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Antigen–antibody biorecognition events as discriminated by noise analysis of force spectroscopy curves

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Abstract

Atomic force spectroscopy is able to extract kinetic and thermodynamic parameters of biomolecular complexes provided that the registered unbinding force curves could be reliably attributed to the rupture of the specific complex interactions. To this aim, a commonly used strategy is based on the analysis of the stretching features of polymeric linkers which are suitably introduced in the biomolecule-substrate immobilization procedure. Alternatively, we present a method to select force curves corresponding to specific biorecognition events, which relies on a careful analysis of the force fluctuations of the biomolecule-functionalized cantilever tip during its approach to the partner molecules immobilized on a substrate. In the low frequency region, a characteristic $1/f^{\alpha}$ noise with α equal to one (flickering noise) is found to replace white noise in the cantilever fluctuation power spectrum when, and only when, a specific biorecognition process between the partners occurs. The method, which has been validated on a well-characterized antigen–antibody complex, represents a fast, yet reliable alternative to the use of linkers which may involve additional surface chemistry and reproducibility concerns.

Keywords: atomic force spectroscopy, $1/f$ noise, biomolecular recognition

(Some figures may appear in colour only in the online journal)

Introduction

The formation of a complex between biomolecular partners is foregone by a searching process in the hypersurface energy landscape, during which encounter complexes may be temporarily formed before reaching a final binding state [1]; such a process being controlled by forces acting at the nanoscale [2]. The recently acquired capability to follow the interaction between single biomolecules has allowed details on the underlying molecular mechanisms to be gathered and even subtle phenomena usually hidden in ensemble measurements to be disclosed [2–7]. Among the nowadays vast repertoire of single molecule techniques, atomic force spectroscopy (AFS) has gained a prominent position since it can provide information on biomolecular complexes with picoNewton force sensitivity, without labeling, at physiological conditions and even at work; becoming also a remarkable tool for biosensing in biology and medicine [8–10].

AFS experiments are usually carried out by an atomic force microscope (AFM) in which the tip, situated at the end of a cantilever, is functionalized with a biomolecule, or even with a cell (with receptors on its surface), and approaching to a substrate covered with the biomolecular partner; the formation of a complex being eventually promoted through a biorecognition process. When the tip is retracted from the substrate, the unbinding of the complex is induced, as visualized in the force-piezo displacement curve by a jump-off to the baseline, and the corresponding unbinding force can be measured through the Hooke law [11]. The AFS force-displacement curves (which consist in an approaching and a retraction stage, figure 2) may encode a wealth of information on single biological complexes (interaction strength, dissociation rate, affinity, energy landscape features, etc).

However, to reliably extract this information, only the AFS curves unambiguously attributed to the unbinding of a specific complex, which are formed during the approaching stage, are to be analyzed in the framework of suitable theoretical models [12–17]. Indeed, the stochastic heterogeneity of the system may lead to nonspecific molecular interactions (due to contact forces, adhesions, multiple events, and so on) between the tip and the substrate. The resulting variability in the force curve shape makes then the attribution of the curves to real specific unbinding events somewhat ambiguous or substantially operator-biased [11, 18]. In an attempt to minimize such an inconvenience, a currently employed empirical strategy introduces in the biological partner immobilization scheme some flexible polymeric linkers, such as polyethyleneglycol (PEG) [19–21]. During the tip retraction and before the rupture of the specific complex, these linkers undergo a molecular stretching as evidenced by a peculiar nonlinear trend of the force-curve just before the jump-off to the baseline occurs. If this nonlinear trend well matches with that predicted by the elongation theoretical models for the used linker, then the occurrence of a specific complex during the partner approaching is assumed [21, 22]. Such a procedure requires, however, quite involved surface chemistry with some reproducibility issues, which might affect the interaction process (geometry and forces), and could hamper a comparison of the affinities of distinct biomolecules for the same receptor [18, 23]. Accordingly, the possibility to use an approach which does not necessarily require the use of a specific linker could be an advantage for some AFS experiments.

Here, we propose a fast, yet reliable method to select force curves based on the evidence that the low frequency power spectrum of the noise displayed by the AFM cantilever close to the contact point during the approaching stage, switches from white to flickering ($1/f$) noise when, and only when, a successful partner recruitment occurs [24].

Our approach, which relies on a careful, reproducible Fourier transform (FT) analysis of the cantilever fluctuations, has been applied to the well characterized complex formed by $\beta_2\mu$globulin and anti-$\beta_2\mu$globulin, involved in the human immune system [25]. The high correlation found between the appearance of $1/f$ noise in the approaching stage and the detection of the peculiar PEG stretching in the retraction stage, validates the reliability of the method. Additionally, we have verified that such a method provides reliable results even without linkers by supporting the capability to single out force curves related to biorecognition events by avoiding lengthy chemical procedures involving the use of molecular linkers and their stretching analysis.

Methods

Sample preparation

The glass slides were cleaned for 5 min in acetone, dried with nitrogen and then UV irradiated for 30 min They were then immersed in a solution of 2% (v/v) APTES (Acros Organics) in chloroform, incubated for 3 min at room temperature, rinsed in chloroform, and dried with nitrogen. The slides were subsequently incubated with a solution of 1% glutaraldehyde (Sigma- Aldrich) in Milli-Q water for 3 min at room temperature, rinsed with Milli-Q water and dried with nitrogen. Fifty μL of a 1 mg ml^{-1} solution of anti-$\beta_2\mu$globulin in 50 mM PBS pH 7.5 were poured onto this amine-reactive surface and incubated overnight at 4 °C. The slides were incubated with 1 M ethanolamine to cap non-reacted *N*-hydroxysuccinimide (NHS) groups for 30 min Finally, they were gently washed with PBS, and stored in buffer at 4 °C. A schematic representation of the slide functionalization is shown in figure 1(a).

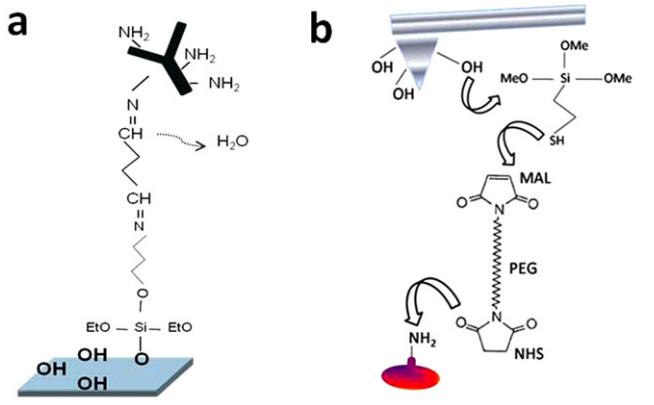

Figure 1. Surface chemical strategies for binding: (a) anti-$\beta_2\mu$globulin on a glass substrate; (b) $\beta_2\mu$globulin to the AFM silicon nitrite tip with the introduction of a 10 nm long PEG linker. The same procedure shown in (a) has also been used for binding $\beta_2\mu$globulin to the tip without the use of PEG.

Silicon nitride cantilevers (Veeco Instruments) were cleaned in acetone for 10 min, dried with a stream of nitrogen, and UV irradiated for 30 min to expose hydroxyl groups. Tips were then immersed in a solution of 2% (v/v) 3-aminopropyltriethoxysilane (APTES) in chloroform, incubated for 2 h at room temperature, rinsed in chloroform, and dried with nitrogen. Silanized tips were immersed in 1 mM *N*-hydroxysuccinimide-polyethylene glycolmaleimide (NHS-PEG-MAL, MW 1395 Da, 9.5 nm length; Thermo Scientific) and dissolved in dimethylsulfoxide (DMSO) for 3 h at room temperature. The NHS-ester group at one end of the PEG linker reacts with amino-silane molecule to form an amide bond: the maleimide group at the other end, reacts with the sulfhydryl group of cysteine residue linked to the NH2-terminus of proteins. The tips were washed in DMSO to remove the unbound linkers, rinsed with Milli-Q water, dried with nitrogen, and incubated with 50 μL of a 10 μM solution of β_2-μglobulin in 50 mM PBS pH 7.5 overnight at 4 °C. The tips were then incubated with 1 M ethanolamine to cap non-reacted NHS groups for 3 min Finally, they were gently rinsed and stored in buffer at 4 °C. A schematic representation of the tip functionalization is shown in figure 1(b). The functionalization of the AFM tip with β_2-μglobulin without the PEG linker was done by following the same procedure used for glass slides.

AFS measurements

Force measurements were performed with a Nanoscope IIIa/Multimode AFM (Veeco Instruments) in buffer by using a liquid cell. The effective cantilever spring constants, k_{eff}, whose nominal value was $k_{nom} = 0.02\ N\ m^{-1}$, were determined by the thermal noise method, and found in the 0.017–0.045 $N\ m^{-1}$ range. The force curves were acquired as a function of the piezo-displacement by applying the following conditions: (i) an approaching speed, v, of about 50 $nm\ s^{-1}$; (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 $\Delta t = 2.80 \times 10^{-4}$ s, as derived from the relationship $\Delta x = v\Delta t$, where v is the above mentioned approaching speed. The retraction stages of the force curves were acquired at the approximate speed values of: 50, 150, 400, 1000 and 3000 $nm\ s^{-1}$). Accordingly, the nominal loading rate, given by $R_{nom} = dF/dt = k_{nom}\ v$, resulted to be: 1, 3, 8, 20 and 60 $nN\ s^{-1}$. The corresponding effective loading rates, $R_{eff} = k_{syst}\ v$ were determined by taking into account that the spring constant of the entire system, k_{syst}, could be different from that of bare cantilever because of the presence of molecules attached to the AFM tip. k_{syst} values were calculated from the slope of the retraction stage immediately before the jump-off [26]. At each loading rate, a thousand force curves were recorded and analyzed.

Power spectrum analysis

The spectral content of the cantilever fluctuations detected by the position sensitive photodetector and arising from the fluctuating forces acting on the cantilever, was analyzed by determining the power spectrum for each force curve of the AFS experiment. The power spectrum of the fluctuating forces, $F(t)$, was derived by the FT of the correlation function, according to:

$$S(f) = \int_0^T \langle F(0)\, F(t) \rangle\, e^{2\pi i f t} \frac{1}{f} dt, \qquad (1)$$

where the brackets < > indicate the correlation function, f is the frequency, T the integration time interval and $F(t)$ is the force expressed as a function of time (indeed the force is registered as a function of the piezo-displacement z, which depends on time through the relationship $z = vt$, where v is the retraction, or pulling, speed). In practice, the power spectrum was calculated by the maximum entropy method through the expression [27, 28]:

$$S(f) = \left(\frac{1}{\sum_{k=-M/2}^{M/2} b_k e^{2\pi i f k}} \right)^2, \qquad (2)$$

where M is the number of poles, set to 256.

Noise analysis was carried out on piezo-displacement curve regions extending for about 10 nm (corresponding to a time interval of about 0.2 s) and located at a different part of the force curves. Accordingly, the resulting power spectra ranged from a minimum frequency of 10 Hz to a maximum of 3.5×10^3 Hz with steps of 14 Hz.

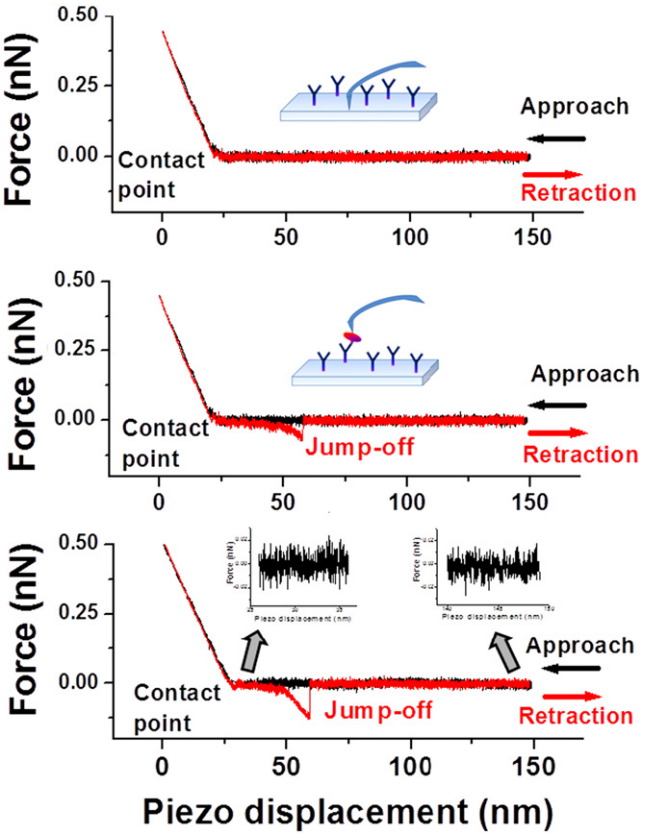

Figure 2. Representative approaching (black) and retraction (red) force-piezo displacement curves from AFS experiments carried out with a glass substrate covered with anti-$\beta_2\mu$globulin approaching: (a) bare tip; (b) tip functionalized with $\beta_2\mu$globulin without PEG; and (c) tip functionalized with $\beta_2\mu$globulin with PEG. Left inset: zoomed region (10 nm long) from the approaching stage just before the contact point. Right inset: zoomed region (10 nm long) at the beginning of the approaching stage.

Analysis of the nonlinear trend by an elongation polymer model

The nonlinear region of the retraction stage before the jump-off (figure 2) was analyzed in the framework of the polymer models in order to find out the elongation features of the PEG under stretching. In particular, we used the free joined chain model which quantitatively describes the behavior of a polymer under stretching, through the following expression [20, 29]:

$$\gamma(F) = L \coth\left[\frac{F\, l_p}{k_B T} - \frac{k_B T}{F\, l_p} \right], \qquad (3)$$

where γ is the molecular extension, F is the applied force, l_p is the persistence length (for PEG $l_p = 0.14$ nm) and L is the contour length of the polymer, i.e. the distance between ends of the linear polymer chain under the application of the force F. The experimental nonlinear trend of F as a function of the piezo-displacement, λ, was turned in terms of the molecular

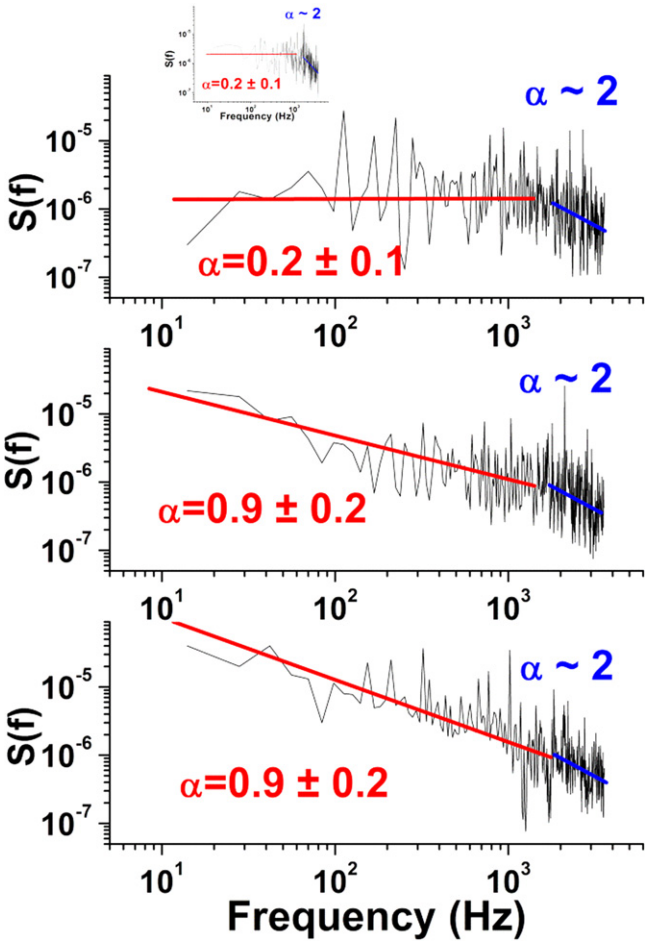

Figure 3. Power spectra derived from 10 nm long regions of the approaching stage located just before the contact point (left inset of figure 2(c)) from AFS experiments carried out with: (a) bare tip; (b) tip functionalized with $\beta_2\mu$globulin without PEG; and (c) tip functionalized with $\beta_2\mu$globulin with PEG. Inset: power spectrum derived from the 10 nm long region located at the beginning of the approaching stage (right inset of figure 2). Continuous lines indicate the best fit through the expression $S(f) \sim 1/f^{\alpha}$, below (red) and above (blue) the cutoff frequency of about 2 kHz; the reported α exponents having been obtained by averaging over a hundred curves.

extension as a function of F; with the molecular extension being derived from the relationship $\gamma = \lambda - \Delta z$, where $\Delta z = F/k$ is the deflection of the cantilever in correspondence to the λ value. Successively, it was fitted by equation (3) to extract the corresponding contour length of the PEG linker. For the 10 nm long PEG linker, used in this experiment, a contour length of (15 ± 5) nm is expected [30, 31]. Finally, a force curve was attributed to a specific biorecognition event if: (i) the experimental nonlinear region of the retraction stage was well-fitted by equation (3) and (ii) the contour length extracted from the fit was found in the (15 ± 5) nm interval.

Results and discussion

As already mentioned, an analysis in the framework of suitable theoretical models of the AFS force curves from a biomolecular complex, recorded at different loading rates in nonequilibrium conditions, allows one to extract the kinetic and thermodynamical parameters regulating the biomolecular interactions at equilibrium [8]. However, the force curves usually exhibit a large variability in their shapes, as shown and discussed for example in [11, 32]. While the approaching stage is practically the same in almost all the cases, the retraction one may display various trends, depending on the different interactions to which the tip and the substrate undergo during their approaching [33, 34]. Just to mention a few examples, force curves in which the retraction stage shows a linear descending trend immediately after the contact point reflect nonspecific interactions (usually attributed to adhesion), between the tip functionalized with a biomolecule and the substrate on which the partner is immobilized. Additionally, force curves in which the retraction stage coincides with the approaching one is indicative of no specific interactions. We have observed the latter trend in the force curves with bare tip (see the curves in figure 2(a)).

Furthermore, the presence of a nonlinear trend in the retraction stage beyond the contact point, indicates the occurrence of a molecular stretching before the detaching of the tip from the substrate, resulting in the jump-off of the cantilever to the baseline (see e.g. curves in figures 2(b) and (c)). Generally, curves exhibiting a nonlinear trend in the retraction stage are believed to be likely candidates to report a specific interaction between the biomolecular partners. In this case, the jump-off would correspond to the unbinding of the complex and it allows extraction of the unbinding force (i.e. the force required to separate the two partners upon the formation of the complex). Accordingly, a reliable scrutiny of the force curves unambiguously related to the unbinding of the specific complex, constitutes a crucial step in the AFS data analysis. With such an aim, the introduction of linker molecules with well-characterized stretching features (such as PEGs) to bind one or both of the biomolecules to the surfaces (tip or substrate) represents a commonly used strategy to single out force curves actually attributable to the unbinding of a specific complex [20, 22]. As already mentioned, such a procedure, although widely used and tested, requires quite involved immobilization protocols with some reproducibility concerns in comparative studies [23, 35].

Examples of AFS curves related to a tip functionalized with $\beta_2\mu$globulin without and with the PEG linker, are shown in figures 2(b) and (c), respectively. In both cases, the retraction curves show a nonlinear trend indicative of the stretching of the involved molecules (biomolecules, linkers etc). However, only when the PEG is involved in the unbinding process, the stretching is expected to exhibit well-defined features which can be put into evidence by a suitable polymer elongation analysis (see the methods section).

Here, we propose a new methodology to single out force curves corresponding to specific biorecognition events. Starting from the recent finding for which a peculiar $1/f$ noise emerges from the cantilever noise power spectrum only when a biorecognition process between the partners occurs [24], we have searched for a quantitative correlation between the

occurrence of this $1/f$ noise in the approaching stage and the expected linker stretching features in the retraction stage.

As shown in figure 2, fast fluctuations with an amplitude of about 10^{-2} nN are clearly evident at zero cantilever deflection; they becoming smaller beyond the contact point because of a lower cantilever sensitivity under higher forces. Generally, these fluctuations arise from thermal and electronic noise with a contribution from the tip-substrate interactions which become more significant as far as the tip and the substrate become closer to each other [24, 36].

On such a basis, we have analyzed the spectral content of 10 nm long regions located at different parts of the approaching stage for different setups. Figure 3 shows representative power spectra from the regions of the approaching stage just before the contact point where the two partners are coming into contact (left inset in figure 2(c)). In particular, figure 3(a) shows the power spectrum from the bare tip, while the inset is related to the region at the beginning of the approaching stage, where the partners are well apart (right inset in figure 2(c)). Figures 3(b) and (c) show the power spectra from a tip functionalized with β_2-μglobulin without and with the PEG linker, respectively; no significant differences in the power spectra of these two systems having been observed.

All the spectra exhibit two distinct linear regimes in the analyzed frequency range. Above a cutoff frequency at about 2 kHz, a linear trend with a slope close to 2 ($S(f) \sim 1/f^{\alpha}$, with α close to 2, see fitting blue lines) is observed in all the spectra. Generally, a $1/f^2$ noise (red noise), reflects a constant correlation function and can be put into relation to cantilever oscillations as described from the Langevin equation taking into account the electronic and thermal noise [36, 37]. The cutoff frequency approximately corresponds to the characteristic frequency of the cantilever, which has been estimated by the dynamic method to be about 2.0–2.5 kHz [24].

At lower frequencies, the power spectrum displays a plateau (i.e. $S(f) \sim 1/f^{\alpha}$, with α around zero) for the bare tip (see fitting red line in figure 3(a)); a similar trend having been observed for the region located at the beginning of the approaching stage (see the inset in figure 3(a)). Such a behavior corresponds to white noise which arises from a δ-correlation function. At variance, the power spectra for most of the force curves characterized by a nonlinear trend in the retraction stage exhibit a linear trend with a slope close to one ($S(f) \sim 1/f^{\alpha}$ with α close to one) (see fitting red lines in figures 3(b) and (c)). A $1/f$ regime, usually called flickering (or pink) noise, derives from a slowly decreasing correlation function, and is a fingerprint of a complex temporal behavior, which occurs in systems characterized by multiple time scale processes [38].

We were then prompted to use this α value to single out force curves corresponding to a specific biorecognition event between the partners. To support the validity of such a choice, we have therefore analyzed the correlation between the $1/f$ noise fingerprint in the power spectrum from the approaching stage, and the PEG elongation features as emerging from a fitting of the nonlinear trend in the retraction stage (carried out as described in the methods).

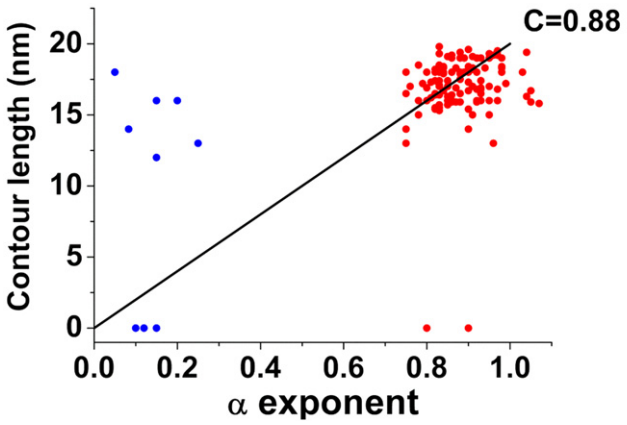

Figure 4. Correlation plot between the α exponent and the contour length of the PEG under stretching (see the text). Red points indicate α values between 0.9 and 1.1 (flickering noise) while the blue points indicate α values between 0.0–0.3 (white noise). The straight line shows a correlation parameter, C, equal to one; the value of the calculated correlation parameter being also reported.

Figure 4 shows the contour length, L, of the PEG plotted as a function of the α exponent, for a collection of N force curves recorded at a loading rate of 8 nN s^{-1}. We found that the α values close to one cluster in correspondence of PEG contour length values in the 15–20 nm range. Additionally, zero L values (i.e. force curves showing no peculiar PEG stretching features) are observed in correspondence of α values lower than 0.2.

We have then evaluated the correlation parameter C between α and L by the expression: $C = \frac{1}{N}\sum_{N}^{i=1} l_i a_i$, where a_i has been set to 1 if α falls in the 0.8–1.1 interval and to zero otherwise; while l_i has been set to 1 when the PEG stretching criteria are satisfied and to zero otherwise. We found $C = 0.88$ for the curves at the loading rate of 8 n N s^{-1} and values higher than 0.85 at the other loading rates; a straight line corresponding to a correlation parameter equal to one having been also plotted in figure 4. These results indicate that the occurrence of $1/f^{\alpha}$ noise with an α exponent close to one in the power spectrum from the approaching stage, is highly correlated with the expected stretching features of the used PEG upon pulling. Therefore, $1/f$ noise analysis constitutes a reliable and fast method to single out force curves corresponding to specific biorecogniton events.

We remark that a $1/f^{\alpha}$ trend with an α exponent close to 1 has been observed in many different processes occurring in electronic devices, membrane channel conduction, protein dynamics, economic processes, and so on [38–40]. Interestingly, a $1/f$ noise has been recently observed in a field-effect transistor for ultra-sensitive detection of a biomolecular recognition [41]. Moreover, an analysis of thermal fluctuations in biorecognition experiments, carried out by a biomembrane force probe, has allowed some authors to extract direct information on the dissociation events of biomolecular complexes [42].

In protein systems, $1/f$ noise was traced back to trapping/escaping within local minima in the energy landscape generated by slightly different conformations, which are involved

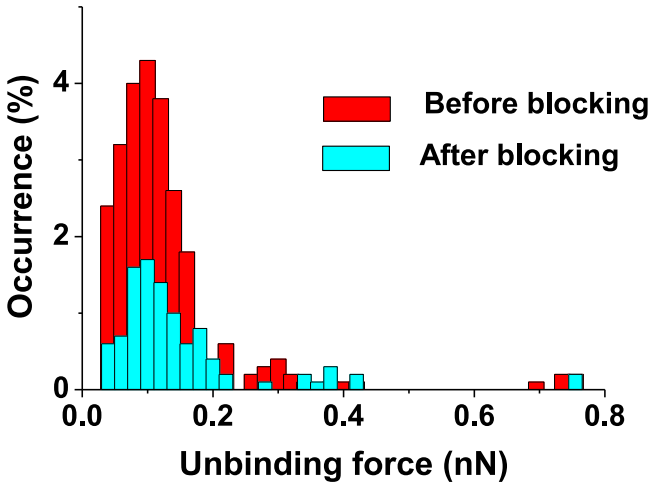

Figure 5. Histograms of the unbinding forces for the $\beta_2\mu$globulin/anti-$\beta_2\mu$globulin complex at the nominal loading rate of 8 nN s^{-1} for the AFS experiments involving a 10 nm long PEG linker, before (red columns) and after blocking (cyan columns), in which the substrate functionalized with $\beta_2\mu$globulin has been incubated with free anti-$\beta_2\mu$globulin. For each experiment, the data have been extracted from a collection of a thousand of the force curves.

in the regulation of biological processes, such as catalysis, exchange of ligands, folding and even high sensitivity bior-ecognition [24, 43–46]. Starting from the hypothesis that proteins explore local minima in the rough energy landscape generated by slightly different conformations [47, 48], we can assume that the biomolecular partners undergo a continuous trapping and escape from these shallow minima, before reaching their final binding state. Such a diffusive exploration may result in characteristic fluctuations of the interaction forces between the tip and the substrate which can then modulate the cantilever noise spectral content.

The AFS curves have been then analyzed within the theoretical context of the Bell–Evans model to extract kinetic and energy landscape parameters at equilibrium [12, 13]. In this framework, the most probable unbinding force, F^*, is expected to linearly increase with the natural logarithm of the loading rate, R, as follows [13]:

$$F^* = \frac{k_B T}{x_\beta} \ln\left(\frac{R x_\beta}{k_{off} k_B T}\right), \quad (4)$$

where k_{off} is the equilibrium dissociation rate constant, x_β is the width of the energy barrier along the direction of the applied force, k_B is Boltzmann's constant and T the absolute temperature; k_{off} and x_β can be then extracted from the F^* versus R by a fit with equation (4).

The most probable unbinding force, at a given R, has been evaluated from the maximum of the corresponding histogram of the unbinding forces which are, in turn, determined from the jump-off in the retraction stage (see figure 2). As an example, figure 5 shows a histogram of the unbinding forces from the AFS curves recorded at a loading rate of 8 nN s^{-1}.

We note a single mode distribution, slightly skewed toward higher force values similarly to those observed for other biomolecular complexes [8]. In the same figure, the unbinding forces recorded after incubation with free anti-$\beta_2\mu$globulin (30 mM), of the substrate functionalized with $\beta_2\mu$globulin (called blocking control experiment) have been also shown (see cyan columns). The marked reduction (61%) of the unbinding frequency (defined as the ratio between the number of the specific unbinding events and the total number of collected force curves) and the similarity in the histogram shape, witness the specificity of the interaction between $\beta_2\mu$globulin and anti-$\beta_2\mu$globulin (see e.g. [49]).

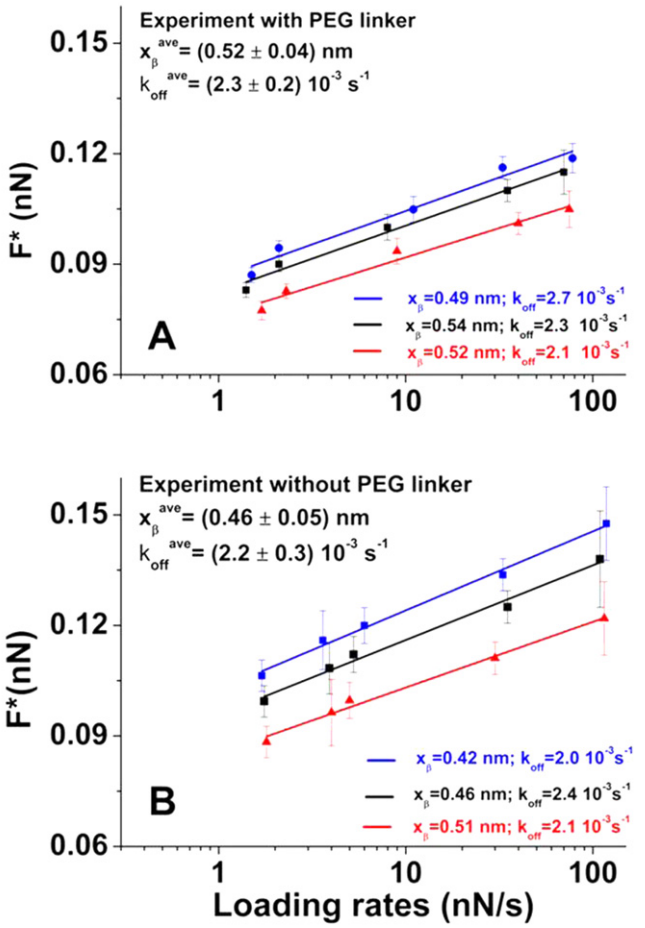

Figure 6. Plot of the most probable unbinding force, F^*, as a function of the logarithm of the loading rate for the three independent AFS experiments: (a) with PEG linker and (b) without PEG linker. The continuous lines are the fit by the Bell–Evans model (equation (4)); the corresponding k_{off} and x_β values extracted from the fit being reported. For each system, the average value of k_{off} and x_β are also reported on the top of the figures.

Figure 6(a) shows the most probable unbinding force, F^*, plotted versus the logarithm of the effective loading rate for three AFS experiments carried out using a PEG linker. In all the cases, we note a linear trend in agreement with the Bell–Evans model for a single energy barrier [13]. The values of k_{off} and x_β, as extracted from a fit by equation (4), show a variability which is quite commonly observed in AFS experiments on biomolecular complexes (figure 6(a)) [8, 11]. The average and the corresponding standard deviation of k_{off} and x_β over the three experiments (figure 6(a)) are in the range

usually encountered for specific biomolecular complexes with a rather high stability [8].

Remarkably, the averaged k_{off} value is in good agreement with that obtained by surface plasmon resonance measurements ($k_{off} = (2.0 \pm 0.2) \times 10^{-3}\ s^{-1}$)) from a setup in which $\beta_2\mu$globulin has been flowed over a functionalized gold substrate covered with anti-$\beta_2\mu$globulin [50].

Although the Bell–Evans model is widely used to analyze the AFS data, other models have been recently developed to take into consideration aspects neglected by the Bell–Evans model [51–53]. In particular, the model of Friddle *et al* [53] takes into account the possibility of rebinding during the barrier crossing. To evaluate if and how rebinding could influence the kinetic properties of the complex, we have analyzed the unbinding forces versus the logarithm of the effective loading rate for the AFS experiments carried out with the PEG linker in the framework of this model. We found a linear trend for the fit according to the experimental data. The extracted $k_{off} = (2.5 \pm 0.2) \times 10^{-3}\ s^{-1}$) and $x_\beta = (0.53 \pm 0.04)$ nm) values are in very good agreement with those found by the Bell–Evans model. Therefore, at our loading rate values, the eventual presence of rebinding does not affect the kinetic properties of our system.

Figure 6(b) shows the most probable unbinding force, F^*, plotted versus the logarithm of the effective loading rate for three AFS experiments carried out without using the PEG linker to bind $\beta_2\mu$globulin to the tip. In this case, the selection of the unbinding force curves to be attributed to a specific unbinding event have been done by searching for the 1/*f* noise fingerprint in their power spectrum. For all three sets of data, we found again a linear trend. The k_{off} and x_β values, extracted from a fit by the Bell–Evans model, exhibit some variability, similarly to what was observed in the presence of the PEG linker. However, the averaged k_{off} and x_β values are in good agreement with those obtained for AFS experiments carried out with the PEG linker. Such an agreement, besides confirming the reliability of the 1/*f* noise method to select force curves from an AFS experiment, highlights the possibility of carrying out reliable AFS experiments without the use of additional linkers.

Conclusions

AFS constitutes an extremely powerful technique to investigate biomolecular complexes at single molecule level, allowing informative interaction properties to be extracted. However, the effective applicability of AFS could be somewhat hampered by the necessity of a reliable selection of force curves corresponding to biorecognition events. A widely used strategy to address such a requirement consists in the use of suitable polymer linkers with well-defined stretching features under pulling. However, this approach is not exempt from some drawbacks because of the quite involved surface chemistry and of reproducibility issues in comparative studies. We have demonstrated here that a reliable and fast selection of force curves, corresponding to specific biorecognition events, can be performed by searching for the characteristics 1/*f* noise in the power spectrum from the force fluctuations during the approaching stage. The high correlation between the occurrence of such a 1/*f* noise fingerprint and the peculiar stretching features of the PEG linker, provides a validation of the method for a specific complex. The capability of the proposed 1/*f* noise analysis to reliably select forces in AFS experiments without using specific linkers, has also been verified in complex partner immobilization without the use of polymeric linkers. Collectively, these results indicate that 1/*f* noise detection represents a powerful approach which may extend the potentialities of the AFS technique to study biological systems. If implemented in commercial AFMs as a routine software facility, it would lead to an increased efficiency, and to a limitation of the operator bias, providing then a statistically robust, yet reliable ground for more routine applications in biological and clinical assays.

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References

[1] Nooren I M A and Thornton J M 2003 Structural characterization and functional significance of transient protein–protein interactions *J. Mol. Biol.* **325** 991–1018
[2] Robert P, Benoliel A M, Pierres A and Bongrand P 2007 What is the biological relevance of the specific bond properties revealed by single molecule studies? *J. Mol. Recognit.* **20** 432–47
[3] Neuman K C and Nagy A 2008 Single-molecule force spectroscopy optical tweezers, magnetic tweezers and atomic force microscopy *Nat. Methods* **5** 491–505
[4] Junker J P, Ziegler P and Rief M 2009 Ligand-dependent equilibrium fluctuations of single calmodulin molecules *Science* **323** 633–7
[5] Thomas W 2008 Catch bonds in adhesion *Annu. Rev. Biomed. Eng.* **10** 39–57
[6] Barkai E, Jung Y J and Silbey R 2004 Theory of single molecule Spectroscopy *Annu. Rev. Phys. Chem.* **55** 457–507
[7] Rico F and Moy V T 2007 Energy landscape roughness of the streptavidin-biotin interaction *J. Mol. Recognit.* **95** 495–505
[8] Bizzarri A R and Cannistraro S 2012 *Dynamic Force Spectroscopy and Biomolecular Recognition* (Boca Raton, FL: CRC Press)
[9] Janshoff A, Neitzert M, Oberdorfer Y and Fuchs H 2000 Force spectroscopy of molecular systems single molecule spectroscopy of polymers and biomolecules *Angew. Chem. Int. Ed.* **39** 3212–37
[10] Dufrêne Y F, Martínez-Martín D, Medalsy I, Alsteens D and Müller D J 2013 Multiparametric imaging of biological systems by force-distance curve–based AFM *Nat. Methods* **10** 847–54
[11] Bizzarri A R and Cannistraro S 2010 The application of atomic force spectroscopy to the study of biological complexes undergoing a biorecognition process *Chem. Soc. Rev.* **39** 734–49
[12] Bell G I 1978 Models for the specific adhesion of cells to cells *Science* **200** 618–27

[13] Evans E 2001 Probing the relation between force-lifetime and chemistry in single molecular bonds *Annu. Rev. Biophys. Biomol. Struct.* **30** 105–28
[14] Hummer G and Szabo A 2003 Kinetics from nonequilibrium single molecule pulling experiments *Biophys. J.* **85** 5–15
[15] Dudko O K, Filippov A E, Klafter J and Urbakh M 2003 Beyond the conventional description of dynamic force spectroscopy of adhesion bonds *Proc. Natl. Acad. Sci. USA* **100** 11378–81
[16] Friddle R W 2008 Unified model of dynamic forced barrier crossing in single molecules *Phys. Rev. Lett.* **100** 138302–5
[17] Jarzynski C 1997 Nonequilibrium equality for free energy differences *Phys. Rev. Lett.* **78** 2690–3
[18] Pincet F and Husson J 2005 The solution to the streptavidin-biotin paradox the influence of history on the strength of single molecular bonds *Biophys. J.* **89** 4374–81
[19] Baumgartner W, Hinterdorfer P, Ness W, Raab A, Vestweber D, Schindler H and Drenckhahn D 2000 Cadherin interaction probed by atomic force microscopy *Proc. Natl. Acad. Sci. USA* **97** 4005–10
[20] Oesterhelt F, Rief M and Gaub H E 1999 Single molecule force spectroscopy by AFM indicates helical structure of poly (ethylene-glycol) in water *New J. Phys.* **1** 6
[21] Sulchek T A, Friddle R W, Langry K, Lau E Y, Albrecht H, Ratto T V, DeNardo S J, Colvin M E and Noy A 2003 Dynamic force spectroscopy of parallel individual Mucin1–antibody bonds *Proc. Natl. Acad. Sci. USA* **102** 16638–43
[22] Hinterdorfer P and Dufrene Y F 2006 Detection and localization of single molecular recognition events using atomic force microscopy *Nat. Methods* **3** 347–55
[23] Ray C, Brown J R and Akhremitchev B B 2007 Correction of systematic errors in single-molecule force spectroscopy with polymeric tethers by atomic force microscopy *J. Phys. Chem.* B **111** 1963–74
[24] Bizzarri A R and Cannistraro S 2013 $1/f^{\alpha}$ noise in the dynamic force spectroscopy curves signals the occurrence of biorecognition *Phys. Rev. Lett.* **110** 0481041
[25] Yang J D H, Peng B and Ding J 2009 Structural modeling and biochemical studies reveal insights into the molecular basis of the recognition of β-2-microglobulin by antibody BBM 1 *J. Mol. Recognit.* **22** 465–73
[26] De Paris R, Strunz T, Oroszlan K, Guntherold H J and Hegner M 2000 Force spectroscopy and dynamics of the biotin-avidin bond studied by scanning force microscopy *Single Mol.* **1** 285–90
[27] Hegger R and Kantz K 1999 Practical implementation of nonlinear time series methods the TISEAN package *Chaos* **9** 413–35
[28] Press W H *et al* 1992 *Numerical Recipes in C* (Cambridge: Cambridge University Press)
[29] Flory P J 1988 *Statistical Mechanics of Chain Molecules* (Muenchen: Hanser)
[30] Ray C and Akhremitchev B B 2005 Conformational heterogeneity of surface-grafted amyloidogenic fragments of alpha-synuclein dimers detected by atomic force microscopy *J. Am. Chem. Soc.* **127** 14739–44
[31] Ratto T V, Langry K C, Rudd R E, Rodney L, Balhornz M J L, Allen M J and McElfresh M W 2004 Force spectroscopy of the double-tethered concanavalin-a mannose bond *Biophys. J.* **86** 2430–7
[32] Taranta M, Bizzarri A R and Cannistraro S 2008 Probing the interaction between p53 and the bacterial protein Azurin by single molecule force spectroscopy *J. Mol. Recognit.* **21** 63–70
[33] Bizzarri A R and Cannistraro S 2009 Atomic force spectroscopy in biological complex formation strategies and perspectives *J. Phys. Chem.* B **113** 16449–64
[34] Celik E and Moy V T 2012 Nonspecific interactions in AFM force spectroscopy measurements *J. Mol. Recognit.* **25** 53–6
[35] Marshall B T, Sarangapani K K, Lou J H, McEver R P A and Zhu C 2005 Force history dependence of receptor-ligand dissociation *Biophys. J.* **88** 1458–66
[36] Butt H J, Cappella B and Kappl M 2005 Force measurements with the atomic force microscope technique, interpretation and applications *Surf. Sci. Rep.* **59** 1–152
[37] Proksch R, Scheffer T E, Clevland J P, Callahan R C and Viani M B 2004 Finite optical spot size and position corrections in thermal spring constant calibration *Nanotechnology* **15** 1344–50
[38] Shlesinger M, Zavlaskii G and Frisch U 1999 *Levy Flights and Related Topics in Physics* (Berlin: Springer)
[39] Siwi Z and Fulinski A 2002 Origin of $1/f^{\alpha}$ noise in membrane channel currents *Phys. Rev. Lett.* **110** 0481041
[40] Deway T G and Bann J B 1998 Protein dynamics and 1/f noise *Biophys. J.* **64** 594–8
[41] Zheng G, Xuan X P A and Lieber C M 2010 Subthreshold regime has the optimal sensitivity for nanowire FET biosensors *Nano Lett.* **10** 3179–83
[42] Chen W, Evans E A, McEver R P and Zhu C Monitoring receptor-ligand interactions between surfaces by thermal fluctuations *Biophys. J.* **94** 694–701
[43] Garcia A E, Blumenfeld R, Hummer G and Krumhansl J A 1997 Landscape paradigms in physics and biology concepts, structures and dynamics *Physica* D **107** 225–39
[44] Carlini P, Bizzarri A R and Cannistraro S 2002 Temporal fluctuations in the poptential energy of proteins $1/f^{\alpha}$ noise and diffusion *Physica* D **165** 242–50
[45] Eliazar I and Klafter J 2009 A unified and universal explanation for Levy laws and 1/f noises *Proc. Natl. Acad. Sci. USA* **106** 12251–4
[46] Khatri B, Yew Z T, Krivov S, McLeish T and Paci E 2010 Fluctuation power spectra reveal dynamical heterogeneity of peptides *J. Chem. Phys.* **133** 015101–6
[47] Frauenfelder H, Sligar S G and Wolynes P G 1991 The energy landscape and motions of proteins *Science* **254** 1598–603
[48] Frauenfelder H, Chen G, Berendzen J, Fenimore P W, Jansson H, McMahon B H, Stroe I R, Swenson J and Young R D 2009 A unified model of protein dynamics *Proc. Natl. Acad. Sci. USA* **102** 126638–16643
[49] Funari G, Domenici F, Nardinocchi L, Puca R, D'Orazi G, Bizzarri A R and Cannistraro S 2010 Interaction of p53 with Mdm2 and azurin as studied by atomic force spectroscopy *J. Mol. Recognit.* **23** 343–51
[50] Santini S, Bizzarri A R and Cannistraro S unpublished data
[51] Dudko O, Hummer G and Szabo A 2008 Theory, analysis, and interpretation of single-molecule force spectroscopy experiments *Proc. Nat. Acad. Sci. USA* **105** 15755–60
[52] Dudko O, Hummer G and Szabo A 2006 Intrinsic rates and activation free energies from single-molecule pulling experiments *Phys. Rev. Lett.* **96** 108101–4
[53] Friddle R, Noy A and De Yoreo J 2012 Interpreting the widespread nonlinear force spectra of intermolecular bonds *Proc. Nat. Acad. Sci. USA* **109** 13573–8