Analytical Biochemistry 387 (2009) 271–275

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

A cell-based sensor system for toxicity testing using multiwavelength fluorescence spectroscopy

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ARTICLE INFO

Article history:
Received 9 December 2008
Available online 10 February 2009

Keywords:
Autofluorescence
HL-1 cell line
Cardiomyocytes
Cytotoxicity screening
Drug testing
Microtiter plate
Tryptophan fluorescence
In vitro toxicity

ABSTRACT

A novel cell-based fluorometric sensor system for toxicity monitoring is described, which uses functional spontaneously contracting cardiomyocytes (HL-1 cell line) as the biological recognition element. Based on these highly specialized cells, it has the potential of providing a sensitive and relevant analytical in vitro toxicity testing method. The system was configured by propagating the surface-attaching HL-1 cardiomyocytes in the wells of a 96-well microtiter plate and connecting the plate via an optical fiber to a fluorescence spectrometer capable of excitation–emission matrix scanning. The fluorescence data were analyzed using a conventional spectral analysis software program. The performance of the system for detection of general cytotoxicity to the cells was evaluated using three well-known drugs: verapamil, quinidine, and acetaminophen. The dose–response curves were assessed and the EC_{50} values were determined (0.10 ± 0.007, 0.23 ± 0.025, and 12.32 ± 2.40 mM, respectively). Comparison with in vitro and in vivo reference data for the drugs showed good correlations, suggesting that this cell-based sensor system could be a useful tool in pharmacological in vitro drug testing.

Cell-based sensing represents one of the important achievements in biosensor technology. Since the early works by Rechnitz and coworkers during the 1980s [1,2], numerous examples of combinations of a variety of cell species with various transducer devices or systems have been demonstrated. Transducers have predominantly been either electrochemical devices or optical transducer systems. Of the optical systems, fluorescence-based methods have significant advantages because fluorescence spectroscopy is fast and requires only small sample volumes. Furthermore, because measurements can be carried out through a transparent window without directly contacting the analyte sample, fluorescence spectroscopy is of particular interest in cell-based assays where sterility and purity are essential for reliable results. The use of multiwavelength instrumentation allows simultaneous recording of several signals by the same optical system. Therefore, especially the recording of excitation–emission matrix (EEM)[1] spectra, also referred to as two-dimensional (2D) or three-dimensional (3D) fluorescence spectra, can be used to detect different fluorophores at the same time.

Of special interest for the application of cell-based sensors has been assay development for cytotoxicity evaluation. Currently, a large number of bioassays based on fluorescence detection are applied in daily toxicity testing practice. In particular for evaluation of drug candidates, fluorometric methods that have high predictability for hepatotoxicity and other cell-related toxicities are used frequently. In these methods, changes in fluorescence indicate the cytotoxic events in the metabolism or membrane structure. Examples of such bioassays are methods using the indicator dyes 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) [3], Alamar Blue [4], Neutral Red [5], nuclear staining [6], propidium iodide [7], and monochlorobimane [8] as well as methods for specific cellular oxygen uptake [9,10].

The technique of multiwavelength fluorescence spectroscopy has previously been successfully applied in cell suspension cultures. For example, Hisiger and Jolicoeur [11] used 2D scanning to measure autofluorescence from yeast and mammalian cells, and they detected emission from tryptophan, nicotinamide adenine dinucleotide (NADH), and riboflavins as well as fluorescence from green fluorescent protein with a single probe.

In this article, we present a novel cell-based biosensing system for toxicity testing that combines the potential of specialized HL-1 cardiomyocytes with the speed and flexibility of multiwavelength in situ fluorescence spectroscopy. The performance of the system is demonstrated with drug substances of known cytotoxicity. The method is based on the detection of ultraviolet (UV) autofluorescence from the cells directly in microtiter plates with the benefit of not requiring the addition of any indicator dye. Moreover, be-

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[1] *Abbreviations used:* EEM, excitation–emission matrix; 2D, two-dimensional; 3D, three-dimensional; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NADH, nicotinamide adenine dinucleotide; UV, ultraviolet; KRG buffer, Krebs–Ringer phosphate buffer with glucose; SD, standard deviation; NADPH, nicotinamide adenine dinucleotide phosphate; hESC, human embryonic stem cell.

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doi:10.1016/j.ab.2009.01.027

cause of the flexibility of the optical system, the sensor has the potential to be further developed for simultaneous detection of additional fluorophores. This may be of particular interest in conjunction with other fluorescence-based approaches.

Materials and methods

Materials

Three established drugs were used to study the performance of the toxicity sensing system: verapamil, a calcium channel blocker; quinidine, an antiarrhythmic agent; and acetaminophen, an analgesic and antipyretic drug also known as paracetamol. All drugs were purchased from Sigma Chemical (USA).

Krebs–Ringer phosphate buffer with glucose (KRG buffer) was composed of 120 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, 1.7 mM KH_2PO_4, 8.3 mM Na_2HPO_4, 10 mM glucose, and 1 mM $CaCl_2$ (pH 7.3). To avoid precipitation of $CaPO_4$ when preparing this buffer, the $CaCl_2$ was separately dissolved in one-tenth of the total volume and added to the rest of the ice-cold solution under stirring.

The 96-well microtiter plates transparent for UV light (UV plate 3695, Costar) were purchased from Corning (USA). This kind of plate features a transmission of more than 80% down to wavelengths of 230 nm, according to the manufacturer.

Cell culture

A cell line derived from murine atrial cardiomyocytes, HL-1, was provided by William C. Claycomb of Louisiana State University [12]. The cells were propagated according to procedures described previously [12,13]. The HL-1 cells were maintained in Complete Claycomb Medium (Sigma Chemical) supplemented with 10% fetal bovine serum (lot 6L0730, JRH Biosciences, UK), 10 μM norepinephrine (Sigma Chemical) dissolved in 30 mM L-ascorbic acid, 2 mM L-glutamine (Invitrogen, USA), 100 U/ml penicillin, and 100 U/ml streptomycin (Gibco, USA). Flasks and multiwell plates were coated with 0.2% gelatin (BDH Prolabo, Germany) and 5 μg/ml fibronectin (Sigma Chemical). All cultures were incubated at 37 °C at 5% CO_2.

The HL-1 cells were grown to confluency in T-25 flasks (Sarstedt, Germany), trypsinized, and seeded into 96-well plates (UV plate 3695) at a ratio of 1:3, where 200 μl per well gave approximately 2×10^4 cells per well. The nonsterile plates were first disinfected with 70% ethanol.

Cell-based sensor system setup

The toxicity testing system described in this article makes use of an HL-1 cardiomyocyte preparation as the biosensor recognition element whose autofluorescence is detected by an F-4500 fluorescence spectrophotometer (Hitachi, Japan). Fig. 1 gives an overview of the experimental setup. The fluorescence measurements are performed through the transparent bottom of a 96-well microtiter plate, where the cells are propagated into a confluent layer. A bifurcated silica fiber bundle (Schölly Fiberoptic, Germany) was mounted on the instrument to connect the spectrophotometer and biorecognition element. The excitation light from the light source of the instrument is reflected into one branch of the bifurcated fiber end and guided to the cells. Because the common end of the fiber bundle is of approximately the same diameter as the wells in a 96-well plate, the excitation covers virtually the entire area and the obtained spectrum represents an average for the cell culture. Parts of the fluorescence light emitted on excitation reenter the fiber bundle and are detected by a photomultiplier tube behind the second branch of the bifurcated fiber end. To position the optic fiber under the microtiter plate, a holder was constructed, enabling reproducible measurements in all 96 wells by stepwise moving of the plate. The positions of all fiber ends and the angle between the fiber and the plate can be adjusted to optimize the signal. Excitation scans or emission scans can be recorded, as can EEM spectra where both wavelengths are varied.

Fluorescence measurements

Except for the EEM spectrum, all fluorescence measurements described in this article were performed in excitation scan mode with fixed emission wavelength. Excitation spectra were recorded by measuring the fluorescence intensity at intervals of 0.2 nm. To compensate for background fluorescence from the microtiter plate material, the signals from reference wells filled with buffer were subtracted from each spectrum. Fluorescence intensities given as single values at 285 nm (Figs. 3 and 5) have been calculated from excitation spectra by averaging all data points within a 10-nm-wide interval ranging from 280 to 290 nm. To enhance accuracy of the method, six wells of the microtiter plate were used for every measurement and the spectra from those were averaged. The standard deviations (SDs) between these measurements are indicated by error bars in the corresponding diagrams.

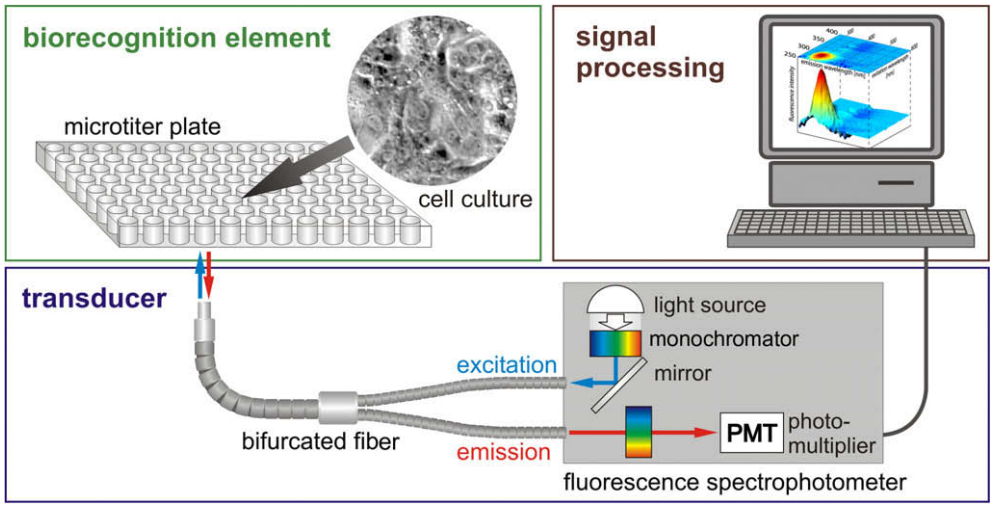

Fig. 1. The experimental setup.