

Label-free sensing and atomic force spectroscopy for the characterization of protein–DNA and protein–protein interactions: application to estrogen receptors[†]

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In this paper we describe a new surface plasmon resonance (SPR) biosensor dedicated to potential estrogenic compounds prescreening, by developing an estrogen receptor (ER) specific DNA chip. Through the covalent binding of a DNA strain wearing the estrogen response element (ERE) to an activated 6-mercapto-1-hexadecanoic acid and 11-mercapto-1-undecanol self-assembled monolayer on gold surface, the SPR biosensor allows to detect specifically, quickly, and without any labeling the binding of ER in the presence of estrogen. In parallel, we investigated the ER interaction with itself, in order to study the formation of ER dimer apparently needed to activate the gene expression through ERE interaction. For that, we engaged force spectroscopy experiments that allowed us to prove that ER needs estrogen for its dimerization. Moreover, these ER/ER intermolecular measurements enabled to propose an innovative screening tool for anti-estrogenic compounds, molecules of interest for hormono-dependant cancer therapy. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: estrogen receptor; force spectroscopy; DNA sensor

INTRODUCTION

Estrogens, and particularly estradiol-17- β (E_2), are female hormones involved in development, growth, and maintenance of reproductive tissues (Diel, 2002). These hormones interact with estrogen receptors (ER) which are transcription factors activated by ligand binding (O'Malley, 2005). These activated nuclear receptors modulate expression of estrogen-responsive genes after the interaction with palindromic DNA sequences called estrogen response element (ERE; Klinge, 2001). Many molecules could interact with these ER, as well as plant derived compounds called phytoestrogens (genistein, glucopyranosylchrysin, *etc.*; Maggiolini *et al.*, 2001; Berthier *et al.*, 2007), synthetic drugs (ICI 182,780, Tamoxifen, *etc.*; Wakeling *et al.*, 1991; Ahn and Sheen, 1997), or endocrine disturbers (polychlorobiphenyls, parabens, *etc.*; Okubo *et al.*, 2001; Ho *et al.*, 2008). Interactions of these compounds have an impact on the conformational state of the receptors. Thus, the agonist or antagonist nature of the bonded compounds allows or blocks receptor dimerization, DNA interaction, and coregulators recruitment (Arbuckle *et al.*, 1992; Brzozowski *et al.*, 1997; Shiao *et al.*, 1998; Klinge, 2000; Gruber *et al.*, 2002). All estrogenic or anti-estrogenic compounds could act on the animal and human health and treatments: (i) phytoestrogens as lignans could be used to prevent the hormonally dependent cancer arrival or to limit climacteric complaints (Thompson, 1998; Power and Thompson, 2003; Bergman Jungstrom *et al.*, 2007; Feng *et al.*, 2008); (ii) drugs like Tamoxifen or fulvestran[®] (ICI 182,780) were applied to cancers and menopause treatments (Love *et al.*, 1994; Landgren *et al.*, 2002; Martino *et al.*, 2004a,b; Jacobsen *et al.*, 2008; McCormack and Sapunar, 2008; Saji and Kuroi, 2008). At present, public and politic attentions are focussed on a new class of

molecules called endocrine disturbers. These compounds, resulting from an environmental pollution (drugs, pesticides, and industrial waste) or from our lifestyle (plastics, cosmetics, *etc.*), affect fertility and enhance risk of cancer (Inadera, 2006; Vanparys *et al.*, 2006; Ho *et al.*, 2008; Soto *et al.*, 2008; Tiemann, 2008).

All these data incite to identify some new potential estrogenic or anti-estrogenic compounds essential to protect human health (therapy, prevention) and environment.

Classically, the identification of new potential estrogenic compounds was based on cellular or animal models. However, the ER dimerization and its interaction with DNA being the initial steps of the estrogenic genomic mechanism, a prescreening of

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Table 1. 5'amine modified oligonucleotides (D1 and D4) and complementary single strand (C3 and C6) sequences

Names	Sequences	Double strand sequences
D1	5'-H ₂ N-(CH ₂) ₁₂ -ATATATAGTTCTTTGATC <u>AGGTCAC</u> CTG <u>TGACCT</u> GAACTTGCT-3'	ERE
C3	AGCAAGTTC <u>AGGTCAC</u> AGT <u>TGACCT</u> GATCAAAGAACT	
D4	5'-H ₂ N-(CH ₂) ₁₂ -ATATATGTCCAAAGTCAATCGCCAGCAGATGATCAAAGTCC-3'	Control
C6	GGACTTTGATCATCGTGTGGCGATTGACTTTGGAC	

The ERE (bold and underlined) and control sequences were created by hybridization of D1/C3 and D4/C6 oligonucleotides, respectively.

molecules of interest could be processed upstream, in order to reduce time and money consumptions.

Surface plasmon resonance (SPR) biosensors and/or on-chip force spectroscopy analysis, which allow real time biomolecular interaction detections and quantification, can be applied to these prescreening steps. These biosensors are based on the immobilization of biomolecules on a gold chip directly or indirectly by physical or chemical adsorption (Boireau *et al.*, 2002; Aoyagi *et al.*, 2008). In order to get free of non-specific protein adsorption, a passivation step of the metallic film is essential (Rella *et al.*, 2004). This process implies the development of indirect grafting strategies. Actually, many kinds of surfaces were provided to SPR biosensor's users, for instance 3D hydrogels like commercial carboxymethyl dextran (CMD) or 2D self-assembled monolayers (SAMs).

For the ER/ERE interaction studies, several authors have oriented their DNA grafting strategies on the CMD based chips (Cheskis *et al.*, 1997; Kostelac *et al.*, 2003; Asano *et al.*, 2004; Habauzit *et al.*, 2008). However, the CMD based surfaces were known to create some kinetic disturbing as mass transport and rebinding (Schuck, 1996; Yarmush *et al.*, 1996; Fong *et al.*, 2002). Thus, to get free of these artifacts, one of the grafting alternative was based on a mixture of 16- mercapto-1-hexadecanoic acid (MHA) and 11- mercapto-1-undecanol (MUO). Previous works have shown that carboxylic functions of the MHA allowed molecular grafting and that the hydroxyl groups of the MUO reduced the non-specific adsorption phenomenon (Frederix *et al.*, 2003; Huang *et al.*, 2005). The grafting of molecules like modified DNA and proteins on such SAMs presenting a primary amine group was enabled by this latest surface choice after carboxylic function activation.

To address the question of ER dimerization, we analyzed interactions between an ER functionalized gold surface and an ER modified AFM tip by force spectroscopy. Indeed, AFM force spectroscopy is able to monitor intermolecular interactions in different medium conditions. Recording the applied force over the stretching distance revealed a characteristic sawtooth-like pattern of force peaks. The appearance of a "repetitive" pattern of force peaks while retracting ER tip to the ER surface confirmed (1) that ER interacts with itself and (2) that this interaction, estrogen specific and repetitive, described a specific pathway. These observations fit with a dimerization of ER in the presence of estrogen.

In this paper we have described a new SPR biosensor dedicated to potential estrogenic compounds prescreening, by developing an ER specific DNA chip, the ERE chip. In parallel, force spectroscopy experiments allowed us to (1) prove that ER needs estrogen for its dimerization and (2) propose an innovative screening tool for anti-estrogenic compounds.

MATERIALS AND METHODS

Materials

DNA immobilization was allowed by a primary amine grafted via a 12 carbons alkyl chain on 5' extremities of D1 and D4 single strand oligonucleotides. Modified oligonucleotides (D1 and D4) were able to create ERE or double strand control sequence by hybridization with complementary sequence C3 and C4. All DNA sequences were presented in Table 1 (Eurogentec, Liege, Belgium).

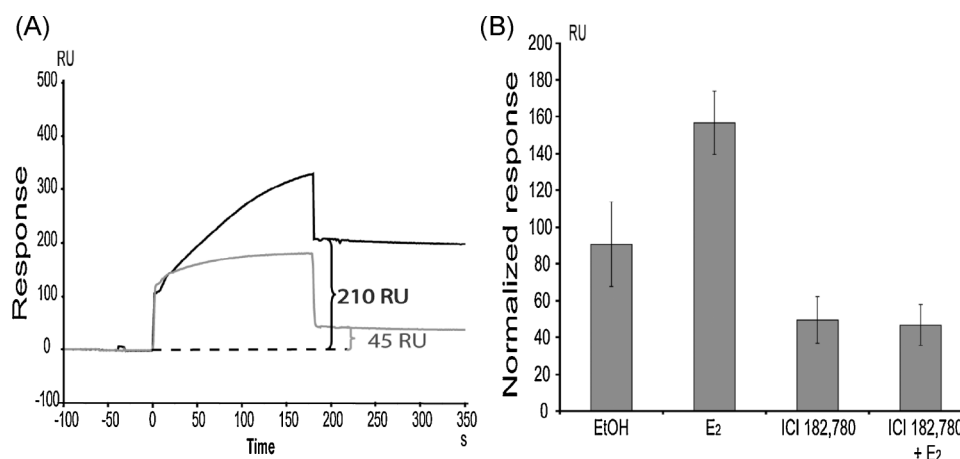


Figure 1. (A) Detection of ER/DNA specific interaction. The ER α was incubated with 10 nM E2 (30 min at room temperature) and injected (90 μ l at 30 μ l/min) on ERE (black) or control DNA sequences (gray). (B) Ligand impact. The ER α was incubated with ligand solvent (ethanol), with equimolar proportion of E2, or with an excess of ICI 182 780 supplemented or not by E2 (10 nM). The specific interaction signals were the control interaction signal subtracted to the ERE signal. Data presented as histograms were the means of three independent experiments.

Human recombinant estrogen receptor- α (ER α ; PanVera, Invitrogen Corporation, Carlsbad, USA) was conserved at -80°C into 10 μl aliquots to limit the number of freeze-thaw cycles. Estradiol-17- β (E2; Sigma–Aldrich, St. Louis, USA) and ICI 182 780 provided by AstraZeneca (Reims, France) was prepared in ethanol at 1 nM and 1 μM , respectively.

The MUO and MHA (Sigma–Aldrich, St. Louis, USA) were prepared in ethanol. MHA carboxylic functions were activated with amine coupling kit consisted of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxy-succinimide (NHS) 1:1 mix (GE Healthcare Life Sciences, Pittsburgh, USA).

Sensor chips

Gold chips were processed as previously described (Mangeat *et al.*, 2009). Briefly a 2 nm thick chromium layer is deposited on a glass wafer (diameter: 13 mm and thickness: 0.17 mm) with plasma sputtering technology to improve the adherence of gold to the substrate. Then a 40 nm thick gold layer is sputtered on the top of the Cr layer. After cleaning of the surface (ethanol/water), the chip was functionalized with a mixed SAM. This SAM was

processed by an overnight chip immersion in a 1 mM MUO/MHA (90:10 by mol) bath. Such functionalized gold surfaces were either used for DNA or ER immobilization, in order to study DNA/ER sensing by SPR or ER/ER dimerization by force spectroscopy, respectively.

SPR experiments

All SPR experiments were run on BIAcore 2000 apparatus at 25°C using BIACORE 2000 Control Software version 3.2 (GE Healthcare Life Sciences).

DNA immobilization

Modified DNA immobilizations were processed with HBS-N running buffer (10 mM Hepes pH 7.4, NaCl 150 mM). First, the surface was washed with 15 μl of 40 mM Octyl-Glucopyranoside (OG, Sigma–Aldrich, St. Louis, USA) at 30 $\mu\text{l}/\text{min}$. Then, the MHA carboxylic functions were activated with two injections (70 μl at 10 $\mu\text{l}/\text{min}$) of EDC/NHS v/v mix. In order to reduce the surface charge repulsion, 10 μM of amine-modified oligonucleotides were diluted in a 10 mM Hepes buffer pH 8.4 supplemented by 0.4 mM hexadecyltrimethylammonium

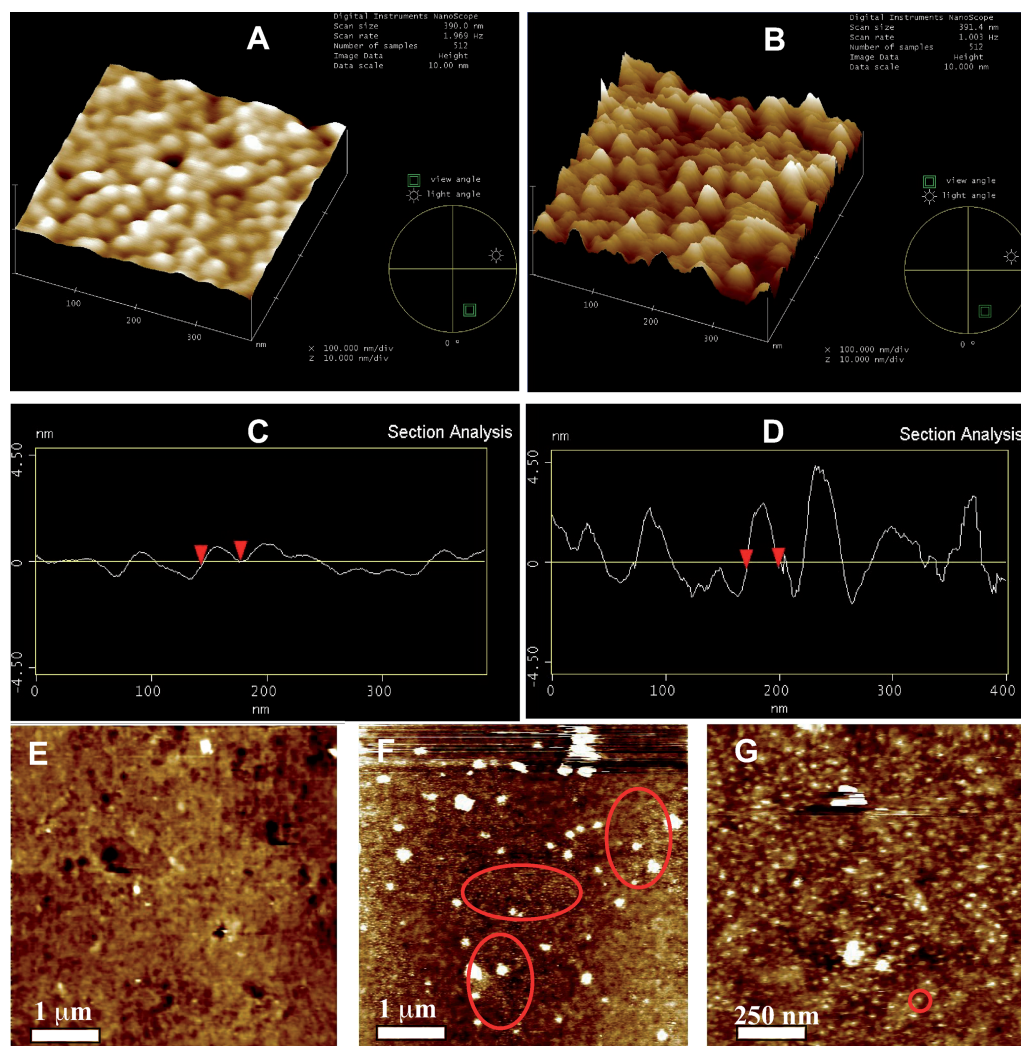


Figure 2. Characterization of ER bio-functionalized surfaces. Thiols functionalized gold chip before (A and C) and after (B and D) ER graftings. AFM images and their corresponding section analysis. Pieces of APTES/glutaraldehyde functionalized silicon nitride, before (E) and after (F and G) ER graftings. The image presented in G is the zoom of one red delimited area in image F. Z range is 10 (E and F) or 5 (G) nm.

bromide (CTAB). Oligonucleotides were then injected on the chip at 10 $\mu\text{L}/\text{min}$ for 15 min. The excess of NHS-ester function was inactivated with 1 M ethanolamine (140 μL at 10 $\mu\text{L}/\text{min}$).

For the hybridization process, the running buffer was replaced by HBS-EP buffer (GE healthcare Life Sciences). Complementary oligonucleotides (2.5 μM) were injected at 20 $\mu\text{L}/\text{min}$ for 2 min. At the end of injection, the surface was cleaned by a 15 μL pulse of OG at 30 $\mu\text{L}/\text{min}$.

Force spectroscopy

ER was immobilized on MUO/MHA (90:10 by mol) functionalized gold surface. For that, the surface was washed with 40 mM Octyl-Glucopyranoside. Then, the MHA carboxylic functions were activated with two injections of EDC/NHS v/v, and the gold surface was incubated during 30 min in the presence of ER solution (20 nM). The surface was then washed with ER buffer.

The AFM used was a Nanoscope III (Veeco, Santa Barbara, CA). Imaging was performed in contact mode using NPS-oxide sharpened silicon nitride probes (Veeco) exhibiting spring constants of 0.3 N/m. For the force spectroscopy measurements, the AFM tip, made of silicon nitride, was functionalized by 1% APTES in toluene during 2 h, washed extensively with toluene then with ethanol. The second step consisted in an incubation in 0.2% glutaraldehyde solution during 10 min, followed by extensive washing with water. The modified tips were then incubated in 20 nM ER solution during 30 min, then washed with ER buffer. For all the curves, a 200 nm ramp size and an interaction time of 1 ms were used. The loading rate was varied, from 30 000 to 520 000 pN/s, in order to study the dependency of the forces necessary to rupture specific bonds on the loading rate.

ER/DNA interactions

For the protein/DNA interaction studies the running buffer was replaced by the interaction buffer (40 mM Hepes pH 7.4, 100 mM NaCl, 10 mM MgCl_2 , 1 mM DTT, and 0.2% Triton X-100). First, 10 nM ER α was incubated with 1 nM E2 or 1 μM ICI 182 780 for 30 min at room temperature in interaction buffer. Then 90 μL of activated receptor were injected at 30 $\mu\text{L}/\text{min}$ on ERE or control channels. Finally, the DNA surface was regenerated by 10 min injection of 0.1% SDS, 100 mM EDTA denaturing solution (at 5 $\mu\text{L}/\text{min}$).

RESULTS AND DISCUSSION

ERE chip processing

The 5' amine modified single strand oligonucleotides D1 and D4 (10 μM) were immobilized on activated mixed SAM. Then complementary oligonucleotides (C1 and C4) were injected to create double strand ERE specific sequence or control sequence, respectively. After 2 min of injection the D1/C3 (ERE) and D4/C6 (control) hybridization degrees were 69.3 ± 6.9 and $78.8 \pm 6.5\%$, respectively (data not shown). Double strand SPR signals were 150 ± 30 RU (Response Unit) for ERE and 244 ± 55 RU for control sequence (data not shown).

ER/ERE interaction validation

The specificity of the ER/DNA interaction was determined by an injection of E2 activated ER on control and ERE sequences. The ER

(10 nM) interaction signal, in presence of 10 nM E2, was 3.63 ± 0.8 fold higher on ERE than on control sequence (for example see Figure 1A). Thus, we can conclude that our ERE chip constitutes a valid tool for specific ER/DNA interaction detection.

In order to determine the ligand impacts on ER/DNA interactions, the receptor has been injected with or without ligand. In presence of E2, the specific interaction signal was 1.7 fold higher than in absence of ligand (157 RU vs. 91 RU, respectively). When ER has been incubated with 1 μM ICI 182 780, supplemented or not by 10 nM E2, the specific interaction was 1.8 fold reduced compared to ligand free condition (Figure 1B).

ER dimerization characterization by force spectroscopy

(a) Characterization of ER biofunctionalized surfaces

The quality of the working surfaces, the ER modified silicon nitride AFM tip and the ER modified gold chip, was assessed using AFM to prove the presence of ER on both surfaces (Figure 2).

The surface of the MHA/MUO functionalized gold chip was compared before and after ER graftings. The surface presented

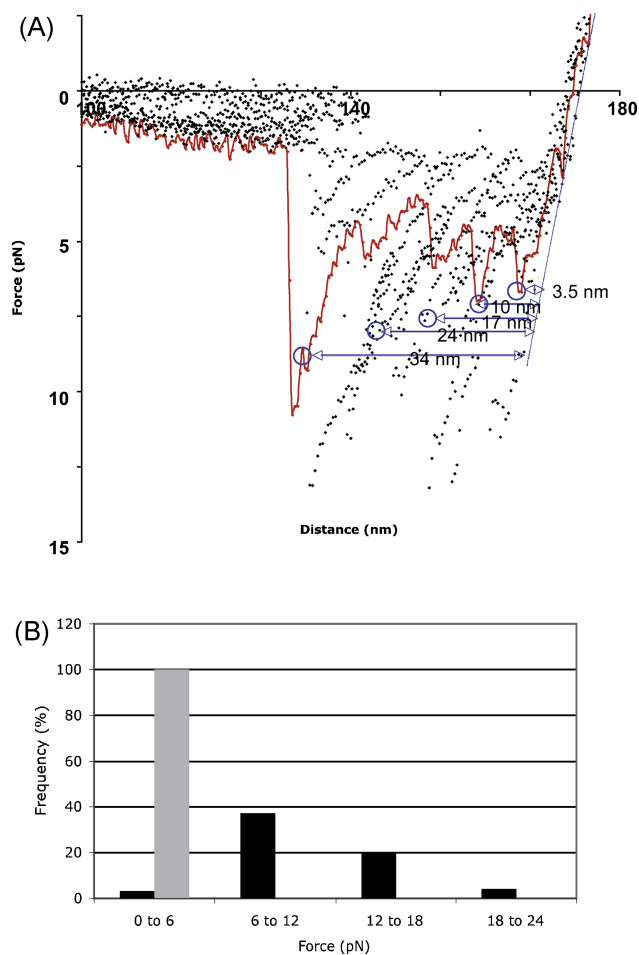


Figure 3. Force curves measured between ER tip and ER gold chip. (A) Scattered spectra represent superimposition of force curves, that highlight common features. A typical force curve is highlighted in red. Blue circles and arrows indicate the average peak position and the rupture distance. Loading rate: 150 000 pN/s, in the presence of 1 nM E2. (B) Histogram presenting the repartition of rupture forces measured in the absence (ER/ER; in gray) or presence (in black) of 1 nM E2 (ER/ER + E2) in the medium.

an increase in rms value (on $400\text{ nm} \times 400\text{ nm}$ surface area), from 0.2 nm ($r_{\text{max}} = 0.9\text{ nm}$) to 0.9 nm ($r_{\text{max}} = 3.4\text{ nm}$) before and after protein grafting, respectively, that reveals the presence of protein material on the gold surface (Figure 2A–D).

We also wanted to prove the presence of ER on AFM tip, made in silicon nitride. For that, we prepared pieces of silicon nitride and functionalized them with APTES and glutaraldehyde and controlled the surface by AFM before and after ER grafting. After incubation of the functionalized surface in ER and a washing step, we distinguished motifs on the surface and also some bigger aggregates. While zooming on such aggregates, it appeared that these bigger complexes were composed of motifs of $12\text{--}15\text{ nm}$ in diameter (Figure 2E–G). Then, the majority of the surface is covered by proteins, with a good homogeneity and only few aggregates of the protein are present. Thus, we can evaluate that the APTES + glutaraldehyde surface enables the grafting of ER protein on the silicon nitride surface, meaning that the ER is effectively bound to the silicon nitride tip when ER tip is employed in force curves experiments.

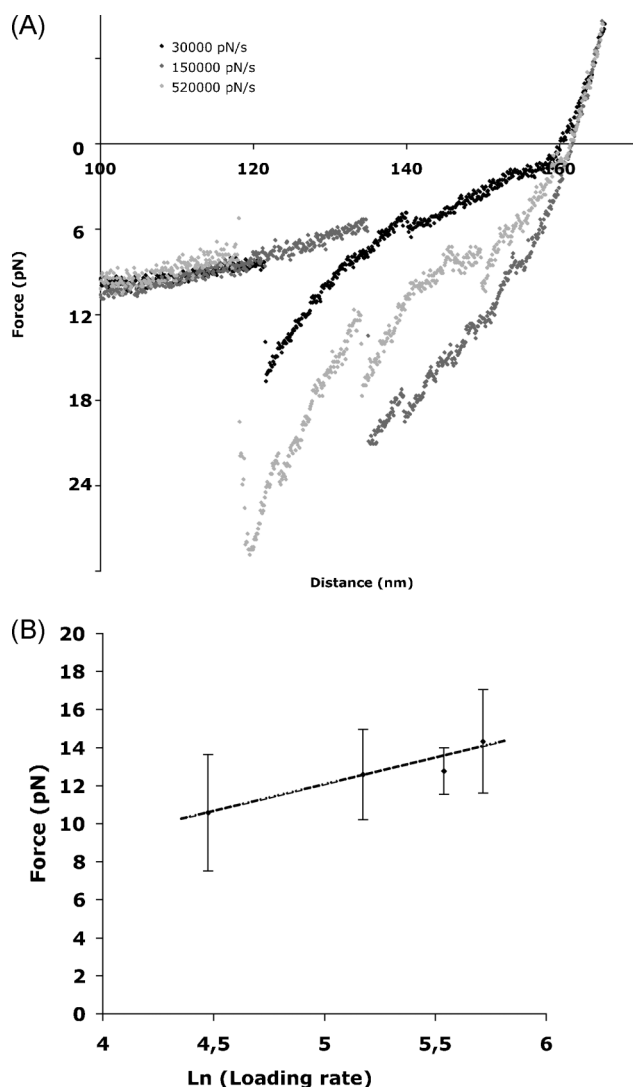


Figure 4. Dynamics of ER/ER interaction. (A) Typical force curves registered in the presence of 1 nM E2, at three different loading rates ($30\,000$, $150\,000$, and $520\,000\text{ pN/s}$) in black, dark gray, and light gray, respectively).

(b) Force spectroscopy measurements

Here we used single molecule force spectroscopy to investigate the molecular forces driving ER/ER interactions. If an ER dimer is formed, under conditions favorable for interaction between the two molecules, single molecule force spectroscopy should detect it while retracting the tip from the surface. In the case of dimerization, when an ER modified AFM tip interacts with an ER modified surface, the dimer is ruptured at the retraction step upon removal of the tip from the surface. Such an event produces a specific rupture signature on the force curve. We observed that when the ER modified AFM tip is used on the ER biofunctionalized gold chip surface after a preliminary 10 min

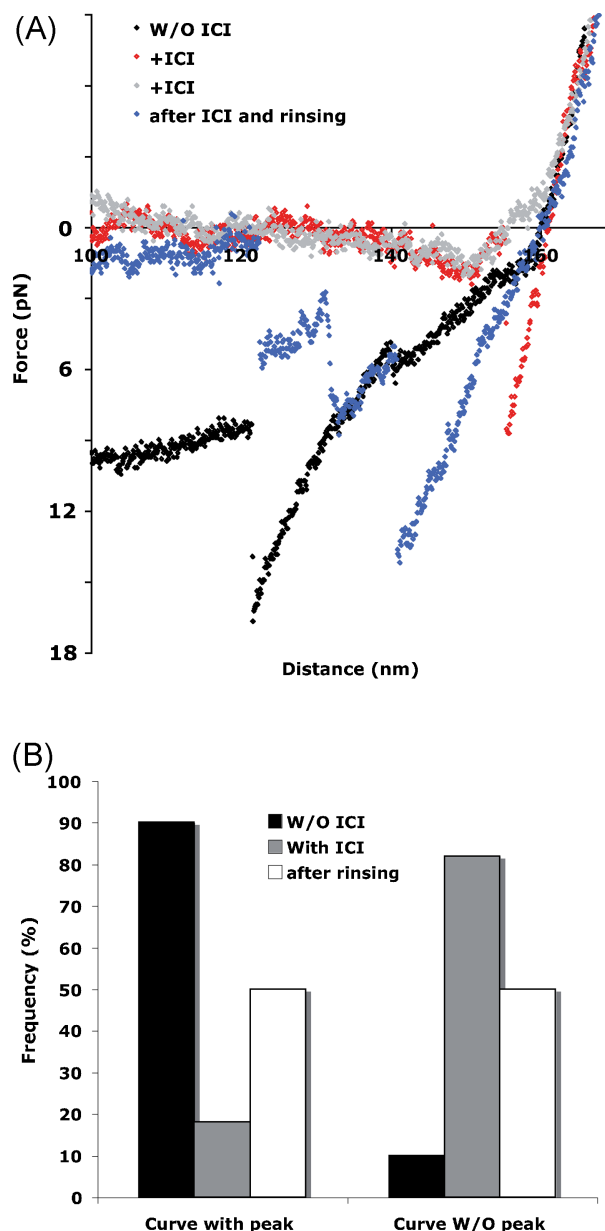


Figure 5. Inhibition and reversibility of ER/ER interaction. (A) In buffer and in the presence of 1 nM E2, forces curves were registered before inhibitor addition (in black), in the presence of $1\text{ }\mu\text{M}$ ICI 182 780 (in red and gray) and after an extensive rinsing and again in buffer containing 1 nM E2 (in blue). (B) Histogram presenting the proportion of curves presenting peaks or not in the different tested conditions.

incubation in the presence of 1 nM E2, interaction events can be recorded. We collected then several force curves (50) in this condition (loading rate 150 000 pN/s), i.e., in the presence of 1 nM E2. We obtained the scattered spectra presented in Figure 3A. Scattered spectra represent superimpositions of force curves obtained in the same conditions. Superimpositions highlight common features (a "five peaks" typical curve) of the unfolding events and reduce deviations, which may occur in individual spectra. More than 90% of force curves presented interaction peaks in the presence of 1 nM E2. The peak intensity was around 10 pN. The results are different in the absence of 1 nM E2, since the majority of force curve present no peak. Moreover, when a peak appeared in these conditions, its intensity was really low, close to zero (Figure 3B, the single gray bar).

Moreover, the forces necessary to rupture specific bonds are known to depend on the loading rate (Merkel *et al.*, 1999). Consistent with this study, 100 force curves were registered at four different loading rate, from 30 000 to 520 000 pN/s. Typical force curves obtained for three different loading rates are represented in Figure 4A. We found that the binding force between the ER tip and the ER surface, in the presence of 1 nM E2 in the solution, increased linearly with the logarithm of the loading rate (Figure 4B).

In our force spectroscopy experiment, we also tested the effect of ICI 182 780, a molecule known as an inhibitor of ER/ER dimerization. While the buffer contained 1 nM E2 and 1 μ M of ICI 182 780, we registered 50 curves and from them, very few curves were presenting peaks, and in this case only a single peak at short rupture distance was observed (Figure 5A, in red). Nevertheless, the majority of curves even presented no more interaction peaks (Figure 5A, in gray). Then, the presence of ICI 182 780 seemed to have inhibited the interaction between the ER tip and the ER surface. After this, we rinsed our surface, tip and AFM fluidic cell extensively with buffer, added again 1 nM E2 and proceeded again in force curves experiments. We observed that force curves presented again several peaks (3–5 peaks), and that the stretching distance fit with the stretching distance registered before addition of the inhibitor (Figure 5A, in blue).

Nevertheless, after recording 50 force curves in these conditions ("after ICI incubation and rinsing"), we noticed that only 50% of curves roughly presented this pattern of peaks (Figure 5B). This observation could mean that the ER/ER interaction is "quasi-reversible" after ICI 182 780 incubation, as if the inhibitor remained partially fixed on ER after washing. This is probably the case. Indeed, to ensure the inhibition of ER/ER interaction, we used a 1 μ M inhibitor concentration, compared to 1 nM concentration of E2. But, it is known that the binding

affinity of ICI 182 780 to the ER is 89% that of oestradiol (Howell, 2006). Then, such a large excess of inhibitor, having an affinity binding comparable to the ligand E2, could induce adsorbed molecules left on ER. This could explain that the interaction between the tip and the surface was reduced after ICI incubation. Moreover, this result indicates that the absence of peaks on curves obtained in the presence of 1 μ M ICI 182 780 was effectively due to an inhibition of the tip/surface interaction, and not to an artifact of experiments.

Then these unbinding events between ER tip and ER gold chip seem to be specific since:

- (1) No interaction is detected between a naked tip and the ER surface (data not shown).
- (2) Only few and weak interactions appeared between the ER modified tip and ER modified gold surface in the absence of E2.

In the presence of 1 nM E2:

- (1) A recurrent pattern of 3–5 peaks appeared, attesting that the ER/ER interaction follows a highly controlled and specific multiple-steps mechanism.
- (2) The forces needed to rupture the ER/ER interaction is loading rate dependent, meaning that these interactions are ER specific.
- (3) The addition of the ICI 182 780 inhibitor prevents the ER/ER interaction.

CONCLUSION

This SPR biosensor, consisting in ERE presenting DNA sequence, allows the selection of molecules which induce ER/ERE interaction. These preselected compounds could be enough to isolate compounds which are able to induce gene transactivation. The estrogenic activity should then be studied with an animal or cellular model. These xeno-estrogens could be used for example to menopause treatment. However, hormone-dependant cancer therapy needs molecules which reduce target gene expressions by ER dimerization and/or its interaction with DNA inhibition. The differentiation of anti-estrogenic and inefficient compounds will be impossible using the SPR ERE biosensor. The force spectroscopy assays represent then a relevant and complementary tool since this strategy allows to preselect quickly anti-estrogenic compounds which inhibit ER dimerization (initial step of gene transactivation).

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