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Poly-N-Isopropylacrylamide/acrylic Acid Copolymers for the Generation of Nanostructures at Mica Surfaces and as Hydrophobic Host Systems for the Porin MspA from *Mycobacterium smegmatis*

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Abstract

The work presented here aims at utilizing poly-N-isopropyl-acrylamide/acrylic acid copolymers to create nanostructured layers on mica surfaces by a simple spin-casting procedure. The average composition of the copolymers determined by elemental analysis correlates excellently with the feed composition indicating that the radical polymerization process is statistical. The resulting surfaces were characterized by Atomic Force Microscopy (magnetic AC-mode) at the copolymer/air interface. Postpolymerization modification of the acrylic acid functions with perfluoro-octyl-iodide decreased the tendency towards spontaneous formation of nanopores. Crosslinking of individual polymer chains permitted the generation of ultraflat layers, which hosted the mycobacterial channel protein MspA, without compromising its channel function. The comparison of copolymers of very similar chemical composition that have been prepared by living radical polymerization and classic radical polymerization indicated that differences in polydispersity played only a minor role when poly-N-isopropyl-acrylamide/acrylic acid copolymers were spincast, but a major role when copolymers featuring the strongly hydrophobic perfluoro-octyl-labels were used. The mean pore diameters were 23.8±4.4 nm for P[(NIPAM)_{95.5}-co-(AA)_{4.5}] (PDI (polydispersity index)=1.55) and 21.8±4.2 nm for P[(NIPAM)_{95.3}-co-(AA)_{4.7}] (PDI=1.25). The depth of the nanopores was approx. 4 nm. When depositing P[(NIPAM)₉₅-co-(AA)_{2.8}-AAC_{8F₁₇}]_{2.2}] (PDI=1.29) on Mica, the resulting mean pore diameter was 35.8±7.1 nm, with a depth of only 2 nm.

Introduction

Novel efficient methods for the design of bioelectronic devices¹, such as biosensors², bio fuels cells³ and memory storage devices⁴, have to be developed to permit their broad application in

the near future. This pertains to the generation of 2-D nanostructures at surfaces^{5–7} (e.g. nanoscopic cavities of a low polydispersity) as well as ultraflat organic surfaces that can be used as host layers for proteins.⁸ Since many proteins have to associate with natural or artificial membranes to retain their biological functions, the development of adaptable and cost efficient artificial hosting systems for proteins is of great importance for the technical implementation of bioelectronic devices.⁹ The use of self-assembled monolayers (SAMs) for protein adsorption and protection is prevalent.^{10,11} Recently, several procedures for the preparation of nanolayers of oligopeptides that were able to stabilize proteins¹² and protein complexes¹³ have been reported. Polymer layers, such as Poly-N-isopropylacrylamide on HOPG surfaces were used as well to provide protein-stabilization.⁷

The work presented here aims at utilizing poly-N-isopropyl-acrylamide copolymers to create nanostructures on Mica surfaces by a simple spin-casting procedure. The resulting surfaces were characterized by using Atomic Force Microscopy (magnetic AC-mode).¹⁴ We have synthesized a series of pH and temperature responsive P(NIPAM-co-AA) (NIPAM: N-isopropyl-acrylamide, AA: acrylic acid) copolymers by free radical¹⁵ and living free radical copolymerization¹⁶ of N-isopropylacrylamide and acrylic acid. Postpolymerization modification was achieved by reacting the free carboxylates with perfluoro-8-iodooctane.¹⁷ The polymers were spin-cast¹⁸ onto freshly cleaved Mica surfaces, followed by incubation in an environmental chamber under defined temperature and humidity. Crosslinking of individual polymer chains permitted the deposition of ultraflat nanolayers, which were able to host the mycobacterial channel protein MspA, retaining its channel function. The MspA porin from *M. smegmatis* is an extremely stable protein, retaining its channel structure even after boiling in 3% SDS, heating to 100°C or extraction with organic solvents.¹ MspA is the only mycobacterial porin that can be purified in mg quantities.²⁰ Another distinct advantage of MspA is its amphiphilic nature.²¹ Not only is the interior channel surface much more hydrophilic than its exterior, the exterior is subdivided in two distinct zones. MspA features a very hydrophobic “docking region” at the stem of its “goblet”, whereas its “rim” section is formed by alternating hydrophilic and hydrophobic residues so that it is much more hydrophilic.²¹ The geometric dimensions of the “docking region” are 3.7 nm in length, and 4.9 nm in diameter. It is our goal to prepare hosting nanolayers for MspA in a simple and straightforward experimental procedure. It is noteworthy that MspA does not require a stabilizing layer²², however the presence of an insulating layer between a channel protein and an electrode is mandatory for many bioelectronic and sensing applications. We have demonstrated in our earlier work that MspA is an ideal host for photonic and magnetic nanoparticles, which can be bound within its hydrophilic vestibule.^{22,23}

Results and Discussion

Polymers responsive to external stimuli such as pH, temperature, ionic strength and electric field have been the focus of many studies in view of the potential applications in diverse fields, such as medicine or bioelectronics.⁸ PNIPAM exhibits thermo-reversible phase separation behavior in aqueous solution, which makes the polymer useful as a thermoresponsive material and is characterized by a lower critical solution temperature (LCST).²⁴ On the molecular level PNIPAM has been used in many forms including single chains, macroscopic gels, microgels, latexes, thin films, membranes, coatings, and fibers.²⁵

The phase transition behavior of PNIPAM in aqueous solution has been widely investigated by calorimetric, turbidimetric and spectroscopic techniques.^{26–29} An interesting feature common to other thermosensitive polymers lies in the possibility of tuning the LCST by adding cosolvents²⁹, salts³⁰, surfactants³¹ or polyelectrolytes³¹ to the PNIPAM solutions or by incorporating comonomers with variable degree of hydrophilicity.^{32,33} Increasing or decreasing the hydrophilic content of a copolymer will result in an increase or decrease of

PNIPAM's LCST, respectively. Furthermore, polymerizing N-isopropylacrylamide (NIPAM) with weakly ionizable comonomers allows us to obtain intelligent polymers capable of responding to both temperature and pH variations; interestingly, owing to the variation of their degree of ionization with pH, systems with pH dependent LCST^{34,35} are obtained. Polymers bearing carboxylic acid functional group such as polyacrylic acid are pH sensitive, as they adopt a coiled conformation in solutions of low pH where the carboxylic acid groups are protonated, and an extended conformation in solutions of high pH where the negatively charged carboxylates undergo strong electrostatic repulsion. Consequently, pH and temperature of aqueous solutions for spin-casting of P(NIPAM-co-AA) polymers are very important parameters that have to be closely observed to achieve reproducibility of the spin-casting experiments reported here. In the work reported here, we synthesized a series of copolymers of N-isopropylacrylamide with the comonomer (acrylic acid) and investigated the influence of both temperature and pH on their solution behavior.

Synthesis of Poly(N-isopropylacrylamide-co-Acrylic acid) Copolymers

Four P(NIPAM/AA) copolymers with molar ratios 99, 98, 95 and 90 of NIPAM and 1, 2, 5 and 10 mol. % of AA were synthesized. All syntheses were carried out at 70°C in t-BuOH by free radical polymerization initiated by AIBN.³⁶ The initiator AIBN undergoes thermal homolytic dissociation around 40–60°C under nitrogen. The formed free radical, namely dimethylcyanomethyl radical, reacts with monomers and forms a chain initiator, which then undergoes a series of chain growing reaction (chain propagation) with monomers and finally leads to the formation of a copolymer. The termination of the polymerization reaction takes place by both recombination and disproportionation reactions.

The purification of the copolymer is very important and the most difficult task as it contains residual monomer, initiator and solvent as impurities. The copolymers were purified by repeated precipitations of tetrahydrofuran solutions into n-hexane, a non-solvent for copolymers but a good solvent of all the monomers. The purity of the copolymers was ascertained by GPC analysis, which shows the absence of low molecular weight species (i.e. monomers) in the purified copolymers. Classic CHN analysis³⁷ was used to determine the composition of the copolymers: NIPAM is the only monomer that contains nitrogen. Therefore, the ratio of carbon and nitrogen is indicative of the polymer composition. A more thorough description of these measurements is provided in reference 38. The results from these measurements are reported in Table 1. They indicate that NIPAM and acrylic acid possess very similar propagation constants and, therefore, the ratios of the monomers in the polymer are very similar to the feed ratios. These results are in good agreement with a previous publication.³⁹ Furthermore we assume that some of the more polar, acrylic acid rich fractions of the copolymer were lost during the precipitation and washing procedures.

We have applied the method of *living radical chain polymerization*⁴⁰ to decrease the polydispersity of the P(NIPAM-co-AA) copolymers. In this reaction, the initial 2-cyanoprop-2-yl radicals react with 2-mercaptoacetic acid to form the corresponding thiyl radical, which then slowly initiates polymerization. This approach leads to a defined number of growing radical chains, which compete for the available monomers. The resulting polydispersities ($P_D=1.18$ to 1.25) are significantly lower than without using 2-mercaptoacetic acid.

Our experimental approach of comparing copolymers of the same composition and similar macromolecular masses, but different polydispersities is guided by the paradigm that the physical properties of all polymers is influenced by their macromolecular mass distribution, although this influence is negligible in some cases. The copolymers from AIBN-initiated radical polymerization feature polydispersities typical for polyacrylates⁴⁰, whereas it is

apparent that the method of *living radical chain polymerization* leads to distinctly narrower molecular weight distributions (see Table 1).

A postpolymerization modification reaction was performed to introduce strongly hydrophobic labels. Perfluoroalkyl-bearing copolymers were obtained from both types of P(NIPAM-co-AA) copolymers by reacting their carboxylate groups with perfluoro-iodo-octane in DMF using sodium carbonate as a strong base. Their compositions are summarized in Table 2.

Spincasting Experiments

Copolymer Superstructures on Mica

The P[(NIPAM)-co-(AA)] and P[(NIPAM)-co-(AA)-co-AAC₈F₁₇] copolymers were dissolved in water (1.0 gL⁻¹) and stored at 295K. Before the spincasting procedure, 1.0 mL of the stock solution and 1.0mL of freshly distilled MeOH were mixed. The presence of the cononsolvent MeOH remarkably decreases the LCST (lower critical solution temperature).²⁹ Consequently, all copolymers formed precipitates under these conditions. These mixtures of cononsolvent and precipitate were allowed to ripen for 10min. and then spincast onto freshly cleaved Mica at 3000 rpm. After drying in air for 1 h, the polymer-coated Mica plates were incubated in an environmental chamber (Agilent) at 295 K and 50% relative humidity for 24 h. It is noteworthy that the pH of the aqueous copolymer solution had a remarkable influence on the morphology of the spincast polymer nanofilms. We have determined the pK_a of P(NIPAM-co-AA) copolymers to 4.60, which is very close to the pK_a of polyacrylic acid (pK_a=4.55).⁴¹ The pH has been adjusted by adding aliquots of HCl or NaOH and measuring the pH with a pH-electrode (Methrohm). Above pH=5 of the aqueous stock solution all samples showed the presence of non-structured polymer deposits on mica. However at pH=4 and pH=3 the nanostructured nanolayers were found on mica after deposition and subsequent incubation, as our AFM results indicate. We attribute this reproducible behavior to the presence of carboxylic acid functions in the polymer, which can form hydrogen-bonding networks.⁴² We have carried out the spincasting experiments reported here at pH=4.0, because less acid is present. The best results, which are shown here, were obtained by employing copolymers with 95 percent of NIPAM content and 5 percent of either acrylic acid or acrylic acid and its perfluoro-octylester combined. The perfluoro-alkyl-postpolymerization modified copolymers only showed reproducible results when the products resulting from living radical polymerization were employed. In all other cases, the polydispersity of the investigated copolymers did not have a significant influence in the observed surface features!

Ultraflat Polymer Surfaces on Mica

P[(NIPAM)₉₅-co-(AA)₅] was precipitated in aqueous solution (pH=4.0, see above) by raising the temperature to 310 K, which is above its LCST. The precipitate was filtered off, dried in high vacuum and redissolved in freshly distilled THF (c=1.0 g L⁻¹). This solution was mixed 1:1 (v/v) with a freshly prepared solution of dicyclohexyl-carbodiimide (DCC) (0.10g L⁻¹) and N-hydroxy-succinimide (NHS) (0.020 gL⁻¹) and 1,3-diaminopropane (0.0180 g L⁻¹) in THF and spincast at 3000 rpm. The drying and incubation procedure previously described was not changed. A mechanistic hypothesis explaining why the cross-linking procedure employing 1,3-diamino-propane leads to ultraflat polymer surface on mica is shown in Scheme 4: The free carboxylate groups react with 1,3-diamino-propane to amides.⁴³ Crosslinking of individual chains during the spin-casting process apparently leads to extended polymer networks that adhere flatly to the Si-OH groups of hydrophilic mica surface.

Atomic Force Microscopy (AFM) Measurements

AFM images were recorded using the PicoScan 2000 AFM (Agilent Technologies) in the Magnetic A/C-mode (MACmode).¹⁴ The measurements were performed at the polymer/air

interface. AFM can determine the topography of the sample. Furthermore, there exists a discernible sensitivity of the phase of the cantilever oscillations to the tip-sample interaction forces. Changes in the phase of oscillations can be used to discriminate between materials possessing different viscoelastic properties. The thermally induced transitions of poly-N-isopropyl-acrylamide⁴³ and its copolymers with acrylic acid⁴⁵, styrene⁴⁶ and ethylene oxide⁴⁷ on surfaces, as well as poly-N-isopropyl-acrylamide- hydrogels⁴⁸ and LB-films⁴⁹ have been investigated by AFM. Our AFM study aims at a greater spatial resolution and investigates the pattern on copolymer surfaces, which can be formed by supramolecular interaction of spincast poly-N-isopropyl-acrylamide copolymers above their LCST. From the mass of deposited polymers on $1 \times 1 \text{ cm}^2$ mica plates, we have estimated that the thickness of the deposited polymer layer is in the range of 25–50 nm.

Poly-NIPAM-acrylic acid Copolymers

After spincasting P[(NIPAM)_{95.5}-co-(AA)_{4.5}] in water/MeOH (pH=4.0) onto mica, followed by drying in air and incubation at 50 percent relative humidity at 295K for 24h, the resulting polymer layer shows nanoscopic pores.

We have investigated the statistical distribution of the pore diameters employing the program Image, which is available from the NIH.⁵⁰ The mean pore diameters, calculated from the data summarized in Figure 4, are $23.8 \pm 2.4 \text{ nm}$ for the higher polydispersity material (PDI=1.55) and $21.8 \pm 4.2 \text{ nm}$ for the lower polydispersity material (PDI=1.25). This means that within our experimental margin of error, we cannot discern a significant influence of the polydispersity of the employed copolymers on their ability to form nanopores. An AFM-image of P[(NIPAM)_{95.3}-co-(AA)_{4.7}] (PDI=1.25) on mica is shown in the Supplementary Information.

We have recorded AFM-images of the same surfaces with higher resolution to determine the depth and the profile of the nanopores. Figure 5 shows a typical result. The maximal depth of the investigated pore is approx. 4nm. The observed pore curvature is gentle (approx. 0.2nm per nm).

Perfluoro-octyl-ester Modified Poly-NIPAM-acrylic acid Copolymer Layers

We have repeated the spincasting, drying and incubation cycle with the copolymer P[(NIPAM)₉₅-co-(AA)_{2.8}-AAC₈F₁₇ 2.2] (PDI=1.29), featuring the very hydrophobic perfluoro-octyl group in 2.2 percent of its side chains. As already noted earlier, only the low polydispersity material led to meaningful and reproducible results. The resulting pores on the surface of the copolymer layer were larger (mean diameter: $35.8 \pm 7.1 \text{ nm}$, see Figure 7) when employing the more hydrophobic material. They were also less deep (see Figure 8). It should be noted that the discernible phase contrasts are only very minor, indicating that the hydrophilic and hydrophobic groups of the copolymer are almost evenly distributed. There are no indications for demixing effects, as one would expect for a statistic copolymer. The presence of hydrophobic groups enhances the hydrophobic forces in the copolymer, which was deposited above its LCST. Therefore, the supramolecular interaction of the hydrophobic groups (N-isopropyl acrylamide above the LCST and perfluorooctyl acrylate) is improved and the ability to form hydrogen-bonding networks due to the presence of acrylic acid is decreased when compared to P[(NIPAM)_{95.3}-co-(AA)_{4.7}] (PDI=1.25). We do not yet have sufficient experimental information to attempt to quantify the observed morphology changes as a function of the copolymers' chemical composition, but we will attempt to fine-tune this straightforward deposition method.

As Figure 8 indicates, the depth of the pores formed by P[(NIPAM)₉₅-co-(AA)_{2.5}-AAC₈F₁₇ 2.2] is approx. 2 nm, about half than observed when depositing P[(NIPAM)_{95.3}-co-

(AA)_{4,7}]. The curvature decreases to 0.05nm per nm. Consequently, they are more like nano-vales than nano-pores.

Deposition of Ultra-Flat Copolymer Layers on Mica

The next logical step, considering that we have already created nano-pores and nano-vales, is the generation of ultra-flat copolymer layers on mica. We have adapted the spin-casting procedure by adding 1,3-diamino-propane as crosslinker and DCC/NHS as versatile reagents for the formation of stable amide bonds between the copolymer's carboxylate groups. Spincoating of the copolymer without adding the linker did not lead to the formation of ultra-flat surfaces. As it can be seen from Figure 9, the overall structure of the crosslinked copolymer layer is similar to the non-crosslinked copolymer layer. As the phase image indicates, there are no demixing effects (e.g. zones of different nanoelastic properties). However the differences in height are greatly decreased. The observed changes in height are clearly below 0.05nm. It must be noted that AFM techniques are very precise in their height measurements, whereas their spatial resolution is a function of the measurement mode and the employed AFM-tip. Crosslinking of individual copolymer chains (see Scheme 4) seems to force the deposition of the copolymer chains to assemble in one plane or in a plane-by-plane fashion so that relative maxima and minima in height are avoided. This method of simultaneous copolymer deposition and crosslinking offers an easy and inexpensive alternative to self-assembled monolayers of surfactants or oligopeptides for the reconstitution and stabilization of functional proteins. The authors are aware that the apparent flatness of the deposited copolymer layers on mica may – at least partially – result from the visco-elastic properties of the copolymer. The elastic surface may behave like a “cushion” for the AFM tip, resulting in the observation of flat surface.

Reconstitution of the Mycobacterial Channel Porin MspA from *Mycobacterium smegmatis* within the Ultraflat Copolymer Layers on Mica

We were able to reconstitute the mycobacterial porin MspA by immersing the ultraflat (crosslinked) copolymer layer in a phosphate-buffered saline MspA solution for 1h, followed by 10 immersions in ultrapure water. Apparently (see Figure 10), the crosslinking procedure does not generate a tightly linked polymer material, because MspA is still able to reconstitute. As numerous studies have indicated^{7,12,21–23}, the strongly hydrophobic *docking zone*, which is located at the stem of the MspA homo-octamer, facilitates its reconstitution into hydrophobic SAMs and polymers. AFM-imaging of the MspA pore in P[(NIPAM)_{95.3}-co-(AA)_{4.7}] on mica reveals that the channel protein is almost complete immersed into the crosslinked copolymer layer. This is a striking difference to MspA reconstitution in SAMs¹² where usually only the docking zone is in (hydrophobic) contact with the aliphatic chains. Consequently, MspA, which has a length of approx. 10 nm, is towering over the SAM and can be easily detected by AFM. Here, MspA extends only slightly (< 2nm) beyond the copolymer layer. This means that 8nm of MspA are covered, therefore, electrically insulated. This finding is of importance, because gold nanoparticles are known to bind strongly to MspA.²³ Embedding of MspA-nanoparticle composites by copolymers will allow the construction of electrically well-insulated noble metal (or magnetic) nanodevices, which could be reversibly charged and de-charged, leading to observable Coulomb-barriers at room temperature.¹² Furthermore, the immersion of MspA in this copolymer layer is much more similar to its natural reconstitution process in the outer cell membrane of *Mycobacterium smegmatis* than within a SAM. This may lead to the construction of models for mycobacterial surfaces.

Since the tip diameter exceeded the width of the nanopore, the inner pore of MspA is not completely detectable by AFM, even in the magnetic AC mode. Note that the phase image shows only minor changes in depths. This behavior is most likely caused by the experimentally well-known fact that MspA pores are water filled⁷, which leads to the coupling of MspA's wall and pore mobility.

Materials and Methods

All commercial chemicals were purchased from Aldrich Chemical Co. unless otherwise noted. N-isopropylacrylamide, acrylic acid, 2,2'-azoisobutyronitrile, perfluoro-octyl-iodide, 2-mercaptoacetic acid, 1,3-diamino-propane, methanol, tert-butyl alcohol, tetrahydrofuran, n-hexane and diethyl ether were used without purification unless noted otherwise. Mica was freshly cleaved prior to the spin-casting of P(NIPAM-co-AA) and P(NIPAM-co-AA-AC₈F₁₇) solutions. CHN-analysis has been performed on a C, H, N, and S- Analyzer (LECO Instruments) in collaboration with the Department of Organic Chemistry of the University of Saarbrücken/Germany. Gel permeation chromatography (GPC) experiments were carried out with an HP-79911 GP-103 column (PL Gel 10 mm, 100 Å, 7.5 × 300 mm) eluted with THF at a flow rate of 0.50 mL min⁻¹. The concentration of the samples injected ($V_{inj.} = 1.0 \mu\text{L}$) in THF was $1.0 \times 10^{-3} \text{ g L}^{-1}$. Calibration was performed using 9 monodisperse PMMA samples. ($M_w = 2,000; 8,000; 30,000; 50,000; 75,000; 100,000; 150,000; 200,000; 460,000$). The detection of the polymer was achieved using a detection wavelength of $\lambda = 220 \text{ nm}$ as well as refractive index detection.

Atomic Force Microscopy (AFM) Measurements

AFM images were recorded using the PicoScan 2000 AFM (Agilent Technologies) in the Magnetic A/C-mode (MACmode).¹⁴ MacMode type II tips from Agilent Technologies were used (tip radii < 7nm, nominal k value = 2.8 N/m, resonance frequency = 50–75 kHz in air). The size of the images was corrected according to the results from a calibration procedure using tris-homoleptic ruthenium(II)-quaterpyridinium-complexes⁵¹ as model compounds.¹²

Synthesis of P(NIPAM-co-AA) Copolymers

AIBN-Initiated Radical Polymerization

Poly-N-isopropyl-acrylamide-acrylic acid copolymers were synthesized via radical chain polymerization in *t*-butanol using AIBN as radical initiator, as described previously³⁶: A solution of NIPAM (10.0g, 0.088 mol) and a given amount of AA (Table 1) in tert.-butyl alcohol (50 mL) was stirred at room temperature under N₂ for 2 hours. The solution was heated to 70°C and then a solution of AIBN (60.0 mg) in tert.-butyl alcohol (2 mL), which underwent the same purging procedure, was added at once. The mixture was stirred for 15 h at 70°C. The solvent was evaporated and the copolymer was isolated by successive precipitations from THF solution into n-hexane. The product was dried at 40°C for 24 hours. Copolymers of NIPAM and AA possessing 99, 98, 95 and 90 mol % of NIPAM and 1, 2, 5 and 10 mol % of AA were synthesized following this procedure. The amounts isolated after precipitation ranged from 9.0 to 9.5 g.

Living Radical Polymerization

The same procedure was performed as described above, except that 0.050g of mercaptoacetic acid ($5.4 \times 10^{-4} \text{ mol}$) was added together with the AIBN to the monomer solution.

Postpolymerization Modification with Perfluoro-iodo-octane

P(NIPAM-co-AA) (3.0 g) was dissolved in anhydrous DMF (50 mL) and purged with nitrogen for 1h. 1-iodo-perfluoro-n-octane (1.09 g, 0.002 mol) and Na₂CO₃ (2.0 g) were added and the reaction was allowed to proceed for 24 hr at 80°C. The solution was filtered at 60°C in order to remove inorganic salts. After cooling to 10°C, diethyl ether (80 mL) was slowly added to induce polymer precipitation. The polymer was allowed to precipitate at 0°C for 1h. The copolymer was then taken up in 1.0M aqueous HCl at 10°C and precipitated by heating to 50°C. This precipitation procedure was repeated twice. All the P(NIPAM-co-AA/AC₈F₁₇)-

copolymers (Table 2) were synthesized by using this procedure. The yields after precipitation ranged from 2.0 to 2.5 g.

Spincasting Procedure

The P[(NIPAM)-co-(AA)] and P[(NIPAM)-co-(AA)-co-AAC₈F₁₇] copolymers were dissolved in water at pH=4.0 (1.0 gL⁻¹) and stored at 295K. Before spincasting, 1.0 mL of the stock solution and 1.0mL of freshly distilled MeOH were mixed, allowed to ripen for 10min. and then spincast onto freshly cleaved mica at 3000 rpm. After drying in air for 1 h, the polymer-coated mica plates were incubated in an environmental chamber (Agilent) at 293°K and 50% relative humidity for 24 h.

In-Situ Crosslinking Procedure

P[(NIPAM)₉₅-co-(AA)₅] was precipitated was aqueous solution (pH=4.0), dried in high vacuum for 12h and re-dissolved in freshly distilled THF (c=1.0 g L⁻¹). This solution was mixed 1:1 (v/v) with a freshly prepared solution of dicyclohexyl-carbodiimide (DCC) (0.10g L⁻¹) and N-hydroxy-succinimide (NHS) (0.020 gL⁻¹) and 1,3-diaminopropane (0.0180 g L⁻¹) in THF and spincast at 3000 rpm.

Reconstitution of MspA Within the Ultraflat Copolymer Layers on Mica

The mica plates coated with the crosslinked ultraflat copolymer layer were immersed in a phosphate-buffered aqueous solution (5 mM phosphate-buffer, pH=7.02, 100 mM NaCl, 2% SDS) of the mycobacterial channel porin MspA (5 micrograms mL⁻¹) for 1h and then immersed 10 times in ultrapure water, dried in air for 10min. and then incubated in an environmental chamber at 295 K and 50% relative humidity for 24 h.

Conclusions

We have developed a reliable procedure for the deposition of (nanostructured) copolymer nanolayers onto mica surfaces comprising the following steps: A) spincasting of MeOH/H₂O-dispersions of poly-N-isopropyl-acrylamide-co-acrylic acid(-co-perfluoro-octyl-acrylate) copolymers, which are above their respective lower critical solution temperatures (LCSTs) onto mica. B) Drying of the spincast copolymer layers in air. C) Incubation at a defined temperature (295K) in 50 rel. percent humidity. The resulting copolymer layers have been investigated by AFM (magnetic AC mode). Our study has confirmed that the pH of the aqueous copolymer solution prior to deposition is a very important parameter. Copolymer depositions from pH=4.0 gave well-reproducible results. Furthermore, we have investigated the influence of the polydispersity of the synthesized copolymers on their ability to form surface structures. When using N-isopropyl-acrylamide/acrylic acid copolymers, the polydispersity did not have a significant influence: The mean pore diameters were 23.8±4.4nm for P[(NIPAM)_{95.5}-co-(AA)_{4.5}] (PDI=1.55) and 21.8±4.2nm for P[(NIPAM)_{95.3}-co-(AA)_{4.7}] (PDI=1.25). Both nanopores were approx. 4nm deep. Modifying the copolymer composition proved to be the key for the reproducible formation of nanopores. When depositing P[(NIPAM)₉₅-co-(AA)_{2.5}-AAC₈F₁₇]_{2.2}] (PDI=1.29) on mica, the resulting mean pore diameter was 35.8±7.1nm. These nano-pores were only 2nm in depth, therefore we'll refer to them rather as nano-vales than as nanopores. Note that only the low polydispersity copolymer material allowed the reproducible generation of nanostructures polymer layers on mica.

We have interpreted this behavior as follows: the increased hydrophobic interaction due to the presence of the perfluoro-octyl-ester labels has increased the interaction between the individual copolymer strands. Therefore, the copolymer has developed a greater tendency to deposit onto mica according to a layer-by-layer mechanism, which results in larger and shallower pores. The next logical step of this endeavor consisted of the crosslinking of individual polymer chains

by employing 1,3-diamino-propane as diamide-linker. As predicted, ultraflat P[(NIPAM)_{95.3}-co-(AA)_{4.7}] (PDI=1.25) copolymer layers on mica were formed. These layers have been used as host system for the reconstitution of the mycobacterial porin MspA. MspA did almost completely immerse in the ultraflat and weakly crosslinked copolymer layer proving that these polymer layers could be of future use as components in bioelectronic devices.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Willner, I.; Katz, E. *Bioelectronics: From Theory to Applications*. Wiley/VCH; New York, Weinheim: 2005. p. 475
2. Baldrich E, Laczka O, Javier del Campo F, Munoz FX. *Anal Bioanal Chem* 2008;390:1557–1562. [PubMed: 18256810]
3. Calvo EJ, Rothacher MS, Bonazzola C, Wheeldon IR, Salvarezza RC, Vela ME, Benitez G. *Langmuir* 2005;21:7907–7911. [PubMed: 16089399]
4. Roth KM, Dontha N, Dabke RB, Gryko DT, Clausen C, Lindsey JS, Bocian DF, Kuhr WG. *Journal of Vacuum Science & Technology, B: Microelectronics and Nanometer Structures* 2000;18:2359–2364.
5. Niederweis M, Heinz C, Janik K, Bossmann SH. *Nano Letters* 2001;1:169–174.
6. Niederweis M, Heinz C, Janik K, Bossmann SH. *Nano Letters* 2002;2:1263–1268.
7. Bossmann SH, Janik K, Pokhrel MR, Heinz C, Niederweis M. *Surface and Interface Analysis* 2004;36:127–134.
8. Mendes PM. *Chem Soc Rev* 2008;37:2512–2529. [PubMed: 18949123]
9. Tsukruk VV. *PMSE Preprints* 2003;89:260–282.
10. Jonkheijm P, Weinrich D, Schroeder H, Niemeyer CM, Waldmann H. *Angew Chem, Int Ed* 2008;47:9618–9647.
11. Estrela P, Paul D, Li P, Keighley SD, Migliorato P, Laurenson S, Ferrigno PK. *Electrochimica Acta* 2008;53:6489–6496.
12. Wörner M, Lioubashevski O, Basel MT, Niebler S, Gogritchiani E, Egner N, Heinz C, Hoferer J, Cipolloni M, Janik K, Katz E, Braun AM, Willner I, Niederweis M, Bossmann SH. *Small* 2007;3:1084–1097. [PubMed: 17514767]
13. Das R, Kiley PJ, Segal M, Norville J, Yu AA, Wang L, Trammell SA, Reddick LE, Kumar R, Stellacci F, Lebedev N, Schnur J, Bruce BD, Zhang S, Baldo M. *Nano Letters* 2004;4:1079–1083.
14. Han W, Lindsay SM, Jing T. *Appl Phys Lett* 1996;69:4111–4113.
15. Rapoport N. *Progress in Polymer Science* 2007;32:962–990.
16. Chen JP, Cheng TH. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 2007;313–314:254–259.
17. Pokhrel MR, Bossmann SH. *J Phys Chem B* 2000;104:2215–2223.
18. Overney RM, Buenviaje C, Luginbühl R, Dinelli F. *Journal of Thermal Analysis and Calorimetry* 2000;59:205–225.

19. Niederweis M. Mycobacterial porins - new channel proteins in unique outer membranes. *Mol Microbiol* 2003;49:1167–1177. [PubMed: 12940978]
20. Heinz C, Roth E, Niederweis M. *Methods Mol Biol* 2003;228:139–150. [PubMed: 12824550]
21. Faller M, Niederweis M, Schulz GE. The Structure of a Mycobacterial Outer-Membrane Channel. *Science* 2004;303:1189–1192. [PubMed: 14976314]
22. Basel MT, Dani RK, Kang M, Pavlenok M, Chikan V, Smith PE, Niederweis M, Bossmann SH. *ACS Nano* 2009;3:462–466. [PubMed: 19236086]
23. Dani RK, Kang M, Kalita M, Smith PE, Bossmann SH, Chikan V. *Nano Letters* 2008;8:1229–1236. [PubMed: 18318505]
24. Liu RCW, Cantin S, Perrot F, Winnik FM. *Polymers for Advanced Technologies* 2006;17:798–803.
25. Bunker, BC.; Huber, DL.; Manginell, RP.; Kim, B-I.; Boal, AK.; Bachand, GD.; Rivera, SB.; Bauer, JM.; Matzke, CM. *Proceedings of SPIE-The International Society for Optical Engineering*. Vol. 5220. *Nanofabrication Technologies*; 2003. p. 28–36.
26. Schild HG, Muthukumar M, Tirrell DA. *Macromolecules* 1991;24:948–952.
27. Winnik FM, Ringsdorf H, Venzmer J. *Macromolecules* 1990;23:2415–2416.
28. Winnik FM, Ottaviani MF, Bossmann SH, MMG-G, Turro NJ. *Macromolecules* 1993;26:4577–4585.
29. Winnik FM, Ottaviani MF, Bossmann SH, Garcia-Garibay M, Turro NJ. *Macromolecules* 1992;25:6007–6017.
30. Cho C, Jung J, Sung Y, Lee Y. *Macromol Chem Rapid Commun* 1994;15:727–732.
31. Yoo M, Sung Y, Cho C, Lee Y. *Polymer* 1997;38:2759–2766.
32. Taylor L, Ceranowski L. *J Polym Sci, Polym Chem* 1975;13:2551.
33. Priest JH, Murray SL, Nelson JR, ASH. *ACS Sym Ser* 1987;350:255.
34. Hahn M, Görnitz E, Dautzenberg H. *Macromolecules* 1998;31:5616–5623.
35. Chen G, Hoffman A. *Macromol Chem Phys* 1995;196:1251–1260.
36. Ottaviani MF, Winnik FM, Bossmann SH, Turro NJ. *Helv Chim Acta* 2001;84:2476–2492.
37. Benrebouh A, Avoce D, Zhu XX. *Polymer* 2001;42:4031–4038.
38. Pokhrel MR, Bossmann SH. *J Phys Chem B* 2000;104:2215–2223.
39. Bignotti F, Penco M, Sartore L, Peroni I, Mendichi R, Casolaro M, Amore A. *Polymer* 2000;41:8247–8256.
40. Matyjaszewski, K.; Davis, TP. *Handbook of Radical Polymerization*. Wiley-IEEE; New York: 2003. p. 920
41. Das KK, Somasundaran P. *Colloids and Surfaces, A: Physicochemical and Engineering Aspects* 2001;182:25–33.
42. Nair AS, Kimura K. *Langmuir* 2009;25:1750–1756. [PubMed: 19115942]
43. Murphy CJ, Arkin MR, Jenkins Y, Ghatlia ND, Bossmann SH, Turro NJ, Barton JK. *Science* 1993;1025–1029. [PubMed: 7802858]
44. Montagne F, Polesel-Mariss J, Pugin R, Heinzelmann H. *Langmuir* 2009;25:983–991. [PubMed: 19093826]
45. Schmidt S, Motschmann H, Hellweg T, von Klitzing R. *Polymer* 2008;49:749–756.
46. Song M, Guo D, Pan C, Jiang H, Chen C, Zhang R, Gu Z, Wang X. 2008;19:165102/1–165102/7.
47. Heinz P, Bretagnol F, Mannelli I, Sirghi L, Valsesia A, Ceccone G, Gilliland D, Landfester K, Rauscher H, Rossi F. *Langmuir* 2008;24:6166–6175. [PubMed: 18484752]
48. Lapeyre, V.; Renaudie, N.; Dechezelles, J-F.; Saadaoui, H.; Ravaine, S.; Ravaine, V. *Langmuir*. ASAP; 2009.
49. Liu RCW, Segui F, Viitala T, Winnik FM. *PMSE Preprints* 2004;90:105–106.
50. <http://rsb.info.nih.gov/nih-image/>
51. Shi A, Pokhrel MR, Bossmann SH. *Synthesis* 2007;4:505–514.

Figure 1.
 $P(NIPAM_x-co-AA_y-coAAC_8F_{19}_z)$ -copolymers for the spincasting of nanothin layers on Mica.

Figure 2. Structure of the porin MspA from *M. smegmatis* (side view). The arrow depicts the constriction zone; green: hydrophilic amino acids; yellow: hydrophobic amino acids; dimensions given in nm. The coordinates were taken from the crystal structure of MspA (PDB code: 1UUN).²¹

Figure 3.
Nanostructured layer of P[(NIPAM)_{95.5}-co-(AA)_{4.5}] (PDI=1.55) on mica.

Figure 4.

Statistical distribution of the apparent pore diameters from five independently prepared samples of P[(NIPAM)_{95.5}-co-(AA)_{4.5}] (PDI=1.55) and P[(NIPAM)_{95.3}-co-(AA)_{4.7}] (PDI=1.25) on mica (F: fraction of pores of the diameter indicated on the x-axis). The mean pore diameters are 23.8 ± 4.4 nm for PDI=1.55 and 21.8 ± 4.2 nm for PDI=1.25.

Figure 5. Nanostructured layer of P[(NIPAM)_{95.5}-co-(AA)_{4.5}] (PDI=1.55) on mica. Right: topology image; left: phase image.

Figure 6. Nanostructured layer of P[(NIPAM)₉₅-co-(AA)_{2.5}-AAC₈F₁₇ 2.2] (PDI=1.29) on mica. Right: topology image; left: phase image.

Figure 7.

Statistical distribution of the apparent pore diameters from three independently prepared samples of P[(NIPAM)₉₅-co-(AA)_{2.5}-AAC₈F_{17 2.2}] (PDI=1.29) on mica (F: fraction of pores of the diameter indicated on the x-axis). The mean pore diameters are 35.8 ± 7.1 nm.

Figure 8. Nanostructured layer of P[(NIPAM)₉₅-co-(AA)_{2.8}-AAC₈F₁₇ 2.2] (PDI=1.29) on mica. Right: topology image; left: phase image.

Figure 9. Ultraflat layer of P[(NIPAM)_{95.3}-co-(AA)_{4.7}] (PDI=1.25) on mica. Right: topology image; left: phase image.

Figure 10. MspA in a 1,3-diamino-propane crosslinked ultraflat layer of P[(NIPAM)_{95.3}-co-(AA)_{4.7}] (PDI=1.25) on mica. Right: topography image; left: phase image.

Scheme 1.
Radical statistical polymerization of P(NIPAM-co-AA) copolymers.

Scheme 2.

Thiyl-initiated living radical chain polymerization of N-isopropyl-acrylamide (NIPAM) and acrylic acid (AA).

Scheme 3.

Postpolymerization modification of P(NIPAM-co-AA) with perfluoro-iodo-octane in DMF in the presence of sodium carbonate as base.

Scheme 4.
Linking of individual polymer chains by introducing amide-crosslinking.

Table 1

Synthesized N-isopropyl-acrylamide/acrylic acid copolymers. Determination of the polymer composition by CHN and their polydispersity by using gel permeation chromatography, M_n , number average macromolecular mass, M_w : weight average macromolecular mass, N_i : mol fraction, M_i : weight fraction

Copolymers by radical statistical copolymerization	Composition according to CHN analysis	$M_n = \frac{\sum M_i N_i}{\sum N_i}$	$M_w = \frac{\sum M_i^2 N_i}{\sum M_i N_i}$	$PDI = \frac{M_w}{M_n}$
P[(NIPAM) ₉₉ -co-(AA) ₁]	P[(NIPAM) _{99.2} -co-(AA) _{0.8}]	52,400	73,900	1.41
P[(NIPAM) ₉₈ -co-(AA) ₂]	P[(NIPAM) _{98.4} -co-(AA) _{1.6}]	48,200	69,900	1.45
P[(NIPAM) ₉₅ -co-(AA) ₅]	P[(NIPAM) _{95.5} -co-(AA) _{4.5}]	46,500	72,100	1.55
P[(NIPAM) ₉₀ -co-(AA) ₁₀]	P[(NIPAM) _{90.4} -co-(AA) _{9.6}]	47,300	71,900	1.52
Copolymers by living statistical copolymerization				
P[(NIPAM) ₉₉ -co-(AA) ₁]	P[(NIPAM) _{99.3} -co-(AA) _{0.7}]	72,500	87,700	1.21
P[(NIPAM) ₉₈ -co-(AA) ₂]	P[(NIPAM) _{98.6} -co-(AA) _{1.4}]	75,500	89,100	1.18
P[(NIPAM) ₉₅ -co-(AA) ₅]	P[(NIPAM) _{95.3} -co-(AA) _{4.7}]	74,100	92,600	1.25
P[(NIPAM) ₉₀ -co-(AA) ₁₀]	P[(NIPAM) _{90.5} -co-(AA) _{9.5}]	69,800	85,200	1.22

Table 2

N-isopropyl-acrylamide/acrylic acid copolymers after postpolymerization transformation by reacting a part of the acrylic acid functions with perfluoro-iodo-octane. Determination of the polymer composition by CHN and their polydispersity by using gel permeation chromatography, M_n , number average macromolecular mass, M_w : weight average macromolecular mass, N_i : mol fraction, M_i : weight fraction

Copolymers by Radical statistical copolymerization	Composition according to CHN analysis	$M_n = \frac{\sum M_i N_i}{\sum N_i}$	$M_w = \frac{\sum M_i^2 N_i}{\sum M_i N_i}$	$PDI = \frac{M_w}{M_n}$
P[(NIPAM) ₉₉ -co-(AA) _{0.5} -AAC ₈ F ₁₇ 0.5]	P[(NIPAM) ₉₉ -co-(AA) _{0.5} -AAC ₈ F ₁₇ 0.3]	59,100	83,300	1.41
P[(NIPAM) ₉₈ -co-(AA) _{1.0} -AAC ₈ F ₁₇ 1.0]	P[(NIPAM) ₉₈ -co-(AA) _{1.0} -AAC ₈ F ₁₇ 0.6]	50,000	73,500	1.47
P[(NIPAM) ₉₅ -co-(AA) _{2.5} -AAC ₈ F ₁₇ 2.5]	P[(NIPAM) ₉₅ -co-(AA) _{2.7} -AAC ₈ F ₁₇ 1.8]	49,500	77,800	1.57
P[(NIPAM) ₉₀ -co-(AA) _{5.0} -AAC ₈ F ₁₇ 5.0]	P[(NIPAM) ₉₀ -co-(AA) _{5.0} -AAC ₈ F ₁₇ 4.6]	55,700	86,300	1.55
Copolymers by living statistical copolymerization				
P[(NIPAM) ₉₉ -co-(AA) _{0.5} -AAC ₈ F ₁₇ 0.5]	P[(NIPAM) ₉₉ -co-(AA) _{0.4} -AAC ₈ F ₁₇ 0.3]	73,200	88,600	1.21
P[(NIPAM) ₉₈ -co-(AA) _{1.0} -AAC ₈ F ₁₇ 1.0]	P[(NIPAM) ₉₈ -co-(AA) _{0.8} -AAC ₈ F ₁₇ 0.9]	77,900	91,900	1.18
P[(NIPAM) ₉₅ -co-(AA) _{2.5} -AAC ₈ F ₁₇ 2.5]	P[(NIPAM) ₉₅ -co-(AA) _{2.5} -AAC ₈ F ₁₇ 2.2]	80,100	103,300	1.29
P[(NIPAM) ₉₀ -co-(AA) _{5.0} -AAC ₈ F ₁₇ 5.0]	P[(NIPAM) ₉₀ -co-(AA) _{5.4} -AAC ₈ F ₁₇ 4.1]	80,500	99,900	1.24