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RESEARCH PAPER

Prêt-à-porter nanoYESα and nanoYESβ bioluminescent cell biosensors for ultrarapid and sensitive screening of endocrine-disrupting chemicals

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Abstract

Cell-based assays utilizing reporter gene technology have been widely exploited for biosensing, as they provide useful information about the bioavailability and cell toxicity of target analytes. The long assay time due to gene transcription and translation is one of the main drawbacks of cell biosensors. We report the development of two yeast biosensors stably expressing human estrogen receptors α and β and employing NanoLuc as the reporter protein to upgrade the widely used yeast estrogen screening (YES) assays. A viability control strain was also developed based on a chimeric green-emitting luciferase, PLG2, expressed for the first time in *Saccharomyces cerevisiae*. Thanks to their brightness, NanoLuc and PLG2 provided excellent sensitivity, enabling the implementation of these biosensors into low-cost smartphone-based devices. The developed biosensors had a rapid (1 h) response and reported on (anti)estrogenic activity via human estrogen receptors α and β as well as general sample toxicity. Under optimized conditions, we obtained LODs of 7.1 ± 0.4 nM and 0.38 ± 0.08 nM for E2 with nanoYESα and nanoYESβ, respectively. As a proof of concept, we analyzed real samples from plants showing significant estrogenic activity or known to contain significant amounts of phytoestrogens.

Keywords Bioluminescence · Biosensor · Luciferase · Smartphone · Estrogen receptor

Introduction

Endocrine-disrupting compounds (EDCs) are chemicals able to disrupt normal endocrine function, thus causing adverse effects on human and animal health [1]. Many of these compounds, either of natural and synthetic origin, affect the hormone systems of living organisms through very different mechanisms of action. Long-term exposure to these substances, even at low

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doses, may lead to severe pathological conditions including obesity, endometriosis, infertility, and cancer [2]. Several pathways are involved, including androgens, estrogens, and thyroid axes. In particular, interaction of EDCs with the estrogenic receptors ERα and ERβ alters the normal hormone biosynthesis, signaling, or metabolism, affecting the growth and differentiation of many organs such as the ovary, mammary gland, prostate, nervous system, cardiovascular system, and bones [3]. The cost of EDC-associated diseases in the European Union was estimated to be in the range of € 81–269 billion per year. The most considerable costs were associated with IQ loss and intellectual disability after prenatal organophosphate exposure and adult obesity attributed to phthalate exposure [4].

Therefore, there is a growing demand for new, fast, and reliable methods suitable for EDC detection both in laboratory settings and for on-site analyses [5]. Many analytical methods such as high-performance liquid chromatography associated with mass spectrometry (HPLC-MS) have been used in recent years, providing low limits of detection and high reproducibility. However, these techniques require sophisticated equipment and extensive sample processing. Furthermore, they do not provide information about bioactivity of the detected substances and are unsuitable for real-time, cost-effective on-field testing [6].

Such information can be obtained using biosensors employing a properly chosen biological recognition element [7]. Indeed, whole-cell biosensors relying on living cells provide information about the bioavailability of analytes or classes of analyte that is the fraction of analyte that is able to permeate the cell membrane and interact with specific molecular targets. Cell-based assays or bioassays are able to measure receptor-mediated effects due to the reprogramming of cells to express a reporter protein after activation of a specific receptor. Several bioluminescent (BL) bioassays and biosensors have been developed by genetically engineering cells with reporter proteins, providing a BL signal proportional to the activation of the target molecular pathway [8]. Furthermore, advances in light detection technology have made it possible to integrate these BL biosensors into portable analytical devices [9–12] suitable for environmental monitoring, food control, and anti-doping screening [13–16].

Moreover, such systems are able to detect chemicals according to their mechanism of action, irrespective of their chemical structure, and also to provide information on their combined effect (synergistic and/or antagonist and toxicity). This capability is highly valuable considering that new molecules are added every year to the watch list of EDCs, with many compounds still unclassified. Also, the US Environmental Protection Agency (EPA) highlighted the importance of high-throughput screening (HTS) with the Toxicity ForeCaster (ToxCast™) Program, obtaining data about thousands of chemicals in hundreds of mammalian-based HTS assays [17].

Mammalian-based HTS are formidable tools to provide data about toxicity and biological activity; however, it is well known that these assays can be highly demanding in terms of assay cost and time. Laboratories need to be equipped with cell culture facilities and incubation times from 5 to 48 h may be necessary for the production of reporter protein [18]. Although mammalian cells provide highly valuable data, yeast cells have many advantages, such as increased robustness, low cost of reagent, absence of endogenous estrogen receptor, and easier handling. Yeast-based assays and biosensors for EDCs generally show lower sensitivity with limits of detection about one order of magnitude higher than those of analogous assays using vertebrate cells [19]. Extraction and pre-concentration of the samples is thus generally required for EDC monitoring in environmental samples with yeast-based assays. Inevitably, this pre-analytical step increases the assay time and may cause higher aspecific cytotoxicity that, if not correctly assessed, could cause artifacts.

We tried to address these limitations in terms of the sensitivity, portability, and response time of yeast biosensors for EDCs by exploiting a newly developed luciferase, NanoLuc, to obtain a set of robust biosensors capable of detecting EDCs acting on estrogen receptors α and β. The applicability of a yeast codon–optimized variant of NanoLuc luciferase (yNLucP) as a reporter protein for yeast biosensors has been previously demonstrated [20, 21]. We immobilized the yeast biosensors in a 3D-printed cartridge and implemented the assay in a portable configuration by exploiting a compact camera as the detector [20]. Here, we report on the development of a new engineered strain expressing the yNLucP under the regulation of ERβ. We also engineered a second reporter strain with a chimeric luciferase, named PLG2, containing the N-domain of *Photinus pyralis* luciferase joined to the C-domain of *Luciola italica* luciferase [22]. PLG2 is a thermostable luciferase that produces a bright BL signal and is resistant to low pH shifting, making it well suited for cell-based assays. The second reporter strain constitutively expressing PLG2 luciferase was used as a viability reporter to correct the analytical signal according to cell viability.

We demonstrated the suitability of these recombinant strains for high-throughput sensitive analysis both inside and outside the laboratory using a luminometer and a mobile phone camera as detectors. We obtained good analytical performance and very rapid response times with incubation times ranging from 30 min to 1 h. The suitability of these biosensors for the analysis of real samples was also investigated using soybean and alfalfa extracts. Estrogenic activity of plant extracts determined with the smartphone biosensor well correlated with estrogenic activity measured with benchtop instrumentation.

Materials and methods

Chemicals and reagents

All the reagents required for yeast cell culture maintenance were purchased from Sigma-Aldrich (St. Louis, MO). Synthetic complete (SC) liquid medium was prepared containing 6.7 g/L yeast nitrogen base w/o amino acids, 1.4 g/L yeast synthetic drop-out medium supplement, and 10 mL/L adenine hemisulfate solution. The solution was autoclaved and then 40 mL/L of glucose 50% *w*/*v* solution (0.22-μm filter sterilized) was added. SC medium was supplemented with the required amino acids L-histidine (2 g/L), L-leucine (0.1 g/L), L-tryptophan (0.02 g/L), and uracil (0.02 g/L). The chemicals required for validation, all of analytical grade, and all reagents required for bacterial cell culture, the kit for plasmid extraction, and *Escherichia coli* (JM-109)-competent cell were purchased from Sigma-Aldrich (St. Louis, MO). FastDigest restriction enzymes, FastAP, and T4 DNA ligase required for cloning and yeast protein extraction reagent (YPER) were from Thermo Fisher Scientific (Waltham, MA, USA). The bioluminescent substrate furimazine was from Promega (Madison, WI, USA). All other chemicals were purchased from Sigma-Aldrich.

NanoLuc estrogen α and β responsive *Saccharomyces cerevisiae*

The reporter vector pRSII426-ERE-yNLucP containing five copies of an ERE response element (-AGGTCAgagTGACCT-) upstream of a minimal cytochrome C promoter (CYCmin) driving the expression of the yNLucP coding sequence has been described elsewhere [20]. The yeast expression plasmid pBEVY-L (Leu2 marker) was from Addgene (Cambridge, MA, USA). The sequence encoding the human estrogen receptor β was amplified with polymerase chain reaction (PCR) using the template pSG5hERβ [23]. The sequence was cloned into pBEVY-L vector under the control of constitutive ADH1 promoter, using *KpnI* and *EcoRI* sites. The generated vector was called pBEVY-ERβ-L.

The yeast codon–optimized version of the NanoLuc-PEST luciferase (yNLucP) has been previously described in [21]. The sequences were confirmed by restriction analysis and sequencing. The yeast *S. cerevisiae* BMA64-1A (MATa, ura3-52, trp1Δ2, leu2-3, 112his3-11, ade2-1, can1-100) wild-type strain [24] was transformed with plasmids pRSII426-ERE-yNLucP and pBEVY-ERβ-L using the LiAc/SS-DNA/PEG method [25]. Colonies were selected in SC-ura-leu plates after incubation at 30 °C for 4 days; 15% glycerol stocks of the recombinant strain were prepared and stored at − 80 °C. The procedure for obtaining *S. cerevisiae* harboring plasmids pBEVY-L-ERα and pRSII426-ERE-yNLucP is described elsewhere [20]. The NanoLuc yeast estrogen screen α and β strains were named nanoYESα and nanoYESβ, respectively.

Laboratory-based nanoYESα and nanoYESβ assays

For the nanoYESα and nanoYESβ assays, an overnight culture of a single colony was used to inoculate 5 mL SC medium containing the selected amino acids to optical density (OD_{600}) of 0.6. This culture was grown at 30 °C with orbital shaking at 200 rpm for about 4 h until $OD_{600} = 1$ was reached. Then, 160 μL of the culture was dispensed in 96-well microplates and incubated at 30 °C with different concentrations of E2 (from 0.001 to 100 nM) at 1% ethanol final concentration. One percent ethanol final concentration was used as control (CTR). Different incubation times (from 5 to 120 min) were tested. Then, 50 μL of YPER-Nano BL substrate [20] was added and BL emission kinetics were recorded using a Varioskan Flash multimode reader (300-ms integration time). Light emission was expressed as relative light units (RLU). The detection limit is defined as the E2 concentration that corresponds to the blank signal plus three times the standard deviation. All experiments were performed in triplicate and repeated at least three times.

In-house validation of nanoYESα and nanoYESβ assays

The Endocrine Disruptor Screening Program Test Guidelines from the EPA (https://www.regulations.gov/document?D=EPA-HQ-OPPT-2009-0576-0006) were used as guidelines for in-house validation. We tested 17β-estradiol (50-28-2; ≥ 97%), 17α-estradiol (57-91-0; ≥ 98%), 17α-methyltestosterone (58-18-4; ≥ 97.0%), diethylstilbestrol (56-53-1; 99.5%), 17α-ethinylestradiol (57-63-6; ≥ 98%), hexestrol (84-16-2; ≥ 98%), genistein (446-72-0; ≥ 98%), estrone (53-16-7; ≥ 99%), butyl paraben (94–26-8; ≥ 99%), 1,3,5-Tris(4-hydroxyphenyl)benzene (15797-52-1; 97%), dibutyl phthalate (84-74-2; 99%), atrazine (1912-24-9; 97.4%), and tamoxifen (10540-29-1; ≥ 99%).

Stock solutions of all reagents required for validation were prepared in ethanol (final vehicle concentration 1%) directly before use. Each plate contains wells treated with 1-nM final concentration of 17β-estradiol as positive controls, and a negative control (CTR) of 1% ethanol (final concentration). For the anti-estrogenic activity screening, chemicals were co-incubated with 1 nM of 17β-estradiol. All chemicals were tested as shown above, with an incubation time of 30 min. The detection limit is defined as the concentration that corresponds to the blank signal plus three times the standard deviation. All experiments were performed in triplicate and repeated at least three times.

Construction of BL *S. cerevisiae* strain constitutively expressing PLG2

The yeast expression plasmid pRSII425 (Leu2 marker) and p405TEF1 were from Addgene (Cambridge, MA, USA). The TEF1 promoter was cloned from p405TEF1 into pRSII425 with *Sac*I and *Xba*I. PLG2 luciferase was amplified by polymerase chain reaction (PCR) and cloned into pRSII425 using *BamHI* and *XhoI*. The final vector was named pRSII425-TEF1-PLG2. The sequences and vectors were confirmed by restriction analysis and sequencing. Yeast *S. cerevisiae* BMA64-1A (MATa, ura3-52, trp1Δ2, leu2-3, 112his3-11, ade2-1, can1-100) wild-type strain was used as the recipient strain [24] and transformed with plasmid pRSII425-TEF1-PLG2 using the LiAc/SS-DNA/PEG method. Colonies were selected from SC-leu plates after incubation at 30 °C for 4 days, and 15% glycerol stocks of the recombinant strain were prepared and stored at − 80 °C. The obtained strain was named ToxYLuc.

Laboratory-based toxicity assay with ToxYluc

An overnight culture from a single colony was used to inoculate SC-leu medium at an optical density (OD_{600}) of 0.6. This culture was grown at 30 °C with orbital shaking at 200 rpm for about 4 h until $OD_{600} = 1$ was reached. Then, 160 μL of cell culture was incubated with different concentrations of all chemicals at 30 °C for 30 min, in 96-black well microplates. The BL signal was acquired using a Varioskan Flash multimode reader (5 min with 300-ms integration time) after the addition of 50 μL of YPER-Bright-Glo™ BL substrate, containing 500 μL of Bright-Glo™ diluted in 500 μL of YPER lysing reagent (YPER-Glo). Light emissions were expressed as RLU. All experiments were performed in triplicate and repeated at least three times.

Smartphone-based biosensor with 3D-printed cell minicartridge and smartphone adaptor fabrication

The minicartridge and smartphone adaptors were fabricated using a desktop 3D printer Makerbot Replicator 2X (Makerbot, Boston, MA, USA) using thermoplastic acrylonitrile butadiene styrene (ABS) polymer as previously described [20]. The open-source Tinkercad browser-based 3D design platform (Autodesk, Inc.) was used to create 3D models. MakerWare v. 2.4 software was used to set up printing options. The cartridge contains an array of 16 wells of 50 μL each (3.5 mm × 3.5 mm × 4.5 mm). The adaptor, providing a dark box to avoid ambient light interference, was designed to fit the OnePlus 5 smartphone (OnePlus, Shenzhen, China).

Smartphone-based biosensor procedure

The OnePlus 5-based platform have been used for integration of nanoYESα, nanoYESβ, and ToxYluc into a portable device for ultrarapid on-field analysis. Briefly, nanoYESα, nanoYESβ, and ToxYluc overnight cultures were diluted in the morning to optical density (OD_{600}) of 0.6 and grown for about 4 h until an $OD_{600} = 1$ was reached. To prepare a cartridge integrating nanoYESα, nanoYESβ, and ToxYluc, the cultures were centrifuged and concentrated 10× in SC medium. Subsequently, 90 μL of cell suspension (about 9.0×10^5 cells/well) and 10 μL of samples (0.1% ethanol final concentration) were added in each well and incubated for 1 h at room temperature (24 °C). A 50-μL volume of YPER-Nano was added to wells containing nanoYESα, nanoYESβ, and a 50-μL volume of YPER-Glo substrate was added to wells containing ToxYluc. BL emission was acquired with OnePlus 5 camera for 30 s, ISO 3200. ImageJ software was used for BL signal measurement by selecting square regions of interest (ROI) around each well.

Analytical performance of the developed platform was assessed by incubating yeast estrogen biosensors α and β with increasing concentrations (from 0.001 to 40 nM) of E2, selected as the model estrogenic analyte. All measurements were performed in duplicate and repeated at least three times with different cell cartridges.

Real samples

Nine plant samples were analyzed for (anti)estrogenic activity with nanoYESα and nanoYESβ.

Five were soybean (*Glycine max* L.) samples: A, 95% methanol (*v*/*v*) extract of the same liquid cell culture previously analyzed by Sansanelli et al. [26] (2014); B, 95% methanol (*v*/*v*) extract analogues of a freshly grown liquid cell culture; C, 95% methanol (*v*/*v*) extract of callus culture grown on solid media; SE, industrial water-based seed extract, and DSE, industrial water-based digested seed extract (Phenbiox Srl, Bologna, Italy), treated for 24 h at room temperature with xylanase, α-amylase and glucosidase, as previously reported [26].

In addition, four alfalfa (*Medicago sativa* L.) samples were obtained from an industrial alfalfa aqueous extraction obtained after a double-screw press and squeezing at high pressure (Alfavita Srl, Ravenna, Italy): D, not treated extract, incubated for 2 h at 60 °C; E, extract treated with 1% (g enzyme/g dry weight) Neutrase 0.8 L (Novozymes A/S, Denmark) for 2 h at 60 °C and pH 7.0; F, extract treated with 1% Alcalase 2.4 L (Novozymes A/S, Denmark) for 2 h at 60 °C and pH 7.0; and G, extract treated with 1% papain (Sigma-Aldrich, Milan, Italy) for 2 h at 60 °C and pH 7.0.

Estrogenic and anti-estrogenic activity of all samples were carried out both with the laboratory-based nanoYESα and

nanoYESβ assays and the smartphone biosensor with an incubation time of 30 min, at 30 °C. Sample toxicities were also evaluated using at least two dilutions for each sample (1:10 and 1:1000) as described in the "Laboratory-based toxicity assay with ToxYluc" section. Real samples analysis with smartphone-based platform was performed as described for E2 samples, in duplicate with nanoYESα, nanoYESβ, and ToxYluc. The toxicity strain was integrated with nanoYESα/nanoYESβ into the same cartridge for simultaneous evaluation of cell viability and (anti)estrogenic activity of real samples with co-incubation with 1 nM of E2.

Then, different concentrations of E2 were added to tap water to prepare the spiked samples with low, medium, and high E2 concentrations. The spiked samples were analyzed using both nanoYESα and nanoYESβ laboratory-based assays as previously described.

Data analysis

All calculations were performed using Microsoft Office Excel 2016 and plotted using GraphPad Prism v.5 (GraphPad Software, Inc. La Jolla, CA). Non-linear regression was performed by fitting the experimental data using a four-parameter sigmoidal curve; EC50 values were calculated by the software as the effective concentration that produces the midpoint y value (50%) of the dose-response curve. The detection limit was calculated as the concentration that corresponded to the blank signal plus three times the standard deviation.

The fold induction (FI) of the nanoYES by the sample was calculated as:

$$FI = BLS/BLB$$

where BLS and BLB are the bioluminescence of the nanoYES with sample and blank solvent, respectively (measured in at least three replicates). At least three independent experiments were used to calculate mean FI values. To consider aspecific effects on cell viability, and correct the analytical signal accordingly, a control strain, ToxYLuc, was developed and used to calculate a correction factor (CF):

$$CF = BB/BS$$

where BB is the BL of the control strain ToxYLuc with blank solvent (1% ethanol) and BS is the BL of the control strain with the sample. When CF was in the range 0.5–2.0, the induction coefficient of the nanoYES with the samples was corrected according to the formula:

$$CFI = CF \times FI$$

where corrected fold induction (CFI) is the fold induction corrected for sample toxicity.

When the CF was higher than 2.0, the sample toxicity was considered too high and an additional sample dilution was performed before the measurement All experiments were performed in quadruplicate and repeated at least three times.

Results and discussion

Luciferase selection and assay design

In the present work, we addressed some of the main drawbacks of yeast-based bioreporters for detection and monitoring of endocrine disruptors, i.e., assay time, sensitivity, and portability. This study was prompted by promising results obtained by a recently developed BL yeast-estrogen screen, named nanoYES [20]. Here, we exploited a low-cost, compact camera as a light detector and 3D-printed cartridges and report the development of two new BL yeast strains: a strain for detection of (anti)estrogenic compounds acting on ERβ and a viability control strain for correcting the analytical signal according to aspecific effects on cell viability.

The newly developed yeast-estrogen strain was obtained by genetically engineering *S. cerevisiae* with plasmid pBEVY-ERβ-L for constitutive expression of hERβ under the regulation of ADH1 promoter and pRSII426-ERE-yNLucP carrying the constitutive pRSII426-ERE-yNLucP containing five copies of an ERE response element upstream of minimal cytochrome C promoter (CYCmin) which drives the expression of the yeast codon-optimized NanoLuc luciferase coding sequence (yNLucP). The vectors contain the 2μ origin for high copy number replication during cell division (50 copies/cell) and consistent production of yNLucP and hERβ. To avoid unrelated toxicity and artifacts in the biosensor response, the expression of the human ERβ was placed under a weak promoter (ADH1) and the destabilized version of yNanoLuc luciferase was chosen as the reporter gene. The use of destabilized luciferases enables detection of rapid changes in gene expression (both increases and decreases) which may not be detectable with stable luciferases that accumulate in living cells due to their long half-life [27]. Therefore, yNanoLuc, which has a half-life of 5 min when expressed in yeast cells [21], and was previously demonstrated suitable for implementation into portable devices with mobile camera detection, was selected as the reporter protein. We then selected another bright luciferase, PLG2, as the constitutive reporter for the viability control strain. PLG2 contains amino acid changes providing activity enhancement in comparison to the chimeric PpyLit, partial protection against red-shifting at low pH, and extended glow emission kinetics. The PLG2 luciferase provided about 3.0-fold greater sensitivity than luciferase from *P. pyralis* when expressed in mammalian cell lines (Hek293); however, the expression of this luciferase has not been reported yet in yeast [22]. Figure 1 shows the bioluminescence emission spectrum and kinetics obtained in yeast cells in lysing and non-lysing conditions. A maximum

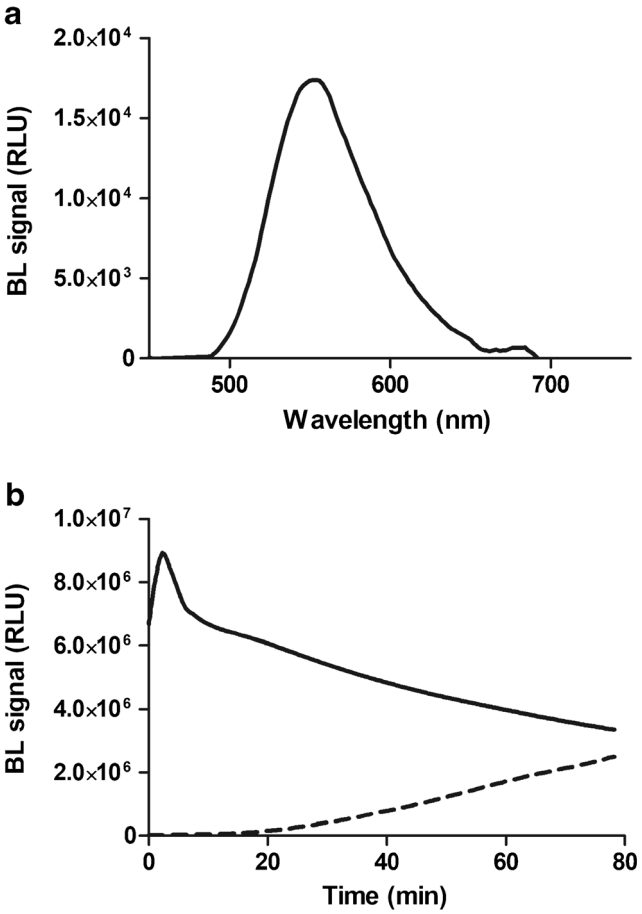

Fig. 1 **a** Bioluminescent emission spectrum obtained with *S. cerevisiae* expressing the PLG2 luciferase. **b** Emission kinetics of PLG2 constitutively expressed in *S. cerevisiae* obtained with YPER-Bright-Glo™ BL substrate (solid line) and Bright-Glo™ BL substrate (dotted line) (see the "Materials and methods" section for experimental details)

emission peak was obtained at 554 nm, consistent with previously reported characterization of this luciferase when expressed in other cell models. Using a commercially available Bright-Glo™ BL substrate, optimized for mammalian cell lines, the kinetic measurements showed non-optimal kinetics, whereas in lysing condition kinetics with a customized YPER-Bright-Glo™ substrate, we obtained a stable measurement that decreases about 50 min after substrate. An optimal acquisition time window was identified between 108 and 192 s, when the mean BL signal is 97 ± 3% of maximum emission.

Laboratory-based assay procedure analytical performance

Dose-response curves for 17β-estradiol (E2) (concentration range 0.0001 to 100 nM) were obtained with different incubation times, from 5 to 120 min, using nanoYESα and nanoYESβ, to identify the best assay conditions (Fig. 2 and Electronic Supplementary Material (ESM) Fig. S1). Unexpectedly, even after 5 min, a dose-response curve was obtained showing a LOD of 1.08 ± 0.02 nM and 0.015 ± 0.001 nM for nanoYESα and nanoYESβ, respectively. It was observed that dose-response curves after 30-min incubation provided similar LOD than those obtained with 120-min incubation with analyte. Longer incubation times lowered the EC_{50}, but did not provide better LOD. Under optimized assay conditions and 30-min incubation times, a LOD of 0.016 ± 0.001 nM and an EC_{50} of 1.47 ± 0.06 nM were obtained for the ERα strain, while a LOD of 0.0011 ± 0.0002 nM and EC_{50} of 0.10 ± 0.01 nM were found for the ERβ strain (Table 1). Values for ERα were similar to those obtained with analogous yeast-based assays. To the best of our knowledge, this response time is the fastest reported to date in yeast-based transactivation assays. In fact, the YES assay requires 72-h incubation at 32 °C with the analyte according to the original protocol based on colorimetric detection developed by Routledge and Sumpter [28] and modified by Rajapakse et al. [29]. Thanks to implementation of chemiluminescent β-galactosidase substrates, the sensitivity and timing of the assay were improved by Balsiger et al., leading to a 4-h assay with yeast bioreporters expressing ERα with a LOD for E_2 of 0.7 nM and an EC_{50} of 0.15 ± 0.01 nM [30]. Concerning yeast bioassays with ERα, recent yeast-based bioreporters based on bioluminescence (lux operon) or fluorescence detection provided similar LOD and EC_{50} values with incubation times ranging from 2.5 to 6 h [31–34].

With the nanoYESβ strain, we significantly improved the LOD in comparison with previously reported assays. Our results agree with those previously obtained by Bovee et al. who reported a maximal activation of the ERβ cytosensor of about 40% of that obtained with the analogous ERα cytosensor [31]. As a result, the EC_{50} values for E_2 decreased (0.06 nM in ref. [31]). The nanoYESβ sensitivity could be explained by the emission properties of the NanoLuc. The use of a customized substrate provided a 20% increase in BL emission when compared to furimazine, with glow-type kinetics (signal half-life > 20 min) compared to NanoGlo® substrate or furimazine alone (signal half-life < 2 min) [20].

Specificity of the nanoYESα and nanoYESβ

The analytical performance was first assessed by measuring the response of the nanoYES to E_2 and other molecules according to EPA Endocrine Disruptor Screening Program Test Guidelines (OPPTS 890.1300: Estrogen Receptor Transcriptional Activation - Human Cell Line HeLa 9903) (https://www.regulations.gov/document?D=EPA-HQ-OPPT-2009-0576-0006). Table S1 (see ESM) shows LOD and EC_{50} values for reference chemicals used in the proficiency test. For the acceptability criteria, we first analyzed positive and negative reference chemicals to verify the responsiveness of the nanoYESα and nanoYESβ using the appropriate

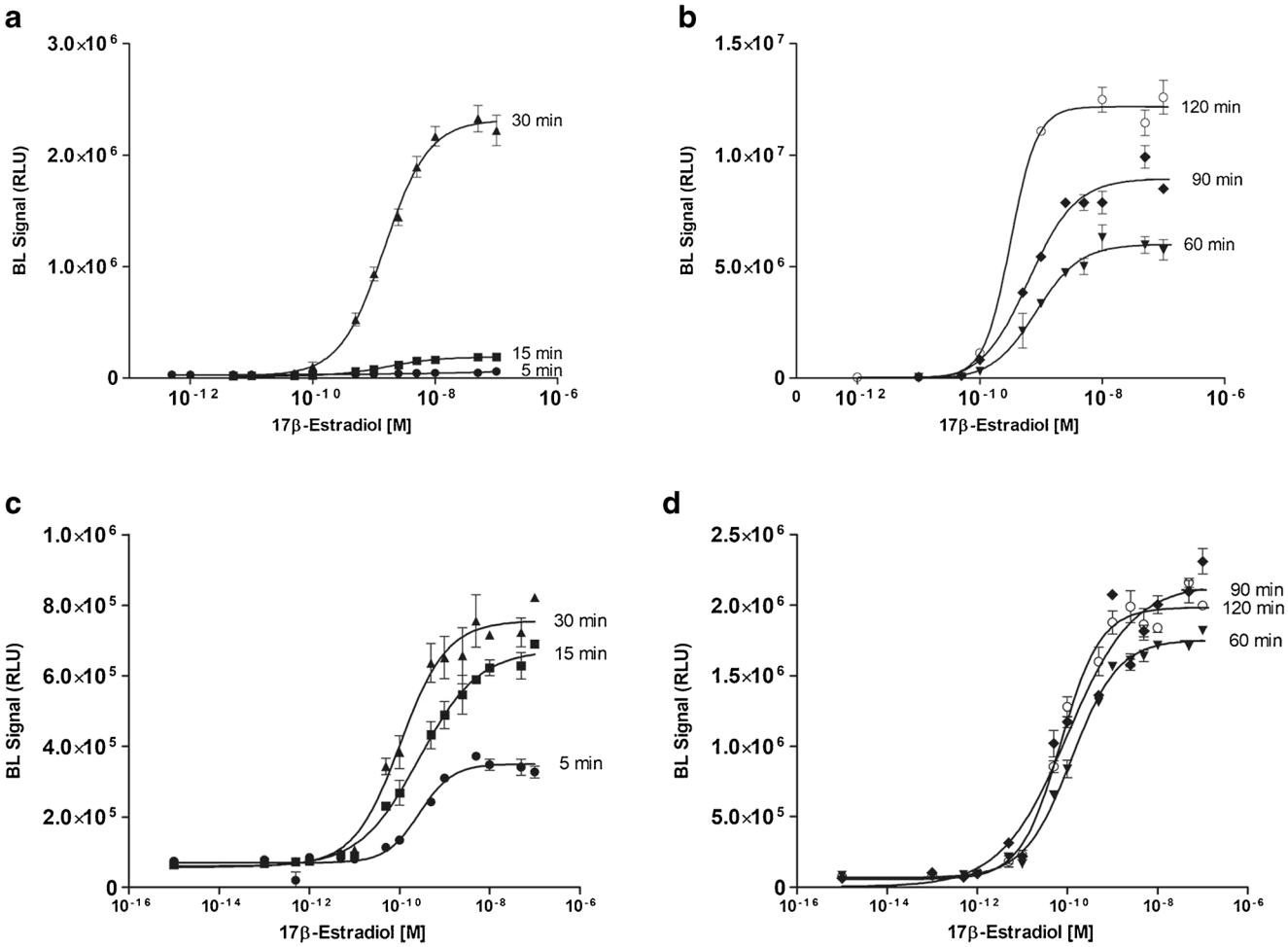

Fig. 2 Dose-response curves for 17β-estradiol obtained using the nanoYESα (**a**, **b**) and nanoYes β (**c**, **d**) at different incubation times (5, 15, 30, 60, 90, and 120 min). Experiments were performed in 96-black well microplate format and benchtop instrumentation using 160 μL of cells and 40 μL of 17β-estradiol solution (concentration range from 0.0001 to 100 nM). Data represent the mean values ± the standard deviation (SD) obtained with three replicates

concentrations of a strong estrogen E2, a weak estrogen (17α-estradiol), a very weak agonist (17α-methyltestosterone) and a negative compound (dibutyl phthalate).

We reported atypical response for some compounds like 1,3,5-Tris(4-hydroxyphenyl)benzene, whose activity, also according to the Endocrine Disruptor Screening Program [1], has a dual estrogenic/antagonistic behavior. Due to this activity, a non-conventional dose-response curve was obtained, and LOD and EC_{50} could not be calculated.

Obtained results (shown in Fig. 3) were within the acceptable range values of the guideline. We calculated the inter- and intra-assay variability (%CV) of the EC_{50} values from individual E2 standard curves and reported %CV of 23.9% and 17.4% for nanoYESα and nanoYESβ, respectively. Anti-estrogenic

Table 1 Limit of detection and half maximal effective concentration obtained with nanoYESα and nanoYESβ in a 96-black well plate format with a benchtop luminometer

Time	LOD	ERα (nM) EC_{50}	LOD	ERβ (nM) EC_{50}
5 min	1.08 ± 0.02	4.06 ± 0.03	0.015 ± 0.001	0.27 ± 0.03
10 min	0.71 ± 0.08	3.05 ± 0.5	0.012 ± 0.001	0.23 ± 0.02
15 min	0.038 ± 0.004	1.69 ± 0.09	0.0007 ± 0.0001	0.27 ± 0.03
30 min	0.016 ± 0.001	1.47 ± 0.06	0.001 ± 0.0001	0.1 ± 0.01
60 min	0.024 ± 0.003	0.83 ± 0.07	0.002 ± 0.0003	0.12 ± 0.01
90 min	0.021 ± 0.003	0.71 ± 0.06	0.0005 ± 0.0003	0.095 ± 0.011
120 min	0.01 ± 0.02	0.32 ± 0.06	0.0011 ± 0.0001	0.077 ± 0.006

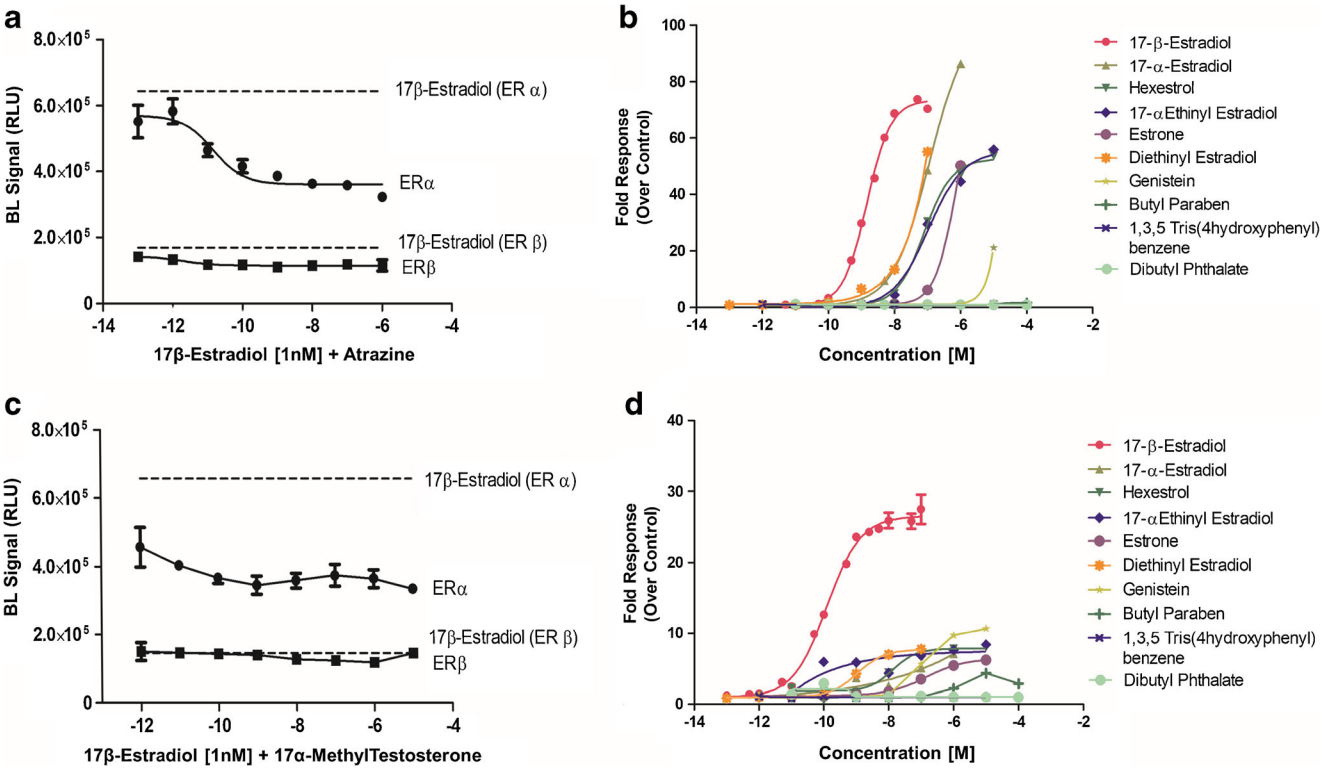

Fig. 3 Evaluation of anti-estrogenic activity of atrazine (**a**) and 17α-methyltestosterone (**c**) obtained by co-incubating nanoYESα and nanoYESβ in 96-black well plate with atrazine (from 0.0001 to 10^3 nM) and 17α-methyltestosterone (from 0.001 to 10^4 nM) with 1 nM of 17β-estradiol for 30 min. BL measurements were obtained after the addition of YPER-Nano BL substrate. Dose-response curves for different chemicals used for the validation of nanoYESα (**b**) and nanoYESβ (**d**) according to EPA Endocrine Disruptor Screening Program Test Guidelines

activity of some well-known anti-estrogenic compounds (i.e., atrazine and 17α-methyltestosterone) was also evaluated (as shown in Fig. 3a and c) by co-incubating nanoYESα and nanoYESβ with 1 nM of 17β-estradiol and different concentrations of the compounds. A decrease of the BL signal inversely proportional to the concentration of the anti-estrogenic compounds was reported for both strains, being more pronounced with nanoYESα. These results confirmed the potential applicability of nanoYESα and nanoYESβ for detecting anti-estrogenic compounds.

As shown in Fig. 3b and d, in agreement with previously published yeast bioassays biosensors, several compounds have different ERα or ERβ agonist activities, such as genistein, a phytoestrogen with a high affinity for estrogen receptor ERβ [35, 36]. We obtained a LOD for genistein of 849 ± 25 nM and 4.68 ± 0.08 nM with nanoYESα and nanoYESβ, respectively. For some chemicals, the relative potencies differed from previous reporter strains. Concerning estrone, which is included in the surface water watch list adopted by the European Commission, we reported a LOD of 7.1 ± 0.5 and 0.009 ± 0.001 with nanoYESα and nanoYESβ, respectively. As an example, we reported a REPβ, calculated as the ratio between the EC_{50} of E2 and the EC_{50} of the compound with ERβ strain, of 0.1 for diethylstilbestrol (ESM Table S1) whereas Bovee et al. reported a REPβ of 2.0 [31]. Despite these discrepancies, that are usually reported for cell-based assays, the LOD for E2 obtained with the nanoYESβ, i.e., 0.0011 ± 0.0002 nM (corresponding to 0.27 ng/L), corroborates the possible use of this biosensor as a rapid screening tool of pharmaceuticals in surface waters according to the European Directive 2013/39/EU, which included E2, estrone, and 17α-estradiol on the watch list. In fact, according to a recent proposal for amending the priority substance list (European Commission 2011), the proposed environmental quality standards (EQS) for E2 of inland surface water are 0.4 ng/L [37, 38].

Smartphone-based biosensor integrating nanoYESα, nanoYESβ, and ToxYLuc

The suitability of using the smartphone camera to detect the BL emitted by living cells has been previously reported by us and others with mammalian cells lines and bacteria [39–41]; however, to date, yeast biosensors have been integrated into devices with portable detectors (e.g., cooled CCD, GoProHero5 CMOS camera) but not coupled with smartphones. Despite yeast's great peculiarities, such as relatively short assay times and the possibility to analyze

environmental complex samples directly without extraction, sterilization, and/or concentration, their implementation in "prêt-à-porter" devices is still an open issue. Therefore, we focused our effort on the development of a very straightforward smartphone-based assay. We adapted a protocol previously developed with a biosensor exploiting the GoPro camera with some changes. The procedure includes incubation of 10 μL of sample (or E2 dilutions in the concentration range from 0.05 to 10 nM) per cartridge well containing 90 μL of cells (about 9.0×10^5 cells/well) for 1 h at room temperature. After the addition of 50 μL YPER-Nano substrate or YPER-Bright-Glo, the cartridge is snapped into the 3D-printed accessory and acquisition is performed with the OnePlus 5 integrated camera, a 1/2.8" 16-MP Sony IMX 398 sensor, using 3200 ISO sensitivity and 30-s acquisition (Fig. 4). We selected the OnePlus 5 smartphone because the shutter speed can be controlled over a longer period time (up to 30 s) and, with our in-house comparison of different smartphone cameras (data not shown), we were able to obtain the best results for low-light applications. This finding was in agreement with a recent inter-smartphone comparison performed by Kim et al. who reported a device and an imaging-processing algorithm to improve the sensitivity of smartphone cameras for low-light detection [40].

To demonstrate the biosensor capabilities and assess the feasibility of the smartphone-integrated CMOS for BL light detection, we integrated in the same cartridge the three strains: nanoYESα, nanoYESβ, and the viability control strain ToxYLuc. Under optimized conditions, we obtained a LOD of 7.1 ± 0.4 nM and 0.38 ± 0.08 nM for E2 with nanoYESα and nanoYESβ, respectively (Fig. 5a, b).

We assessed the reproducibility of the nanoYESα and nanoYESβ biosensors by testing the same concentration of E2 (1nM) and intra-assay variability of 18% and 13% was obtained using the same cartridge for nanoYESα and nanoYESβ, respectively, while an inter-assay variability of 25% and 18% was obtained with different cartridges prepared starting from different cultures.

Real sample analysis

To test the proposed smartphone-based biosensor integrating nanoYESα, nanoYESβ, and ToxYLuc on real samples, we assessed the estrogenic activity of industrial soybean seed extracts and cell suspensions (Fig. 5), previously characterized using the standard analytical technique HPLC–DAD [26], as well as alfalfa extracts.

Sansanelli et al. reported that soybean cell suspensions contain higher amounts of free isoflavones in comparison to seed extracts (i.e., 93.5% vs 16.8%) and they are more bioactive than the glycoside forms. Figure 6 shows results corrected for toxicity obtained with the nanoYESα and nanoYESβ using benchtop instrumentation. Toxicity effects used for correcting the estrogenic activity are shown in ESM Fig. S2. As an example, sample A, containing extract of soybean cell suspensions (dilution 1:100) with a prevalence of aglycones such as

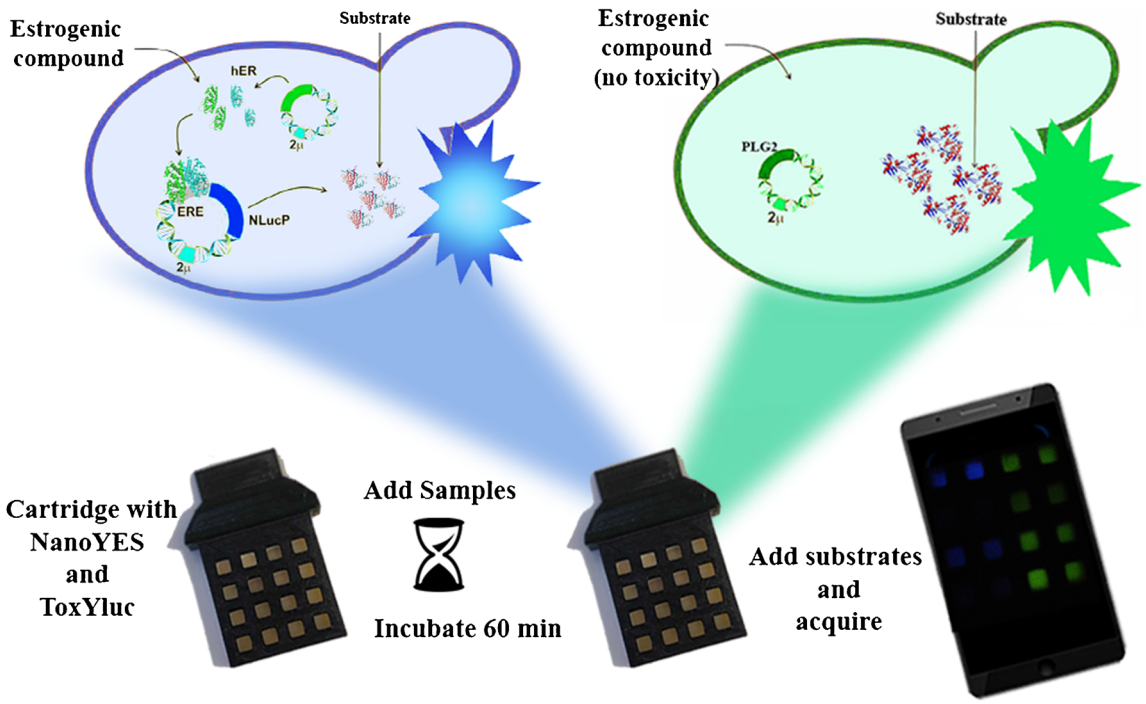

Fig. 4 Schematic representation of the smartphone biosensor principle. The 3D-printed cartridge integrates the three strains: nanoYESα, nanoYESβ expressing the reporter protein Nanoluc under the regulation of estrogen receptor α or estrogen receptor β activation, and the viability control strain ToxYLuc constitutively expressing the green-emitting PLG2 luciferase as reporter protein

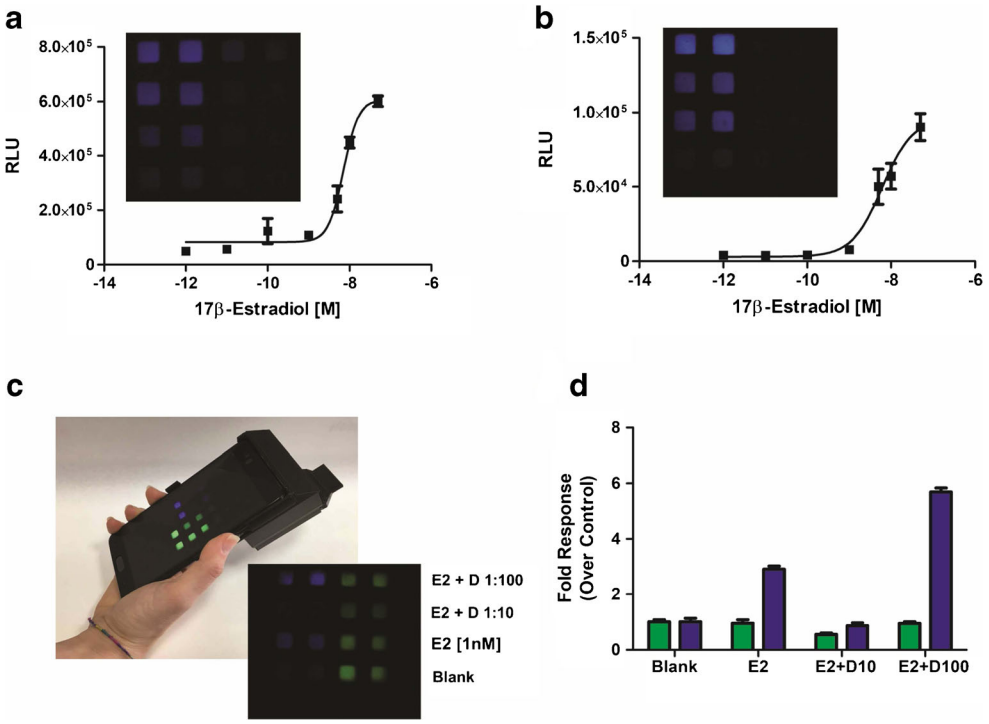

Fig. 5 Dose-response curves for 17β-estradiol with nanoYESα (**a**) and nanoYESβ (**b**) obtained after quantification of BL emissions with ImageJ software. BL images were obtained by incubating the cell-cartridge containing 90 μL of yeast bioreporters with 10 μL 17β-estradiol (concentration range from 0.001 to 100 nM) and by acquiring BL emission with OnePlus camera (30 s at ISO 3200), after addition of 50 μL YPERNano solution. Data are plotted as fold response with respect to control (1% EtOH). (**c**) Picture of assembled device composed by smartphone adaptor and cell cartridge comprising of yeast bioreporters nanoYESβ (in duplicate, left part of the cartridge) and ToxYLuc (in duplicate, right part of the cartridge). Yeast estrogen assays were performed by incubating the cell-cartridge containing 90 μL of nanoYESβ and ToxYLuc with 10 μL of sample. BL signals were obtained with a OnePlus camera acquiring for 30 s at ISO 3200, after addition of either 50 μL YPERNano substrate or YPERBrightGlo. (**d**) Graphical elaboration of sample analysis. Blank sample for estrogenic activity and for cell toxicity was obtained by incubating cells with 1% EtOH. Both BL signals of control wells were set at 1 to normalize results and enable both evaluation of estrogenic activity and toxicity

genistein and daidzein, showed a fold induction corrected for sample toxicity (CFI) of 11.0 ± 0.3 and 51.3 ± 0.3 for ERα and ERβ, respectively.

Conversely, alfalfa extracts (*Medicago sativa* L.) showed estrogenic activity towards both ERα and ERβ due to the presence of phytoestrogens in agreement to Boue et al. [42]. An interesting result was reported for the alfalfa sample digested with 1% Alcalase (Fig. 6b, sample F) which showed a significant increase in estrogenic activity with a CFI of 28.9 ± 0.7 and 28.4 ± 0.5 for ERα and ERβ in comparison to the non-digested extract (Fig. 6b, sample D). A possible explanation for the increase in estrogenic activity could be related to the generation of bioactive peptides, as previously reported for other plant derivatives and extracts with high biotechnological potential [43].

Unexpected results were obtained with some samples, i.e., B and DSE, for which lower dilutions provided higher estrogenic activity than more concentrated solutions, due to high variability in cell viability signals. For example, a normalized estrogenic activity corrected for cell viability of 0.68 ± 0.06 (ERα) and 1.01 ± 0.03 (ERβ) for sample B (1:100) and 1.45 ± 0.10 (ERα) and 2.10 ± 0.03 (ERβ) for sample B (1:1000) was reported. Although cell viability (see ESM) was taken into account for calculating the corrected normalized estrogenic activity, we believe that the high variability of sample B (1:1000) viability signal could have masked the expected decrease in estrogenic activity at higher dilution.

Moreover, to evaluate the ability of nanoYESα and nanoYESβ to detect unknown concentrations of estrogenic compounds, we performed recovery test of spiked 17β-estradiol as a reference compound in tap water samples. Table 2 reports the recovery values obtained when deionized water (blank) and different tap water samples, spiked with low, medium, and high 17β-estradiol concentrations, were analyzed with the nanoYESα and nanoYESβ strains. Satisfactory recoveries were obtained with nanoYESα showing average recovery values ranging from 95 to 116%, while the nanoYESβ from 80 to 90% (Table 2).

As concerns the results obtained with the new smartphone biosensor, a quantitative analysis for both (anti)estrogenic activity and cytotoxicity was obtained by co-incubating the samples with 1 nM E2. Figure 5c shows a typical cartridge used for analyzing two different dilutions (1:10 and 1:100) of sample D (alfalfa aqueous extract) in duplicate with nanoYESβ

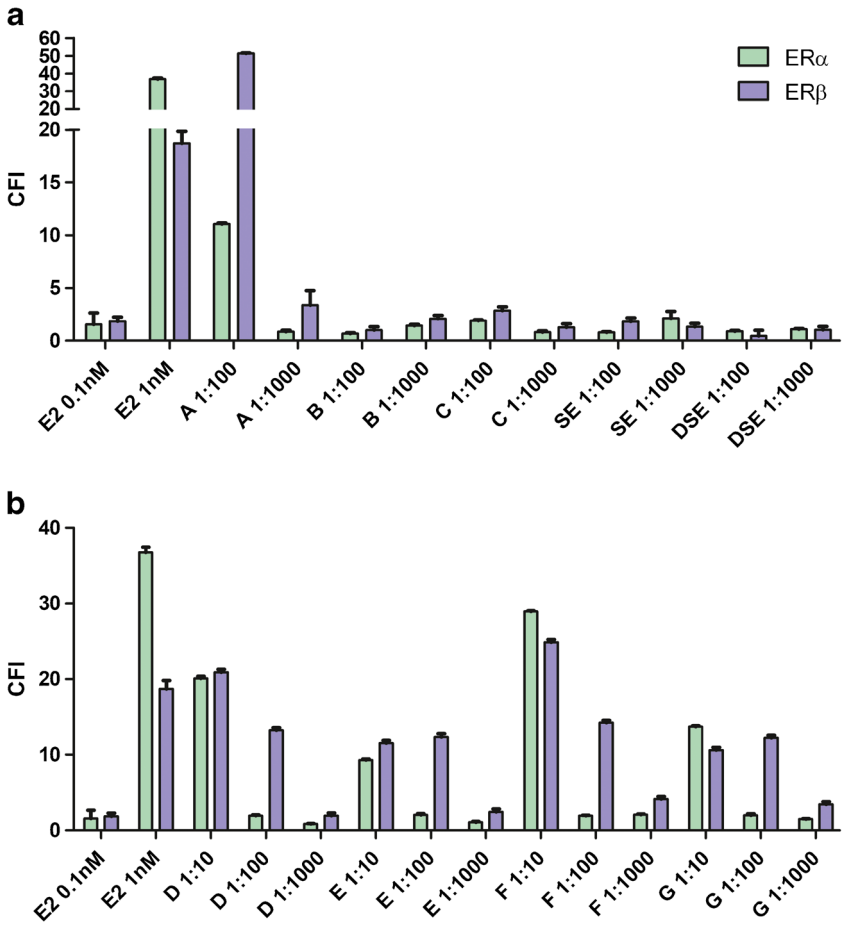

Fig. 6 (**a**) Evaluation of estrogenic activity of industrial soybean seed and (**b**) alfalfa (*Medicago sativa* L.) extracts incubated with nanoYESα (green) and nanoYESβ (violet) in 96-black well plates for 30 min. Data represent the mean values of CFI ± the standard deviation (SD) obtained with three replicates (details of data elaboration are in the "Materials and methods" section)

and ToxYLuc strain. The cartridge also included two reference samples in duplicate wells: 1 nM E2 and blank (vehicle), either water for aqueous extracts or 1% methanol for methanol extracts. The apparent anti-agonistic activity of sample D (1:10 dilution) was due to a significant loss in cell viability (55 ± 2%); conversely, a cell viability of 94 ± 4% was observed with incubation of sample D (1:100) dilution, with significant estrogenic activity (fold response 5.9 ± 0.1). Further characterizations will be required to better understand the bioactive molecules that are responsible for this activity.

These results confirm the importance of including the viability control strain in the same cartridge to correct the analytical signal and avoid artifacts due to sample or matrix-aspecific effects.

Conclusion

Here, we report the development and optimization of a rapid screening assay based on nanoYESα and nanoYESβ and its

Table 2 Recovery obtained by analyzing tap water samples spiked with low, medium, and high concentrations of 17β-estradiol using nanoYESα and nanoYESβ

Sample	Spiked concentration E2 (nM)	Detected concentration E2 (nM)		Recovery% E2 (nM)[a]	
		α	β	α	β
1	0	n.d.	n.d.	–	–
2	1.00×10^{-5}	n.d.	n.d.	–	–
3	1.00×10^{-3}	$1.16 \pm 0.03 \times 10^{-3}$	$0.90 \pm 0.06 \times 10^{-3}$	116%	90%
4	0.10	$0.11 \pm 0.01 \times 10^{-3}$	$0.08 \pm 0.02 \times 10^{-3}$	110%	80%
5	1.00	0.97 ± 0.07	0.81 ± 0.05	97%	81%
6	10.00	9.52 ± 0.09	8.78 ± 0.90	95%	88%

[a] Obtained by interpolating the corrected signal on a corrected dose-response curve for 17β-estradiol

implementation into a smartphone-based biosensor for on-site analysis. The low limit of detection allows the use of the developed yeast estrogen assay for the quantitative detection of estrogenic activity in complex biological samples and water samples. Those limits of detection and EC_{50} are comparable or better than those already reported for yeast estrogen bioassays. We integrated these three strains in a smartphone-based biosensing platform which could provide a first-level bioactivity and toxicity screening for rapid detection of EDCs in environmental and biological samples. Furthermore, those results were achieved with, as far as we know, the shortest incubation time ever used for a yeast biosensor. We envisage potential applications of such biosensor in different scenarios, such as for the analysis of environmental and food samples, and security applications for rapid monitoring of potentially harmful contaminants.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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