NANO LETTERS

2005
Vol. 5, No. 10
1910−1914

Size Sorting of Protein Assemblies Using Polymeric Gradient Surfaces

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Received June 28, 2005; Revised Manuscript Received August 26, 2005

ABSTRACT

We report on a novel approach for the size-dependent fractionation of protein assemblies on polymeric surfaces. Using a simple temperature gradient method to generate one-dimensional gradients of grafted poly(ethylene glycol), we fabricated silicon-oxide chips with a gradually changing surface density of kinesin motor molecules. We demonstrate that such a bioactive surface can be used to sort gliding microtubules according to their length. To our knowledge, this is the first example of the self-organized sorting of protein assemblies on surfaces.

Introduction. Surfaces that change their properties gradually in one or more directions are applied successfully for combinatorial and high-throughput investigations in numerous research areas. Detailed scanning of such gradient surfaces allowed, for example, the thorough investigation of dewetting processes,[1] the morphological and wetting phenomena of polymer thin films,[2−7] as well as the measurement of the cloud point.[1] Thereby, the gradient approach allows for a substantial acceleration of data collection in multiparameter spaces combined with a significant reduction in the variance of the results due to experimental conditions. Moreover, gradient surfaces have recently become increasingly attractive for the investigation of proteins and cells.[8−15] Simultaneous control over the position and density of proteins on a surface constitutes a powerful means to investigate their properties. Until now, gradients of proteins on the surface have been generated by varying the dose of light during protein photoimmobilization,[13] by the controlled adsorption of nanoparticles with immobilized proteins,[12] by adsorption on gradient self-assembled monolayers[16] and on gradient surface-immobilized polymer layers prepared using controlled corona discharge,[17] as well as by ink-jet methods.[18] Such gradient surfaces have, for example, been used to study the effects of ligand/receptor density on biological recognition[9,19] and polyvalency.[20]

In this work, we report on the use of gradient surfaces for the sorting of protein assemblies according to their size. In particular, we demonstrate the sorting of gliding microtubules on surfaces that are coated with a gradually changing density of kinesin motor proteins (Figure 1). Microtubules are hollow, cylindrical protein filaments that can be formed in vitro by self-assembly of tubulin-heterodimers. They have an outer diameter of 25 nm and can be as long as 10−50 μm. Kinesin is an ATP-hydrolyzing motor protein that moves vesicles and organelles along microtubules in the cellular environment.[16,21−26] In vitro gliding motility assays, where

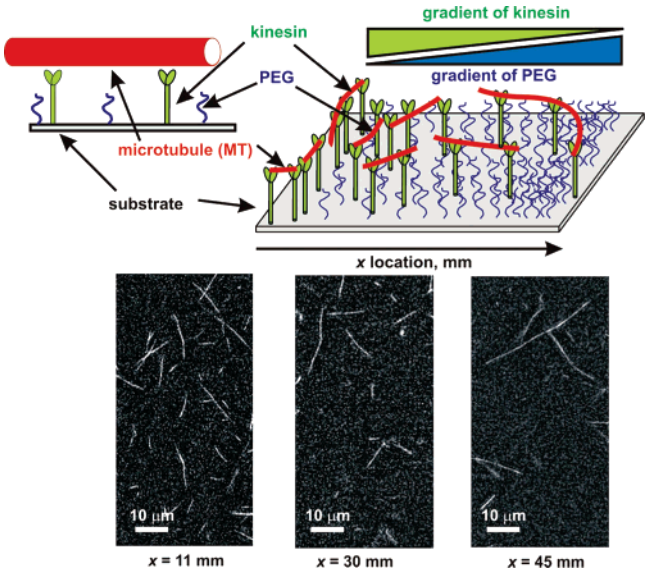

Figure 1. Gliding motility of microtubules on a poly(ethylene glycol) (PEG)-gradient surface with immobilized kinesin. Upper part: schematic diagram of the motility system. Although the grafting density of PEG increases from left to right, the kinesin gradient is formed in the opposite direction. Lower part: fluorescence micrographs of gliding microtubules taken at three different locations along the gradient surface. At lower kinesin density, the number of microtubules per field of view decreases, whereas the average length of the microtubules increases (see also supporting information).

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10.1021/nl051235h CCC: $30.25
Published on Web 09/21/2005

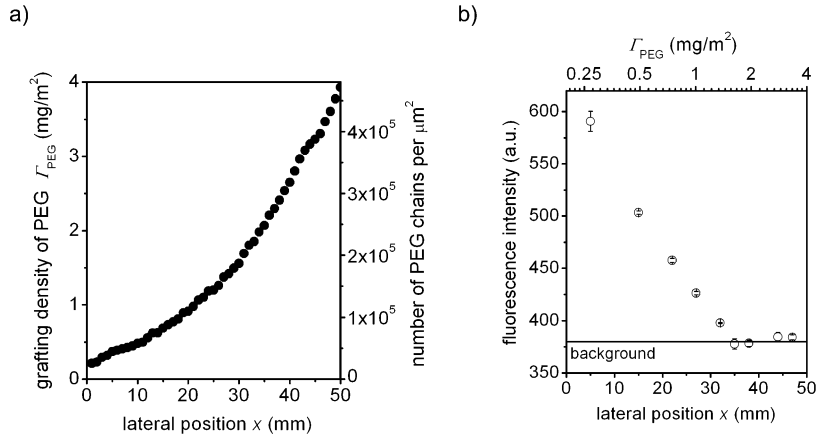

Figure 2. PEG density and GFP-kinesin binding. (a) Grafting density PEG and number of PEG chains per μm^2 as a function of the lateral position on the substrate surface. (b) Fluorescence intensity of surface-bound GFP-kinesin vs lateral position and PEG grafting density. The solid line indicates the background fluorescence measured in the absence of GFP-kinesin.

microtubules are propelled over a glass coverslip by surface-bound kinesin molecules, have been used to study the interaction between motor proteins and microtubules.[27−30] Moreover, the kinesin-microtubule system has been implemented successfully into synthetic environments in order to facilitate tasks such as nanotransport and nanostructuring.[30] In such applications, control over the kinesin surface density and the microtubule length is crucial because the maximum force that can be generated depends strongly on these parameters. In particular, the length distribution of microtubules that are grown in vitro is very broad. Methods to separate microtubules according to their length, while keeping their fragile structure intact, are therefore highly desirable but have, to our knowledge, not been reported to date.

Being a processive motor, a single kinesin molecule can move a whole microtubule over the surface without letting go of it.[25,31] The kinesin surface density and the length of the microtubule then determine if the microtubule subsequently leaves the surface or is grabbed by one or more other motors. Thus, the use of surfaces with gradually increasing distances between kinesin molecules is suited to sort microtubules according to their length.

We prepared kinesin gradients by adsorption of kinesin molecules onto surfaces with previously formed gradients in the grafting density of poly(ethylene glycol) (PEG). Poly(ethylene glycol) prevents the binding of proteins to surfaces and is used widely for the design of protein-resistant coatings.[32−35] Recently, it was demonstrated that the adsorption of proteins on PEG-modified surfaces decreases with increasing PEG grafting density.[36] Therefore, the use of surfaces with a gradually changing density of PEG chains is an appropriate way to prepare surface concentration gradients of proteins. We show that the adsorbed kinesin retains its bioactivity and can be used for the sorting of microtubules. To our knowledge, this is the first report about the active sorting of objects on a surface according to their size.

Fabrication of Gradient Surfaces. Highly polished single-crystal silicon wafers (final chip size 50 × 10 mm^2, {100} orientation, obtained from Semiconductor Processing Co.) with a native silicon oxide layer of 1.6 nm thickness were used as surface substrates. The wafers were cleaned in an ultrasonic bath with chloroform for 30 min, placed in hot piranha solution (3:1 concentrated sulfuric acid and 30% hydrogen peroxide) for 1 h, and rinsed several times with water. Subsequently, a thin layer of polyglycidyl methacrylate (PGMA) (thickness about 1.5 nm) was deposited by spin-coating a 0.01% PGMA solution in methylethyl ketone (MEK, VWR). PGMA (M_n = 84 000 g/mol) was synthesized by free radical polymerization of glycidyl methacrylate (Aldrich) in MEK at 60 °C using azobisisobutyronitrile (Aldrich) as the initiator. The obtained polymer was purified by multiple precipitations from MEK solution in diethyl ether. On top of the PGMA layer, a thin film (thickness about 200 nm) of PEG-NH_2 (2% solution in chloroform, PEG-NH_2, M_n=5 000 g/mol, M_w=5 400 g/mol, Polymer Source, Inc.) was spin-coated and annealed for 1 h on a stage with a 1D temperature gradient. The temperature changed gradually from 40 to 90 °C along the 50-mm length of the sample. Upon heating, the chemical reaction between the terminating amino groups of the PEG and the epoxy groups of the PGMA results in the formation of the grafted PEG layer with a gradient in the grafting density caused by the temperature dependence of the grafting kinetics. The thin layer of PGMA thereby served as a macromolecular anchoring layer.[37] Ungrafted polymer was removed using Soxhlet extraction in chloroform for 3 h. The main advantages of our method to produce PEG-gradient surfaces are that it (i) allows for a precise control of the grafted amount of PEG and (ii) can be performed without any special equipment.

Characterization of the Surface Gradients. The grafting density, Γ_{PEG}, of the one-dimensional PEG-gradient was determined by ellipsometric mapping using a SENTECH SE-402 scanning microfocus ellipsometer at λ = 633 nm at an angle of incidence of 70° with a lateral resolution defined by the beam spot of about 20 μm. Figure 2a shows that the amount of surface-immobilized PEG chains increases gradually with lateral position x on the sample (x = 0 at the edge with the lowest grafting density of the PEG layer).

Kinesin binding experiments were performed in a 5-mm-wide flow cell constructed from a silicon chip containing the PEG gradient,[38] a coverslip[39] (Corning, 50 × 24 mm^2), and two pieces of double-sided sticky tape (Scotch 3M, thickness 0.1 mm). A casein-containing solution (0.5 mg/mL in BRB80) was perfused into the flow cell and allowed to adsorb to the surfaces for 5 min. This step originates from the commonly used protocol for gliding microtubule motility (as applied later on for the sorting application) and serves to reduce the denaturation of kinesin as well as prevent the sticking of microtubules to the surface. To quantify the binding of motor proteins, we perfused 50 μL of motor solution (containing 27.4 μg/mL kinesin labeled with the green-fluorescent protein (GFP) in BRB80) into the flow cell and allowed it to adsorb for 5 min. Afterward, an oxygen scavenger mix (20 mM D-Glucose, 0.020 mg/mL glucose oxidase, 0.008 mg/mL catalase, 1% β-mercaptoethanol) was perfused into the flow cell in order to reduce photobleaching and to remove unbound GFP-kinesin. Fluorescent images were obtained using an Axiovert 200M inverted microscope with a 40x oil immersion objective (Zeiss, Oberkochen, Germany). For data acquisition, a CoolSnap HQ Camera (Photometrics, Tucson, AZ) in 2 × 2 binning mode (yielding a field of view with an effective area of 226 × 169 μm^2) was used in conjunction with the MetaMorph imaging system (Universal Imaging, Downingtown, PA). Figure 2b shows data of the measured fluorescence (each value representing the averaged intensity over 7 fields of view) as function of the lateral position, x. The gradual decrease of fluorescence intensity with increasing grafting density of PEG indicates the formation of a 1D gradient of surface concentration of kinesin. No GFP-labeled kinesin molecules were detected to bind to the surface at PEG densities higher than 1.8 mg/m^2 where the measured fluorescence values corresponded to the background fluorescence measured in the absence of GFP-kinesin. This background is attributed mainly to auto fluorescence in our experimental setup and to residual back-reflected excitation light.

Microtubule Motility on Gradient Surfaces. Surfaces with a gradually changing density of kinesin were prepared as described above using a motor solution containing 2 μg/mL wild-type kinesin (full length drosophila conventional kinesin expressed in bacteria and purified as described in ref 40). Thereafter, motility solution containing rhodamine-labeled taxol-stabilized microtubules[41] (~30 nM tubulin, 1 mM ATP, 1mM $MgCl_2$, 10 μM taxol, and oxygen scavenger mix; all in BRB80 buffer) was perfused into the cell. To perform the motility experiments under conditions where the binding and unbinding of microtubules to/from the surface is in equilibrium, we did not wash out unbound microtubules. Imaging was started 10 min after perfusion of the microtubules into the flow chamber. It was found that the number of microtubules gliding over the PEG-kinesin gradient surface decreases linearly with the lateral position on the chip (Figure 3, green circles). However, the speed of the gliding microtubules was independent of the PEG grafting density and thus the kinesin surface density (Figure 3, red triangles). These observations demonstrate clearly, that (i) the use of a PEG gradient layer allows the fabrication of surface density gradients of functional kinesin molecules and (ii) the activity of individual kinesin molecules is independent of the amount of grafted PEG underneath. In fact, we observed only very few immobile microtubules that were attached to the surface but did not move. This indicates a low amount of inactive motor molecules on the surface because it is known that inactive motors tend to inhibit MT gliding. Notably, we found gliding microtubules at grafting densities of PEG up to $\Gamma = 3$ mg/m^2. This finding is in contrast to the fluorescent measurements of GFP-kinesin (Figure 2b), where the surface seems to block protein absorption for PEG densities of $\Gamma > 1.8$ mg/m^2. This difference might be the result of the limited detection sensitivity in Figure 2b (see above). However, it could also be a sign of steric hindrance effects, that is, kinesin molecules that are labeled with a GFP molecule (diameter about 4 nm) at their tails might need more unblocked surface area to bind to than wild-type kinesin.

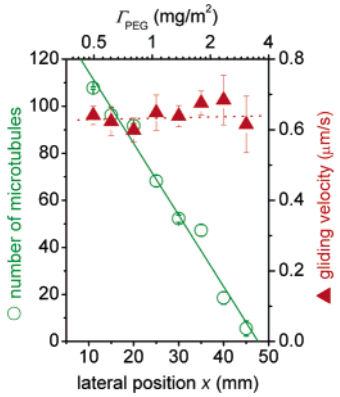

Figure 3. Characterization of gliding motility on PEG-kinesin gradient surfaces. Number of gliding microtubules per field of view (green circles) and gliding velocity (red triangles) vs lateral position and PEG grafting density. Plotted are average values ± standard deviation derived from at least four time-lapse movies at each position.

Size Sorting of Gliding Microtubules. Analysis of the length distribution of the gliding microtubules on the gradient surfaces showed that shorter microtubules disappeared gradually at positions with increased PEG-grafting density (Figure 4a). We found that the average length of microtubules increased from 6 to 13 μm (Figure 4b) at increasing PEG grafting densities from 0.5 to 2.2 mg/m^2. The length of the shortest motile microtubules, which provides an estimate of the distance between active kinesin molecules, was found to increase from less than 1 μm to about 5 μm over the evaluated range of PEG densities.

To explain the obtained results, we can make the following considerations: microtubules whose length, l, is larger than the average spacing, L_K, between neighboring kinesin molecules can continuously glide over the surface (i.e., they will very likely find a second kinesin to be propelled by before detaching from the first one). However, if $l < L_K$, then the probability to find a microtubule of length l on a surface with a kinesin density $\rho_K = 1/L_K^2$ will be proportional to the microtubule landing rate $\eta_{landing} = \alpha \cdot \rho_K \cdot l$ and the

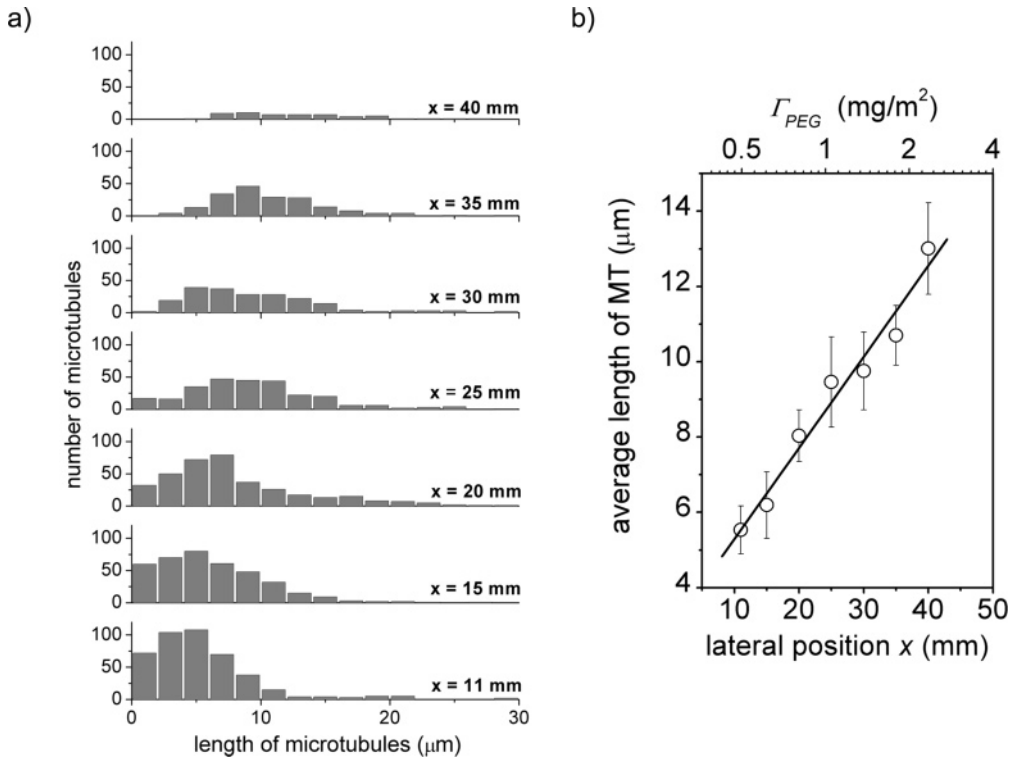

Figure 4. Dependence of the length of motile microtubules on the PEG-kinesin density. (a) Histograms of microtubule lengths at different lateral positions on the substrate (data represent typical length distributions of microtubules in four fields of view) (b) Average microtubule length as function of the lateral position and PEG grafting density. Error bars show the standard deviation for four fields of view at each lateral position.

gliding time on the surface $t_{gliding} = l/u_{gliding}$ with $u_{gliding}$ being the microtubule gliding velocity and α being a constant. Thus, longer microtubules have a higher probability of landing on the surface and a lower probability of leaving it. Consequently, for a given microtubule length (or a range of lengths), the fraction of gliding microtubules will depend linearly on the kinesin surface density, which has been measured in Figure 2b via GFP-fluorescence to be a linear function of the lateral position on the gradient chip. In fact, such behavior can be demonstrated in Figure 5 where the data from Figure 4a (pooled for the 3−6 μm and 6−9 μm length ranges) has been plotted normalized to the number of microtubules present in the initial distribution (as inferred from the $x = 11$ mm data). The number of short microtubules (length range between 3 and 6 μm, open circles) monotonically decreases along the lateral position on the chip. Looking at longer microtubules (length range between 6 and 9 μm, filled triangles), it becomes apparent that the fraction of gliding microtubules first stays constant and only decreases from a certain lateral position on the chip.

In summary, we have developed a novel method for the surface-based sorting of protein assemblies according to their size. As an example, we have demonstrated experimentally that surfaces with a gradually changing density of kinesin molecules can be used for the size fractionation of gliding microtubules. To our knowledge, this approach constitutes the first example of the self-organized sorting of protein assemblies on surfaces. We note that the sorting depended highly on the functionality of the surface-bound kinesin motors, which actively transported the microtubules to be sorted. In this way, motor proteins are very well suited to test the functional implementation of proteins into artificial environments because gliding filament motility can be used readily as read-out of retained protein activity. In regard to nanotechnological applications of the kinesin-microtubule transport system, our method might provide a means to obtain microtubules with narrow length distributions. The motile microtubules could be directed into collection reservoirs by

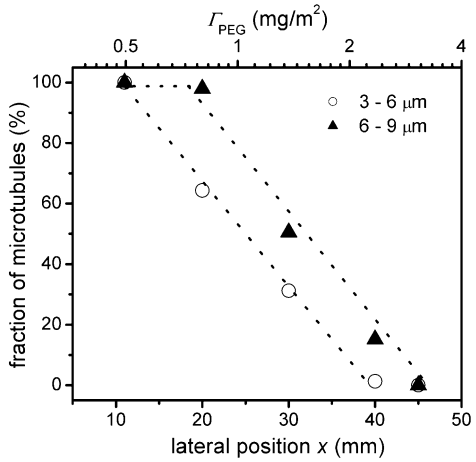

Figure 5. Fraction of gliding microtubules (normalized by the initial distribution) as a function of the lateral position on the gradient surface. Although a linear decrease is observed for 3−6 μm long microtubules starting from $x = 11$ mm, the fraction of microtubules with 6−9 μm length decreases only after some initial plateau. The dotted lines in the figure serve only to guide the eye and shall illustrate the general behavior of the data.

hydrodynamic flow perpendicular to the direction of the gradient in order to collect the different lengths after separation on the surface.[42] In general, we foresee a large potential of the reported method for further investigations of motor protein activity, as well as for high-throughput screening and combinatorial investigations of protein properties and interactions between proteins.

Acknowledgment. We thank C. Reuther, C. Bräuer, and J. Kerssemakers for assistance with the experiments, R. Clark and J. Howard for providing the GFP-labeled kinesin, and G. Brouhard, C. Dinu, J. Helenius, B. Nitzsche, E. Schäffer, and B. Schroth-Diez for helpful comments on the manuscript. We are also grateful to the DFG (grant SFB 287) and the BMBF (grant 03N8712) for financial support.

Supporting Information Available: Movie of gliding microtubule motility at three kinesin densities on the gradient surface. This material is available free of charge via the Internet at http://pubs.acs.org.

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(38) To quantify the amount of surface-bound GFP-kinesin via fluorescence measurements, we used silicon wafers with a 75-nm oxide layer (obtained from GeSiM mbH, Rossendorf) in order to avoid destructive interference effects present at low oxide thickness.
(39) Before usage, the coverslips were immersed in a PEG solution that consisted of 5 mM 2-[methoxypoly-(ethyleneoxy)propyl]-trimethoxysilane (90%, ABCR Karlsruhe) and 0.08% hydrochloric acid (36%) in toluene. After 12 h, the coverslips were rinsed and sonicated in toluene, ethanol, and water. The PEG layer on the glass coverslip prevented kinesin and microtubule binding to that side of the flow cell.
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(42) Although, using the presented method the fraction of the short microtubules would always contain a number of long ones, this could potentially be overcome by flowing the microtubules into the device at high concentration. Because of a higher diffusion coefficient, shorter microtubules would reach the active kinsein binding sites faster than the longer ones. Having bound to the surface at high density, the short microtubules would then prevent the binding of the longer ones because of steric hindrance (note that microtubules are stiff polymers with a persistence length of about 1 mm in solution and that they have to land rather parallel to the surface in order to attach to the kinsein motors).

NL051235H