

Biotemplated synthesis of stimuli-responsive nanopatterned polymer brushes on microtubules

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We report the synthesis of nanostructured stimuli-responsive polymer brushes using atom transfer radical polymerisation initiated on protein filaments of the cytoskeleton. In particular, we used microtubules and prepared thermoresponsive poly-(*N*-isopropylacrylamide) brushes with incorporated fluorescent groups. The use of microtubules as templates for fabrication of “soft” polymeric nanostructures on surfaces opens new possibilities for the design of functional materials.

Nanostructured surfaces are of growing interest for information storage,¹ microfluidics,² design of smart materials,³ biotechnology,⁴ microelectronics,⁵ photonic applications⁶ *etc.* Thereby, the use of biological objects as templates for the design of the nanostructures offers a range of advantages.⁷ In particular, selective molecular recognition and proof-reading during the build-up of biomolecules can provide a high degree of uniformity in the possible structures. Different biomacromolecules and their assemblies including DNA, viral capsids, cytoskeleton filaments and protein crystals were successfully applied as templates for the fabrication of inorganic nanostructured materials (see recent review⁷). On the other hand, design of functional organic nanostructures^{8–10} using biotemplating is still an almost unexplored field.

Microtubules, which are cylindrical protein filaments with outer diameters of about 24 nm and lengths up to tens of micrometres, deserve a particular interests as templates.^{11–14} First, microtubules can be easily formed by self-assembly of tubulin dimers. Second, microtubules are able to form segmented structures with different functionality in a controlled manner.¹⁵ Third, a number of approaches to control position as well as orientation of microtubules on artificial substrates are available.^{16–20} This makes microtubules highly promising for templated synthesis of complex materials.

Here, we demonstrate the fabrication of stimuli-responsive nanopatterned polymer brushes using microtubules as templates. In particular, we report on the “grafting from” synthesis of thermoresponsive poly-(*N*-isopropylacrylamide)—PNIPAM—brushes by atom transfer radical polymerisation (ATRP) initiated on microtubules. The outer surfaces of microtubules contain reactive amino groups providing the possibility for chemical modification with fluorescent and other functional groups. In our approach, we substituted the amino groups with reactive groups capable of

initiating ATRP and grew polymer chains from surface-adsorbed microtubules (Fig. 1).

Rhodamine-labelled microtubules (fluorescent emission in the red wavelength range) were prepared by self-assembly of α,β -tubulin dimers (see Experimental part). The microtubules were then adsorbed on DDS-coated glass surfaces by perfusing them in buffer solution through a narrow channel between two glass cover slips. The adsorbed microtubules were crosslinked by glutaraldehyde, which reacted with lysine residues and formed chemical links between neighbouring amino groups (Fig. 2a). Because part of the glutaraldehyde molecules did not react bifunctionally with the lysine residues, the outer surface of the crosslinked microtubules then contained aldehyde groups. Initiator groups were then immobilized onto the crosslinked microtubules by sequentially treating them with 2,2'-(ethylenedioxy)bis(ethylamine) and bromo-2-methylpropanoyl bromide (see Experimental part for details). The initiator-modified microtubules possessed a rod-like shape, which remained undeformed after multiple rinsings in different organic solvents (Fig. 2b). Using the simplified ATRP developed by Matyjaszewski *et al.*,²¹ we demonstrated the polymerisation of *N*-isopropylacrylamide with small addition (2 wt%) of fluorescein *o*-acrylate (Fig. 2c). Most strikingly, the diameters of the polymer-modified microtubules were substantially increased and their shape was often deformed.

The role of fluorescein *o*-acrylate (fluorescent emission in the green wavelength range) was to make the synthesized polymer brushes

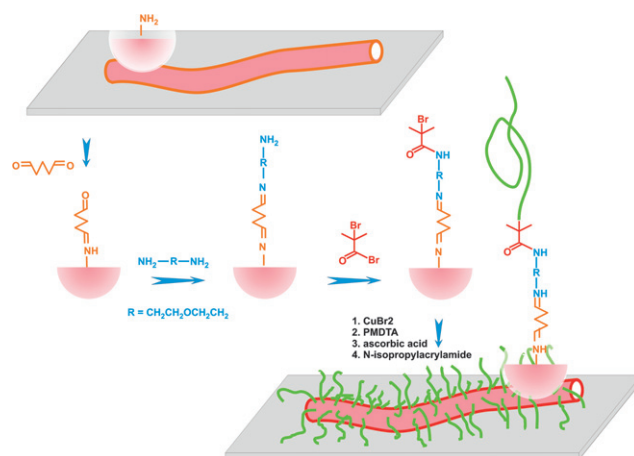


Fig. 1 Schematic diagram of microtubule modification and polymer grafting. The polymer chains were grown from microtubules that were adsorbed on a hydrophobic glass substrate and sequentially crosslinked with glutaraldehyde, amino-functionalised using 2,2'-(ethylenedioxy)-bis(ethylamine) and modified by 2-bromo-2-methylpropanoyl bromide.

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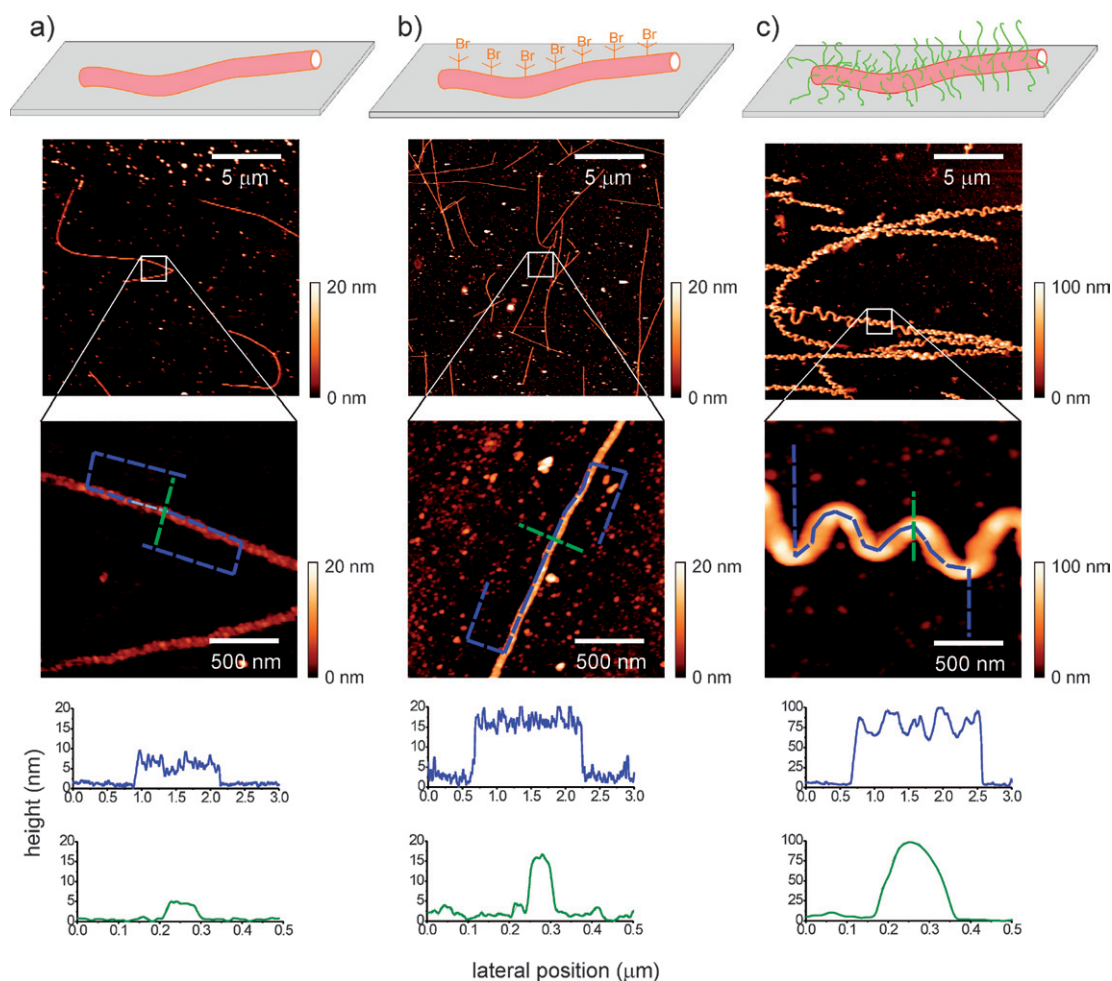


Fig. 2 Morphology of microtubules at different stages of the modification procedure. AFM images (middle panel) of microtubules (a) after crosslinking with glutaraldehyde, (b) after immobilization of initiator, and (c) after grafting of poly-(*N*-isopropylacrylamide–fluorescein *o*-acrylate) ($M_n = 69\,000\text{ g mol}^{-1}$, $M_w = 152\,000\text{ g mol}^{-1}$). Cross-sections (bottom panel) are given along the paths indicated with the corresponding colour on the AFM images. No tip-deconvolution was performed.

detectable by fluorescence microscopy. Verifying the successful synthesis of the polymer chains on the microtubule we found that the originally “red-fluorescent” microtubules became additionally “green-fluorescent” after polymerisation (Fig. 3).

In order to investigate the structural changes of the microtubule in detail, we imaged the same area of interest by AFM before and after the polymerisation (Fig. 4). These measurements revealed that the contour length of the microtubules increased by up to 100%. On the other hand, the end-to-end distance of the surface-attached microtubules remained almost unchanged. We attribute the increase of the contour length to stretching within and between the tubulin dimers caused by steric repulsions between the grafted polymer chains rather than to high temperature and interactions with solvents.²²

We estimated the grafting densities of the polymer chains per microtubule length (Γ_{length}) and area (Γ_{area}) using the following equations:²³

$$\Gamma_{\text{length}} = \frac{S_{\text{SEC}} \rho_{\text{PNIPAM}} N_A}{M_w} \quad (1)$$

$$\Gamma_{\text{area}} = \Gamma_{\text{length}} / C_{\text{SEC}} \quad (2)$$

where S_{SEC} is the cross-sectional area of the polymer-modified microtubule, ρ_{PNIPAM} is the polymer mass density, M_w is the weight averaged molecular weight, N_A is the Avogadro constant, and C_{SEC} is the arc-length over the surface of a given section cut perpendicularly to the microtubule. Thereby S_{SEC} is derived experimentally as the integrated area of the cross-section of a polymer-modified microtubule as shown in Fig. 2c (lowest panel). Here, the contribution from the microtubule before the polymerisation is neglected. C_{SEC} is determined as the circumference of the initiator-modified microtubule as shown in Fig. 2b (lowest panel). For the estimation of S_{SEC} and C_{SEC} the AFM curves have been corrected for the tip radius. We derived grafting densities of $\Gamma_{\text{length}} \approx 40\text{ chains nm}^{-1}$ ($\Gamma_{\text{area}} \approx 0.3\text{ chains nm}^{-2}$) and $\Gamma_{\text{length}} \approx 42\text{ chains nm}^{-1}$ ($\Gamma_{\text{area}} \approx 0.32\text{ chains nm}^{-2}$) for the data presented in Fig. 2 and 4, respectively. The average distance D between grafting sites could then be calculated by $D = \Gamma_{\text{area}}^{-1/2} \approx 1.8\text{ nm}$. This value is substantially smaller than the gyration radius of PNIPAM chains with similar molecular weights in the collapsed state ($R_g \approx 20\text{ nm}$).²⁹ Consequently, the polymer-grafted shell can be considered as a brush-like one.

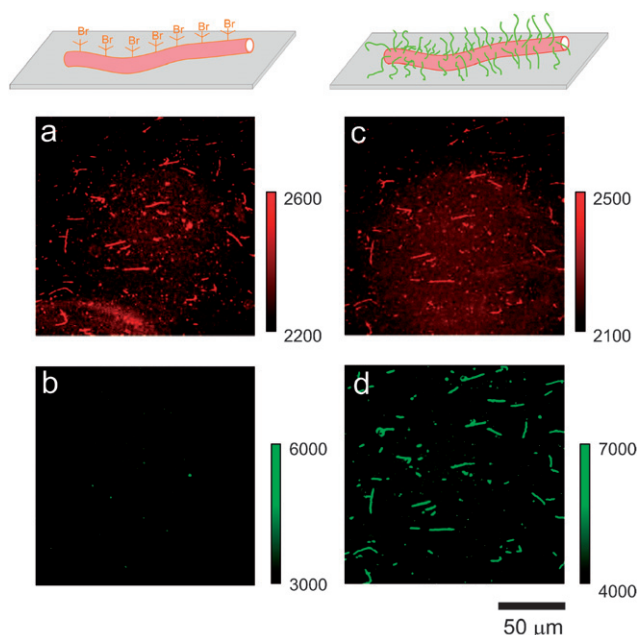


Fig. 3 Epi-fluorescent images of surface-adsorbed microtubules after immobilization of initiator (a: microtubule signal, b: polymer signal) and after grafting of poly-(*N*-isopropylacrylamide–fluorescein *o*-acrylate) ($M_n = 59\,000\text{ g mol}^{-1}$, $M_w = 136\,000\text{ g mol}^{-1}$) (c: microtubule signal, d: polymer signal).

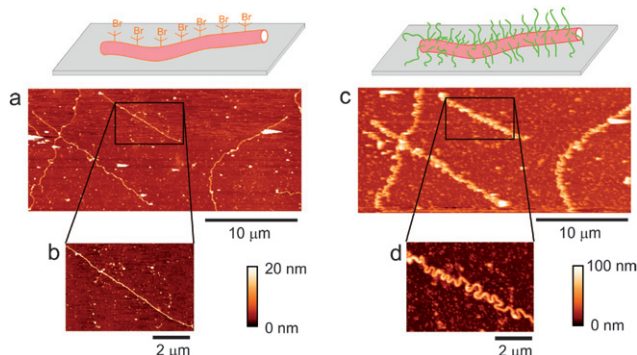


Fig. 4 AFM images of microtubules in the same area of interest before (a, b) and after (c, d) grafting of poly-(*N*-isopropylacrylamide–fluorescein *o*-acrylate) ($M_n = 47\,000\text{ g mol}^{-1}$, $M_w = 122\,000\text{ g mol}^{-1}$). No tip-deconvolution of AFM images was performed.

We explored the correlation between the grafted amount of polymer chains and the number of amino acid residues, which were able to react with α -bromoisobutyl bromide initiator for the polymerisation. Microtubules are composed of about 13 protofilaments made up of α , β -tubulin dimers (repeat length 8 nm). Each α , β -tubulin dimer contains 34 lysine, 42 arginine and 50 serine amino acid residues, corresponding to $N \approx 200$ of potential grafting sites per nanometre of microtubule length. On the other hand, since the contour length of microtubules increased after polymerisation, the apparent density of potential grafting sites decreased by a factor of L_{C-POL}/L_C , where L_{C-POL} and L_C are the contour lengths of the microtubules after and before polymerisation. The apparent density of potential grafting sites N_{POL} was found to be $N_{POL} = 130\text{ nm}^{-1}$

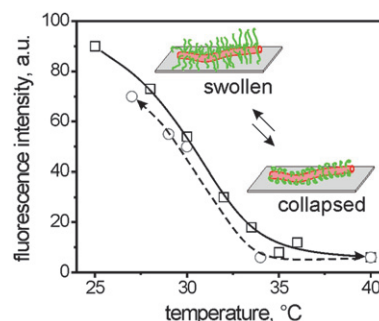


Fig. 5 Switching of PNIPAM grafted on microtubules. Dependence of fluorescence intensity of poly-(*N*-isopropylacrylamide–fluorescein *o*-acrylate) brushes on temperature upon heating (open squares, solid line) and cooling (open circles, dashed line).

(data from Fig. 2) and $N_{POL} = 100\text{ nm}^{-1}$ (data from Fig. 4) meaning that about $\Gamma_{\text{length}}/N_{POL} = 30\%–40\%$ reactive amino acid residues initiated growth of polymer chains²³

Finally, we investigated the switching behaviour of the grafted poly-(*N*-isopropylacrylamide) brushes in an aqueous environment. We found that the fluorescence intensity of the polymer chains (obtained from images as in Fig 3d) gradually decreased with increasing temperature (Fig. 5), reaching almost zero at the low critical solution temperature of PNIPAM ($T = 33\text{ °C}$). The reason for this effect was, most probably, fluorophore quenching by the collapsed polymer chains^{24–26} indicating that the polymer chains were capable of switching. Given the optical resolution of our imaging system, we did, on the other hand, not detect any morphological changes of the PNIPAM-decorated microtubules as a result of the polymer swelling and collapse.

In conclusion, we presented a novel approach for the design of nanopatterned polymer brushes based on the use of biological templates. Using microtubules as templates, we fabricated thermoresponsive nanopatterned poly-(*N*-isopropylacrylamide) brushes which can be further used for design of responsive material systems²⁷ and biomolecular switches.²⁸ We believe that microtubule-based polymeric materials will be of interest for a variety of nanotechnological and microelectronic applications.

Experimental part

Materials

2,2'-(Ethylenedioxy)bis(ethylamine) (Aldrich), glutaraldehyde (Sigma-Aldrich), *N*-isopropylacrylamide (NIPAM, Aldrich), acetone (Aldrich), *N,N,N',N''*-pentamethyldiethylenetriamine (PMDTA, Aldrich), ethyl-2-bromoisobutyrate (EbiB, Aldrich), ethylenediamine (ED, Fluka), dimethyldichlorosilane (DDS, Fluka), anhydrous dichloromethane (Aldrich), 2-bromo-2-methylpropanoyl bromide (BMPB, Aldrich), triethylamine (Fluka), L-ascorbic acid (Sigma), fluorescein *o*-acrylate (FA, Aldrich), copper (ii) bromide (Aldrich) were used as received.

Preparation of DDS-coated glasses

Piranha-cleaned glass substrates were treated by DDS (125 μL) solution in 250 mL of trichloroethylene over a period of 60 min. The DDS-coated glasses were rinsed several times in methanol and water.

DDS was used to prevent the adsorption of ethylenediamine and immobilization of initiator on the glass substrate.

Preparation of microtubules

Rhodamine-labelled microtubules were grown from 10 μL of bovine brain tubulin (4 mg mL^{-1} , mixture of 1 rhodamine-labelled/3 unlabelled tubulin units) in BRB80 buffer (80 mM potassium PIPES, pH 6.9, 1 mM EGTA, 1 mM MgCl_2) with 4 mM MgCl_2 , 1 mM Mg-guanosine 5'-triphosphate sodium salt, and 5% DMSO at 37 °C. After 30 min, the microtubules were stabilized and 100-fold diluted in room-temperature BRB80 containing 10 μM taxol.

Modification of microtubules

Grown microtubules were purified from free tubulin by centrifuging at 40 000 rpm for 10 min. The supernatant was substituted with 10 μM taxol solution in BRB80 buffer. The microtubules were perfused and adsorbed in 2 mm wide flow cells self-built from two DDS-coated coverslips (Corning, 22 $\times$ 22 μm^2 and 18 $\times$ 18 mm^2) and two pieces of double-sided sticky tape (Scotch 3M, thickness 0.1 mm). The non-adsorbed microtubules were washed out by perfusing 10 μM taxol in BRB80. The 1% and 3% solution of glutaraldehyde in 10 μM taxol in BRB80 were subsequently perfused for 5 min in the flow cell to crosslink the microtubules. Final crosslinking was performed by 25% water solution of glutaraldehyde over a period of 20 min. The crosslinked microtubules were subsequently treated with 100% 2,2'-(ethylenedioxy)bis(ethylamine) for 20 min. The initiator was immobilized on microtubules from a solution of BMPB (0.96 g, 4.2×10^{-3} mol) and triethylamine (0.72 g, 7×10^{-3} mol) in anhydrous dichloromethane (100 mL) over a period of 2 h. The modified microtubules were rinsed in dichloromethane 2 $\times$, ethanol 2 $\times$ and dried with nitrogen flux.

Grafting of polymer on microtubules

Polymer was grafted on initiator-modified microtubules as follows: NIPAM (2 g, 1.75×10^{-2} mol), FA (40 mg, 1×10^{-4} mol), EBIB (0.65 mg, 3.3×10^{-6} mol), CuBr_2 (0.35 mg, 1.6×10^{-6} mol), PMDTA (1.3 mg, 8×10^{-6} mol) were dissolved in 2 mL of acetone. The reaction solution was added to the tube containing the DDS-glass substrate with adsorbed initiator-modified microtubules. After the tube was sealed with a rubber septum, a solution of L-ascorbic acid (18 mg, 1×10^{-4} mol) in water (0.06 mL) was injected. The vial was placed in a 70 °C oil bath. The polymerisation was stopped after 1 h. The polymer obtained in solution was collected by precipitation in diethyl ether and used for determination of molecular weight. It is believed that these values should be close to those of the grafted polymer brushes. The substrates with polymer-modified microtubules were rinsed several times in hot chloroform and acetone. DMAC–2% H_2O –LiCl (3 g L^{-1}) was used as eluent for GPC.

Epi-fluorescent microscopy

Fluorescence images were obtained using an Axiovert 200M inverted microscope with a 40 $\times$ objective (Zeiss, Oberkochen, Germany) equipped with FluoArc lamp. For data acquisition standard TRITC (excitation: HQ 535/50; dichroic: Q 565 LP; emission: HQ 610/75, Chroma Technology) and FITC (excitation: HQ 480/40; dichroic: Q 505 LP; emission: HQ 535/50, Chroma Technology) filter sets in

conjunction with a Micromax 512 BFT camera (Photometrics, Tucson, AZ) and a MetaMorph imaging system (Universal Imaging, Downingtown, PA) were used.

Atomic force microscopy

AFM studies were performed with a Dimension 3100 (Digital Instruments, Inc., Santa Barbara, CA) microscope. Tapping mode was used to map the film morphology at ambient conditions.

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