Chapter 35
Biosensors for Detecting Estrogen-like Molecules and Protein Biomarkers

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Abstract A novel evanescent-based biosensor (Endotect^{TM}, ThreeFold Sensors, Inc.) was developed with laser-based fiber optics using fluorescent dye-labeled recombinant human estrogen receptor-α (rhERα) and hERβ as probes. A three-tiered approach evaluating various steps in the formation of the estrogen-receptor complex and its subsequent activity was developed for instrument calibration to detect estrogen mimics in biological samples, water and soil. Using this approach, binding affinities and activities of certain known estrogen mimics were determined for their use as calibrator molecules. Results indicated rhERα and rhERβ may be employed as probes to distinguish estrogen mimics with a broad range of affinities. In addition, application of the biosensor for detecting DNA-binding proteins in human tissue extracts was demonstrated. The later studies suggest the biosensor may be used as a clinical laboratory tool for assessing tumor marker proteins.

35.1 Introduction

Estrogen mimics used in clinical management of breast cancer and osteoporosis, e.g. Tamoxifen and Raloxifene respectively, mediate their therapeutic effects through direct interaction with estrogen receptor proteins. Determination of the extent of estrogen mimicry by new generations of these drugs as well as estrogenic compounds encountered in the environment (endocrine disruptor compounds, EDCs) is essential to estimate risk/benefit ratios.

The effects of putative EDCs on the reproductive functions of human and animal species has become an area of intense concern [1]. The Environmental Protection Agency (EPA) has been mandated by Congress to develop a plan for testing some 87,000 chemicals known to be present in the environment to

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determine their potential for producing endocrine disruption. In response to this mandate, the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) was created by the EPA and has issued recommendations for a multi-tier screening program to be executed on 15,000 chemicals beginning in 1999. Tier 1 screening involves assessment of the effects of test compounds on estrogen receptor mediated functions. Additional EDC activities may be due to cancer therapeutics, such as tamoxifen, which was designed to exert endocrine disrupting effects, useful in treating certain reproductive cancers [2]. A thorough understanding of the mechanisms by which each type of effect occurs is required in order that informed regulatory decisions may be made, and useful new drugs may be developed for treating reproductive cancers.

Evidence supporting an aggressive testing program for endocrine disruptors suggests that the reproductive functioning of both animal and human species is being adversely affected and that environmental exposure to chemicals is likely to be involved [1]. In response to this need, a novel biosensor (Endotect™, ThreeFold Sensors, Inc.) was developed to identify compounds in biological samples which contained EDCs, such as estrogen mimics [3–5].

The physical principles of operation of the sensor instrument are explained in detail in previous publications [3–5]. The biosensor technology utilizes an evanescent field generated on the surface of an optical fiber held in a cartridge. The fiber surface possesses covalently-linked molecules (e.g., steroids, ERE sequences, antibodies), to which the molecules of interest are attracted. In the case of the biosensor described, this is either Cy5-labeled recombinant hERα or hERβ proteins. When the fluorophore-labeled molecules are attracted to the ligand on the fiber surface, the fluorophore will be excited by the evanescent field upon binding. Because of the nature of the evanescent field [3–5], fluorophore-labeled molecules in the surrounding solution are not excited, thus kinetic analyses becomes possible.

35.2 Methods

35.2.1 Biosensor

The essential feature of the fiber-optic evanescent biosensor is confinement of fluorescence sensing to the immediate surface of an optical fiber. It takes advantage of the evanescent field produced by total internal reflection of light propagating within the fiber, as described by Hirchfield [6]. Figure 35.1A illustrates the ligand-based optic fiber, in which estrone-1-glucuronide (E1g) has been chemically attached to the fiber surface. Recombinant hER, acting as the probe for the biosensor, was fluorescently labeled by incubation with Cy5 (Amersham Pharmacia Biotech) for 30 min, and separation of Cy5-hER from free Cy5 was performed using a 10DG gravity flow column (Biorad). Cy5-labeled hER is used as a probe in solution, which flows through the cartridge

around the optical fiber. When bound to the ligand E1g on the fiber surface, the evanescent field excites fluorescence of Cy5-hER. In solution, E1g exhibits an affinity for rhERα with an apparent Kd value of 2.5 × 10[–9 M[3]. Figure 35.1B illustrates the estrogen response element (ERE)-based optic fiber, in which Vitellogenin A2 ERE (5′-GATCCGTCAGGTCACAGTGACCTGATG-3′) is bound to the optic fiber in a system similar to that of the ligand-based fiber.

A three-tiered approach was developed with hERα and hERβ consisting of 1) ligand titration and competition arrays [7], 2) gel mobility shift and super-shift assays [8,9] and 3) yeast cell-based bioassays [10] with various known estrogen mimics for instrument calibration to detect suspected estrogen mimics in biological samples, water and soil. The therapeutic tamoxifen associated with recombinant hERα (Fig. 35.2) with lower affinity (Kd = 0.8–2 × 10^{-7} M) than that of hERβ (Kd = 3–7 × 10^{-8} M). Using this approach, binding affinities of other calibrator substances were determined: 4-Hydroxytamoxifen- 2–4 × 10^{-10} M; Clomiphene- 2–5 × 10^{-7} M; Ethynylestradiol- 2–5 × 10^{-10} M; Nafoxidine- 1–3 × 10^{-8} M; and Raloxifene- 3–8 × 10^{-10} M.

For calibration of the ERE-based fiber, the second tier approach was employed using electrophoretic mobility shift assays (EMSA) to identify hER or other proteins that may bind to ERE sequences located upstream of estrogen receptor target genes. Double-stranded ERE sequences (i.e., VitA2, pS2, h-fos, jun, cath D), were labeled with [a [32] P]dATP (Perkin Elmer) [8,9]. EMSA reactions were performed in 40 mM Tris-HCl buffer, pH 8.0, containing 500

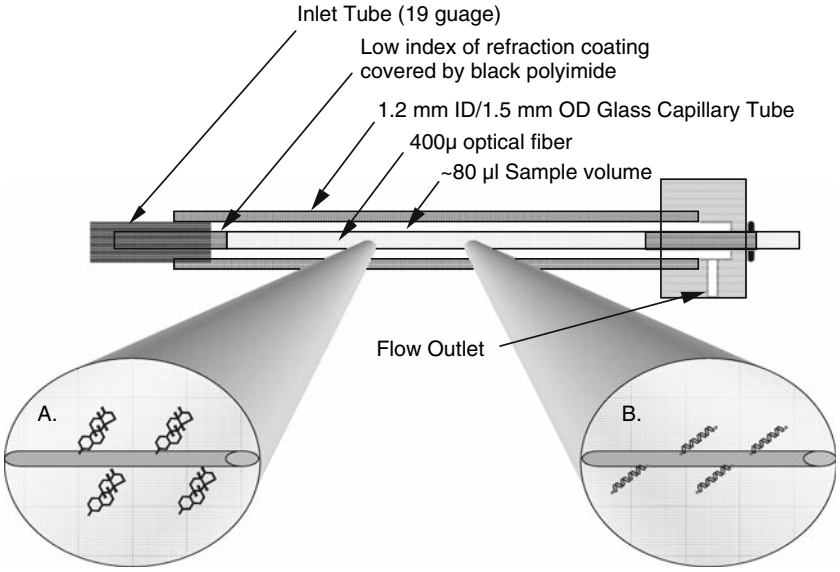

Fig. 35.1 Schematic of biosensor cartridge illustrating the ligand-based (A) and ERE-based (B) optic fibers.

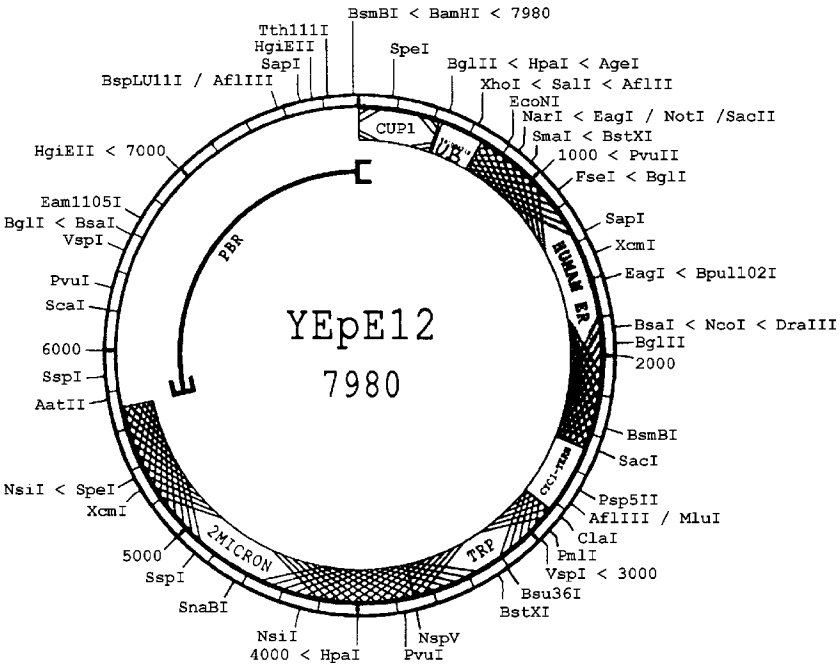

Fig. 35.2 Schematic of plasmid expressing recombinant human estrogen receptor-α (YEpE12) in yeast cells. Adapted from Wittliff et al. 1993 [11].

μM PMSF and 10 μM monothioglycerol [8,9]. rhER and tissue extract preparations were incubated with labeled ERE overnight at 4°C. Electrophoresis was performed as described previously [8,9] using 0.5x TBE running buffer. Gels were dried and exposed to phosphor screens (Perkin Elmer) overnight, and bands representing [[32] P]ERE-protein complexes and free [[32] P]ERE were analyzed using a Cyclone Storage Phosphor System with OptiQuant® software (Perkin Elmer).

These results and others using the three-tiered approach for calibration indicate recombinant hERα and hERβ isoforms may be employed as biosensor probes to distinguish therapeutic estrogen mimics with a broad range of affinities. Currently we are investigating the detection of these and other estrogen mimics using both the ligand-based and the ERE-based fibers with the biosensor with a focus on ascertaining their endocrine-activating and endocrine-disrupting activities.

35.3 Results and Discussion

The biosensor utilizes either a ligand-based or an ERE-based optic fiber to detect estrogen-like compounds in various solutions [3–5]. Figure 35.3A illustrates the association of Cy5-labeled recombinant hER with the ligand-based

fiber producing an increase in fluorescence as detected by the sensor. The ligand-based fiber allows detection of estrogen mimics present in a test solution, which compete for the Cy5-labeled recombinant hER and create a decreased fluorescence. Figure 35.3B illustrates the competition of a test solution for the ERE fiber. The upper curve represents loading of unliganded Cy5-labeled hERα, while the lower curve represents the reaction of Cy5-labeled hERα with an estrogen mimic in a test solution creating diminished fluorescence detected by the sensor. The sensogram results are converted to relative binding affinities using the results of known estrogens and mimics from the three-tiered calibration approach (Table 35.1).

Using the ERE-based fiber-optic biosensor, protein molecules recognizing hormone response element sequences, such as hERα and hERβ may be detected in extracts. The sensograms in Fig. 35.4A indicate the presence of ERE-binding proteins since addition of extracts prepared from two human reference samples decreased Cy5-hERα binding to the ERE fiber. Curves a and b represent two reactions showing association of uncompeted Cy5-hERα (control) with the ERE sequence bound to the optical fiber. Curve c represents a reaction in which an extract of a reference myometrium specimen exhibited 36% inhibition

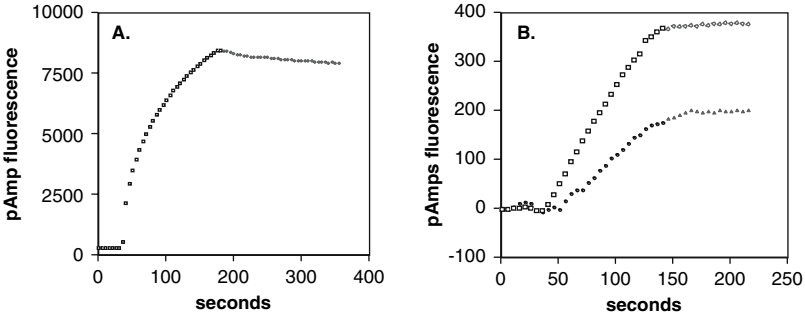

Fig. 35.3 Representative sensograms of Cy5-labeled recombinant human estrogen receptor-α associating with a ligand-based optic fiber (A) and ERE-based fiber (B) with (upper curve) and without (lower curve) competition.

Table 35.1 Comparison of Kd values of binding of various estrogen mimics to rhERα calculated from biosensor data and those obtained by standard radioligand binding methods

Competitor	K_d obtained from biosensor	K_d obtained from radioligand binding
estradiol-17ß	2×10–10 M	1.1×10–10 M
estrone	2×10–8 M	1.8×10–8 M
estriol	2×10–8 M	3.1×10–8 M
DES	2×10–10 M	1.8×10–10 M
zearalenone	2×10–8 M	3.6×10–8 M
tamoxifen	2×10–9 M	1.1×10–9 M

Note: All first digits of the sensor data are 2, because solutions used to determine K_d consisted of concentrations of test compounds which were 2 times some power of 10.

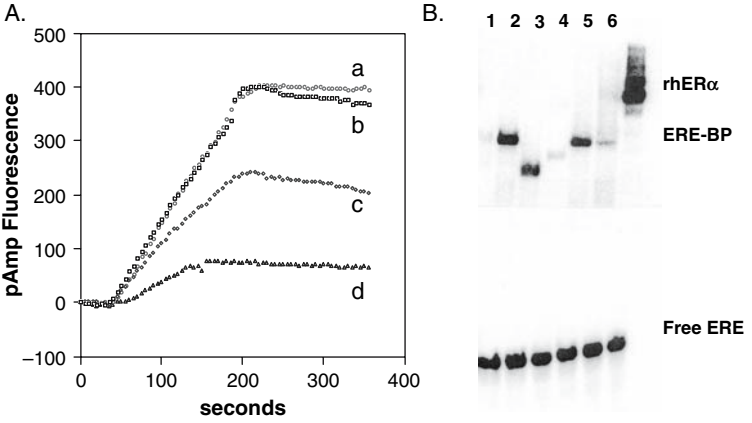

Fig. 35.4 (A) Representative sensogram demonstrating the presence of ERE- binding proteins in extracts of human reference samples of breast tissue. Curves a and b represent loading of Cy5-labeled hERα alone on the ERE-based optical fiber. Curves c and d represent loading of recombinant hERα in the presence of protein extracts of human reference specimens of myometrium (c) and breast cancer (d), showing the decrease in binding indicative of ERE-binding proteins in the tissue preparations. (B) EMSA gel pattern showing migration and distribution of various ERE-binding proteins (ERE-BP) in extracts of five different (lanes 1–5) breast cancers compared to that of recombinant human ERα (rhERα), confirming the results from biosensor measurements.

compared to that of the control. Curve d represents a reaction in which an extract of a reference breast cancer specimen exhibited 68% inhibition.

The degree of inhibition by a tissue extract determined from a sensogram (e.g., Fig. 35.4A) is being calibrated against the results of the same sample obtained by EMSA (e.g., Fig. 35.4B). Preliminary evaluation suggests a good qualitative relationship between measurements taken by the two methods. However, considerably more analyses of various tissue extracts will be required to establish a quantitative relationship. This is due to the physically different conditions of the ERE-binding reactions, i.e., soluble ERE sequences in the EMSA compared to covalently-bound ERE sequences on the optical fiber. Experiments addressing the latter relationship are in progress with the goal to use the biosensor to detect as well as quantify these new biomarker proteins.

Further examination of these activities in human tissue extracts by EMSA revealed the presence of several species of ERE-binding proteins that appear to be unrelated to human estrogen receptor. As shown in Fig. 35.4B, reference tissue extracts exhibited varied abundance of these novel ERE-binding proteins that migrated more rapidly than that of intact recombinant human ERα. Furthermore, certain cytosols contained ERE-binding protein species with different migration properties (e.g., lanes 13). Levels of expression of ERE-binding proteins were determined by scanning each lane of the EMSA gels and using Optiquant^{TM} imaging software for quantification after normalization for the total protein content [8,9]. There was no apparent correlation between the level

of ERE-binding protein and the amount of estrogen receptor in a reference tissue extract, suggesting these proteins recognizing ERE sequences are unrelated to estrogen receptor proteins. Although preliminary, results using super-shift assays of human tissue extracts incubated with monoclonal antibodies prepared against specific epitopes located in various functional domains of rhERα and rhERβ indicated these ERE-binding proteins are not related to known human estrogen receptor isoforms [12].

35.4 Summary and Conclusions

As demonstrated, the biosensor was employed in a laboratory setting to analyze compounds known to express estrogen-like properties, as well as in a practical setting to detect the presence of estrogen mimics with suspected endocrine disrupting activities in lake water, in which deformed frogs were identified [4,5]. Analyses are easily and rapidly performed on extracts to determine the presence of hormone mimics and EDCs in various samples, such as industrial waste, foodstuffs, hospital waste, and ground water. Biosensor instrumentation, optic fiber composition and fluorescent probe selection may be modified readily to analyze other types of molecular interactions including kinetics of ligand-receptor association and dissociation of candidate drugs. Furthermore, our research demonstrated that hormone response element-based fibers may be used as a discovery tool for detecting novel DNA-binding proteins with potential to serve as cancer biomarkers.

The long-term goal of our research is directed toward an understanding of the mechanisms of action by which chemical compounds and ligands exert their influences on receptor-mediated signal transduction. This includes the development and testing of an instrument and methods for screening environmental chemicals for endocrine disrupting potential [1], as a result of their participation in hormone-controlled pathways. Estrogens and certain of their mimics (e.g., phytoestrogens and therapeutic estrogen mimics) appear to influence the rates of association and dissociation of estrogen receptors-α and -β with their cognate accessory proteins (co-activators and co-repressors) as well as the composition of the transactivation complex in the ERE and promoter regions of responsive genes [1]. A new generation of instrumentation is being developed to address these complex issues using a FRET-based evanescent fiber optic sensor (J.L Erb, J. Downward & J.L. Wittliff, unpublished) that provides real time kinetic measurements reflecting association and dissociation of receptors with co-regulatory proteins, response elements and ligands.

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