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Liquid crystal based sensors monitoring lipase activity: A new rapid and sensitive method for cytotoxicity assays

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ABSTRACT

In this work we present liquid crystal (LC) based sensor devices to monitor cell viability. The sensing layer is composed by the LC and a planar monolayer of phospholipids. In the presence of minute traces of phospholipases, which hydrolyze enzymatically phospholipids, the LC–lipid interface is disintegrated. This event causes a change in orientation of the LC, which was followed in a polarized microscope. The lipase activity can be used to measure the cell viability, since members of this enzyme family are released by cells, as they undergo necrosis. The described sensor was used to monitor the presence of the lipases released from three different cell lines, which were either exposed to highly cytotoxic model compounds (sodium azide and paracetamol) or subjected to freeze-thaw cycles to induce cell death by a non-chemical based inducer for apoptosis, such as temperature. Finally, the comparison of lipase activity detected by a state-of-the-art fluorescence assay to the LC based system resulted in the superiority of the LC system concerning incubation time and sensitivity.

1. Introduction

Lipases are hydrolytic enzyme species that act on carboxylic ester bonds. These enzymes are widely found in animals, plants, molds and bacteria (Svendsen, 2000). Their catalytic activities are not limited to hydrolysis but also catalyze esterification, inter-esterification and trans-esterification processes (Waite, 1987). As a result, they have enormous potential in areas such as food technology, detergents, the chemical industries and biomedical sciences. Despite the high potential of lipases, their industrial applications are mainly limited to the detergent industry and the fine chemistry. This limitation is mainly due to the high production and assay costs. The success of the molecular approach involves the proper combination of molecular biological techniques coupled with efficient high-throughput assays for lipases. The future goals of lipase research are to develop inexpensive and fast assays for high throughput screening. No less important is the development of a sensitive lipase detection assays for screening of new chemotherapeutics or for assessing toxic compounds in cell cultures. Lipases are also very important in diagnostic settings. Under pathological conditions, lipases are detected e.g. in body fluids, where they usually are not present. This suggests to make use of these enzymes as markers for processes which destroy cells and/or organs. A well-known example is the presence of high levels of serum lipases in blood as a consequence of pancreatitis: acinar cell membranes become more permeable, allowing much more enzyme to enter the blood circulation (Wong and Dennis, 1990). Lipases are usually removed from the plasma by glomular filtration and metabolized by the renal tubule in the kidney. High levels of active lipases, capable of damaging renal cells can be correlated to the organ failure which may result in renal disease. Additionally, the conversion of cells from a normal to a cancerous state is accompanied by changes in several metabolic pathways such as lipid production, where the monoacylglycerol level increases, which is induced by lipases (Mills and Moolenaar, 2003). Therefore, a robust, simple and portable sensor device that can detect the presence of released lipases is of continuous interest and highly desirable from a diagnostic point of view.

The presence and organization of surfactants, lipids, and polymers at LC interfaces can be observed through changes in the optical appearance of the LCs. Examples concerning more complex interfacial phenomena, such as specific binding events involving proteins, bacteria, viruses, enzymatic reactions, hybridization of DNA, etc. at aqueous–LC interfaces which trigger dynamic transitions

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of orientation in the LCs have also been observed (Bi et al., 2009; Brake and Abbott, 2007; Brake et al., 2003; Chen and Yang, 2010; Gupta and Abbott, 1997; Gupta et al., 1998; Kim et al., 2005, 2000; Kim and Abbott, 2002; Lai et al., 2009; Luk and Abbott, 2003; Price and Schwartz, 2008; Shah and Abbott, 2001; Xue and Yang, 2008). Since lipases act at the oil/water interface, a change in the properties of the interface is an important criterion to measure lipolysis. It is also reported in the literature that in the presence of phospholipases, hydrolysis of the lipid layer takes place at the lipid–LC interface, which can then be directly monitored through the change in the optical appearance of the lipid–LC layer (Brake and Abbott, 2007; Hartono et al., 2008; Hartono et al., 2009). It has been described recently, that bimolecular lipid vesicles formed monolayers spontaneously on top of LC interfaces as the optical properties correlate to perfect lipid membrane architecture (Masutani et al., 2013). The preparation of such a perfect lipid monolayer is a prerequisite for our sensor approach in which we use the optical parameter of the LC/monolayer as optical readout to detect functional activity and thereby the presence of lipases.

Of course there are methods available to detect active lipases based on their hydrolytic activity in bulk phase (Beisson et al., 1999; Hu and Jang, 2012; Moskowitz et al., 1977; Mosmuller et al., 1994; Roberts et al., 1985). However, these conventional methods detect either the products of the reactions, which the lipases catalyzes, such as the release of fatty acids, fatty acid esters or glycerol. Another approach is antibody-based, employing monoclonal and specific anti-lipase antibodies. In both cases, colorimetric assays (Roberts et al., 1985), fluorescence detection (Beisson et al., 1999; Mosmuller et al., 1994), or chromatographic procedures (such as TLC, GC, or HPLC) are used at the respective time and costs (Gupta et al., 2003). A disadvantage which holds true for most colorimetric/fluorescence assays is that ester compounds, serving as substrates, are usually not stable towards pH and/or temperature changes. In case of the LC-based sensor the direct hydrolysis of phospholipids is observed, releasing the products into the bulk solution (polar head group) and LC surface (fatty acids). Under such conditions the LC-based sensor represents a single use system.

This is the first sensor device being reported measuring the lipase activities released from cells directly as a function of change in orientation of the membrane-functionalized liquid crystal layer. Therefore, in this article, an LC-based sensor device is reported detecting the existence and activity of lipases, released from various cells and probing their viability as a function of membrane integrity by monitoring the lipase release. Since the presence of lipases is correlated with the organ failure by e.g. toxic substances, the presented assay is useful as a novel concept for quantitative lipase screening in the context of viability testing and discovery of chemotherapeutic active compounds.

2. Materials and methods

For the reported experiments, following materials were purchased and used. Nematic liquid crystal 4-cyano-4′-pentylbiphenyl (5CB) was purchased from Sigma Aldrich (Germany). Phosphoplipase A1 (PLA1) from *Thermomyces lanuginosus*, Phospholipase C (PLC) from *Bacillus cereus* and Phospholipase D (PLD) Type VII from *Streptomyces chromofuscus* were purchased from Sigma Aldrich (Germany). Glass microscope slides were obtained from VWR (Germany). Gold grids of 150 mesh size were purchased from Plano GmbH (Wetzlar, Germany). *N,N*-Dimethyl-*N*-octadecyl-3-aminopropyltrimethoxysilyl chloride (DMOAP) was purchased from Sigma Aldrich (Germany). Phospholipid, 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids Inc. (Alabama, USA). All aqueous solutions were prepared with deionized water (18 MΩ cm^{-1}), using a Milli-Q water purification system (Millipore, Bedford, MA). Eagle′s Minimum Essential Medium (EMEM), Dulbecco′s Modified Minimum Essential Medium (DMEM), Alpha Minimum Essential Medium (alpha-MEM) with ribonucleosides fetal bovine serum (FBS), horse serum, Antibiotic–Antimycotic solution, Trypsin × EDTA (1X) were purchased from PAA/GE Healthcare (Austria).

2.1. Cell cultures

Human hepatocarcinoma cell line HepG2 cells (ATCC HB-8065) were cultured in EMEM containing 10% FBS, 0.25 mg/l amphotericin B, 0.1 g/l streptomycin. Human pancreatic carcinoma cell line mia PaCa-2 (ATCC CRL-1420) in DMEM supplemented with the same components and 2.5% horse serum in addition. Mice embryonic carcinoma cell line P19 (ATCC CRL-1825) was cultured in alpha-MEM medium with ribonucleosides containing 10% FBS and 0.25 mg/l amphotericin B, 0.1 g/l streptomycin. All HepG2, PaCa-2, and P19 cells were plated in T75 flasks and grown at 37 °C in a humidified 5% CO_2 and 95% air atmosphere. For the experiments, cells were plated at a confluence of 60–80%, in 96-well plates after Trypsin treatment at 37 °C. Cell plating density was varied from 2.0×10^5 down to 6.25×10^3 in sequential two-fold dilutions and cultured for 24 h. To analyze the secretion of lipases, cells were incubated with 100 μl of 0.5% sodium azide (NaN_3) for 2 h, 4 h and 24 h and with 0.05% paracetamol for 30 min, 2 h and 24 h.

2.2. Preparation of glass microscopic slides for liquid crystals alignment

Microscope glass slides, serving as substrate for the LC-sensor, were prepared by soaking in an aqueous 5% decon solution and treated in an ultrasonic bath for 5 min. The slides were cleaned using water and dried with compressed air. Subsequently, the substrates were soaked in an aqueous solution of DMOAP for more than 5 min. This solution was prepared by mixing 250 μl of Octadecyldimethyl(3-trimethoxysilylpropyl)ammonium chloride (60% in methanol, AB111261) and 150 ml of Milli-Q water. After washing with Milli-Q water, the slides were dried first by compressed air and then in a vacuum oven at 100 °C for 15 min. Slides were then taken out and left to cooled down to room temperature. These DMOAP coated slides could be stored for at least 3–4 days under nitrogen atmosphere for an optimal activity. In some cases, the slides were cleaned with oxygen plasma before the DMOAP coating procedure was done, as the surface seemed to retain some unspecific (physisorbed) layer. This treatment was performed with oxygen at 0.2 mbar (100 W) for 1 min using Plasma Prep 5 (GaLa Instruments, Germany).

2.3. Preparation of gold-grids

As container for the LC in a standard LC-based sensor setup served 150 mesh (pitch 150 μm) gold grids. These grids were cleaned by rinsing with copious ethanol, methanol and acetone, before drying at 100 °C in a vacuum oven. The grids were stored under nitrogen atmosphere until use.

2.4. Preparation of phospholipid vesicles

For the preparation of phospholipid vesicles (liposomes), a stock solution of 10 mM DOPC in chloroform (p.A.), was prepared. From this solution, a 1.0 mM solution was prepared by diluting with chloroform. Chloroform was then evaporated with nitrogen gas and the resulting lipid film was dried for 1 h on a vacuum line. This dried lipid film was then rehydrated with 5 ml of PBS buffer