Conformations and cryo-force spectroscopy of spray-deposited single-strand DNA on gold

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Cryo-electron microscopy can determine the structure of biological matter in vitrified liquids. However, structure alone is insufficient to understand the function of native and engineered biomolecules. So far, their mechanical properties have mainly been probed at room temperature using tens of pico-newton forces with a resolution limited by thermal fluctuations. Here we combine force spectroscopy and computer simulations in cryogenic conditions to quantify adhesion and intra-molecular properties of spray-deposited single-strand DNA oligomers on Au(111). Sub-nanometer resolution images reveal folding conformations confirmed by simulations. Lifting shows a decay of the measured stiffness with sharp dips every 0.2–0.3 nm associated with the sequential peeling and detachment of single nucleotides. A stiffness of 30–35 N m⁻¹ per stretched repeat unit is deduced in the nano-newton range. This combined study suggests how to better control cryo-force spectroscopy of adsorbed heterogeneous (bio)polymer and to potentially enable single-base recognition in DNA strands only few nanometers long.
nucleic acids (NA) are among the most studied biomolecules nowadays due to their biological relevance, but also their nanodevice applications or computing. Control over nucleotide sequences as well as knowledge of their folding properties has enabled the rational design of highly elaborate two- and three-dimensional DNA structures, the so-called "DNA origami" programmed by Watson–Crick complementarity. These remarkable advances have been made possible by the accurate determination of nucleotide characteristics beforehand, using noninvasive single-molecule manipulation techniques, such as optical tweezers or magnetic tweezers. Beside these approaches, force spectroscopy based on atomic force microscopy (AFM) also allows direct measurements of mechanical, adhesion, and tribological properties, as well as visualizing self-assembly processes. So far, such force spectroscopic experiments on biomolecules have been conducted under ambient conditions in solutions, mostly up to few tens of picometer resolution. Mechanical properties are then dominated by thermal fluctuations and folding/unfolding of soft parts. Only few AFM studies on long polymers strongly bound at both ends reached the nm force level where thermal fluctuations are largely suppressed. To the best of our knowledge, no features attributable to sub-nanometer structural details have been observed in force versus extension curves recorded under ambient conditions.

Imaging of DNA has also been a long-term challenge. Although numerous groups successfully visualized DNA branches with impressive spatial resolutions in solution under ambient conditions, the highest accuracy has been reached using scanning tunneling microscopy (STM)/AFM imaging at cryogenic temperatures enabling reduced contamination. These experiments further required efficient deposition techniques to successfully transfer the macromolecules from solution onto a substrate while maintaining UVH cleanliness standards. However, the characterization of adsorbed biomolecules at the sub-nm level, specifically of DNA under such conditions, still remains rather unexplored. Notably, the recent advances in frequency modulation AFM under cryogenic conditions have pushed spatial resolution of adsorbed molecules to the single-bond level, while force spectroscopy enables complex manipulations of single molecules at surfaces.

Here, we demonstrate that dynamic AFM-based force spectroscopy in cryogenic conditions (5 K) is a promising method for characterizing the mechanics of single-strand DNA (ssDNA) 20-cytosine oligomers down to the sub-nm level. Similar to the advent of cryo-electron microscopy for structure characterization of biosystems, further investigations along this line could open avenues toward the integration of DNA into solid nanodevices through biomechanical studies at this level of precision.

**Results**

**Real-space imaging of spray-deposited ssDNA.** 20-Cytosine ssDNA oligomers were electrospray-deposited at room temperature on the precleaned Au(111) kept in ultrahigh vacuum (UHV). The surface was then annealed step by step up to a maximum temperature $T_{\text{max}}$ of 500 K. After each step, the resulting structures were subsequently imaged at 5 K using constant-current STM. As shown in Fig. 1a, the surface morphology evolves from large aggregates of several nanometers to 4-nm-long isolated oligomers. Clusters of various dimensions are observed by STM.Similar to previous results on ssDNA/Cu(111) deposited using a pulsed-injection technique, these structures are too large (Fig. 1b) compared to the expected size of a single dehydrated ssDNA 20-mer (Fig. 1c). Before annealing, we therefore suppose that a small amount of solvent molecules might also surround single oligomers.

To promote solvent desorption from the gold surface, we step-by-step annealed the surface that we later imaged by STM after each step (Fig. 1a). This results in a decreased apparent size of the “hydrated ssDNA clusters” ($T = 340$ K) as water molecules desorb from the surface. At $T = 440$ K, only “dehydrated ssDNA oligomers” are observed by STM with a length of about 4 nm (Fig. 1d). Their overall size also corresponds to the structure of a folded oligomer adsorbed on Au(111), as systematically predicted by our simulations performed under different adsorption conditions (see Supplementary Note 5) and superimposed top views in Fig. 1b, d, e. When $T_{\text{max}} \geq 500$ K, these dehydrated oligomers coalesce into several nanometer-long structures (Fig. 1e). Sub-nm contrast could be obtained along individual dehydrated oligomers and their assembly not only by STM, but also by using constant-height AFM with CO-terminated tips. In spite of the potential resolution below the molecular level, the determination of the overall ssDNA conformation is rather difficult.

**Molecular dynamics simulations of ssDNA adsorption.** Instead of simulating the complex electrospray-deposition processes which start and end up in charge-neutral species (see Supplementary Note 1 and Supplementary Fig. 1), in all our simulations, we considered a single ssDNA oligomer together with charge-compensating counterions. For a comparison with Fig. 1b, a 20-cytosine ssDNA oligomer generated from the canonical B-form was inserted into a water droplet, equilibrated, and then allowed to adsorb onto an unreconstructed Au(111) surface at room temperature (see Supplementary Note 3). We first used the ssDNA conformation fully embedded in water (Supplementary Note 4 and Supplementary Fig. 4) that we relaxed only considering the first three surrounding hydration layers, i.e., within ~1 nm around the molecule corresponding to 1332 water molecules (see Supplementary Fig. 5). Within the first 10 ns of the simulation, the droplet size decreases due to the surface tension causing a considerable folding of the ssDNA chain into a compact structure of only 3-nm diameter (see Supplementary Fig. 5). The hydration layer and the ssDNA folded structure then remain stable during the rest of the 100-ns-long simulation. Note that much less folding was observed if the chain was completely immersed in water, having a total length of 6.4 nm (see Supplementary Fig. 4).

The droplet so prepared was placed 2 nm above the surface and let free to adsorb (Fig. 2a). At the first stage of the adsorption, a meniscus is formed between the hydration layer and the gold surface, whereas, after the 100-ns-long simulation, the ssDNA absorbs folded directly on the gold surface, but is elsewhere surrounded by its hydration layer. Compared to the free droplet conformation, the ssDNA structure thus obtained is longer, which is in agreement with the STM images of hydrated oligomers on Au (111) (Fig. 1b). To test the effects of dehydration and thermal annealing on the final adsorption configuration, starting from the adsorbed hydrated ssDNA shown in Fig. 2a, we simulated (i) partial water evaporation by annealing at 450 K (stage 3a in Supplementary Fig. 6), (ii) the effect of removing all water molecules (stage 3b in Supplementary Fig. 7), and (iii) by simulating the adsorption of a single DNA strand at 400 K without any water molecules (stage 4 in Fig. 1c and Supplementary Fig. 7). All adsorption simulations, including stage 3 (hydrated ssDNA at 300 K) led to very similar folded adsorbed conformations ~4 nm in size, in good agreement with the prevalent experimentally observed structures after annealing at 440 K, as shown in Fig. 1.
The systematic nature of the findings can be traced back to the direct ssDNA/Au(111) contact already established at 300 K before dehydration. Concurrently with this direct contact, the interaction of cytosine bases with gold also induces a systematic flattening of the ssDNA structure. Indeed, most bases lie nearly parallel to the surface, similar to optimum adsorption structures for single-nucleotide bases on Au(111) computed in vacuum using a nonlocal van der Waals density functional38, and very recently by MD simulations in vacuum at 300 K39.

The effect of thermally assisted diffusion of ssDNA on Au(111) has been investigated with 500-ns-long MD simulations of two adsorbed ssDNA at temperatures of 400 and 500 K (see stage 5 in Supplementary Note 6). Diffusion and coalescence of the ssDNA oligomers is observed only at ~300 K annealing (Fig. 1e). Properly scaled top views of the representative ssDNA structure are superimposed on the STM image of hydrated ssDNA after spray-deposition at room temperature. The structural model of c is superimposed on the STM image. The systematic nature of the ssDNA conformation from constant-height AFM images with CO-terminated tips (Fig. 1e).

ssDNA cryo-force spectroscopy. To investigate the mechanical properties of ssDNA adsorbed on Au(111), we have attempted to lift single oligomers from the surface (Fig. 1c, d). We used the protocol introduced in refs. 33,40 to pull off single polyfluorene chains with the AFM tip. Experimentally, the tip was first gently indented into Au(111) to sharpen its apex, and then approached to one end of a selected oligomer until the tunneling current suddenly increased, thus indicating a jump to contact. A retraction curve was then recorded slightly beyond the position where the $I_t$ and $\Delta f$ dropped to their noise levels (see Supplementary Fig. 7). The yellow dots shown in the inset of Fig 3b was the point at which the retraction process was initiated at a constant speed of $v = 22 \text{ pm \ s}^{-1}$. The effective stiffness $k \approx k_0 \Delta f / f_0$ ($k_0$ being the deflection sensor stiffness and $f_0$ its resonance frequency) decreases progressively from 23 to about 5 N m$^{-1}$ as the tip–sample separation $Z$ increases. This variation is interrupted by narrow dips observed every 0.2–0.3 nm followed by an abrupt drop to zero when the tip is approximately at 1.4 nm far from the contact point, well below the length of one ssDNA oligomer in its adsorbed conformation (4 nm in Fig. 2). The premature detachment of the ssDNA from the tip (see Supplementary Notes 7 and 8) is confirmed by comparing STM images before and after the lifting experiment showing the entire oligomer still on the surface (inset Fig. 3b). Experimentally, only partial lifting of single ssDNA oligomers either self-assembled or individually adsorbed (see Supplementary Fig. 9) could be achieved. Thus, it appears that the folded ssDNA conformation and its strong adhesion on gold are the limiting factors of the experimental lifting. Indeed,
desorbing a single-folded oligomer with the AFM tip requires not only peeling off the structure from the gold surface, but also unfolding part of the backbone prior to detachment. Each peculiarity of the folding configuration can thus cause an abrupt increase of the required force to lift the molecule and the rupture of the tip–molecule bond.

Steered MD simulations\textsuperscript{41,42} have been run to shed light on the experimental results (see Methods and Supplementary Note 3). A representative theoretical retraction trace is shown in Fig. 3d. To do that, a virtual “tip atom” was connected to the P atom of the backbone between the first two nucleotides by a spring of stiffness $k_{\text{tip}}$ representing the tip–molecule bond and was pulled up at a constant speed of $0.1 \text{ ms}^{-1}$ at 5 K (Fig. 3a). From the recorded variations of the noise-averaged normal force $\langle F(Z) \rangle$ (Fig. 3c), the effective stiffness was extracted as $k = d\langle F(Z) \rangle/dZ$ (Fig. 3d), $Z$ being the distance between the tip atom and the pulled P atom at $t = 0$. In the simulations, the whole ssDNA oligomer detaches from the substrate when the tip has been retracted up to $Z_{\text{off}} = 11.8 \text{ nm}$, which is slightly less than its fully stretched length (see Supplementary Fig. 8). In spite of the discrepancy with the experimental value of $Z_{\text{off}}$, the measured maximum $k$ values exhibit a similar trend in the common Z-range (blue area in Fig. 3d). Not only the stiffness $k$ decreases from comparable initial values of $\sim 15–25 \text{ N m}^{-1}$, but pronounced dips (coinciding with abrupt force drops in the simulations) also appear at repeat distances of about $0.2–0.25 \text{ nm}$. Careful observation of the configurations adopted by the ssDNA atoms during simulated pulling reveals that the repeat distances reflect intermediate stages (peeling, lifting, and detachment) in the successive lifting of cytosine bases. These events are also accompanied by stick-slip-like sliding of the adjacent base over the Au(111) surface (0.28-nm lattice spacing) as well as irregular unfolding of the backbone. Details of such dynamics can be better visualized in Supplementary Movies 1 and 2. Note that the first step of the lifting process involves correlated base detachments and intricate unfolding, which require increased lifting forces. This increase might explain the premature detachment of ssDNA from the tip apex observed experimentally. Similar observations have been reported at room temperature in solution for grafted polymers\textsuperscript{42} and ssDNA adsorbed on carbon nanotubes\textsuperscript{47}.

It is also remarkable that the computed $k$ variations as a function of $Z$ are very different from those previously reported for polyfluorene chains from Au(111) lifted with the same method\textsuperscript{33}. There, the $k$ maxima ($\sim 0.4 \text{ N m}^{-1}$) were constant during retraction. The process ended at a distance corresponding to the number of monomers initially identified by STM on the surface. In that case (and also for graphene nanoribbons\textsuperscript{34}), the much stiffer repeat units weakly adhere to the substrate, allowing nearly frictionless sliding and a complete chain detachment. The oligomer backbone is more flexible and most bases are strongly bonded to the gold surface. As shown in our MD simulation (snapshot of Fig. 3e and Supplementary Movies 1 and 2), the cytosine bases that remain adsorbed do not slide during lifting\textsuperscript{33,34}. As a result, the lifted segment between the tip and the sample becomes essentially straight and inclined as the tip–sample separation increases. This sequential base detachment, similar to peeling off an adhesive tape, also reflects the strong adhesion of adsorbed cytosine bases. Interestingly, “infinite” as opposed to negligible friction was already detected for long ssDNA chains in solution at room temperature when adsorbed on gold\textsuperscript{13} and graphite\textsuperscript{15}, respectively. Easy sliding of short ssDNA oligomers on graphene under cryogenic conditions was recently predicted by steered MD simulations\textsuperscript{42}.

The gradual reduction of the $k$ maxima arises because the stiffness of the lifted segment decreases as it becomes longer. Focusing on the most pronounced $k$ maxima achieved on the longest nearly linear parts of $F(Z)$, we assume local mechanical equilibrium in the springs-in-series model applied earlier to polyfluorene chains and to unzipped dsDNA hairpins. Including the stiffnesses $k_{\text{tip}}$ and $k_{\text{pin}}$ of the segment ends anchored to the tip and to the adsorbed part of the oligomer (Fig. 3e and Supplementary Fig. 9), the envelope of $k$ maxima is expected to satisfy

\begin{equation}
    k = \left[ \frac{1}{k_{\text{ends}}} + \frac{n}{k_{\text{pin}}} \right]^{-1},
\end{equation}

with

\begin{equation}
    \frac{1}{k_{\text{ends}}} = \frac{1}{k_{\text{tip}}} + \frac{1}{k_{\text{pin}}}.
\end{equation}

$k_{\text{f}}$ being the stiffness per repeat distance $b$ in fully stretched ssDNA and $n = \text{int}[Z/(b \cos \theta)]$ is the number of nucleotides detached from the substrate in the range where the lifted segments are straight and
inclined by $\theta$ (almost 18° between $n = 8$ and 18 according to Fig. 3e and the corresponding Supplementary Movies 1 and 2). The resulting fit (red curve) is superimposed on the computed $dF/dZ$ trace in Fig. 3d obtained for $k_{\text{tip}} = 35$ N m$^{-1}$. The resulting parameters are $k_1 = 32.6 \pm 3.9$ and $k_{\text{pin}} = 18.3 \pm 3.3$ N m$^{-1}$. This confirms that ssDNA is strongly adsorbed and that fully stretched ssDNA is much more compliant than polyfluorene, presumably because the stretched ssDNA backbone stiffness is dominated by bond angle bending. As a consequence, $k_{\text{DNA}} = k_1/19 = 1.7$ N m$^{-1}$ would be the stiffness of the fully stretched 20-cytosine oligomer with one base at each end subject to an average tension of $\sim$2 nN. This load is one to two orders of magnitude larger than those applied at room temperature with single-molecule force spectroscopy on hundreds of nanometer-long ssDNA.

A drawback of the present system for experimental lifting is the complex folding of the ssDNA oligomers in comparison to polymeric systems in similar conditions. This limits the lifting heights to only a fraction of the extended length. Compared to $k_{\text{pin}} = 0.7$ N m$^{-1}$ obtained for polyfluorene chains, a complete detachment of ssDNA might be achieved by using end linkers allowing to reinforce the bond between the oligomer and the AFM tip in UHV. Although more difficult than solution chemistry, this task appears feasible in view of the successful detachment of PTDCA from Au(111) following contact to a carboxylic oxygen atom. In future experiments, we will focus on controlling the adsorption of the ssDNA, using, for example, appropriately functionalized tips and patterned surfaces. We believe that such strategy might enable the complete lifting of linearly adsorbed oligomers and permit a meaningful statistical analysis of their detachment. Another alternative is to perform measurements on less adhesive surfaces. Nevertheless, our results suggest that cryogenic force spectroscopy has the potential to study strongly adsorbed biomolecules or similar nano-sized synthetic systems with sub-nanometer resolution under tensile loads up to a few nanonewtons, i.e., 10–100 times higher than hitherto applied in most single-molecule force spectroscopy studies under ambient conditions.
conditions. Such studies might better characterize the diffusion properties or enable single-base distinction.

Methods

Sample preparation and electrospay deposition. An Au(111) single crystal purchased from Mateck GmbH was cleaned by several sputtering and annealing cycles in a ultrahigh vacuum (UHV). The single-strand 20-mer DNA molecules were purchased from Microsynth (Switzerland). The solution provided was subjected to high-performance liquid chromatography (HPLC), thus ensuring that only the organic molecules present were 20-cytosine ssDNA oligomers. Subsequently, dialysis was performed in the presence of an excess of NaCl which guaranteed that any other salts generated during synthesis are exchanged by NaCl. The delivered solution contained 14.7 nM of ssDNA and a NaCl concentration sufficient to provide more than twice the required amount of Na+ neutralizing counterions. This solution was further diluted to a concentration of around 1 nM in water and then sprayed in UHV (see Supplementary Note 1). Depending on the applied voltage, the deposition time varies from 1 to 30 min at constant solution flux controlled by a syringe pump with a speed of 2–10 × 10⁻⁶ l min⁻¹.

Further details on the electrospay-deposition apparatus and characterization can be found in refs. 43–45.

STM/AFM microscopy. The STM/AFM experiments were carried out at ~5 K with an Omicron GmbH low-temperature STM/AFM controlled by a Nanonis ICS electronics. We used an commercial tuning fork sensor, the qPlus configuration, e.g., one prong-fixed, the other with an etched tungsten tip exsposed at the end (superbunched frequency is 26 kHz, quality factor Q=10000–25000 in UHV, and nominal spring constant k=1800 N m⁻¹). These tips were sharpened by slight indentation into the gold surface; some were then terminated by a CO molecule at the apex packed up from the surface. All measurements were performed with 0.5 s residence time per sample bias with respect to the tip. The constant-height AFM images were acquired with CO-terminated tips using the noncontact mode by driving the free prong on resonance while maintaining a constant tip oscillation amplitude A=50 pm. For such small oscillation amplitude, the frequency shift Δf induced by a smoothly varying force acting on the tip is to a good approximation proportional to the gradient (effectiveness) of the conservative force along the oscillation direction (perpendicular to the sample surface). The signal-to-noise ratio is then nearly optimal.

Lifting experiments. Pulling experiments were performed under the same conditions with gold-decorated tips while simultaneously recording the tunneling current at a typical bias of 40 μV. The ssDNA oligomers were picked up by gently pressing the AFM tip to the molecule at one of its extremities. Attachment of the molecule to the apex is revealed by an abrupt jump in the force and current signals, as shown in Supplementary Fig. 7. Force spectroscopic measurements upon retraction were performed at a velocity of 22 pm s⁻¹. In contrast to such measurements on biomolecules in ambient conditions, the gradient of the force along the oscillation direction rather than the pulling force itself is thus measured here.

Atomic-level models and force fields. In our simulations, we considered one Au(111) slab composed of three atomic layers-thick slab, where the positions of the atoms in the lowest layer are fixed during the MD runs using a harmonic restraint of 5 kJ mol⁻¹. Furthermore, we considered surfaces of two different sizes, i.e., 16 × 16 nm² (Fig. 2 and Supplementary Fig. 5) and 18 × 22 nm² (see Supplementary Fig. 6). The dialysis and self-assembly of the ssDNA molecule was generated using the software NAB48, thus obtaining a double helix with the canonical B-form, as shown in Supplementary Fig. 2. Then, we removed the complementary sequence and used only one single-stranded 20-cytosine oligomer with the charged phosphate groups in the backbone of the ssDNA and 19 sodium counterions. The ssDNA atoms were described using both the parmbsc047 and the xOL1 refinements48 of the Cornell ff99 force field49. The choice of this force field is motivated by its accuracy to describe the mechanical properties of DNA50 as well as adsorption of biomolecules to surfaces51. The sodium counterions were described using the recently improved Lorentz-Berthelot mixing rules were used to determine the interaction parameters between different atoms. In all vacuum simulations, the volume of the system was kept fixed and the temperature was adjusted by means of a Langevin thermostat with a damping rate of 1 ps⁻¹ ensuring fast thermalization with a minimal effect on the fast slip dynamics. The SHAKE algorithm was used to constrain bonds containing hydrogen, thus allowing us to use an integration time step of 2 fs. Coordinates were saved every 1000 steps. In all our simulations, we observed that the final configuration was stable as it did not change during the last 40 ns of simulations (which was corroborated by the low, i.e., <0.2 nm, root-mean-square deviation). In the steered MD simulation results shown in Fig. 3 and Supplementary Fig. 10, the conservative force (FQ) was computed as kQ (z–Zd) and its thermal average f(x) was approximated by a running average over 100 ps, an interval adjusted to obtain a smooth dependence without distortions except close to force drops. The resulting dF/dz can be safely compared to the measured effective stiffness k between negative slips in the intervals where k is positive.

Simulation protocols. In total, we have performed eight different MD simulations shown in Fig. 3 and Supplementary Fig. 3, each labeled as a stage and described in Supplementary Note 3 and Supplementary Fig. 3. Here we briefly outline each stage. Stage 1: ssDNA oligomer fully embedded in water at 300 K. Stage 2: ssDNA inside a water droplet in vacuum at 300 K. Stage 3: Adsorption of a water droplet containing one ssDNA onto a Au(111) surface at 300 K. Stage 3a: Water evaporation at 450 K from the same adsorbed droplet. Stage 3b: Evolution of a fully dehydrated ssDNA adsorbed on Au(111) in vacuum at 400 K. Stage 4: Adsorption of a single DNA strand in vacuum onto Au(111) at 400 K. Stage 5: Diffusion and self-assembly of two ssDNA molecules adsorbed on Au(111) in vacuum at 400 and 500 K. Stage 6: Lifting a ssDNA molecule adsorbed on Au(111) in vacuum at 5 K. In all simulations, Na+ counterions per ssDNA molecule were included to ensure overall charge neutrality.

Data availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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