

An advanced biosensor for the prediction of estrogenic effects of endocrine-disrupting chemicals on the estrogen receptor alpha

Peter Fechner · Florian Pröll · Mats Carlquist · Günther Proll

Received: 26 August 2008 / Revised: 13 October 2008 / Accepted: 14 October 2008 / Published online: 1 November 2008
© Springer-Verlag 2008

Abstract A label-free and time-resolved biosensor based on reflectometric interference spectroscopy (RIfS) has been developed to evaluate the agonistic or antagonistic effects of potential ligands with unknown behavior. The biosensor utilizes the specific interaction between the estrogen receptor α (ER α) and short specific peptides. The unique feature of these peptides allows the investigation of the behavior of ligands and the discrimination between the agonistic and antagonistic effects caused by conformational changes of the receptor. Thus, this developed biosensor allows not only the differentiation between ligands and nonligands of a receptor, but also the potential of these ligands to influence conformational changes in the receptor, leading to activation or inhibition of the receptor-dependent pathways. Owing to the robustness of the direct optical detection principle used, the biosensor is applicable to complex biological matrices, even crude cell extracts. Moreover, the reliability of the biosensor, including regeneration steps when performing subsequent measurements, has been verified.

Keywords Reflectometric interference spectroscopy (RIfS) · Endocrine disruptor (EDC) · Estrogen receptor (ER) · Biosensor · Small interacting peptides · Label free

Introduction

Endocrine-disrupting chemicals (EDCs) are defined as chemicals that can potentially interfere with the human hormone system. EDCs belong to a variety of chemical classes ranging from naturally produced components in plants to man-made pesticides. One of the main targets of EDCs are the nuclear receptors (NRs), a superfamily of proteins playing a crucial role in the human hormone system. To date, 48 NRs have been identified [1]. These NRs are involved in many hormone-dependent pathways making them an interesting subject of commercial and academic research.

A major group of EDCs are chemicals that can exert or prevent estrogenic effects on the human body. These EDCs are called xenoestrogens if they are man-made, or phytoestrogens if they are produced in plants. Examples for xenoestrogens are bisphenol A and vinclozolin. Bisphenol A has been found in plastics used to produce baby bottles, water bottles, as a coating in food and beverage cans, as well as in medical devices [2]. Vinclozolin is a pesticide known for its antiandrogenic effects and connections with cancer. Phytoestrogens can be found in high concentration in flax and soy [3] and therefore also in baby food.

The target of these xeno- or phytoestrogens is the estrogen receptor (ER). The existence of a protein able to bind β -estradiol was discovered in the 1950s [4], but it took until 1996 to detect the second isoform of the ER in human tissue [5]. Today these two isoforms are known as estrogen receptor α (ER α) and estrogen receptor β (ER β). While the sequence in the DNA binding domain of those two

Dedicated to Prof. Günter Gauglitz on the occasion of his 65th birthday.

Electronic supplementary material The online version of this article (doi:10.1007/s00216-008-2480-3) contains supplementary material, which is available to authorized users.

P. Fechner (✉) · F. Pröll · G. Proll
Institute of Physical and Theoretical Chemistry (IPTC),
Eberhard Karls University of Tübingen,
Auf der Morgenstelle 8,
72076 Tübingen, Germany
e-mail: peter.fechner@ipc.uni-tuebingen.de

M. Carlquist
Karo Bio AB,
Novum 141 57 Huddinge, Sweden

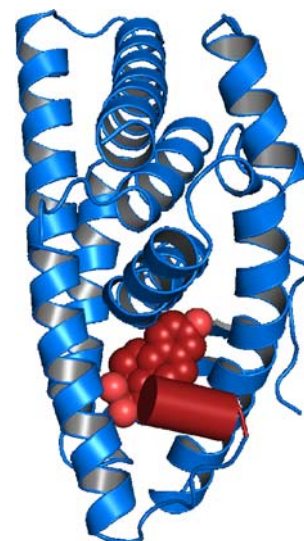
isoforms is almost the same (97% similarity), the ligand binding domain (LBD) has a much lower similarity (56%). The expression of ER α and ER β depends strongly on the tissue or even on the different cell types of the same tissue. While in some tissues both isoforms are expressed on a similar level, ER α is mainly expressed, e.g., in the uterus, prostate (stroma), ovary (theca cells), bone, and breast tissue. In contrast ER β is mainly expressed, e.g., in the colon, prostate (epithelium), ovary (granulosa cells), testis, salivary gland, and vascular endothelium [6]. The different expression pattern of the two isoforms might also play an important role in cancer development. Some data suggest that ER β acts as a tumor suppressor in the breast [7], while the lack of ER β leads to invasive and more proliferative breast cancer cells [8]. Further examples of diseases linked to the ER are prostate cancer [9], osteoporosis [10], cardiovascular diseases [11], and metabolic diseases like type 2 diabetes [12].

The monitoring of EDCs in the environment affecting human health is a major challenge not only for governmental institutions. Furthermore, since there is an obvious connection between ER and disease development, research into ER modulators, agonists, and antagonists has become a billion dollar investment for the pharmaceutical industry. First-generation selective estrogen receptor modulators (SERMs) used in cancer treatment, like tamoxifen, are highly tissue-specific and exert an antagonistic effect in the breast while acting as an agonist in the uterus and bone, thereby preserving the bone density [13]. Second-generation SERMs show an even higher tissue selectivity. Raloxifene has been approved for the treatment of postmenopausal osteoporosis.

To monitor EDCs in the environment and to help understand the mechanisms by which EDCs interact with their potential target(s), the NRs, the development of novel investigation methods has become an important task. Biosensors have shown the potential to monitor EDCs, using NRs as recognition elements for their corresponding EDCs. Normally these biosensors are suitable to detect the EDCs quantitatively, but they cannot discriminate what effect the chemical has on the receptor. For example, β -estradiol is a ligand of ER α , causing the receptor to adopt a conformation typically caused by a bound agonist (Fig. 1), whereas raloxifene or tamoxifen, both ligands of ER α , cause the receptor to adopt a conformation typically caused by a bound antagonist (Fig. 2).

This agonistic or antagonistic behavior is based on the conformational changes the ligand induces in the ER α . Agonists induce a conformation of helix 12 (red in Fig. 1), forming a binding site for coactivators. In contrast, antagonists such as the SERMs raloxifene or tamoxifen induce a conformation in which helix 12 (green in Fig. 2) is displaced, thus preventing coactivator interaction. Other antagonists such as ICI 182,780 force helix 12 to adopt a disordered conformation, while the side chain blocks the

Fig. 1 Structure of ER α -LBD with a bound agonist. Helix 12 (red) is in a conformation typical for ER α with a bound agonist. 1ERE [14] from the RCSB Protein Data Bank

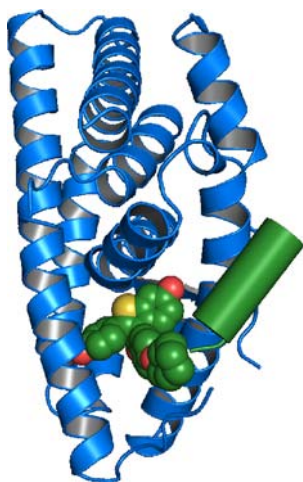


coactivator binding groove [15]. While in nature coactivators or corepressors interact with this specific binding site of the ER α , it is generally possible to replace the part of the coactivator or corepressor with small peptides, mimicking the interaction site of the coactivator or corepressor. This interaction site is a potential target for the development of new peptide-based pharmaceuticals [16]. In the past few years whole libraries with such interacting peptides have been built up [17].

Label-free direct optical biosensors based on techniques such as surface plasmon resonance (SPR) [18] and reflectometric interference spectroscopy (RIFS) [19] are widely used for the investigation of biological processes. The major advantage of these methods, apart from the avoidance of labels which might interfere in the biological processes, is the ability to determine kinetic and thermodynamic data of the monitored interactions [20]. The drawbacks of these technologies are a worse limit of detection, especially compared with fluorescence-based methods [21], and a problem in distinguishing between specific and nonspecific binding to the sensor surfaces. Thus, a suitable surface chemistry with low nonspecific binding is mandatory [22].

RIFS is a simple and robust direct optical detection method for the label-free and time-resolved investigation of biomolecular interactions [23]. The binding of molecules to sensitive bionic interfaces is monitored through the change in the apparent optical thickness of the sensing layer. The method is based on white light interference at thin solid films. The light is guided to the sensor surface, and the reflected light beams coming from different interfaces interfere with each other, forming a specific interference spectrum dependent on the physical thickness and the refractive index of the sensing layer. The product of the refractive index and the physical thickness is called the optical thickness. The shift in the reflectance spectrum is dependent on changes in these two parameters caused by binding events to the sensor

Fig. 2 Structure of ER α -LBD with a bound antagonist. Helix 12 (green) is in a conformation typical for ER α with a bound antagonist. 1ERR [14] from the RCSB Protein Data Bank



surface. Owing to its simple setup and temperature independence [24], RfS offers the possibility of hyphenation with important techniques such as electrophoresis and mass spectrometry [25]. For applications in cell-based assays, RfS offers advantages over other label-free detection principles as a result of the higher penetration depth compared with evanescent-field-based methods such as SPR or grating coupler devices [26].

Using these small interacting peptides, we have developed a label-free time-resolved biosensor based on RfS that allows one not only to discriminate between ligands and nonligands of the ER α , but also between agonists and antagonists of the ER α .

Experimental

Materials

RfS transducer chips of 1-mm-thick D263 glass substrate with a first layer of 10 nm Ta₂O₅ and a layer of 330 nm SiO₂ on top were obtained from Schott AG (Mainz, Germany).

Common organic compounds and biochemicals were purchased either from Fluka (Neu-Ulm, Germany), Sigma-Aldrich (Deisenhofen, Germany), or Merck (Darmstadt, Germany). 3-Glycidyloxypropyltrimethoxysilane (GOPTS) and diisopropylcarbodiimide (DIC) were purchased from Fluka (Neu-Ulm, Germany). Diaminopoly(ethylene glycol) (DAPEG) with molecular mass 2,000 Da was purchased from Rapp Polymere (Tübingen, Germany). D-Biotin and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) were purchased from Sigma-Aldrich (Deisenhofen, Germany). Purified (single band on native-PAGE) human ER α -LBD was kindly provided by Karo Bio AB, Sweden [27].

The human ER α -LBD (residues 301–553) was expressed in *Escherichia coli* BL21 Star™ (DE3) cells (Invitrogen), using the pET11a expression system. Fermentation was

carried out in batch culture (2 × Luria Bertani medium, 22 °C), and expression of the recombinant protein was induced by the addition of 0.5 mM isopropyl- β -D-thiogalactoside at A_{600} =5.8. Three hours after induction the cells were harvested by centrifugation and the pellet was frozen at –70 °C. The lysate was obtained by passing 10 g cells suspended in 60 mL 100 mM Tris-HCl pH 8.4, 10% glycerol, and 4 mM TCEP through an M-110 L microfluidizer (Microfluidics, MA, USA).

Biotinylated peptide α/β I [17] with the amino acid sequence Ser-Ser-Asn-His-Gln-Ser-Ser-Arg-Leu-Ile-Glu-Leu-Leu-Ser-Arg was purchased from Thermo Scientific (Ulm, Germany).

Surface chemistry

Surface cleaning and activation

The sensor surface was cleaned by treatment with 6 M KOH for 1 min, followed by ultrasonication with freshly prepared piranha solution (60 vol% concentrated H₂SO₄ and 40 vol% H₂O₂) for 20 min. The slides were then thoroughly rinsed in Milli-Q water and dried under a stream of nitrogen.

Silanization

A 15 μ L aliquot of GOPTS was pipetted onto a freshly activated dry slide and covered with another freshly activated slide (“sandwich technique”). After silanization for 60 min, the slides were rinsed with dry acetone and dried under a stream of nitrogen.

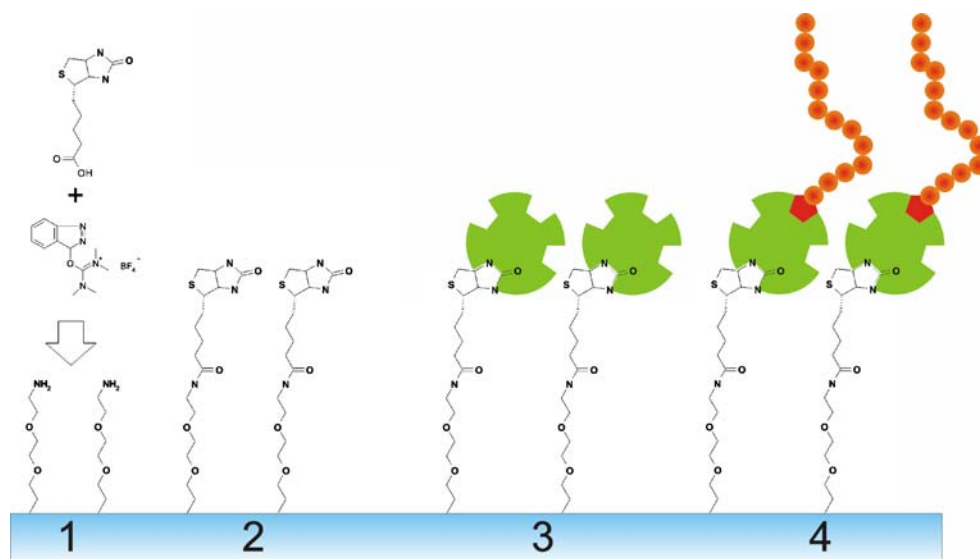
Polymer immobilization

For immobilization of diaminopoly(ethylene glycol), 15 μ L of a 4 mg/mL DAPEG/methylene chloride solution was pipetted onto the slides. The slides were transferred into an oven where immobilization took place overnight in an open-topped vessel at 70 °C. The slides were then thoroughly rinsed with Milli-Q water and dried under a stream of nitrogen.

Biotin immobilization

Biotin was immobilized by using TBTU activation: D-biotin (1 mg, 4 mmol), TBTU (1.4 mg, 4.4 mmol), and *N,N*-diisopropylethylamine (4 mL, 23.3 mmol) were mixed with DMF (50 mL). The solution was pipetted onto a transducer with immobilized DAPEG. After a reaction time of 1 h in a saturated DMF atmosphere, the sandwich was separated and both transducers were rinsed with DMF and water [28]. This process is summarized in Fig. 3 (steps 1 and 2).

Fig. 3 Scheme of the immobilization strategy on the sensor surface. The biosensor with immobilized DAPEG was covalently modified with D-biotin (1,2). Streptavidin was bound to the immobilized biotin (3). Biotinylated peptide α/β I was bound to the immobilized streptavidin via its biotin tag (4)



Peptide immobilization

Immobilization of streptavidin and then biotinylated peptide was achieved by flushing a 30 μ L aliquot of 1 mg/mL streptavidin solution or 3 μ L of the 1 mg/mL biotinylated peptide solution over the sensor surface, respectively. Both immobilization processes were monitored online using RIfS (Fig. 3, steps 3 and 4).

Ligands

Ligands were dissolved in DMSO, yielding a 1 mg/mL stock solution of the corresponding ligand. Stock solutions were stored at 4 °C in the dark until use.

Reaction mixtures

A 10 μ L aliquot of the ligand stock solution was diluted with 990 μ L buffer containing 500 mM Tris, 100 mM potassium chloride, and 1 mM EDTA with a pH of 7.4 to a total concentration of 10 μ g/mL. A 27 μ L aliquot of this solution was then incubated with 3 μ L of a 1 mg/mL solution of ER α for 30 min at 4 °C in the dark before measurement.

Characterization with RIfS

The RIfS setup used has been described elsewhere [29] and consists of a halogen white-light source and a Y-optical fiber which guides the light to the previously described transducer. The reflected light is guided via the same optical Y-fiber to a diode array spectrometer (Spekol-1100, Analytik Jena, Germany). Data acquisition and evaluation were performed using internal software. The liquid handling system consists of a Hamilton diluter Microlab

(Hamilton, Switzerland) with two syringe pumps and a four-way valve.

The interaction of the ER α -LBD with the peptide-coated surface was monitored with RIfS. The principles and the experimental setup of this technique for monitoring binding events at interfaces have been discussed in detail [30, 31]. The change in apparent optical thickness of a thin silica layer (ca. 330 nm) upon analyte binding is detected by interference of white light reflected at the interfaces of a multilayer system using a diode array spectrometer. Binding curves were recorded as apparent optical thickness (nm) versus time (s).

All measurements were carried out in 500 mM Tris buffer containing 100 mM potassium chloride and 1 mM EDTA with a pH of 7.4 at room temperature (ca. 23 °C).

The binding of the receptor to the surface was monitored by incubating 30 μ L of the reaction mixture for 250 s after a 100 s baseline period, followed by a 300 s to 600 s dissociation phase and a regeneration step with 6 M guanidinium hydrochloride pH 2 and a 240 s baseline period.

Results and discussion

The transducers were coated with DAPEG, functionalized with D-biotin as described above, and tested for nonspecific binding. This is important because all label-free optical detection methods detect any kind of binding. Nonspecific binding has to be suppressed for two reasons: nonspecific binding causes unclear binding curves, making an interpretation more difficult; and excessively high nonspecific binding may interfere with specific binding processes, e.g., blocking binding sites for specific analytes.

For the test for nonspecific binding, ovalbumin at high concentration was used in the normal acquisition protocol

to monitor the change in optical thickness in real time. Figure 4 shows the change of optical thickness versus time. After a stable baseline has been achieved, the sample is injected, leading to an increase of optical thickness by the binding of the receptor to the peptides immobilized on the surface (black curve). After reaching the maximum surface loading, buffer is rinsed over the biosensor, leading to a dissociation phase. Preventing false positive binding signals by nonspecific absorption of molecules to the surface is crucial before obtaining data. To check if nonspecific binding is suppressed sufficiently, ovalbumin at a high concentration was used as sample to check for nonspecific binding.

After 380 s, 30 μ L of ovalbumin solution with a concentration of 1 mg/mL was injected. Nonspecific binding would have caused a noticeable increase in optical thickness over time, starting at 380 s. However, no indication of nonspecific binding could be observed.

After ensuring that the surface was properly shielded against nonspecific binding, the next layer was immobilized. A solution of 1 mg/mL streptavidin was bound to the biotinylated surface via the specific and very stable biotin–streptavidin interaction (Fig. 3). The binding of streptavidin to the surface was monitored online until the maximum surface loading could be observed (see Fig. S1, [Electronic supplementary material](#)).

The transducers were finalized by binding the biotinylated peptide α/β I to the streptavidin-coated surface (Fig. 3, step 4). The peptide α/β I was flushed over the surface at a concentration of 1 mg/mL until the maximum surface

loading could be observed online (see Fig. S2, [Electronic supplementary material](#)).

To evaluate the binding properties of the generated surface, ER α –LBD was incubated with or without different ligands, and rinsed over the surface. ER α –LBD (0.1 mg/mL) was incubated with 10 μ g/mL of ligand for 30 min at 4 °C in the dark. As a control, buffer containing 1% (v/v) DMSO was used. No sample exceeded the amount of DMSO as solvent of 1% (v/v). The obtained data are shown in Fig. 5 and are in very good agreement with the following theory: β -estradiol causes the ER α to adopt a conformation which enables an interaction between the receptor in solution and the peptide immobilized on the surface; this results in the highest slope and surface loading. In contrast, Tamoxifen causes the receptor to adopt a conformation less recognized by the peptide and results in the lowest slope and surface loading. A binding curve between these two extrema occurs in the absence of ligands.

A major challenge in biosensor development is reproducibility and therefore the need for a reusable surface. We regenerated the surface of our biosensors over 40 times without noticeable decrease of performance.

Figure 6 shows a series of regeneration cycles to test the stability of the sensor surface. Each peak corresponds to an injection of guanidinium hydrochloride, as was also used in the normal acquisition protocol. Neither a decrease in optical thickness between the single peaks nor between the first and the last regeneration cycle could be observed. We concluded that the surface was therefore stable, since a

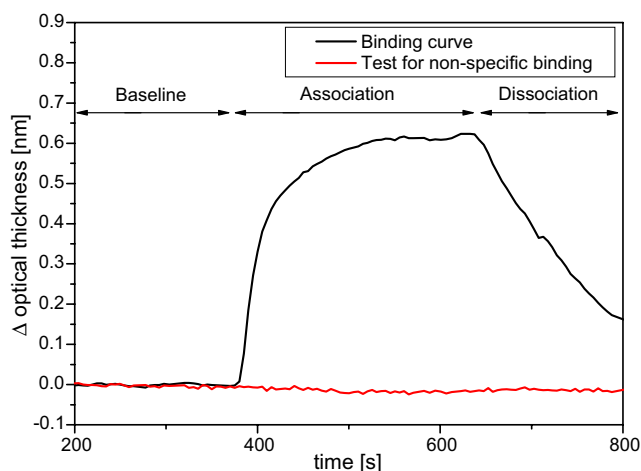


Fig. 4 Acquisition protocol and testing for nonspecific binding. Typical binding curve, consisting of the baseline, an association phase where the sample is pumped over the surface, and a dissociation phase (black curve). After the dissociation phase, the surface is regenerated until a stable baseline is obtained (data not shown). A 30 μ L aliquot of 1 mg/mL ovalbumin was injected at 380 s. No nonspecific binding could be observed (red curve). Nonspecific binding would lead to an increased optical thickness

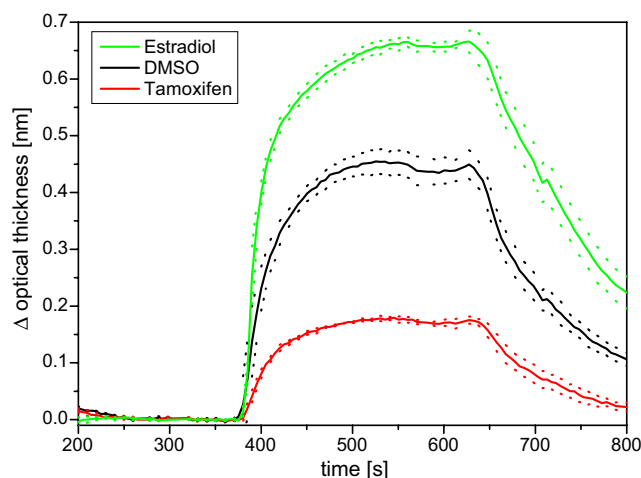


Fig. 5 Binding curves of ER α –LBD. Different responses of the biosensor to different ligand treatments with ER α –LBD. Each curve contains the information of three consecutive measurements on the same transducer with the corresponding standard deviation. The samples contained the 0.1 mg/mL ER α –LBD incubated with 10 μ g/mL β -estradiol (green curve), with 10 μ g/mL tamoxifen (red curve), or 1% DMSO (black curve)

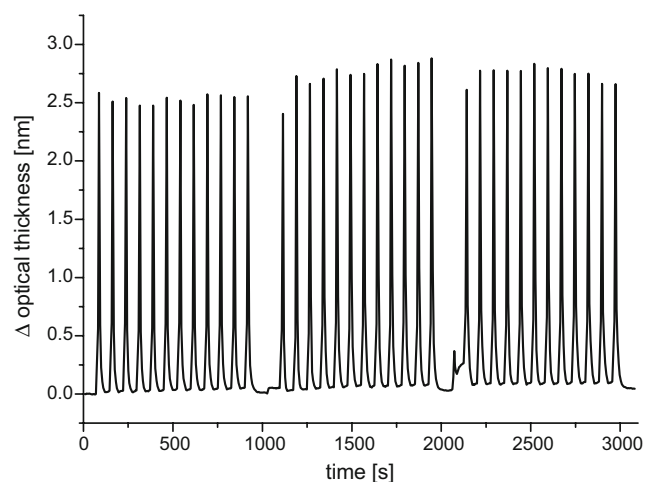


Fig. 6 Stability over multiple regeneration cycles. Stable baseline from regeneration cycle 1–36

degradation of the surface would have led to a noticeable decrease in optical thickness. To be entirely sure that the surface was still functional, sample measurements were undertaken to compare the slope and the maximum surface loading between multiple regeneration cycles. In Fig. 7 two binding curves between the 20th and the 40th regeneration cycle are compared with each other. Both curves show almost exactly the same properties in slope and maximum surface loading. The binding curve obtained after 20 regeneration steps (black curve) shows slightly higher maximum surface loading at the same slope as the binding curve obtained after 40 regeneration steps (red curve). The small gap between the baseline before and after sample injection can be explained by different pumping speeds, which equilibrates between the single measurements.

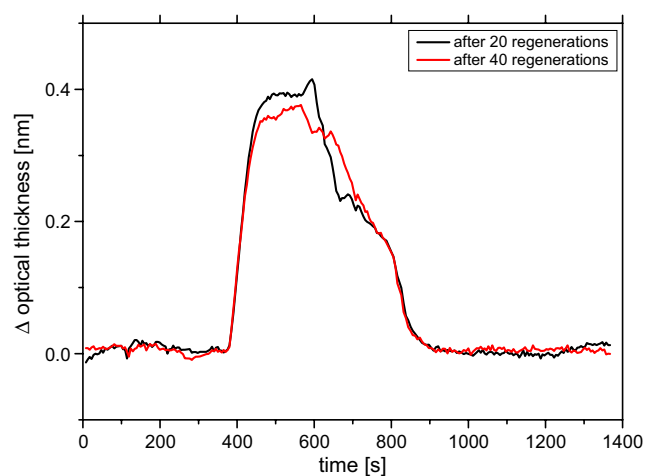


Fig. 7 Reproducibility of binding curves. Only a small deviation of the whole binding curve can be observed between multiple regenerations. Shown here are binding curves after 20 and 40 regeneration cycles

Figures 6 and 7 together clearly demonstrate the high reproducibility upon multiple measurements using the same transducer. The next logical step to test the possibilities of this biosensor was its application to samples containing complex matrices. For this purpose, whole cell lysate from ER α -LBD-expressing cells was used instead of purified ER α -LBD.

Lysate containing 0.01 mg/mL ER α -LBD was incubated with 10 μ g/mL of ligand for 30 min at 4 $^{\circ}$ C in the dark. As a control, buffer containing 1% (v/v) DMSO was used. No sample exceeded the amount of DMSO as solvent of 1% (v/v). The obtained data are shown in Fig. 8 and are in good agreement with those obtained from the measurements using the purified ER α -LBD (Fig. 5). Minor deviations in the behavior of the lysate compared with the purified receptor (e.g., the slightly decreased dissociations) are negligible for the applications of the biosensor.

Conclusions

We were able to show that this biosensor can discriminate between agonists and antagonists of the ER α . The surface chemistry used is highly flexible and can be easily adapted for peptides representing other possible interaction sites. The surface is very stable, even against harsh regeneration treatment, which leads to a high reproducibility of the obtained data. This high specificity allows us to use cell lysates with a rather low concentration of ER α -LBD (0.1 mg/mL; 300 ng per measurement) instead of purified ER α -LBD.

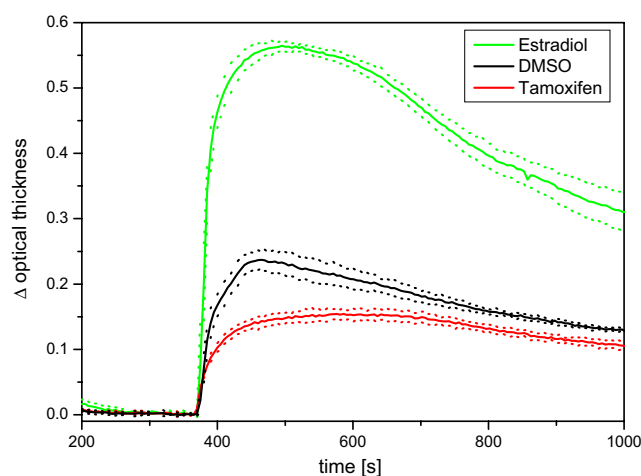


Fig. 8 Binding curves of ER α -LBD-containing lysate. Different responses of the biosensor to different ligand treatments with ER α -LBD-containing lysate. Each curve contains the information of three consecutive measurements on the same transducer with the corresponding standard deviation (dotted line). The samples contained lysate equivalent to 0.01 mg/mL ER α -LBD incubated with 10 μ g/mL β -estradiol (green curve), with 10 μ g/mL Tamoxifen (red curve), or 1% DMSO (black curve)

Beside the advantage of being able to discriminate between different conformations of the ER α caused by different potential EDCs, this biosensor offers a fast and reliable alternative to the established assay formats. In the future, the principle of this assay might be extended not only to other peptide sequences, but also to other receptors.

The possibility of using cell lysate yields another advantage which might play an important role for future biosensor development: certain receptors (e.g., arylhydrocarbon receptor) would be highly unstable without their stabilizing proteins. This problem could be easily overcome by the ability to use lysate (naturally containing these stabilizing proteins) instead of having to rely on purified receptors.

Acknowledgements We kindly acknowledge financial support by the European Union project "CASCADE" FOOD-CT-2004-506319. Special thanks go to Björn Kauppi from Karo Bio AB for supporting us with his great experience in PyMOL and for preparing the images.

References

1. Gronemeyer H, Gustafsson JA, Laudet V (2004) *Nat Rev Drug Discov* 3:950–964
2. Hileman B (2007) *Insights* 2007:38
3. Thompson LU, Boucher BA, Liu Z, Cotterchio M, Kreiger N (2006) *Nutr Cancer* 54:184–201
4. Jensen EV, Jordan VC (2003) *Clin Cancer Res* 9:1980–1989
5. Kuiper G, Enmark E, PeltoHuikko M, Nilsson S, Gustafsson JA (1996) *Proc Natl Acad Sci USA* 93:5925–5930
6. Dahlman-Wright K, Cavailles V, Fuqua SA et al (2006) *Pharmacol Rev* 58:773–781
7. Garinis GA, Patrinos GP, Spanakis NE, Menounos PG (2002) *Hum Genet* 111:115–127
8. Lazennec G, Bresson D, Lucas A, Chauveau C, Vignon F (2001) *Endocrinology* 142:4120–4130
9. Taylor RA, Cowin P, Couse JF, Korach KS, Risbridger GP (2006) *Endocrinology* 147:191–200
10. Sims NA, Dupont S, Krust A et al (2002) *Bone* 30:18–25
11. Mendelsohn ME, Karas RH (1999) *N Engl J Med* 340:1801–1811
12. Bailey CJ, Ahmedsrorour H (1980) *Diabetologia* 19:475–481
13. Jordan VC, Phelps E, Lindgren JU (1987) *Breast Cancer Res Treat* 10:31–35
14. Brzozowski AM, Pike ACW, Dauter Z et al (1997) *Nature* 389:753–758
15. Pike ACW, Brzozowski AM, Walton J et al (2001) *Structure* 9:145–153
16. Watt PM (2006) *Nat Biotechnol* 24:177–183
17. Paige LA, Christensen DJ, Gron H et al (1999) *Proc Natl Acad Sci USA* 96:3999–4004
18. Homola J, Yee SS, Gauglitz G (1999) *Sens Actuators B* 54:3–15
19. Leipert D, Nopper D, Bauser M, Gauglitz G, Jung G (1998) *Angew Chem Int Ed* 37:3308–3311
20. Gauglitz G (2005) *Anal Bioanal Chem* 381:141–155
21. Mallat E, Barzen C, Klotz A et al (1999) *Environ Sci Technol* 33:965–971
22. Mehne J, Markovic G, Proll F et al (2008) *Anal Bioanal Chem* 391:1783–1791
23. Hänel C, Gauglitz G (2002) *Anal Bioanal Chem* 372:91–100
24. Pröll F, Möhrle B, Kumpf M, Gauglitz G (2005) *Anal Bioanal Chem* 382:1889–1894
25. Proll G, Steinle L, Pröll F et al (2007) *J Chromatogr A* 1161:2–8
26. Mohrle B, Kohler K, Jaehrling J, Brock R, Gauglitz G (2006) *Anal Bioanal Chem* 384:407–413
27. Hegy GB, Shackleton CHL, Carlquist M et al (1996) *Steroids* 61:367–373
28. Birkert O, Haake HM, Schutz A et al (2000) *Anal Biochem* 282:200–208
29. Mohrle BP, Kumpf M, Gauglitz GN (2005) *Analyst* 130:1634–1638
30. Brecht A, Gauglitz G, Nahm W (1992) *Analisis* 20:135–140
31. Schmitt HM, Brecht A, Piehler J, Gauglitz G (1997) *Biosens Bioelectron* 12:809–816