Site-specific binding and stretching of DNA molecules at UV-light-patterned aminoterpolymer films

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Abstract

Site-specific deposition of nanoparticles and DNA onto micro-patterned aminoterpolymer films is reported. The chemical patterning of the film surface is accomplished by an easy-to-handle, one-step procedure. Photolabile protection groups are locally removed by applying UV light through an optical mask. This causes exposure of amino groups to the surface to which charged nanoparticles can then associate. End-specific binding of DNA at the surface-exposed amino groups is obtained at optimum pH conditions. This allows a site-specific attachment and stretching of single DNA molecules at the patterned polymer film surface.

1. Introduction

Advanced methods for the fabrication of micrometre and submicrometre structures become more and more essential for novel developments in the emerging field of bio-nanotechnology, where site-specific immobilization of nanoparticles or functional biomolecules is often an important issue for the processing or diagnostic techniques applied. Several methods, such as photo- or electron-beam lithography, offer one the ability to build up the necessary surface pattern, but in most cases the substrate processing is a relatively complicated multi-step procedure, because the 'geometrical' and the 'chemical' patterns are processed subsequently, e.g., first metal films are patterned, which are then chemically or biologically treated to create a desired surface functionality. One well known exception is microcontact printing [1], which allows us to fabricate a chemical surface pattern or even direct deposition of molecules onto a substrate in one step in a massively parallel fashion. UV-light patterning of ultra-thin self-assembling monolayers (SAMs) of organic molecules as recently reported for SAMs prepared from amines protected with photolabile groups [2, 3] has the same potential. However, the preparation of SAMs for this purpose turned out to be laborious—special molecules have to be designed, and monolayer quality and stability cannot be taken for granted on all substrates. Another, recently developed, method to create a chemical surface pattern with nanometre-scale perfection directly is dip-pen lithography [4]. However, this method does not yet have the capability of massive parallel fabrication.

Here, we report on a novel method to fabricate a functional surface pattern for a site-specific deposition of charged nanoparticles and biomolecules, based on optical exposure of a thin, spin-coated aminoterpolymer (ATP) film, and intrinsically capable of massive parallel processing. The amino groups of the polymer film are protected by UV-labile nitroveratryloxycarbonyl (NVOC) groups [5]. Thus, the film surface can be easily chemically patterned by applying UV light which causes photo-cleavage of the NVOC groups. As a result, the amino function is created at the UV-light exposed area, ready to use for a site-specific immobilization of nanoparticles. In particular, using the molecular combing technique originally developed by Bensimon *et al* for the deposition of stretched DNA onto non-patterned silane-terminated glass surfaces [6], we have studied the chemical
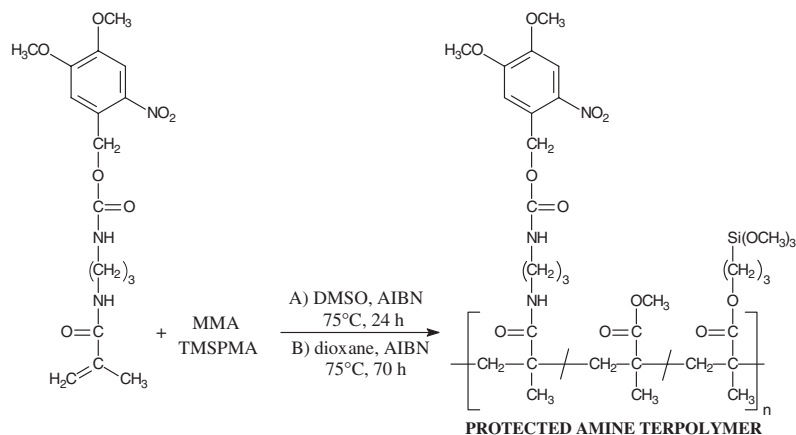

Figure 1. Scheme of the terpolymer preparation.

conditions for an end-specific binding of DNA to the patterned ATP films. The results obtained under optimum conditions clearly demonstrate that DNA anchoring takes place specifically at the UV-exposed areas. The strength of the bonding is large enough to overstretch the deposited DNA molecules.

2. Materials and methods

2.1. Preparation of the terpolymer

The experiments are carried out with the aminoterpolymer poly(methylmethacrylate/N-(N-NVOC-aminopropyl) methacrylamide/(3-trimethoxysilyl-propyl)-methacrylate). It is prepared via a free radical polymerization in 1,4-dioxane using AIBN (2,2′-azoisobutyronitrile) as initiator (see figure 1) as described in [5]. For the preparation of the polymer, the three monomers MMA (methylmethacrylate), amine, and TMSPMA (3-(trimethoxysilyl)propylmethacrylate, acting as silane anchor group) are employed in equimolar amounts. The polymer is precipitated by pouring the reaction mixture into a dry diethyl ether solution. Thereafter, the precipitate is filtered and dried. The final composition of the resulting terpolymer determined by ^{1}H-NMR is 46 mol% MMA, 18 mol% amine and 36 mol% TMSPMA. The ATP is dissolved in DMSO and used for film preparation.

2.2. Film preparation and patterning

Glass substrates are cleaned with Piranha solution and sonicated at 70 °C using the standard procedure described in [7]. Immediately after cleaning, the dried substrates are coated with ATP films by spin coating, using a 2 wt% terpolymer solution in DMSO at 3000 rpm for 30 s. Subsequently, the films are annealed for 2 h at 120 °C in air. Non-attached polymer is removed by sonicating in Millipore water for 5–10 min at room temperature. Then the films are dried in vacuum. The typically obtained film thickness—determined by ellipsometry—is about 30 nm. The obtained ATP films are hydrophobic. The surface roughness of the films, determined by scanning force microscopy (SFM) using a NanoScope IIIa (Digital Instruments), is less than 1 nm.

Lithographic patterning of the film is accomplished by UV irradiation through a mask, using an Hg arc lamp (Oriel, Stratford, CT, USA) with a power of about 100 mW cm^{-2}. The irradiation time has been varied between 1 and 5 min. (For comparison, a detailed description of the parameters for the irradiation of the ATP films with a UV laser can be found in [5].) For irradiation test purposes, we use TEM grids with squared holes of 53 μm × 53 μm as masks. Smaller structures are created by employing different masks with circular holes with diameters between 1 and 7 μm with centre-to-centre distances from 2 to 13 μm, respectively. After the UV patterning the films are employed for the decoration and binding experiments without any additional washing.

2.3. Decoration of the exposed patterns with nanoparticles

Negatively charged 5 nm gold colloids (Sigma, G-1402) are bound to the irradiated ATP film regions with the aim of verifying the successful cleavage of the protection groups, and the consequent exposure of positively charged amino groups to the surface. For this a drop of 50 μl colloid solution (pH = 7.0) is placed onto the patterned surface. After an incubation time of 10 min the sample is thoroughly rinsed with Millipore water. The remaining, bound colloids are used as nucleation centres for a subsequent gold enhancement [8]. For this, 60 μl KAuCl$_4$ (23 mg ml^{-1}) are mixed with 60 μl KSCN (potassiumthiocyanate) (60 mg ml^{-1}) followed by a centrifugation at 5000 g for 1 min. The pellet is dissolved in 480 μl 1 M sodium phosphate buffer (pH 5.5). Then 120 μl of 5.5 mg ml^{-1} hydrochinone are added. About 300 μl of the solution are placed for about 3.5 min onto the sample and subsequently rinsed with H$_2$O.

2.4. Binding of DNA

Fluorescently labelled DNA from salmon testes (Sigma) with an average molecule length of about 2000 base pairs (bp), corresponding to about 600 nm, is used in the decoration experiments. First, the DNA is dissolved in TE buffer (DNA concentration 1 mg ml^{-1}, 10 mM Tris, 1 mM EDTA, pH 8.0 adjusted with HCl). Then, 5 μl DNA solution is diluted in 1 ml of TE, containing the fluorescence dye (see section 2.5). A 300 μl drop of this solution is placed onto the patterned
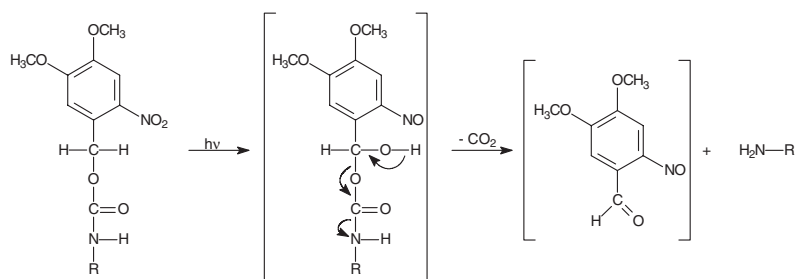
Figure 2. Scheme of the UV cleavage reaction of the nitroveratryloxycarbonyl (NVOC) protecting group, resulting in a primary amino function.

- UV light
- optical mask
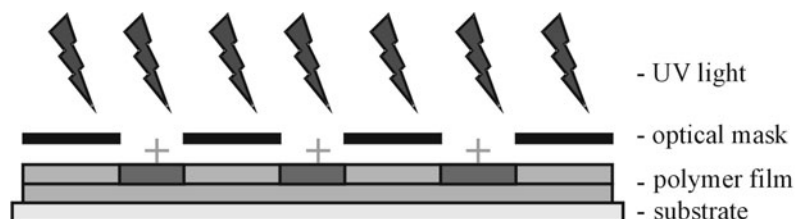- polymer film
- substrate

Figure 3. Schematic view of the selective deprotection of a thin terpolymer film by UV irradiation through an optical mask.

surface. After an incubation time of 20 min the sample is rinsed with 10 mM TE to remove excess DNA from the surface.

About 48 kbp long λ-DNA (New England Biolabs, N3011, concentration 0.5 μg μl^{-1}) with a length of about 16.1 μm is used in the experiments, where we stretch DNA molecules at the surface using the method of the receding meniscus [6, 9]. For this, 100 μl fluorescently labelled DNA solution is diluted in 3 ml of 10 mM AMPSO buffer (pH 8.8, adjusted with KOH). Then the cover slip with the patterned ATP film is immersed in the DNA solution, incubated for 10–15 s, and after this very slowly removed from the solution (a speed of about 5 mm s^{-1}). Because the ATP films are hydrophobic, no specific drying procedure after the immersion is needed.

2.5. DNA imaging

For optical imaging, the DNA is fluorescently stained for 30 min in 10 mM TE buffer (pH 8.0) with YOYO-1 iodide (Molecular Probes, Y-3601, 1 mM stock diluted 1:100 in 1 × TBE). In the case of salmon testes DNA, 5 μl of DNA are stained in 1000 μl TE buffer containing 30 μl of the diluted YOYO-1. λ-DNA is less intensively stained. Here, 2 μl of DNA are stained in 100 μl of TE buffer with 4 μl of the diluted YOYO-1 solution.

The labelled DNA is imaged with an Axiovert 200 M fluorescence microscope (Zeiss, Germany) using Hg-lamp excitation and filter set 9 from Zeiss (excitation, 450–490 nm; beam splitter, 510 nm; emission, long pass 515 nm).

3. Results and discussion

The prepared aminoterpolymer consists of different functional groups, namely anchor groups and photolabile protected amine groups. Spin coated on glass substrates, it forms a thin, homogeneous film of about 30 nm thickness. By subsequent annealing, a covalent attachment of the polymer film to the glass substrate is achieved because the trimethoxysilane anchor groups hydrolyse, and form a siloxane network structure with

the silanol groups of the glass substrate. The prepared films have a very smooth surface with a surface roughness below 1 nm. Upon UV irradiation, the nitroveratryloxycarbonyl (NVOC) protecting group can be cleaved, forming back a primary amino function (figure 2). Photo-cleavage of protecting groups in thin ATP films by irradiation with UV lasers has been recently demonstrated [5]. There, the deprotection of NVOC groups was verified by fluorescence labelling of the free amino groups. Using a similar concept, diazosulfonate terpolymers were patterned by UV lasers, and the remaining diazosulfonate groups were verified by metal salt complexation [7, 10].

Here we focus on the binding of nanoparticles and DNA onto patterned ATP films. These investigations allow us to answer the question of whether deprotected amino groups are exposed to the surface upon UV irradiation with a sufficient high density to obtain a strong electrostatic binding of charged nanoparticles. Within this approach the patterning will be characterized not only regarding changes in chemistry, which could preferentially or even solely happen 'inside' the—compared to SAMs—relatively thick ATP films, but also with regard to changes in surface charge. With this aim, we investigate the selective decoration of the deprotected areas with negatively charged gold colloids and fluorescently labelled DNA fragments. Moreover, we study molecular combing of λ-phage DNA at the patterned film surfaces. This method allows us to estimate the bonding forces between the deprotected amino groups and the negatively charged DNA ends.

3.1. Patterning of the polymer film

The prepared ATP films are lithographically patterned by selective UV exposure through optical masks with characteristic structure sizes between 2 and 50 μm (figure 3). These structures can be readily transferred into the polymer film as we demonstrate, e.g., by fluorescence staining of the patterned film with very diluted DNA solution. Figure 4 shows
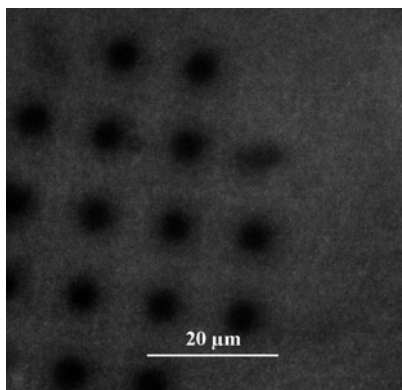
Figure 4. Fluorescence microscopy image of a patterned ATP film, stained with a very diluted YOYO-1 solution. The areas exposed to UV light appear dark. The fluorescent dye binds selectively to the area which was protected by the mask during irradiation.

a fluorescence microscopy image of an ATP film irradiated through a mask with periodically arranged 5 μm holes after treatment with very diluted YOYO-1 solution. The UV-light-exposed areas appear dark, whereas the area protected by the mask appears light, indicating that the fluorescent dye used selectively decorates the non-exposed area. We assume this selective staining effect to be caused by a repulsion of the YOYO dyes from the irradiated, charged ATP areas. On the one hand, this behaviour is opposite to the results reported by Braun *et al* [5] for the staining with fluorescein isothiocyanate (FITC), where areas exposed by UV light were labelled through a selective reaction of the FITC dye with the deprotected amino function. On the other hand, labelling of the untreated film area as observed here is an extremely useful feature, because in this way the fluorescent dye applied to make the obtained film pattern visible certainly does not interact with the amino function of the UV-exposed areas, which is thought to be used for other purposes, such as, e.g., for the binding of nanoparticles. We stress the importance of visualizing the obtained film pattern by optical methods, because otherwise the applied UV treatment does not cause any change in the film morphology unless very high doses are applied. In particular, the roughness of the ATP films measured by SFM is less than 1 nm, and does not change upon UV exposure up to 90 s. This exposure time has been found to give best results for the local deprotection of the amino groups in the following decoration experiments.

3.2. Decoration of the patterned films

The charge of surfaces covered with layers of ionizable groups, like amines, can be adjusted by the pH value of the solution which is in contact with this surface. At a pH below the pK_a value, protonation of the functional groups takes place (i.e., $NH_2 + H^+ \leftrightarrows NH_3^+$), thus, the surface is positively charged. The lower the pH the higher the charge density, providing a tight adsorption of negatively charged particles at the surface. This effect is used in the decoration experiments with both charged colloids and DNA.

3.2.1. Decoration with nanoparticles. The UV-irradiated ATP films are exposed to solutions of negatively charged gold
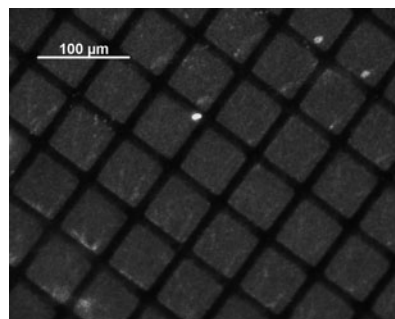

Figure 5. Light microscopy image of an ATP film patterned by using a TEM grid as optical mask. After the UV exposure gold nanoparticles were allowed to adsorb at the film surface and treated with a gold enhancement solution to grow a continuous metal film. The gold film appears bright, whereas the undecorated film surface appears black. The size of fabricated gold squares is 53 μm $\times$ 53 μm.
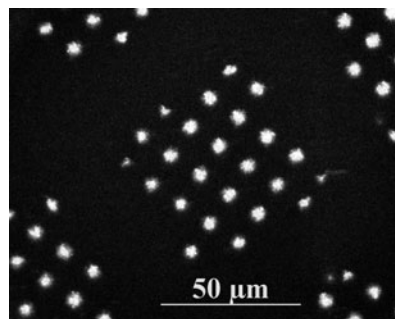

Figure 6. Fluorescence microscopy image of a UV-patterned ATP film after treatment with a solution of salmon testes DNA. The areas where the DNA binds are seen as bright spots.

colloids. After subsequent washing steps, the films are treated with a gold-enhancement solution. Figure 5 shows the result for the case where a 400-mesh TEM grid is used as the optical mask. The gold film is selectively deposited at the irradiated areas. Since the gold enhance can only develop where gold colloids are present, this result gives clear evidence of both the successful photo-cleavage of the NVOC protection groups and the consequent exposure of positively charged amino groups to the surface to which the negatively charged gold colloids bind selectively.

3.2.2. Local decoration of the patterned films with salmon testes DNA. Following the same concept, patterned ATP films have been decorated with DNA fragments from salmon testes. Figure 6 shows a fluorescence microscopy image of a polymer film, which was irradiated through a mask with 7.2 $\pm$ 0.5 μm hole size and a centre-to-centre distance of 13.5 $\pm$ 0.5 μm. The negatively charged DNA molecules bind selectively to the deprotected film areas, which appear in the form of bright spots. Note that the brightness is maximum in the centres of the spots and decreases towards the edges of the structures, indicating a lower decoration density at the edges than in the centre of the structures. The circumferences of the circular structures do not appear smooth but frayed. The average diameter of the spots is 6.2 $\pm$ 1.7 μm, which is slightly smaller than the size of the holes in the mask. These effects could be explained by the fact that the deposited
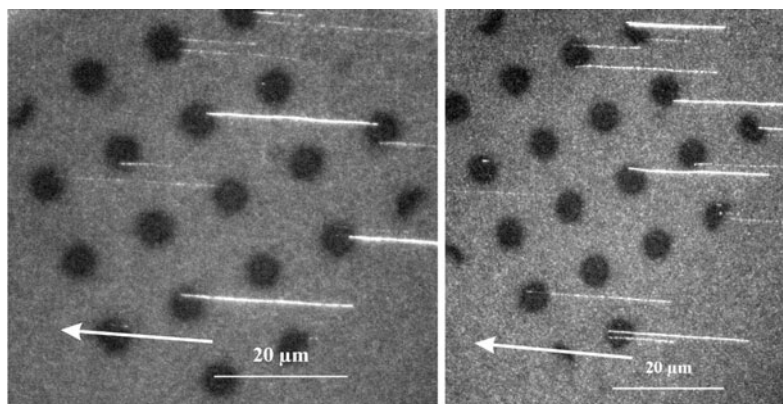
Figure 7. Fluorescence microscopy images of a UV-light patterned sample after immersion into a λ-DNA solution of pH = 8.8 and slow removal from the solution. The arrows indicate the direction of the removal of the sample from the liquid. The DNA molecules are stretched by molecular combing. The length of the DNA varies between 10 and 25 μm. Please note that the sites where the DNA attach spontaneously to the surface (left-hand end of the molecules in this case) are exclusively located at the UV-exposed areas, which appear dark due to the YOYO-staining effect of the ATP film, described in section 3.1 (cf figure 4).

molecules have a finite size which is considerably larger than the deposited gold colloids. Alternatively, differences in the UV-light intensity in the middle and at the edge of the dots due to scattering effects at the edges of the used mask could also explain the lower deposition density at the edges of the dots. Consistently with the sizes of the mask, the centre-to-centre distance of the decorated dot areas is found to be 13.5 ± 0.6 μm. As can be seen in figure 6, the DNA binding at the patterned ATP films is highly site specific. The unspecific binding outside the UV-exposed areas is extremely low.

3.3. Site-specific binding and stretching of λ-DNA

Since the ability of specific binding of DNA to a surface by its extremities has a profound application potential—from biophysical studies of single molecules up to gene mapping [11–13], and to demonstrate manipulation of single DNA molecules, we have investigated molecular combing of λ-phage DNA on micro-patterned ATP films. The technique of molecular combing allows us to align and to stretch single DNA molecules at surfaces by a receding meniscus of the buffer liquid [6, 9, 11, 14]. However, combing of DNA only takes place when at least one extremity of the molecule spontaneously binds to the surface and when the strength of the bond is sufficient to withstand the acting capillary force. Only in this case will the DNA molecules, which exhibit a coiled conformation in solution due to entropic origin, be extended by the receding air–water interface and left to dry on the surface in an aligned manner. Therefore, this method can be used to detect the end-specific binding of DNA. Moreover, it allows us to estimate the characteristic binding and stretching forces involved.

In order to obtain an end-specific anchoring of DNA at the amino-terminated areas of the UV-light-patterned ATP films, their surface charge has to be balanced in such a way that exclusively the DNA ends will stick to these surface areas. This condition can be achieved by adjusting the pH of the buffer solution to a value where the negatively charged DNA midsegment is no longer attracted to the amino-terminated surfaces, whereas the unmodified extremities, which carry a higher charge than the midsegment of the molecule [15], still

adsorb strongly enough to withstand the forces generated by the receding meniscus. In our particular case this condition is fulfilled at an optimum pH value of 8.8. Below this, the molecules adsorb strongly over their whole length. Therefore, they do not become extended. At pH above the optimum value their adsorption capability decreases rapidly. Figure 7 shows two fluorescence images of cover slips with patterned ATP films after they have been immersed in the DNA solution at the optimum pH of 8.8, incubated for 10–15 s, and then slowly removed from the solution. The fluorescently labelled DNA molecules are stretched by molecular combing in the direction opposite to the direction of the removal of the sample from the solution. The latter is indicated by an arrow. Please note that the sites where the DNA extremities first anchor to the surface (left-hand ends of the depicted molecules in this case) are exclusively located at the UV-exposed areas. The latter can be easily identified, because they appear dark due to the YOYO-staining effect of the ATP film, described in section 3.1 (cf figure 4). Anchoring of DNA molecules at the non-UV-treated, hydrophobic ATP film area is not observed. This highly selective effect of DNA anchoring can be understood by taking into account that the optimum pH value for an end-specific DNA anchoring at hydrophobic surfaces is much lower; e.g., Allemand *et al* [11] reported an optimum pH of about 5.5 for the molecular combing of DNA at polystyrene surfaces. Above this value, the end-specific attachment of molecules strongly decreases with increasing pH. Therefore, at pH = 8.8, where the UV-patterned areas have their optimum for the specific anchoring of the unmodified DNA extremities, no DNA attachment takes place at the untreated, hydrophobic area, thus leading to the observed high contrast in the site specificity of binding between the UV-exposed and the protected film area.

The length of the combed λ-DNA molecules varies from 10 μm up to 29 μm with a maximum in the length distribution at 25 μm. This value is a factor of about 1.5 longer than the contour length of a λ-DNA which is about 16.1 μm. The measured length distribution is similar to that reported by Bensimon *et al* [9] for the combing of λ-DNA on silanated, purely hydrophobic surfaces. This behaviour can be easily explained, taking into account that the action of the receding meniscus appears to be localized at the air–water
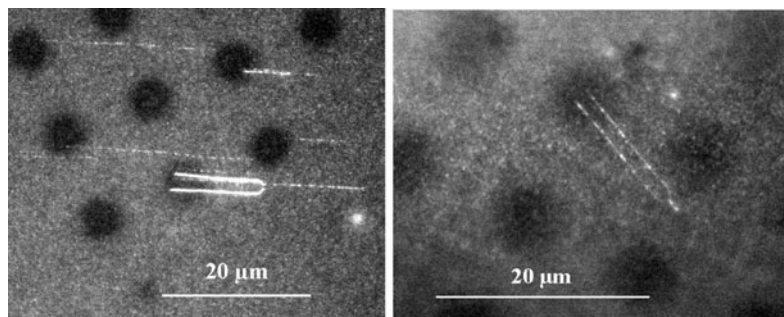
Figure 8. Fluorescence microscopy images of bound and stretched λ-DNA. The molecules were stretched from the left- to the right-hand side in the left-hand image and from the top left to the bottom right in the right-hand image. In both cases one molecule is anchored to the UV-treated ATP-film area with both of its extremities. The deposited molecules exhibit a typical U-shape. Both are broken at the very 'bottom' point of the U.

interface. Therefore, although in our case molecule extremities selectively bind to the UV-exposed, amino-terminated areas of the film, the larger parts of the combed molecules are deposited on the untreated, hydrophobic film area (cf figure 7). This causes the λ-DNA molecules to be overstretched as on purely hydrophobic surfaces. The force to extend a DNA molecule to about 150% of its contour length, l_0, can be estimated from its bulk elastic behaviour: $F = EA(l/l_0 - 1)$, where $E = 1.1 \times 10^8$ N m^{-2} is its Young modulus [16] and $A = 2.3 \times 10^{-18}$ m^2 its cross-sectional area. For $l/l_0 = 1.5$ one obtains $F \sim 130$ pN. This value is about twice the force which has been measured to extend double-stranded DNA. According to the measured force–extension curve of double-stranded DNA [17], the force which is needed to overstretch a DNA molecule by a factor of 1.5 is about 60–70 pN.

However, since the forces generated by the receding water–air interface act very locally, we have to take into account that these values can only be considered as upper bounds for the binding force of one DNA extremity to the surface. The initial binding of the molecule takes place locally at the amino-terminated ATP-film areas. According to Bensimon *et al* [9], the degree of extension on hydrophilic surfaces is less. In this case, the molecules are only extended to their contour length by capillary forces which counteract the entropic forces in solution. In this case we obtain $F \sim 35$ pN from the measured force–extension curve [17].

In some cases we observe combed DNA molecules which show a typical U-shaped morphology. This appears when the molecule anchors at the surface with both of its extremities simultaneously. Figure 8 shows that in this case also the anchoring sites are located at the UV-treated areas of the patterned film, supporting the results obtained for the linearly combed DNA molecules. The formation of the U-shape has been thoroughly analysed by Besimon *et al* [9]. First, as the meniscus moves across the anchoring points, the doubly attached molecule is stretched in the same way as a singly attached molecule. Therefore, at the beginning the molecule forms two segments aligned in parallel. At the same time the part of the molecule which is still located in solution and which connects the two linearly deposited segments rapidly shortens. Finally, this part of the molecule has to pass the water–air interface of the receding meniscus, which leads to a very high tension in the crossing loop. If the tension is above a critical value, the loop breaks. The estimated force needed to break a

DNA molecule is larger than 400 pN [9]. After breaking, both formed ends of the broken molecule are further aligned like two independent molecules, that is, in parallel to the segments of the molecule which are deposited first (see the right-hand image of figure 8).

In summary, the observed results clearly demonstrate the capability of a micro-patterned ATP film to selectively anchor DNA at its unmodified extremities to film areas which have been deprotected by UV-light patterning. The obtained binding forces are large enough to allow extension and manipulation of individual DNA molecules.

4. Conclusion

We have demonstrated that UV-light-induced deprotection of aminoterpolymer films leads to a local exposure of primary amino groups to the film surface. This allows the highly site-specific binding of negatively charged gold colloids to the exposed polymer film areas. This behaviour was used to fabricate a patterned metal layer by applying a gold enhancement to the deposited gold colloids. A highly site-specific binding of DNA to the patterned aminoterpolymer layer was observed, which shows—in general—that patterned ATP films are well suited for a site-specific immobilization of negatively charged biomolecules. The surface charge of the deprotected ATP-film areas can be tuned to obtain a highly selective, end-specific DNA binding. The obtained binding forces are large enough to allow single-molecule manipulation of DNA which is one important prerequisite for biological or bio-physical studies at single-molecule level.

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