Communications

Force Spectroscopy of Hyaluronan by Atomic Force Microscopy: From Hydrogen-Bonded Networks toward Single-Chain Behavior

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The conformational behavior of hyaluronan (HA) polysaccharide chains in aqueous NaCl solution was characterized directly at the single-molecule level. This comunication reports on one of the first single-chain atomic force microscopy (AFM) experiments performed at variable temperatures, investigating the influence of the temperature on the stability of the HA single-chain conformation. Through AFM single-molecule force spectroscopy, the temperature destabilization of a local structure was proven. This structure involved a hydrogen-bonded network along the polymeric chain, with hydrogen bonds between the polar groups of HA and possibly water, and a change from a nonrandom coil to a random coil behavior was observed when increasing the temperature from 29 ± 1 to 46 ± 1 °C. As a result of the applied force, this superstructure was found to break progressively at room temperature. The use of a hydrogen-bonding breaker solvent demonstrated the hydrogen-bonded water-bridged nature of the network structure of HA single chains in aqueous NaCl solution.

Introduction

Numerous studies have been carried out to obtain an understanding of the effects of the molecular structure of proteins on their biological functions.[1] Yet, little is known of how the functions of polysaccharides might be affected by their chain conformation. In general, there is a growing need to elucidate the intimate relation between the chemical and the conformational structure of macromolecules and their biological activity. Hydrogen bonds play an important role in the local conformation of stereoregular polysaccharides as many of them adopt a helical conformation in the solid state, held together by the corresponding specific supramolecular forces. In aqueous solution, under specific thermodynamic conditions, they adopt an "ordered conformation", assumed to be helical in many cases, as revealed by circular dichroism, optical rotation, or NMR spectroscopy,[2] traditionally used for conformation analysis. For many of these polysaccharides, a helix−coil conformational transition is induced when increasing the temperature, as a result of the diminution of the attraction by hydrogen bonds when the temperature increases. Another option is to decrease the ionic concentration, giving rise to electrostatic repulsion between ionic groups of the chain.[3]

Hyaluronan (also called hyaluronic acid, HA) (Figure 1) is a glycosaminoglycan that is present in the extracellular matrix of most vertebrate connective tissues as well as in some bacterial capsules.[4] HA is a linear polysaccharide with repeating disaccharide units of 2-acetamido-2-deoxy-β-D-glucose and β-D-glucuronic acid linked (1,3) and (1,4), respectively. HA is more and more emerging as a key molecule in the regulation of many cellular and biological processes.[5] Nowadays, an increasing number of possibilities for its current applications in the biomedical field lie in the exploitation of its specific biological and bioactive properties. This is a result of HA directly communicating with proteins and cells present in tissues. However, most of its functions are based on its physical properties, such as hydration, viscosity, and space filling.[6]

It is well-known that the HA polysaccharide adopts a helical conformation in the solid state.[7] Meticulously performed IR spectroscopy studies on HA films in the dried and hydrated states have demonstrated the formation of a structure going from an intramolecular hydrogen-bonded organization (dried state) to a hydrogen-bonded intermolecular structure where "water wires" bridge the chains (hydrated state).[8] Figure 1 shows a representation of the intrachain hydrogen-bonded structure that exists in the dried state.

The first depiction of HA in aqueous solution was suggested after performing studies by classical methods of physical chemistry of polymer solutions. HA was here represented as a comparatively stiff random coil.[9] Later, certain indications, gathered by Scott,[10,11] suggested that the formerly proposed random coil HA (aqueous) had a secondary structure stabilized by extensive intraresidue hydrogen bonding. Moreover, the formation of a tertiary structure of aqueous HA was shortly

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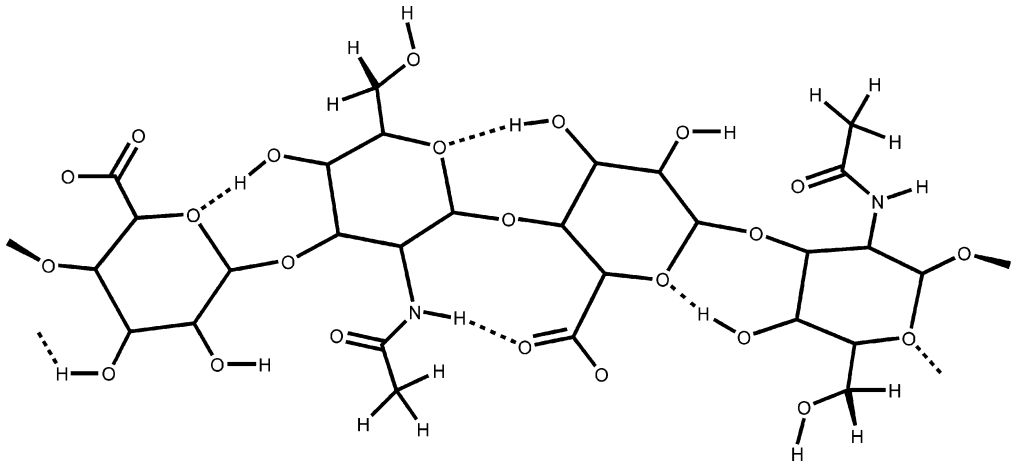

Figure 1. Local conformation of a fragment of hyaluronan and a representation of intrachain hydrogen bonds existing in the dried state.

thereafter discussed by Scott and Heatley on the basis of ^{13}C NMR data.[12]

Additionally, Haxaire et al.[13] reported on ^{1}H NMR and viscosity measurements of HA aqueous solutions in the presence of NaCl that revealed a semirigid structure for the HA chains related to the formation of a hydrogen-bonded network structure. Furthermore, a progressive decrease of stiffness when increasing the temperature beyond ca. 40 °C was detected and ascribed to a destabilization of the hydrogen-bonded network.

Nevertheless, conclusions concerning the structural conformation of individual aqueous HA chains have generally been drawn via extrapolation of ensemble-average results to infinite dilution, implicitly removing intermolecular contacts and specific interactions. Since the molecular structure of HA is at the basis of its properties, the purpose of the present study is to further characterize and comprehend the conformational behavior of HA polysaccharide chains in aqueous solution directly at the single-molecule level, thereby complementing the up-to-date knowledge on the HA molecular conformation. Moreover, the influence of the temperature on the stability of this conformation was also investigated, following the observations stated above for HA in aqueous solutions.[13] The present comunication reports on one of the first single-chain AFM experiments performed at variable temperatures, connecting temperature-responsive single-chain elastic behavior with bulk properties across a predicted phase transition.

Results and Discussion

The atomic force microscopy-based single-molecule force spectroscopy (AFM-SMFS) technique allows the characterization of elasticity and conformation of single polymer chains.[14] Among its many applications, certain are of particular interest, e.g., the study of interactions between complementary recognition units in guest−host complexes,[15] hydrogen-bond-driven recognition,[16] and bond stability in dimers and supramolecular polymers,[17] interactions between macromolecules and solvents, i.e., hydrogen-bonded structures in water[18−20] or other small molecules,[21,22] force-induced conformational transitions, such as the chair−boat transition of α-(1,4)- and α-(1,6)-linked polysaccharides[23] and characteristic rotations of the exocyclic groups in β-(1,6)-linked polysaccharides,[24] and the rupture of secondary structures of individual polysaccharide chains and multistrand complexes.[22,25] The AFM-SMFS technique has the advantage of combining the possibility of locating and probing single molecules under environmentally controlled conditions (e.g., solvent, temperature, ionic strength, or electrochemical potential). In the present work, AFM-SMFS was used under conditions of variable and controlled temperature and solvent.

For the nanomechanical AFM-SMFS characterization of single HA chains (physically adsorbed onto a glass substrate), an AFM tip (attached to the end of a cantilever) was used to pick up and stretch the single macromolecules. Upon separation of the tip and the substrate, the linking macromolecule was first uncoiled and stretched. Laser light was focused at the back of the cantilever, and the reflected light as well as the corresponding deflection of the cantilever were detected by a position-sensitive photodiode. The registered profile of the piezoelectric displacement−cantilever deflection was subsequently transformed into force−extension data corresponding to each single-molecule stretching experiment.

The elastic responses of the HA chains were studied in a 0.1 M NaCl solution at two temperatures, i.e., $T_1 = 29 \pm 1$ °C and $T_2 = 46 \pm 1$ °C. The temperatures were chosen below and above 40 °C due to the progressive destabilization of the hydrogen-bonded network observed for HA solutions when increasing the temperature beyond ca. 40 °C.[13] Figure 2 shows the force−extension traces of individual HA molecules measured at T_1 (Figure 2a) and T_2 (Figure 2c) as well as the corresponding superposition of the normalized force curves (Figures 2b and 2d). To perform the SMFS experiments, the polymer chains were, in this case, physically adsorbed onto the glass substrates. Consequently, the points at which the polymer chain was adsorbed to the AFM force probe tip and to the substrate when contact was established were randomly distributed along the chain. This caused the extension length of the molecule to vary for each pulling event (below a maximum of 2045 nm, estimated from the M_w), which was reflected in various effective contour lengths observed in each individual experiment.[26] Therefore, with the intention of comparing the resulting force−extension curves at each temperature, data were normalized by the extension corresponding to a certain, previously chosen, common force value (300 pN in this case), and subsequently, the normalized force−extension curves were plotted together as shown in Figures 2b and 2d for T_1 and T_2, respectively. The fact that the normalized curves superimpose well is an indication that single molecules were stretched, since the elastic properties scale linearly with the contour length.[27]

To evaluate the behavior of the single HA molecules at the two temperatures, the results were compared with predictions by models for single-chain elasticity of random coiled polymer chains, derived from statistical mechanics. The modified freely

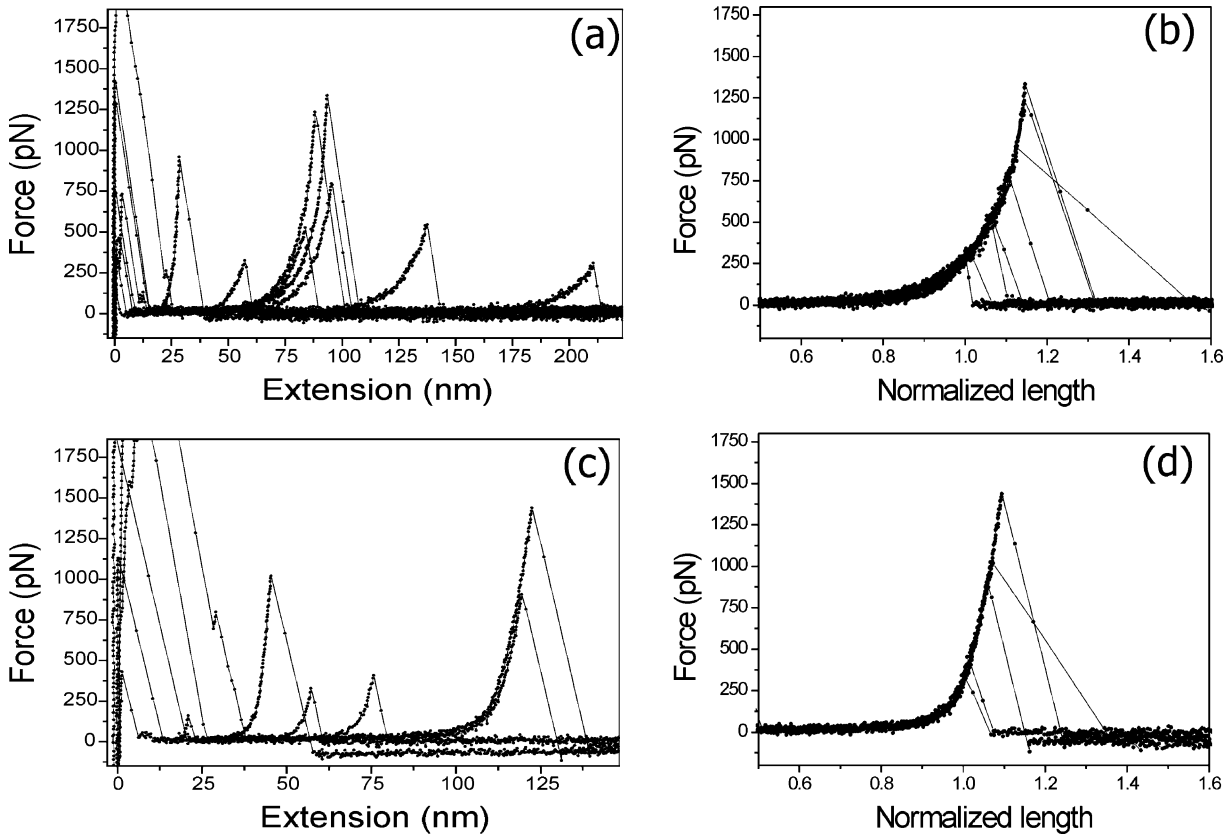

Figure 2. Force−extension traces of individual HA molecules measured at (a) 29 ± 1 °C (T_1) and (c) 46 ± 1 °C (T_2) in 0.1 M NaCl and the corresponding (b and d) superposition of the normalized force curves (normalized at 300 pN).

jointed chain model (m-FJC), which is an extension of the FJC model, was employed. This model describes the macromolecular extension as a function of force[28] considering the macromolecule as n identical elastic springs in a series, introducing the segment elasticity K_s, and hence taking into account the deformation of bonds and bond angles while the polymer is stretched to large extensions (high forces). The corresponding m-FJC expression is

$$z(F) = L_c\left[\coth\left(\frac{Fl_K}{k_BT}\right) - \frac{k_BT}{Fl_K}\right]\left(1 + \frac{F}{K_sl_K}\right) \qquad (1)$$

where z is the extension, F is the force, L_c is the contour length of the polymer,[26] l_K is the Kuhn segment length, k_B is the Boltzmann constant, and T is the temperature. As is depicted in Figure 3a, this model describes very well the elastic behavior of the HA polymer chains in aqueous NaCl solution for the high-temperature range T_2 (46 ± 1 °C). The fit with eq 1 was obtained using a nonlinear regression method based on the Levenburg−Marquardt algorithm.[29] Nevertheless, no theoretical model for random coiled polymer elasticity (neither the FJC nor the wormlike chain (WLC)[30]) could describe the force−extension results of the HA in aqueous NaCl solution close to room temperature, i.e., T_1 (29 ± 1 °C). This suggests that the behavior of the polysaccharide differs from an isolated, single-chain random coil at room temperature in aqueous NaCl solution. However, the m-FJC behavior was simulated at this temperature using the elasticity parameters obtained for the experiments at T_2 (l_k and K_s), with the corresponding contour length values (L_c)[26] for the results at T_1. When the simulation and experimental data (for T_1) were compared (Figure 3b), it was clearly demonstrated that, in the high force regime (after ca. 700 pN), the m-FJC model offered a very good description of the stretching behavior also at T_1. Moreover, when the force curves for T_1 and T_2 were normalized (at 900 pN) and compared, as can be seen in Figure 3c, they could undoubtedly be superimposed in the high force regime.

Accordingly, the conformation of HA at room temperature differs from an isolated, single macromolecule random coil. Instead, it adopts a superstructure in aqueous NaCl solution, i.e., a local structure involving a hydrogen-bonded network along the polymeric chain, with hydrogen bonds between the polar groups of HA and possibly water as well as water-mediated intramolecular bonds. This structure clearly becomes increasingly destabilized when the temperature is raised to T_2 (46 °C). Furthermore, the deviation in the middle force regime suggests that, at low temperature (T_1), the superstructure (tertiary structure) breaks progressively when increasing the force, leading to the same conformational structure that HA chains adopt at T_2, as is demonstrated by the superposition of the curves at high force values. These observations are comparable to a SMFS study of poly(ethylene glycol) (PEG) in various solvents carried out by Gaub's group.[19] As a result of the deformation of the superstructure within the polymer, i.e., a nonplanar superstructure stabilized by water bridges, marked deviations from the ideal behavior in the transition region from the entropic to enthalpic elasticity were found. A similar behavior has been reported for poly(*N*-vinyl-2-pyrrolidine) in water[20] and was also ascribed to the formation of hydrogen bonds between the solvent molecules and the polymer, thereby establishing a water bridge between the carbonyl groups of pyrrolidone rings in water. For poly(vinyl alcohol) (PVA) in water,[18] the deviation in the middle force regime of the force curves was attributed to the existence of a hydrogen-bonded superstructure (supramolecular assembly).

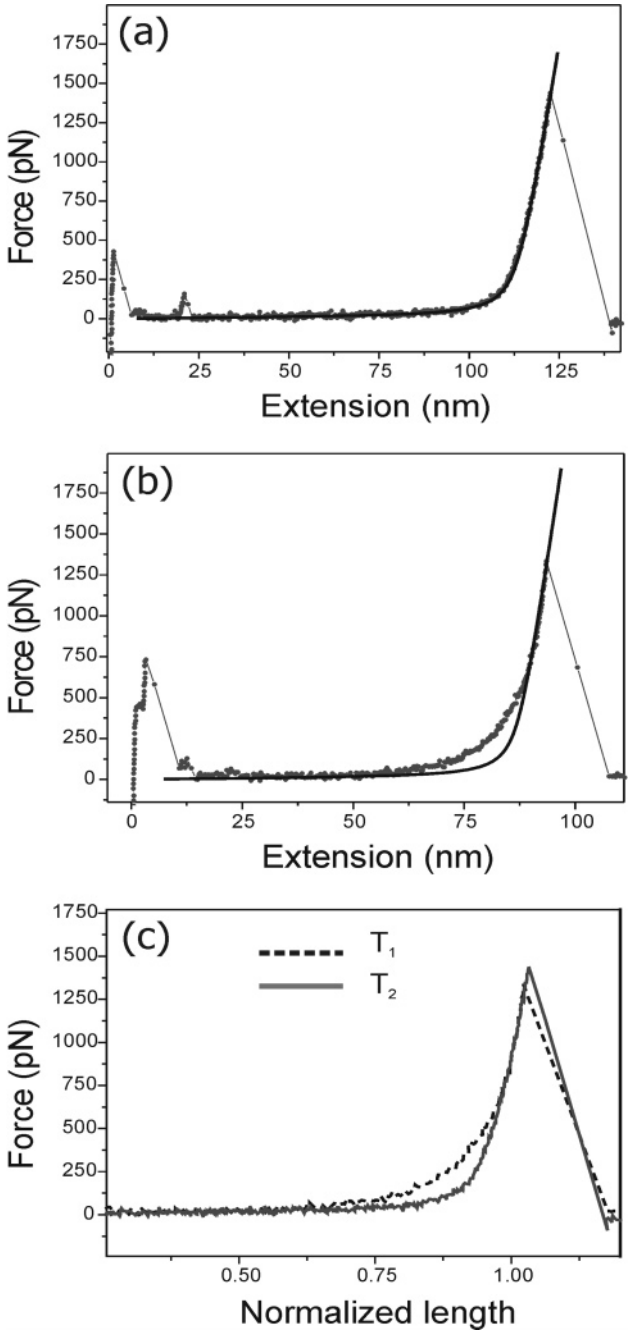

Figure 3. (a) Force−extension trace of an individual HA molecule measured at 46 ± 1 °C (T_2) in 0.1 M NaCl. The continuous dark line corresponds to the fitting with the m-FJC model (l_K = 0.53 nm, K_s = 30.85 nN/nm, L_c = 113.40 nm).[26] (b) Force−extension trace of an individual HA molecule measured at 29 ± 1 °C (T_1) in 0.1 M NaCl. The continuous dark line corresponds to the simulation with m-FJC model, using the segment parameters obtained in the fitting of the results at 46 ± 1 °C and the corresponding contour length (l_K = 0.53 nm, K_s = 30.85 nN/nm, L_c = 87.10 nm).[26] (c) Superposition of the normalized force curves of individual HA molecules measured at 29 ± 1 °C (T_1) (dotted line) and 46 ± 1 °C (T_2) (continuous line) in 0.1 M NaCl (normalized at 900 pN).

In an analogous way, Marszalek's group[31] used AFM-SMFS to study and determine an inter-residue hydrogen-bonded structure for amylose in a nonaqueous solution.

An investigation of the elastic response of the single HA molecules was also carried out at T_1 (29 ± 1 °C) in dimethylsulfoxide (DMSO), an effective breaker of hydrogen bonds. This study was aimed at proving that the existing superstructure in aqueous NaCl solution is directly related to the formation of a

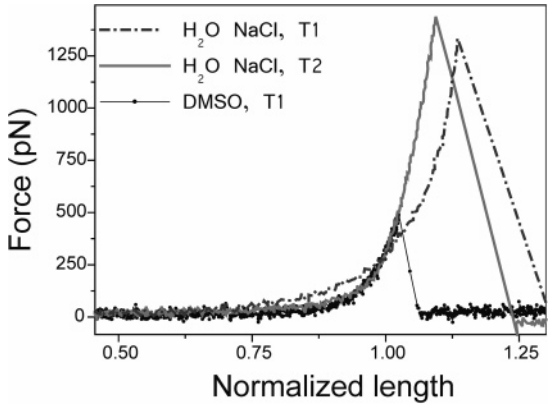

Figure 4. Superposition of normalized force curves of individual HA molecules measured in DMSO at 29 ± 1 °C (T_1) as well as in 0.1 M NaCl at 29 ± 1 °C (T_1) and 46 ± 1 °C (T_2) (normalized at 300 pN).

hydrogen-bonded network where intramolecuclar hydrogen bonds as well as intramolecular tertiary water-bridged bonds (defining a tertiary structure) play the main role, as suggested by the ^{1}H NMR and viscosity results for HA aqueous (NaCl) solution.[13] The force−extension traces of several individual HA molecules were recorded, and afterward the data were normalized by the extension corresponding to 300 pN (not shown). The normalized curves superimposed well, indicating that single molecules were stretched. In Figure 4, a representative normalized force curve obtained in DMSO is compared to the ones corresponding to T_1 and T_2 in aqueous NaCl solution. The behavior of the single HA chains in DMSO at T_1 is in excellent accordance with the behavior in NaCl aqueous solution at T_2 (46 ± 1 °C), when the tertiary structure was destabilized. In other words, the presence of DMSO suppressed the formation of water-bridged intramolecular tertiary hydrogen bonds and led to a structure identical to the one the macromolecule adopts in NaCl (aq) at T_2. These results are in agreement with the structure of HA described by Scott's group,[11] where intramolecular hydrogen bonds are present in the structure of HA in DMSO solution (in the same way as in Figure 1), and "water bridges" (that lead to tertiary structures) are generated when DMSO is partly replaced by water. In water, the hydrogen bond between the N−H and C═O groups of HA is modified because water molecules are inserted between the two groups. The same water-mediated, long-range, hydrogen-bonded structure starts to break when the temperature increases to over 40 °C, while the intrachain hydrogen bonds between adjacent groups remain.

Conclusions

AFM-SMFS with a temperature-controlled setup proved to be an invaluable tool for studying the temperature dependence of the conformation of single HA molecules in aqueous NaCl solution. The observation of a change from a nonrandom coil to a random coil behavior when increasing the temperature from 29 ± 1 to 46 ± 1 °C proved the temperature destabilization of a local superstructure. A progressive breaking of this superstructure was observed at room temperature, as a result of the applied force. The use of a hydrogen-bonding breaker solvent demonstrated that the superstructure of HA single chains in aqueous NaCl solution had a hydrogen-bonded, water-bridged nature, i.e., a hydrogen-bonded network along the polymeric chain, with hydrogen bonds between the polar groups of HA and possibly water.

Experimental Section

Bacterial hyaluronan was produced by Soliance (Pomacle, France) and carefully purified in the Na salt form as described elsewhere.[32] The weight-average molar mass (M_w) obtained by size exclusion chromatography (SEC) with on-line light scattering and differential refractometer detectors was found to be 820 000 g/mol with a polydispersity index (PDI) of ~1.3.

For the single-chain nanomechanical analysis, the hyaluronan molecules were physically adsorbed onto glass substrates by overnight incubation in a solution of ca. 0.5 mg/L in 0.1 M NaCl. Prior to this, the solution was filtered with a 0.2 μm pore size filter to avoid aggregates. After incubation, the samples were rinsed with 0.1 M NaCl solution and dried in a stream of N_2.

Single-molecule force spectroscopy experiments were performed with a Nanoscope IVa MultiMode AFM (Veeco/Digital Instruments (DI), Santa Barbara, CA) equipped with a DI fluid cell. For the experiments in DMSO, the solvent was exchanged in the fluid cell by injecting DMSO and displacing the NaCl aqueous solution. The samples were not dried between these steps. The temperature control was to within ±1 °C. A DI MultiMode heater/cooler (−35 to 100 °C) was used, with a scanner cooling system that cooled the piezo crystal inside the scanner from the heat generated (or transferred) by the heater/cooler. The temperature was measured in the solution, close to the tip. Commercially available V-shaped Si_3N_4 cantilevers (DI) were used, and each cantilever was calibrated according to the thermal oscillation technique.[33] A scan rate of 1 Hz was utilized (velocity, 720−800 nm/s).

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