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Polymer-immobilized ready-to-use recombinant yeast assays for the detection of endocrine disruptive compounds

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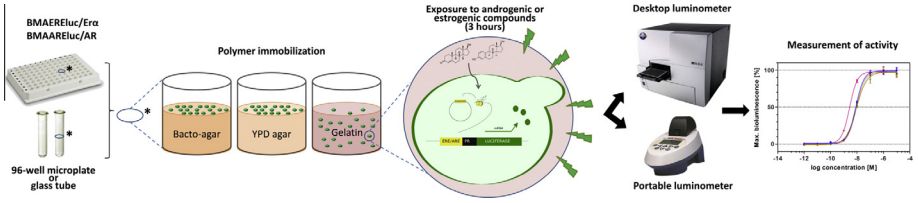
HIGHLIGHTS

- Immobilization techniques applied to develop rapid ready-to-use assays.
- Immobilized recombinant yeast used for detection of androgens and estrogens.
- Recombinant yeast cells were immobilized in gelatin, Bacto agar and YPD agar.
- Gelatin was the best immobilization matrix.
- Immobilized yeast stored in fridge maintained sensitivity for at least 90 d.

GRAPHICAL ABSTRACT

Scheme of recombinant yeast assays using three types of immobilizing matrices in a microplate or tubes.



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ABSTRACT

Recombinant yeast assays (RYAs) constitute a suitable tool for the environmental monitoring of compounds with endocrine disrupting activities, notably estrogenicity and androgenicity. Conventional procedures require yeast reconstitution from frozen stock, which usually takes several days and demands additional equipment. With the aim of applying such assays to field studies and making them more accessible to less well-equipped laboratories, we have optimized RYA by the immobilization of *Saccharomyces cerevisiae* cells in three different polymer matrices – gelatin, Bacto agar, and Yeast Extract Peptone Dextrose agar – to obtain a ready-to-use version for the fast assessment of estrogenic and androgenic potencies of compounds and environmental samples. Among the three matrices, gelatin showed the best results for both testosterone (androgen receptor yeast strain; AR-RYA) and 17β-estradiol (estrogen receptor yeast strain; ER-RYA). AR-RYA was characterized by a lowest observed effect concentration (LOEC), EC_{50} and induction factor (IF) of 1 nM, 2.2 nM and 51, respectively. The values characterizing ER-RYA were 0.4 nM, 1.8 nM, and 63, respectively. Gelatin immobilization retained yeast viability and sensitivity for more than 90 d of storage at 4 °C. The use of the immobilized yeast reduced the assay duration to only 3 h without necessity of sterile conditions. Because immobilized RYA can be performed either in multiwell microplates or glass tubes, it allows multiple samples to be tested at once, and easy adaptation to existing portable devices for direct in-field applications.

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1. Introduction

Endocrine disrupting compounds (EDCs) are defined as exogenous substances that cause adverse effects in an organism, or its progeny, subsequent to changes in the endocrine system (European Commission, 1996). This definition covers a wide-range of substances, both man-made and natural, able to interfere with wildlife and human endocrine systems at very low concentrations, potentially leading to physiological anomalies (Sumpter and Johnson, 2005; Brander et al., 2013). EDCs are now widespread all over the world in various matrices (Rotchell and Ostrander,

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2003; Bainy, 2007; Novák et al., 2009; Jarque et al., 2014). Moreover, new chemical compounds that may show endocrine disrupting activity are produced and released into the environment every year, which demands new tools for their fast detection and subsequent risk assessment (Ezechiá et al., 2014).

Recombinant yeast assays (RYAs) have been demonstrated to be suitable tools for environmental monitoring (Brix et al., 2010; Jarošová et al., 2014; Mesquita et al., 2014). They consist of engineered yeast strains which respond to compounds with endocrine disrupting activities. They harbor two foreign elements: a vertebrate receptor able to recognize the analyte of interest, and a reporter gene under transcriptional control of the receptor (Michelini et al., 2005). Because the transcriptional response of the reporter gene is proportional to the receptor activation, it is possible to determine the equivalent concentration of standard ligand by measuring the activity of the reporter gene. In this work, the bioreporter yeast strains are based on the human androgen/estrogen receptor-mediated expression of *luc* reporter gene (Leskinen et al., 2005). Thus, the luminescence of recombinant yeast cells increases in the presence of compounds with estrogenic/androgenic activity.

Compared to other *in vitro* models, RYAs are easy to perform, are usually less time-consuming, show good sensitivity and high reproducibility, and are relatively inexpensive. Moreover, because yeast cells are relatively tolerant to environmental chemicals, they can be used for the detection of the hormonal activity of samples without any pre-treatment (Leskinen et al., 2005). However, the fact that yeast require previous reconstitution from frozen stock and cultivation in sterile conditions complicates the applicability of RYA in less well-equipped laboratories and in-field studies.

One of the critical steps for the development of ready-to-use whole-cell biosensors is the effective immobilization of living cells, which ideally should not affect the performance of the assay (Michelini et al., 2013). Biologically modified ceramics, also known as biocers, and cell arrays organized in defined patterns were developed to encapsulate unmodified cells (Böttcher et al., 2004; Krol et al., 2005). Recently, immobilizations to three-dimensional biocompatible gel matrices such as calcium alginate or polyvinyl alcohol (PVA) have been discussed as effective yeast cell entrapment methods (Fine et al., 2006). Similarly, polymeric matrices have been used as a support for cell immobilization with the aim of developing portable biosensors (Roda et al., 2011). Although they represented important advantages, most of these approaches significantly diminished the sensitivity of immobilized yeast cells by at least one order of magnitude compared to the non-immobilized versions (Fine et al., 2006; Roda et al., 2011). Moreover, cell viability after immobilization usually decreases due to the low stability and durability of the supporting matrices, resulting in lower yields after about one month (Fine et al., 2006). As a consequence, new immobilization strategies are needed.

The goal of this study was to develop an effective ready-to-use yeast bioassay that is easily applicable to field studies and in less well-equipped laboratories. This goal can be achieved by the immobilization of transgenic yeast in an appropriate matrix that holds the yeast responsive for several months, and is also compatible with commonly used microplates or tubes used in portable luminometers. We compare several novel approaches for the long-term immobilization of recombinant yeast cells by applying three different polymers, Yeast Extract-Peptone-Dextrose (YPD) agar, Bacto agar and gelatin. The sensitivities and durabilities of cells were compared among the bioassay versions using different immobilization strategies. Applicability of immobilized RYA in environmental samples was evaluated by the assessment of estrogenic and androgenic activities of extracts from river water, since presence of endocrine disrupting compounds in river water, especially in industrial or urbanized areas, is of high significance worldwide (Jálová et al., 2013; Gorga et al., 2014; Chou et al., 2015).

2. Experimental

2.1. Materials

Testosterone and 17β-estradiol, D-luciferin sodium salt, citric acid monohydrate and trisodium citrate dihydrate were purchased from Sigma–Aldrich (USA). Luciferin solution 1 mM was prepared by dissolving D-luciferin sodium salt into 0.1 M citric acid and 0.1 trisodium citrate dihydrate. Gelatin from porcine skin (No. 48724, Sigma–Aldrich, USA), Yeast Extract Peptone Dextrose (YPD) agar (No. Y1500, Sigma–Aldrich, USA) and Bacto agar (No. 214010, BD, USA) were used as immobilization polymers. Gelatin liquid solution was obtained by dissolving gelatin powder in synthetic dextrose (SD) complex medium to a final concentration of 20%. Agars were prepared according to the manufacturer's recommendations by dissolving the agar powder in SD complex medium.

2.2. Yeast cell cultures and standard assay

BMAEREluc/ERα (ER-RYA) contains the coding sequence of human estrogen receptor alpha (hERα) cloned into the constitutive expression vector pG-1 and a reporter plasmid carrying a truncated form of *Photinus pyralis* luciferase regulated by the estrogen responsive element (ERE), which serves as a reporter gene (Leskinen et al., 2003). BMAAREluc/AR (AR-RYA) has a similar construction but contains human androgen receptor (hAR) and androgen responsive element (ARE) in the reporter plasmid (Michelini et al. 2005).

The detection of EDCs is based on the measurement of firefly luciferase luminescence from intact living yeast cells (Leskinen et al., 2003). Estrogenic or androgenic compounds diffuse into the cell and bind to the hormone receptor. The resulting activated receptor complex translocates into the nucleus and activates the specific responsive promoter, which results in the expression of the *luc* reporter gene. By the external addition of D-luciferin, light is emitted and measured by a luminometer.

The standard RYA was performed according to the protocol from Michelini et al. (2008) with minor changes. Briefly, yeast from frozen stock (stored at −80 °C) were reconstituted on agar plates and incubated for three days at 30 °C. One colony was picked and grown overnight in complex SD medium at 30 °C and 180 rpm. The OD_{600} of the grown culture was adjusted to 0.4 and the culture was re-grown again for 2 h to reach an OD_{600} of 0.65, which is the mid-exponential phase, when cells are more sensitive to environmental stressors. 100 µl of yeast culture were transferred per well onto a 96-well microplate (Grainer Bio-One GmbH, Germany) and subsequently exposed to the tested chemicals. 17β-estradiol (1.5×10^{-11}–3.3×10^{-8} M) and testosterone (1×10^{-12}–1×10^{-5} M) in methanol (1% v/v) were used as positive induction controls. Methanol was used as the vehicle control. The microplates were incubated for 2.5 h at 30 °C and shaking at 160 rpm. After incubation, 100 µl of D-luciferin solution were added into each well by using an automatic dispenser, and the plates were briefly shaken. After one minute, luminescence was measured using a luminometer (BioTek, Winooski, Vermont, USA) with a controlled temperature of 30 °C.

With the aim of making RYA more accessible to *in situ* measurements, we adapted the assay to a portable luminometer. The procedure was the same as described for microplates with the following modifications: 200 µl of yeast culture with an OD_{600} of

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