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Energy landscape investigation by wavelet transform analysis of atomic force spectroscopy data in a biorecognition experiment

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Abstract Force fluctuations recorded in an atomic force spectroscopy experiment, during the approach of a tip functionalized with biotin towards a substrate charged with avidin, have been analyzed by a wavelet transform. The observation of strong transient changes only when a specific biorecognition process between the partners takes place suggests a drastic modulation of the force fluctuations when biomolecules recognize each other. Such an analysis allows to investigate the peculiar features of a biorecognition process. These results are discussed in connection with the possible role of energy minima explored by biomolecules during the biorecognition process.

Keywords Atomic force spectroscopy · Wavelet transform · Biotin–avidin · PACS

1 Introduction

Upon specific recognition events, biomolecules give rise to associations driving a large variety of cellular processes [1–3]. These highly controlled and hierarchical processes are regulated by the forces acting at the molecular scale, based on a combination of non-covalent interactions. Atomic force spectroscopy (AFS), which allows measuring the forces acting between individual biomolecules, is a rewarding technique helping to understand the molecular mechanisms of biorecognition [4, 5]. Generally, AFS experiments on a complex are carried out by atomic force microscope (AFM) equipment in which the tip, situated at the end of a cantilever, is functionalized with one biomolecule and approached to a substrate covered with the biological partner; the formation of a complex being eventually promoted [6]. When the tip is retracted from the substrate, the unbinding of a previously formed complex is induced. Upon selecting those force curves effectively related to specific biorecognition events by applying

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specific criteria [6], the corresponding unbinding force can be measured and analyzed in the framework of suitable theoretical models to extract information on the biorecognition process, such as the dissociation rate constant, the affinity, and the width of the energy barrier between the unbound and bound states [7–10]. Additionally, the theoretical approach based on Jarzinski inequality makes possible the evaluation of the free energy of the unbinding process from the determination of the work performed along many different non-equilibrium pathways [11, 12]. On the other hand, measurements carried out at different temperatures allow evaluating the roughness of the energy landscape along the pathway connecting the unbound and bound state [13, 14]. In this framework, the biorecognition can be viewed as a diffusion-like process in which the individual biomolecules thermally explore the energy hypersurface with a continuous trapping and escape from shallow minima before reaching the final binding state.

We recently analyzed the force fluctuations of a cantilever recorded in AFS experiments during the approach of a tip functionalized with a molecule to the substrate charged with the biological partner. Surprisingly, it was found that 1/f noise appears in the power spectrum of the force fluctuations only for a successful partner recruitment [15]. 1/f noise is a fingerprint of a complex temporal behavior, observed in many different processes from electronic devices, economic processes, membrane channels, and protein dynamics [16]. Generally, the presence of 1/f noise can be traced back to the exploration of different local minima of the energy hypersurface leading also to anomalous diffusive properties [17]. Such a finding has opened the opportunity to study the energy landscape regulating the biorecognition processes by following the force fluctuations in AFS biorecognition experiments. Accordingly, a wavelet transform analysis was applied to AFS data; such a method being based on the calculation of a Fourier transform on an interval moved along a time series [18]. Briefly, wavelet transform "analysis allows localizing events in the time domain from multiscale dynamical processes when standard signal analysis, such as the Fourier transform, fails [19]. Wavelet transform analysis has been applied to many different systems including biological systems (e.g., DNA sequences [20]). Recently, wavelet transform analysis was used to investigate single molecule tracking experiments, resulting in the establishment of the biological process [21, 22]. Here, the wavelet transform was applied to analyze data from AFS experiments on a biotin–avidin complex whose previous analysis showed the presence of 1/f noise in the power spectra of force fluctuations [15]. We remark that the biotin–avidin complex, which is characterized by one of the strongest non-covalent interactions in nature, deserves much interest in biochemical, biophysical, pharmaceutical, and biotechnological constructs [23]. Furthermore, it represents a sort of benchmark for investigating the mechanisms regulating the formation of biomolecular complexes.

The region at zero force of the approaching stage of each force curve was analyzed by wavelet transform. It was found that high values appear in the local wavelet power spectrum of the force fluctuations when a specific interaction between the biomolecules takes place. Such a finding indicates that the biorecognition induces a strong modulation of the force during the approach of the two biomolecules. Furthermore, a comparison between the wavelet maps of the curves corresponding to specific and non-specific biorecognition events localized where the biorecognition process takes place. These results confirm that a biorecognition process is characterized by a complex behavior involving the exploration of the local minima of the energy landscape. A suitable

analysis of AFS data recorded in a biorecognition experiment allows to appropriately investigate such a complexity.

2 Materials and methods

2.1 Sample preparation

Silicon nitride cantilevers (Veeco Instruments) and glass slides, cleaned in acetone, were UV irradiated for 30 min and then immersed in a solution of 2% 3-aminopropyl-triethoxysilane (APTES, Acros Organics) in chloroform, for 1 h at room temperature, then rinsed by chloroform and dried with nitrogen. The silanized tips and substrates were immersed in a solution of 1% glutaraldehyde in MilliQ water for 10 min at room temperature, rinsed with MilliQ water, and dried with nitrogen. The tip was incubated in a solution at 10 μM of biotin–bovin serum albumin (hereupon called biotin) while the substrate is incubated in a solution of neutravidin at 10 μM (called avidin) (see also ref. [15]).

2.2 Atomic force spectroscopy measurements

Force measurements were performed with a Nanoscope IIIa/Multimode Atomic Force Microscope (Veeco Instruments) in a buffer at pH 7.5 by using a liquid cell. The effective cantilever spring constants, k_{eff}, whose nominal value was k_{nom}=0.02 N/m, were determined by the thermal noise method [24] and were in the 0.017–0.045 N/m range. The force curves were acquired as a function of piezo-displacement by applying the following conditions: (i) an approaching and retraction speed, v, of about 50 nm/s; (ii) a relative trigger of 23–35 nm to limit at 0.7 nN the maximum contact force exerted by the tip on the protein monolayer; (iii) a ramp size of 150 nm; and (iv) an encounter time (interval between the approaching and retraction stages) of 100 ms. The spatial resolution, Δx, of the approaching stage was 1.46×10^{-2} nm corresponding to a temporal resolution Δt= 2.80×10^{-4} s, as derived from the relationship Δx=v Δt. A thousand force curves were recorded and analyzed.

2.3 Wavelet transform analysis

The wavelet transform is a mathematical tool suitable for analyzing time series that contain non-stationary power at many different frequencies. The wavelet transform calculates the local integral of time series over various scales with weighting defined by the wavelet basis function. Different scales generate a time series of wavelet transform coefficients corresponding to different time scales [18]. For a discrete time series, x_n (n=0, 1 … N–1) with a time spacing dt, the wavelet transform can be expressed by:

$$W_n(s) = \sum_{n=n'}^{N-1} x_{n'} \psi^* \left[\frac{(n'-n)}{s} \right] \quad (1)$$

where ψ* is the complex conjugate of the wavelet basis function, centered at the time corresponding to n′ and with a width s, which corresponds to the wavelet scale, s being along the time index n. The wavelet basis function must have zero mean and be localized in both

time and frequency space. Here, a Morlet function was used, consisting in a plane wave modulated by a Gaussian function:

$$\psi(\eta) = \pi^{1/4} e^{i\omega_o \eta} e^{-\eta^2/2} \quad (2)$$

where ω_o is the non-dimensional frequency (here fixed to 6) and η is a non-dimensional time parameter. In practice, the wavelet transform was carried out by the routines implemented in the website [25], as described in ref. [26]. The wavelet maps were plotted as a function of time and at different samplings (provided by the adimensional period parameter) of the power spectrum. The effective time, τ, corresponding to the period P can be obtained from $\tau = \Delta t * P$ where Δt is the time step $\Delta t = 2.80 \times 10^{-4}$ s of the recorded data. Although P parameters up to values corresponding to a half of the total analyzed time interval could be used, here P was fixed at 256 to obtain a good sampling of even the wavelet features extending for long times. Before applying the wavelet transform, each set of data was checked to assure a zero average. Data that do not fully satisfy this condition were adjusted through the addition or subtraction of a constant value.

3 Results and discussion

The AFS experiment on the biotin–avidin complex was carried out by vertically approaching and retracting the biotin–BSA functionalized tip towards the substrate charged with Avidin molecules. The force (proportional to the cantilever deflection) is recorded as a function of the piezo displacement (related to the tip–substrate distance); an example of force curves is shown in Fig. 1. During the approaching stage, the force fluctuates around a zero value up to the contact point after which the repulsive forces between the tip and the substrate yield an upward deflection of the cantilever (black line in Fig. 1). After reaching a fixed value of the force, F_c, the approach is stopped and the tip is retracted (red line in Fig. 1). In the retraction curve, the force initially follows the same trend as in the approach. Beyond the contact point, a strong deviation with a marked non-linear trend is observed; such a trend arising from a stretching of the

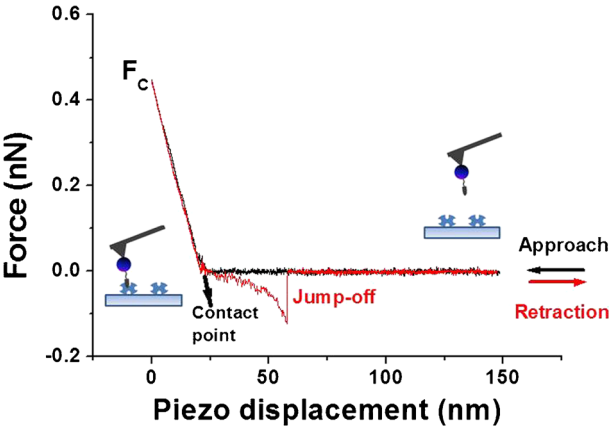

Fig. 1 Representative approach (*black*) and retraction (*red*) force-piezo displacement curves from an AFS experiment with the tip functionalized with biotin and the substrate with immobilized avidin molecules. From the jump-off the unbinding force of the complex can be estimated

molecules involved in the functionalization procedure of the tip and the substrate [5, 27]. Successively, the curve exhibits a rapid jump to the baseline, which marks the detaching of the tip from the substrate; from this jump, an estimation of the unbinding force of the complex can be obtained. An analysis of the unbinding forces in the framework of theoretical models allows determining the kinetic and energy parameters of the unbinding process [7, 8]. It should be remarked that force curves acquired in sequence may exhibit retraction trends markedly different from that shown in Fig. 1; e.g., a linear trend just after the contact point (non-specific adhesion) or no hysteresis (no interaction between the tip and the substrate).

Such a heterogeneity can be attributed to the variability emerging at the single molecule level (for a discussion of these aspects, see, for example, [5, 28]). On the other hand, not all the curves with the same features as shown in Fig. 1 are necessarily related to a specific biorecognition event. Accordingly, a preliminary scrutiny of all the curves is required to select those ones effectively related to the formation of a specific complex. With such an aim, a polymer linker is commonly used to bind one or both the molecules to the substrates; a fitting of a non-linear trend in the retraction curve by appropriate polymer elongation models being then performed. Only the curves showing the features of the linker (e.g., for PEG) are assumed to correspond to specific events [26]. An alternative approach to discriminate force curves related to specific events by analyzing the force fluctuations in the approaching stage has been recently proposed and validated [15, 29]. In particular, the power spectrum of the force fluctuations recorded in a region of the force curve before the contact point has been analyzed. The appearance of 1/f noise in this power spectrum has been related to the occurrence of a specific biorecognition event between the partners. Remarkably, such a method is promising as routine software, avoiding the use of specific linkers to bind biomolecules to substrates.

Starting from the underlying idea that force fluctuations, besides thermal noise, mechanical vibrations, drift effects, electronic noise etc., also include a contribution from the interaction between the tip and the substrate, the analysis of the force fluctuations were extended to extract more information. In particular, a wavelet transform analysis, which is an appropriate tool to investigate signals evolving in time, was carried out. Accordingly, the force curves were first analyzed in terms of 1/f noise, by following the procedure given in refs. [15, 29]. Briefly, the power spectrum calculated from the portion of the force curves, at zero deflection and extending for 10 nm before the contact point, was plotted as a function of frequency. Then, the low-frequency region (below 500 Hz) was fitted by a $1/f^{\alpha}$ expression, by evaluating the α exponent [15]. By following the criterion for which the value of the α exponent allows to attribute the force curves to specific (α =1) or non-specific (α=0) events, a preliminarily discrimination of the force curves was carried out. Successively, the same set of force curves was analyzed by calculating the wavelet transform. In particular, for each curve, a wavelet transform analysis was performed on the portion of the approaching curve starting when the tip and substrate are at the maximum piezo-displacement distance and ending when the tip and substrate become in contact (contact point); such a portion of the approaching stage being characterized by a zero cantilever deflection.

Representative sets of data from force curves corresponding to specific and non-specific biorecognition events are shown in Figs. 2 and 3, respectively. The top panels in both the figures show the force fluctuations plotted as a function of time from zero (corresponding to the maximum piezo-displacement distance of about 120 nm), up to about 2200 ms (corresponding to the contact point). In both cases, we note a similar trend with fluctuations covering a rather wide range of amplitudes and frequencies. The maps of the wavelet coefficients plotted as a function of time for progressively higher periods (from top to bottom) are shown in the middle

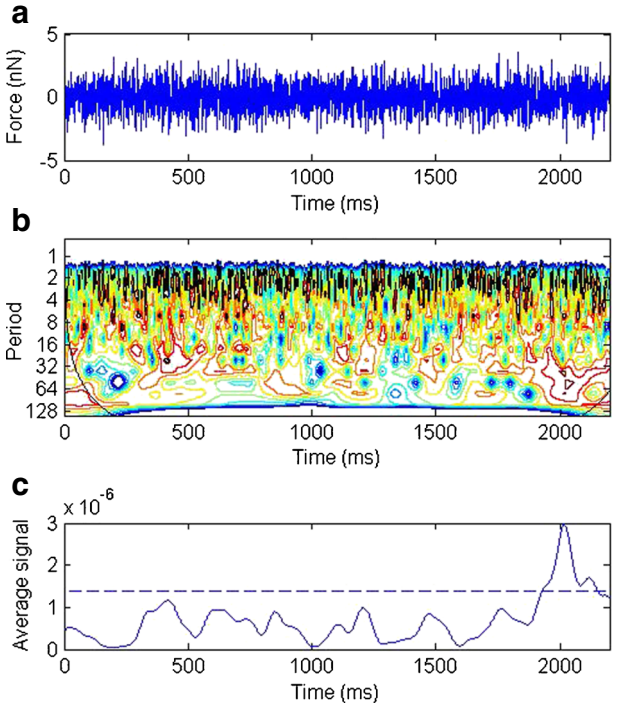

Fig. 2 **a** Force signal at zero deflection, plotted as a function of time, from the approaching stage of a force curve corresponding to a specific biorecognition event. The time step is $\Delta t=2.80 \times 10^{-4}$ s. **b** Map of the wavelet coefficients, plotted as a function of time, for progressively higher periods from top to bottom. The *colored contours* correspond to wavelet coefficients with the same value. The period is an adimensional parameter, corresponding to the number of time steps used for the sampling of the power spectrum calculation. **c** Wavelet coefficients, plotted as a function of time, averaged over the 32–64 periods. The *dashed line* marks the value corresponding to two standard deviations over the average

panels of Figs. 2 and 3. At short periods (high frequencies), common features are clearly evident in both the maps. In particular, at the highest frequencies (with periods lower than 8), rapid oscillations with a substantially homogeneous behavior in time are observed irrespectively of the fact that force curves correspond to specific or non-specific events. Such a behavior is indicative that changes in the fluctuations continuously occur in the analyzed temporal windows.

At lower frequencies (periods between 8–32), the presence of small regions, characterized by rather high values (blue and red contours), emerges in both the maps; these islands appear in the whole spectrum although they are non-homogeneously distributed in time. These features are related to the occurrence of transient changes in the force fluctuations, likely arising from mechanical, thermal or electronic contributions in the whole time interval.

For lower frequencies (periods between 32–64) larger regions appear in both the maps, however, with marked differences for force curves corresponding to specific or non-specific events. Indeed, these islands are distributed in the whole time window and with rather low values for the wavelet coefficients of the non-specific event curve (Fig. 3). At variance, islands characterized by rather high values of wavelet coefficients, appear in the last part of the map (i.e., at high times) for periods even at 128 for curves corresponding to specific biorecognition events (Fig. 2). Such a difference can be better visualized in the bottom panels showing the

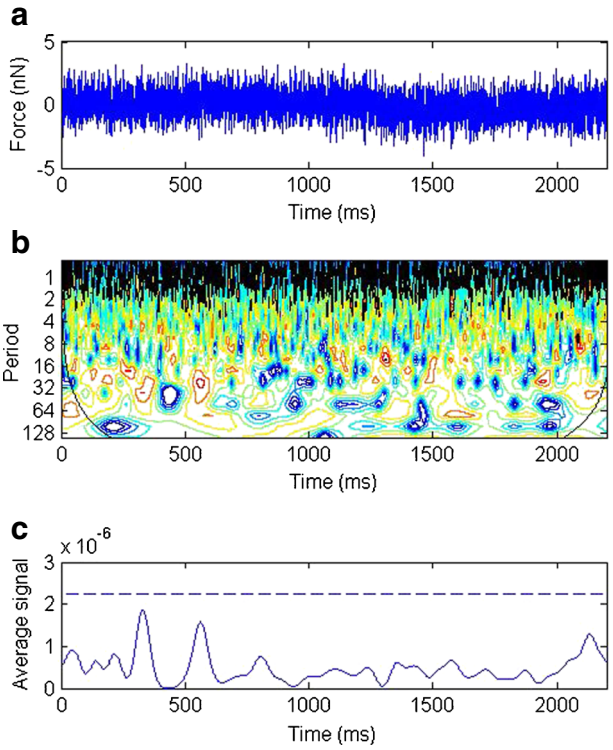

Fig. 3 **a** Force signal at zero deflection, plotted as a function of time, from the approaching stage of a force curve attributed to a non-specific biorecognition event. The time step is $\Delta t = 2.80 \times 10^{-4}$ s. **b** Wavelet map plotted as a function of time calculated for progressively higher periods from top to bottom. The *colored contours* correspond to wavelet coefficients with the same value. The period is an adimensional parameter, corresponding to the number of time steps used for the sampling of the power spectrum calculation. **c**Wavelet coefficients, plotted as a function of time, averaged over the 32–64 periods. The *dashed line* marks the value corresponding to two standard deviations over the average

average of the wavelet coefficients over periods in the 32–64 interval and plotted as a function of time. A strong peak over the threshold (dashed line) is clearly evident at times around 2050 ms (corresponding to a distance of 15–20 nm with respect to the initial point); all the other peaks being below the threshold in Fig. 2. Notably, the period values of 32–64 correspond to a frequency of about 50–100 Hz (see the Materials and methods section), which is close to the range (below 500 Hz) in which 1/f noise (i.e., $\alpha = 1$) was observed in the power spectrum [15].

By remarking that wavelet coefficients with large absolute values correspond to marked transient changes in the dynamics of the process, such a result indicates that a modulation of the tip–substrate interaction takes place in this region of the force curve. Although the open loop configuration of the AFM equipment does not allow to estimate the effective distance between the tip and the substrate, it can be reasonably hypothesized that the peak observed in the average of the wavelet coefficient, before the contact point, is located in the region where the two biomolecules start to interact. Finally, at the lowest frequencies, corresponding to the highest periods, (generally higher than 128), only partial islands are evident in the spectra; this being due to poor sampling that does not allow a complete description of the spectral features in the analyzed temporal window.

Features similar to those shown in Figs. 2 and 3 were observed in the wavelet maps of other force curves. In particular, the presence of large islands with diffusion coefficients with high values in the 32–64 period, or even longer times, is indeed shared by the maps from curves related to biorecognition events. To quantitatively investigate the interplay between the presence of the peak in the wavelet map and the occurrence of a biorecognition event, a correlation analysis was performed. In particular, for each force curve, together with the α exponent as previously described, a Boolean index, B, was derived; fixed to be one if the wavelet map exhibits a peak exceeding the threshold for periods of 32–64 and at times higher than 1500 ms, zero otherwise. The plot of the B index vs. the α exponent is shown in Fig. 4, for a sample of a hundred curves corresponding to non-specific events and a hundred curves related to specific events. The largest part of the curves corresponding to specific events (about 92%) is characterized by α exponents and B values around one (red circle). At the same time, about 90% of force curves corresponding to non-specific events are characterized by α exponents and B values around zero (blue circle). A few curves have instead high α values together with low B values or vice versa. These results clearly indicate a high correlation between the presence of 1/f noise and the presence of peaks in the wavelet maps confirming that the observation of peculiar features in the wavelet maps is related to the occurrence of specific biorecogniton events. In other words, two biomolecules undergoing a biorecognition process can give rise to a significant modulation of the force fluctuations whose effects are over imposed on those arising from other contributions, such as thermal, electronic, effects etc., which are instead common to all the curves. Accordingly, an analysis of the force fluctuations by the wavelet transform could make accessible some peculiar features commonly hidden in the whole signal. In this respect, some general considerations about the biorecognition process, seen as a diffusive process over the energy landscape, can be done. Briefly, the exploration of the energy landscape could result in a trapping of local minima, leaving then a fingerprint in the force fluctuations. Interestingly, AFS measurements at different temperatures allow estimating an energy barrier of 6–10 k_BT for the biotin–avidin complex [13, 14]. Additionally, the roughness of the energy landscape could yield a slowing of the diffusion of the biomolecule, resulting in a power law for the time distributions, as observed for the biotin–avidin complex in [15].

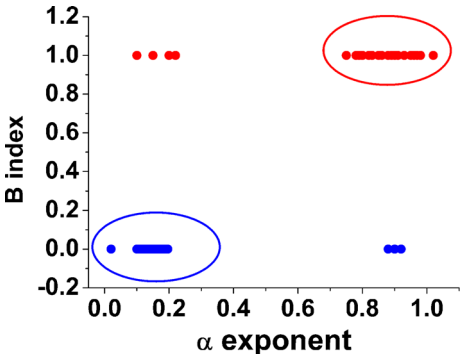

Fig. 4 Plot of the B index derived from the wavelet maps and the corresponding α exponent derived from the trend of the 1/f noise of the power spectrum (see text) for a hundred force curves corresponding to specific biorecognition events (*red points*) and for a hundred force curves corresponding to non-specific events (*blue points*). A *red circle* marks data with both the α and B index around one, while a *blue circle* marks data with both the α and B index around zero

The existence of local minima in the energy landscape is a widespread feature of biological systems, shared with other complex systems [30]. In particular, it has been experimentally demonstrated that different proteins in their functional state continuously explore local minima, called conformational substrates [31]. The involvement of these energy minima in biology has become progressively more important to understand some aspects of the behavior of biological systems. For example, the folding state of intrinsically disordered proteins can be related to the existence and to the exploration of different states that may correspond to different capabilities to bind a variety of targets [32]. Concerning the possible role of the exploration of the local energy minima in the regulation of the biorecognition process, it can be hypothesized that the different energy minima might be related to different binding states. Accordingly, their exploration could have a direct impact on the kinetic parameters regulating the association and dissociation rates. In other words, the exploration of the energy minima could allow a fine tuning of the biological response to a large variety of stimuli coming from the environment (such as solvent composition, crowding, etc.). On the other hand, the exploration of the local energy landscape has been found to be connected with force fluctuations, responsible for multiple successive bonds [33]. In this respect, knowledge of the details of the energy landscape and their influence on kinetics could help to understand how two biomolecules approach and recognize each other and how they regulate their reaction rates. These details may provide information useful to designing more effective drugs. Therefore, our approach, based on the wavelet transform analysis, represents a suitable tool for investigating the energy landscape of biomolecular complexes, with a large advantage over other AFS approaches since it does not require additional measurements or sample preparations.

4 Conclusions

Force data recorded in AFS biorecognition experiments generally encode a large amount of information whose decodification requires suitable analysis procedures.

Up to now, the majority of AFS studies have focused on the unbinding process, occurring after two biomolecules have been in contact and formed a complex.

Accordingly, the analysis of a complex formation could well complement the study of the unbinding process by obtaining new insights into the mechanisms regulating how two biomolecules recognize each other. Starting from the evidence that force fluctuations recorded during the approaching of a tip to a substrate, besides including electronic, acoustic, and mechanical noise, might also reflect the specificity of tip–substrate interactions, fluctuations were analyzed by a wavelet transform, which allows localizing and quantifying the changes in the behavior of a temporal series. Such an analysis showed that drastic transient changes appear in force fluctuations only when a specific biorecognition event takes place. This confirms that force fluctuations experience a strong modulation when two biomolecules undergo a specific interaction leading to a complex formation. These results indicate that the complexity regulating the biorecognition process can be addressed by AFS experiment data, opening new perspectives in the study of the mechanisms regulating the approaching of two biomolecules. Furthermore, they indicate that the wavelet transform is a powerful and appropriate tool for investigating AFS data with a high potential, which should be further explored in the future.

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