conditions. The environmental triggers can be temperature, ionic strength, pH, light or the presence of an electric field [9]. This paper explores the possibility of using these compounds as fouling-release surfaces. Our model system employs poly(*N*-isopropylacrylamide) (PNIPAAM), a polymer exhibiting a lower critical solution temperature (LCST) [11]. It is soluble in water below 32°C. Above this temperature, PNIPAAM is insoluble in water. This property has been exploited for a number of biotechnological applications, including use of PNIPAAM for *in situ* drug delivery [10], neural cell patterning [3,14] and control of protein–ligand interaction [18]. We chose PNIPAAM as a model system because it is commercially available and its environmental trigger is easily and unambiguously controllable in a laboratory setting. We view PNIPAAM as a model for other polymers that may be useful as fouling-release agents and that can be designed to exhibit critical solubility transitions at desired temperatures or in response to a desired stimulus. In this paper we report initial studies of thin films of PNIPAAM produced by dip coating. Further work will focus on attached ultrathin films of PNIPAAM produced by covalent surface modification.

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Received 26 September 1997; accepted 29 November 1997

Materials and methods

Bacterial strains

Halomonas marina (basonym *Deleya marina*, Baumann *et al*, 1983) [8,17] ATCC 2534 was obtained from the American Type Culture Collection, Bethesda, MD, USA. Twenty per cent glycerol stocks of this organism were maintained at −70°C and revived on Marine Agar 2216 (Difco, Detroit, MI, USA) slants. At the beginning of each experiment, *H. marina* was transferred from the slant to 50 ml of Marine Broth 2216 (Difco) and grown at room temperature overnight (18–20 h) with constant, low-speed stirring.

Sea water

Bay water (BW) was collected on the south part of Tulalip Bay in Puget Sound, near Marysville, WA, USA. Samples were collected during ebb tide in early August of 1997. The collection vessels were clean plastic milk containers. After collection the containers were sealed and shipped to our laboratory by express delivery. Upon receipt, the samples were stored at 4°C until use.

Sample preparation

Glass coverslips (22×50 mm, thickness No. 3, Erie Scientific, Portsmouth, NH, USA) were soaked in ethanol for 15 min followed by a rinse in water (referred to as d-H_2O), deionized in a RO-Pure/Nanopure System (Barnstead/Thermolyne, Rapid City, SD, USA) and dried in a stream of nitrogen. The slides so cleaned were subjected to an argon plasma (pressure 75 μm Hg) in a Harrick RDC-32G plasma cleaner/sterilizer. Stock solutions of PNIPAAM (Polysciences Inc, Warrington, PA, USA) were prepared as 5% w/v in ethanol/d-H_2O (80/20). Stock solutions were made fresh weekly. Working solutions were 1/5 dilutions of the stock in ethanol/d-H_2O (80/20). Freshly cleaned slides were dipped into the 1% PNIPAAM for 20 min followed by withdrawal at a rate of 10–15 mm s^{-1} using a Chemat 201 dip-coating machine (Chemat Technologies, Northridge, CA, USA). Samples were allowed to air dry 15–20 min before being placed into incubation.

Film characterization

Films were also generated on plasma-cleaned silicon wafers using the protocol described above for glass slides; these films were characterized by ellipsometry using a spectroscopic ellipsometer (M-44, JA Wollum Co, Lincoln, NE, USA) at a wavelength range of 400–700 nm and an angle of incidence of 75°. Measurement of PNIPAAM as a Cauchy layer ($\eta_D = 1.5$ and extinction coefficient = 0) indicated that this layer was ~400 nm thick. Coated samples were incubated in 37° artificial sea water (ASW; [13]) for 18 h and rinsed as they would be in a normal detachment experiment (see below). Subsequent thickness measurements indicated that PNIPAAM was totally removed.

The advancing water contact angles of deposited PNIPAAM were taken on a Rame-Hart (Mountain Lakes, NJ, USA) goniometer. The samples were maintained on a heating tape at approximately 45°C to prevent the water droplet from cooling and consequently dissolving the PNIPAAM. The water contact angle of PNIPAAM was $43 \pm 2.5°$ and that of the plasma cleaned glass was $15 \pm 1°$.

Detachment studies

Samples were incubated in approximately 25 ml ASW containing ~4×10^8 cells ml^{-1} *H. marina* or unfiltered BW (cell titer: 4×10^6 cells ml^{-1}) that had been pre-warmed at 37°C for at least 30 min prior to use. Samples were incubated for 2 or 18 h depending upon the type of experiment that was performed. Control experiments were conducted on slides that had been plasma-cleaned but not coated with PNIPAAM. After incubation, the slides were quickly rinsed in pre-warmed (37°C) d-H_2O to remove loosely attached cells and salt and the number of cells counted. The slides were rinsed with 60 ml 4°C water delivered from a 60-ml syringe, followed by a rinse in d-H_2O. The slides were then dried under a stream of nitrogen and the cells remaining counted.

Cells were enumerated by examination of slides under phase contrast microscopy at 60× magnification with a Nikon Labophot microscope. The microscope was attached to a Panasonic CCTV camera and images were captured using UTHSCSA Image Tool image processing software (IT, Version 1.27 developed at the University of Texas Health Science Center at San Antonio, TX, USA and available from the Internet by anonymous FTP at URL http://ddsdx/uthscsa.edu/dig/itdesc.html) and a Data Translations DT3155 frame grabber card. One in-focus and one out-of-focus image were captured for each field of view. The out-of-focus frame was then subtracted from the in-focus frame using IT. This step eliminated optical aberrations introduced by the microscope and isolated the bacteria from the background. The images were processed through a low pass filter, converted to a binary image using the 'threshold' function and counted using the 'find objects' protocol. Individual cells were not separated well in those samples severely fouled with *H. marina* from overnight incubations. Therefore, the number of pixels in the binary image comprising the area of the field of view covered by the bacteria were counted. The number of pixels was then divided by 71 to estimate the number of bacteria in those areas. Seventy-one pixels represented the average image area covered by a single bacterium in 14 different slides containing less than 20 well-separated *H. marina* cells. A heterogeneous collection of cell forms and sizes accumulated on the surfaces exposed to BW, making it impossible to generalize a particular cell size and the total number of pixels covered by the attached material were counted. The number of cells or pixels in 10 fields of view were counted and averaged for each experiment. Five replicate experiments were performed for each combination of incubation condition and surface.

Results

The results of the detachment studies are summarized in Table 1 and Figure 1. All data are presented as the average of five experiments ± standard error. As can be seen in Table 1, there was a high degree of variability in the number of cells attached and remaining after the 4°C ASW wash between replicate experiments in which the samples were incubated in *H. marina*, and this is reflected in the large standard errors. These large standard errors made it impossible to quantify a difference in initial attachment of

Table 1 Number of bacteria attached to test surfaces initially and after 4°C wash

		Number of cells attached mm^{-2}	Number of cells remaining mm^{-2} after 4°C wash
Halomonas marina	Glass	136 ± 58	94 ± 52
2-h attachment	PNIPAAM	142 ± 70	6 ± 2
Halomonas marina	Glass	632 ± 283	392 ± 250
18-h attachment	PNIPAAM	2830 ± 1490	239 ± 14
Bay water	Glass	6947 ± 1341	4598 ± 818
18-h attachment[a]	PNIPAAM	8273 ± 2313	176 ± 26

All numbers are expressed as the average ± standard error of five replicate experiments.
[a]Because of the heterogeneity of the attached cells from incubation in bay water, these data are presented as number of pixels covered in a digitized image of the field of view.

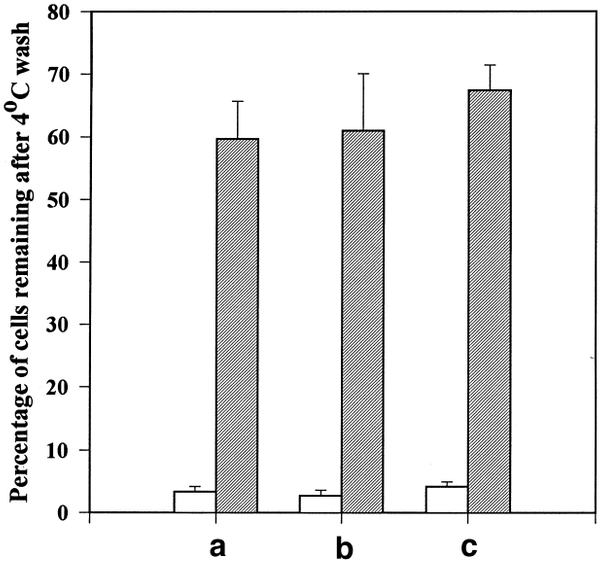

Figure 1 Detachment of biofilms from solid surfaces: □, PNIPAAM; ▨, glass. Percentage of cells retained after removal of PNIPAAM layer by rinsing layer with 4°C ASW. Biofilm forming conditions: (a) *Halomonas marina* 2-h attachment at 37°C. (b) *H. marina* overnight attachment at 37°C. (c) Tulalip Bay water overnight incubation at 37°C. Data points represent averages and standard errors of five replicate experiments.

cells attached to the two surfaces. In the comparisons of the averages, the student *t*-test showed no statistically significant difference between PNIPAAM and glass ($P > 0.05$). When considering the percentage of release from surfaces incubated in *H. marina*, however, a significant difference was seen (Figure 1). The average reductions for PNIPAAM incubated in *H. marina* for 2 h and overnight were 96 ± 0.8% and 97 ± 1% respectively, whereas the numbers for glass were 40 ± 6% and 41 ± 1%.

The variability for overnight incubation in bay water was less pronounced and the difference between glass and PNIPAAM was significant (*t*-test: $P < 0.05$). Cells of a variety of sizes and shapes attached to both surfaces exposed to BW. Because of the heterogeneity of the attached material, it was difficult to determine an average cell size in order to estimate the number of cells in the binary images. Therefore, for these experiments, the data collected were the total number of pixels covered by attached material and this information was used to determine the percent detachment. There were 307 200 pixels comprising a field view with a total area of 0.24 mm^2. The data are an expression of the reduction in the number of pixels covered by attached cells, rather than the number of those cells. The average percentage reduction in surface coverage after 4°C wash in ASW was 95.2 ± 0.8% for PNIPAAM and 32.5 ± 4% for glass.

In all cases, release of the PNIPAAM reduced bacterial attachment by at least 90%. It is possible that the few remaining bacteria in each case were washed 'onto' the newly-exposed glass surface. The biofilm formed on the surfaces subjected to overnight incubation in *H. marina* was often visible to the naked eye. Slides containing PNIPAAM were only partially covered due to the nature of the coating procedure, and upon rinsing a clear boundary could be seen between regions where the PNIPAAM had been present and regions where it had not. An example of this boundary is shown in Figure 2. This micrograph of the most severely fouled sample was taken after the surface was rinsed with 4°C ASW. The original number of bacteria attached to this slide was 8000 ± 529 cells mm^{-2}. After the 4°C ASW wash, the number of cells retained on the side from which the PNIPAAM was removed was 85 ± 16 cells mm^{-2}, whereas the amount remaining on the glass side is estimated to be 3000 cells mm^{-2}.

Discussion

Both PNIPAAM and plasma-cleaned glass were able to support biofilm formation. In all cases, biofilms attached to PNIPAAM were removed when the PNIPAAM layer was dissolved from the glass support. The actual number of *H. marina* cells attached to either surface varied widely between each replicate experiment and prevents us from drawing conclusions regarding the relative affinities of cells toward the surfaces. The percentage of the originally adhering cells detached after the 4°C ASW wash was, however, consistent from experiment to experiment. The variability in the number of cells attached was smaller in BW experiments. Although the difference between the average number of pixels covered by adhering cells in the glass and PNIPAAM samples was not significant by a paired student *t*-test ($P = 0.44$), the differences between averages of each sample before and after the 4°C ASW rinse were significant ($P < 0.05$), as were the differences between the average number of pixels after the 4°C wash for the glass and PNIPAAM surfaces. Like the samples incubated in *H. marina*, the differences in percent reduction between the glass and the PNIPAAM after the 4°C rinse were consistent from repetition to repetition in the samples incubated in BW.

At least two factors may have an effect on a wide variability of actual number of attached cells in the *H. marina* experiments. First, it is apparent that the surface hydrophobicity of the PNIPAAM was not consistent from sample to sample. The advancing water contact angles on PNIPAAM ranged from 37–52°. The water contact angle is reflective of the surface-free energy, and has been implicated as a factor in bacterial attachment [1]. The range in contact angles may be the result of dissolution of the PNI-

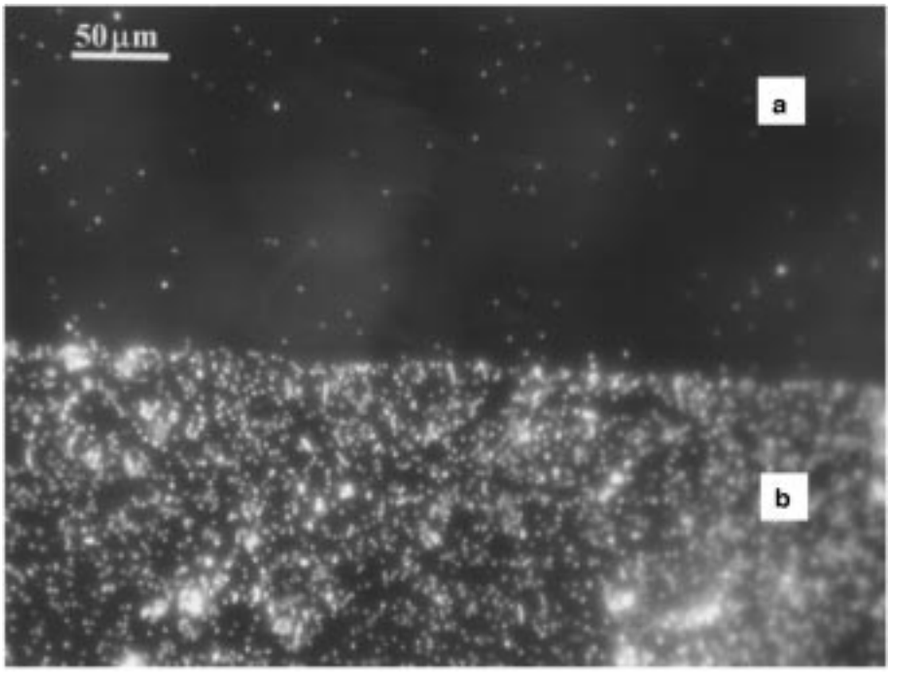

Figure 2 Boundary between glass (b) and the area formerly covered by PNIPAAM (a) showing visible signs of removal of the biofilm upon removal of PNIPAAM. The sample was incubated overnight in ASW containing *H. marina* at 37°C and rinsed with 4°C ASW. The micrograph was taken under darkfield illumination.

PAAM into water during contact angle measurement, despite attempts to keep both the water and the surface well above 32°C. Second, the bacteria were grown in batch culture, and their surface composition may not have been uniform from experiment to experiment. It is known that the relationship between the interfacial and cell hydrophobicity of the cell surface plays a role in the adsorption of cells to surfaces [1].

A major limitation to the utility of dip-coated films as fouling-release agents is that removal of the biofilm requires removal and subsequent replacement of the coatings. That the release properties may be maintained upon covalent attachment of the polymer to a support is suggested by studies in which PNIPAAM was grafted onto polystyrene tissue culture dishes and endothelial cells grown at 37°C followed by release of the cells into the medium upon lowering of the temperature to 10°C for 30 min [15]. The mechanism for this process was proposed to be the change from a hydrophobic to a hydrophilic environment and an increase in the hydration of the PNIPAAM chains. Based on the tissue culture data [15] and our studies using PNIPAAM, we believe that environmentally responsive polymers show promise as candidates for bacterial fouling-release coatings. We are currently studying the properties of these coatings when grafted to a surface and have found that PNIPAAM can preserve its fouling-release properties, even when not removed by the cold water rinse [Ista and Lopez, unpublished data]. We are also exploring the possibility of combining this polymer with a known fouling prevention agent, oligo(ethylene glycol) [12].

Acknowledgements

We would like to thank Dr JM Gosau and Mr H Fan for their suggestions and advice. This research was supported by ONR grants N00014-95-1-1315, N00014-95-1-0901 and NSF grant HRD-9450473.

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