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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

(pH 7.0) prior to a treatment by 3–4 freeze-thawing cycles in liquid nitrogen and a water bath at 37 °C. Aliquots of 500 μl each were stored in 1 ml eppendorf tubes in a freezer at −20 °C. Shortly before use, the solutions were thawed, diluted 1:10 with PBS (0.1 mM) and extruded through a 100 nm polycarbonate membrane (Avestin Europe GmbH, Germany) for 21 times using a hand-held extruder (Avestin™). The resulting small unilamellar lipid vesicles were used for lipid monolayer formation on top of LC assemblies by spontaneous fusion. The formation of a lipid monolayer was monitored as a function of change in optical properties of the assembly, visible under polarized light in a standard light microscope.

2.5. Preparation of optical LC-based sensor

For the preparation of standard LC-based sensors, several 150 mesh gold grids were placed on a DMOAP coated glass slide. Then, 2 μl of 5CB were applied on each gold grid and the excess LC was removed by capillary actions using a micro syringe tip. After impregnating the gold grid with 5CB, the slide was placed for 10 min in an oven at 50 °C and then cooled down slowly to room temperature. The oven temperature was above the phase transition temperature from nematic to isotropic orientation of the LC (35 °C for 5CB), in order to achieve a uniform homeotropically (or vertically) aligned LC (Fig. 1 a). These sensors were checked under a microscope, and any gold grid with no proper alignment of 5CB was discarded. The sensors meeting the criteria were used within two days.

Prior to lipase experiments, 2–5 μl of a 0.1 mM phospholipid vesicle suspension were added to the LC-based sensor inducing homeotropic alignment of 5CB and resulting in a decreased light transmission (Fig. 1b). The sensor could be stored, under conditions preventing the drying effect of the solution on top of the LC. In all presented experiments observations were made within 30 min.

2.6. Microscopic measurement setup for LC based sensor

A polarized optical microscope (DMRX-HC, Leica) was used to image the optical textures of the LC-based sensor. Calibration of the cross-polarized microscope brightness was set as the following: 100% transmittance was defined in a setup without sample and in a parallel configuration of polarizer and analyzer. 0% transmittance was set when the light source was turned off. All recorded images were obtained using a 10× objective lens between the crossed polarizers. The optical appearance of the LC was recorded using a digital camera (pco.1600, PCO camera) attached to the polarized optical microscope. The images were captured at a resolution of 1600 × 1200 pixels, a gain of 1.00× and a shutter speed of 1/10 s.

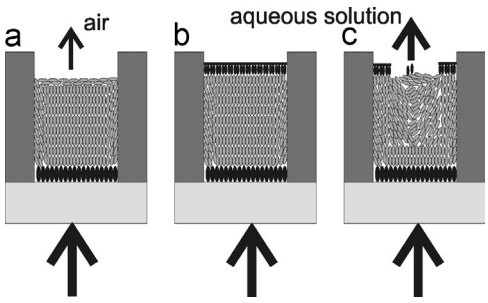

Fig. 1. Schematic representation of the LC-based sensor, consisting of a gold grid compartment, a silanized glass substrate and the LC, the arrows indicate the transmission of light: (a) towards air, showing an alignment towards the substrate and slight disorder towards the air interface. (b) Shows the homeotropic alignment of the LC towards an aqueous environment mediate by the lipid layer. (c) Represents the destruction of the lipid layer resulting in a disorder of the LC.

2.7. Fluorescence based lipase assay

The lipolytic activity of lipases secreted by HepG2, P-19 and mia PaCa-2 cells were measured in vitro using high sensitive lipase activity fluorometric Assay Kit III (BioVision, Milpitas, CA). In the assay, lipase hydrolyzes a specific substrate to generate methylresorufin, which is in turn detected fluorometrically at *Ex*/*Em* = 529/600 nm. For these assays, cell suspensions were incubated with cytotoxic compounds and treated for 1 s in an ultrasonic bath. Then, samples were centrifuged at 13,000 rpm for 10 minutes to remove insoluble material. Aliquots of 50 μl of supernatant were used for the assays. The fluorescence intensity changed as a function of lipase activity. The assay and calculation of lipase activity were done as described in the manufacturer's manual. Experiments have been obtained from six individual experiments performed in triplicate and variations within the results are represented by the error bars.

3. Results and discussion

For the preparation of a lipase sensitive sensor device, a phospholipid species was employed to form a monolayer on the surface, to surface-align the LC. These lipids are widely present in membranes in diverse structural variations and exhibit common structural features. These common features are the polar head groups and the non-polar acyl chains. Lipases are responsible for the extracellular degradation and the intracellular metabolism of the lipids. The selectivity and activity of a certain lipase towards a specific lipid depends on structural properties as e.g. the chain lengths, stereoisomers, interfacial characteristics of the water–lipid interface. The enzymatic activity of lipases can be enhanced or stopped by using specific co-factors.

As model cell lines for lipase mixes served the murine progenitor cell line (P19), human hepatocytes (HepG2) and a human pancreatic cell line (PaCa-2). As positive control, commercial lipases were employed, such as PLA1, *Thermomyces lanuginosus,* PLC from *Bacillus cereus* and PLD Type VII, isolated from *Streptomyces chromofuscus*.

In the investigations LC–lipid layers were prepared (Masutani et al., 2013). Briefly, the liquid crystals were homeotropically (vertically) aligned on top of a LC (Fig. 1b) that was supported by a DMOAP alignment layer which was applied to the substrate in advance. To such sensors, DOPC vesicles in a PBS solution were applied, promoting the LC molecules to form a homeotropic alignment at the aqueous interface too (Fig. 1b). The formation of LC–lipid layer was observed within a few seconds from the change in the light transmittance (Masutani et al., 2013).

Once the preparation of sensor device was completed and a stable conditions were achieved samples could be applied. As described above, three different cell lines (P-19 cells, HepG2 cells and PaCa-2 cells) were used for the release of lipases. For following experiments, these cells were cultured to obtain a certain cell number before being processed to undergo cell death in order to release lipases. Both physical (freezing/thawing) as well as chemical (NaN_3 and paracetamol treatment) methods were used to rupture the cells mechanically and show cytotoxicity.

3.1. Application of freeze-thaw stress on cells

Before applying the physical method to induce lipases release, HepG2 cells were grown under standard cell culturing conditions.

In a typical procedure, 5×200 μl of cells samples (3.0×10^5 cells/ml) were collected, after trypsinization and stored in separate vials. These samples were frozen in liquid nitrogen then thawed in a water bath (37 °C). After several freeze-thaw cycles (2, 4, 6, 8 and 10 times), the samples were kept in an ultrasonic bath at 37 °C for 5 min. This ultrasonic treatment was implemented as it showed to be necessary to promote the reproducibility of the results of lipase detection. As a control, a sample containing living cells was also subjected under the same conditions to an ultrasonic treatment showing no detectable influence on the LC sensor. Under these conditions, ultrasonic treatment seemed not to induce cell lysis, but assured homogenous distribution of already released lipases in the supernatant. In order to show whether all cells were dead, 20 μl of the cell sample were used to stain with 20 μl of Trypan blue solution (0.4%), which allowed assessing the viability of the cells. In this case, viable cells rejected the blue dye while diffusion into porous membranes of dead cells was possible, which was observed under the microscope. After confirming the efficient cell death by such staining, the remaining cell samples were centrifuged at 3500 rpm for 5 min and the supernatant was collected from the centrifuged samples. The lipase activity was analyzed by adding 5 μl of the supernatant to the LC–lipid layer. Fig. 2 shows micrographs of LC sensors subjected to cell supernatants after an increasing number of freeze-thaw cycles. The experiments have been performed in triplicate, showing the same results. The change in light transmittance is caused by a change of the alignment of the liquid crystal layer directly correlating with the lipase activity disrupting the lipid layer through hydrolysis (Figs. 2 and 1c schematically). The alignment change of LCs was completed within 5 min, resulting in the successful detection of active lipases released from dead cells. To support the hypothesis, that only active lipases are responsible for the optical change, further control experiments have been made. Commercially acquired PLC was inactivated by low pH (pH=1 and 3, respectively) and by a heat method, autoclaving the lipase. The addition of the inactivated enzymes to the LC–lipid layer architecture did not show any effect. Another type of control experiments was to test cell culture mediums and supplements for possible unspecific interactions with the phospholipid–LC architecture. Again, changes in optical transparency of the architecture could not be detected. These experiments gave further support for the hypothesis that the observed alignment changes above were due to the existence of active lipases only hydrolyzing the DOPC layer.

3.2. Determination of the cytotoxic effect of sodium azide and paracetamol on cells

Sodium azide is a known cytotoxic compound to induce cell death in cell culture. In the present work the compound was used to determine a release of lipases due to toxic effects. In order to ensure that NaN_3 did not deactivate lipases, experiments incubating single and mixtures of lipases with NaN_3 (0.5% NaN_3 in Milli-Q water for 24 h at 37 °C) had to be performed. For these experiments, 30 μl PLA1 (80 U/ml in PBS), 30 μl PLC (30 μM in PBS) and a mixture of 10 μl PLA1, 10 μl PLC and 10 μl PLD (100 nM in sodium acetate buffer pH=5.6) was used.

As can be followed from the polarized micrographs shown in Fig. 3, NaN_3 did not show any inhibitory effect on the enzyme activity (comp. a, b, and c to d, e, and f). In Fig. 3, two series of polarized light micrographs are presented. The top row (a, b and c) shows the effect of active lipases once they got in contact with LC–lipid layers, while the second row (d, e and f) represents the same types of lipases which were incubated for 24 h with 0.5% sodium azide. This finding is clearly demonstrating that the enzymes did not lose their activities upon NaN_3 treatment. However, a weak alternation between the corresponding micrographs is visible. In Fig. 3, the micrographs a, b, c, and f show the formation of fully developed point defects in the middle of the compartment with four line defects, so-called Schlieren, directed towards the walls of the compartment, which is typical for an LC-based detector lacking a full amphiphilic layer (Lockwood et al., 2008). The form of the defects is caused by the reorganization of the LC molecules, the

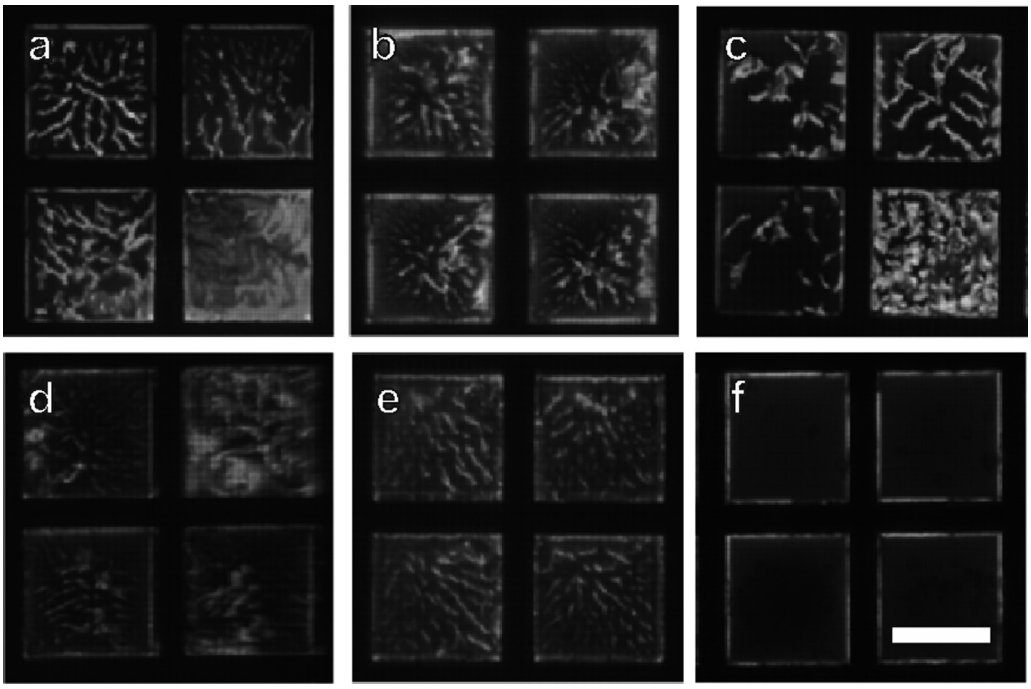

Fig. 2. Polarized light micrographs showing lipase activity on a LC-based sensor after the formation of a DOPC lipid layer (0.1 mM in PBS) and the addition of cell culture supernatants from HepG2 cells. The supernatants were obtained after an increasing number of freeze-thaw cycles (a) 2 (b) 4 (c) 6 (d) 8 and (e) 10 cycles. (f) Represents the supernatant of non-treated cells that served as negative control. The scale bar represents 100 μm.

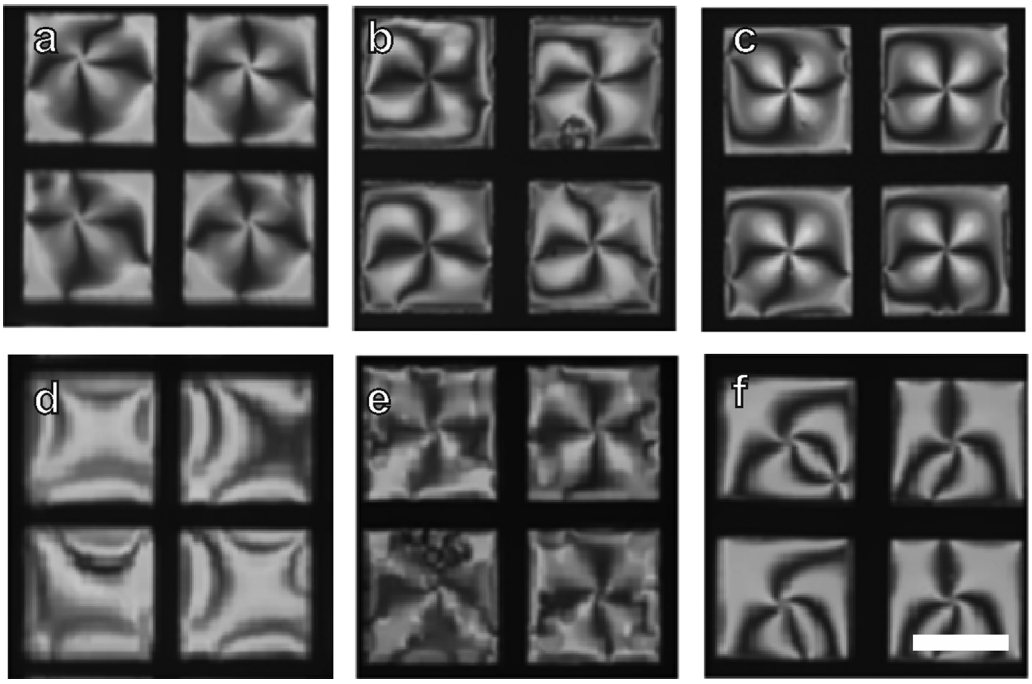

Fig. 3. Polarized light micrographs showing the interaction of lipases on LC-based sensors after the formation of a DOPC lipid layer (0.1 mM in PBS). The top row represents (a) a lipase mix of PLA1, PLC and PLD, (b) PLC and (c) PLA1. The bottom row shows (d) a lipase mix of PLA1, PLC and PLD, (e) PLC and (f) PLA1 after a 24 h incubation with 0.5% NaN_3 at 37 °C. The scale bar represents 100 μm.

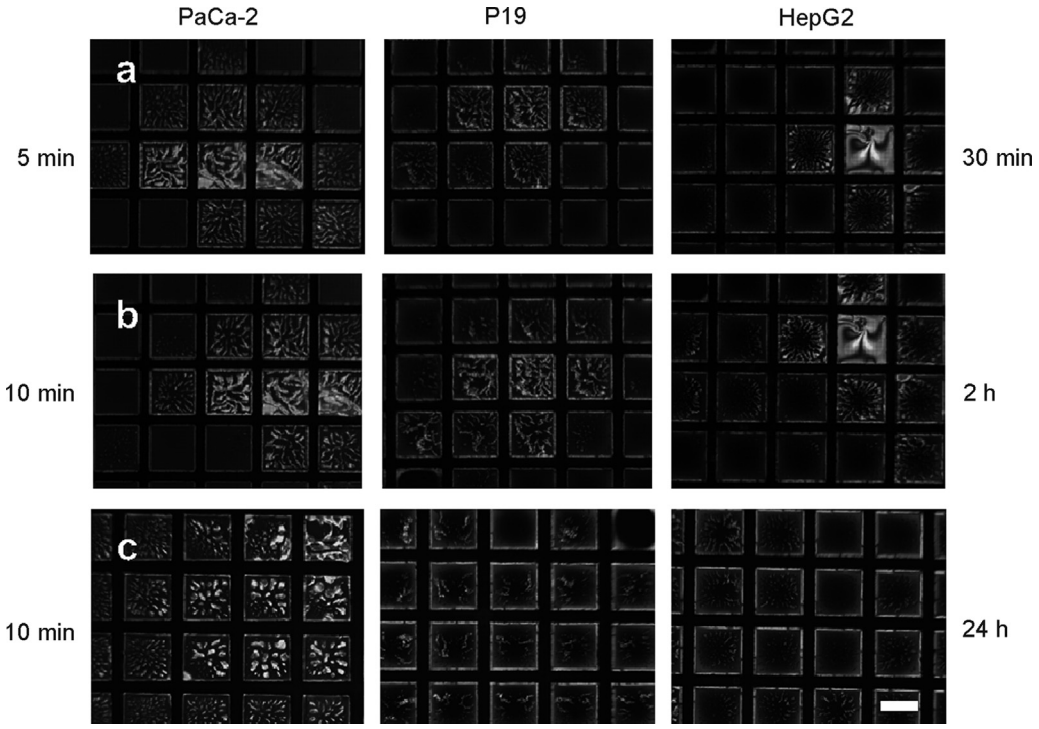

Fig. 4. Polarized light micrographs showing the interaction of cell supernatants of different cells on LC-based sensors after the formation of a DOPC lipid layer (0.1 mM in PBS). From left to right, the results of cell supernatants from PaCa-2, P19 and HepG2 are presented. The numbers on the left side represent the time point when the micrograph was obtained and those on the right side the incubation time in NaN_3 solution for the respective cell type. The scale bar represents 100 μm.

degradation products of the enzymatic reaction and polar PBS buffer solution. In contrast, the micrograph in Fig. 3d shows only Schlieren on the sides and was recorded at an earlier time point, so that the previously described reorganization could not take completely place. Fig. 3e represents the intermediate step. These reorganization phenomena take place on a time scale of a few minutes.

As the abundance and activity of expressed lipases depend individually on the type of cell, three different cell lines have been selected: the pancreatic cell line PaCa-2 and hepatocytes line HepG2 were expected to express a considerable high amount of lipase, due to their origin, compared to the P19 cell line which is assumed to express only moderate amounts.

The cytotoxicity investigations were performed on 96-well plates using the following protocol. An amount of 2.0 × 105 cells, after over night cultivation, was incubated with NaN_3 for 30 min, 2 and 24 h under cell culture conditions. The controls were incubated in media lacking NaN_3. At the end of these time intervals 60 μl of the supernatant from each cell well was taken out and a small amount of this solution was used for a Trypan blue staining to proof there, eventually, the existence of dead cells. The remaining solution was treated in an ultrasound bath for 5 min at 37 °C and centrifuged. 5 μl supernatant were taken out and applied on the LC-based sensor. The results of these experiments, which were performed in triplicate, showing the same result and represented here by only one micrograph, are shown in Fig. 4. There, polarized micrographs are presented that show the formation of transmitting areas on LC–lipid layers which were a direct indication for the presence of lipases. The first and second horizontal set of micrographs show the hydrolysis activity of released lipases after 30 min and 2 h incubation period of the corresponding cells in 0.5% NaN_3 solution. The series in (a) shows a droplet pattern of increased transmittance as direct indication of an enzymatic reaction already 5 min after the application of the samples. In (b), after 10 min, the further development of this reaction can be observed as an increase of bright spots in already affected areas and a spreading to neighboring LC compartments.

The third horizontal row (c) shows the interaction of supernatants of the three different cell types after 24 h of incubation and 10 min after the addition to a LC-based sensor. The obtained data indicate that PaCa-2 cells affect the lipid on the LC-based sensor the strongest compared to the HepG2 and P19 cells. This is also valid for the 4 h incubation (not shown here). The lipase activity seems to be independent of the incubation time for PaCa-2 (Fig. 4). Interestingly, this observation is different for P19 and HepG2 cells which show decreased interactions with the sensor after an incubation of 24 h (Fig. 4c), compared to 2 h (Fig. 4b). This indicates, that PaCa-2 lipases can maintain their activity for a longer time. Additionally, the hydrolyzing potential of PaCa-2 lipases is also higher than that of P19 and HepG2 enzymes. However, this method is not able to elucidate whether the observations are caused by differences in quantity, enzymatic activity or stability of the lipase.

In continuation of the investigation on cytotoxic effects of NaN_3 and detection of released lipases from various cells, experiments with paracetamol were performed. Paracetamol was chosen as reference substance as it is widely used as active ingredient in several drugs and has a proven cytotoxic effect on HepG2 cells. First, the sensitivity of the lipase release depending on the concentration of paracetamol was investigated. Therefore, HepG2 cells, P19 cells and PaCa-2 cells were incubated with 0.15%, 0.10% and 0.05% paracetamol in culture medium. However, only the results for HepG2 in 0.05% paracetamol containing mediums were presented in Fig. 5 that were performed in triplicate and are represented here by one micrograph only. There, polarized micrographs show the interaction between LC sensors and released lipases, depending on the incubation time with the cytotoxin and the cell number. Overall, a lipase activity was detected down to the very low cell number of 6250. In terms of incubation time with the cytotoxin, the lipase release was observed already after 30 min. A slightly stronger interaction of lipases from HepG2 with the lipid layer was observed for the 2 h incubations. Regarding the incubation time, the longer period of 24 h did not influence the enzymatic activity. This means, that a possible lipase release is achieved after 30 min and retains its activity for at least 24 h. An exception represents the sample of 2×10^5 cells, where a maximal activity was observed after 2 h that decreased after an incubation of 24 h (Fig. 5). This particular result correlates with the findings for NaN_3 (Fig. 4). For all tested concentration lipases could be identified. However, the strength of the effect varied in case of the 30 min samples (comp. 2.5 and 1.25×10^4 cells).

3.3. Comparison of the LC based sensor results with commercial lipase activity assay kit (Biovision III)

The detection of lipase content in the supernatant after cell treatment with cytotoxic compounds was measured using LC-based sensors with a phospholipid sensing layer and a commercially available high sensitive fluorescence kit, Biovision III. In the first case, the free lipase activity was determined from the changes of light intensity transmitted by the LC-based sensor using a polarized light microscope. The variation of transmitted light was caused by lipase-induced breakdown of a DOPC lipid layer and hence related to the lipase contents (vide supra). This method allowed the detection of the presence of lipases. In the second case, the lipase activity was measured with the Lipase Activity Assay Kit Biovision III. The lipases hydrolyze a specific substrate to generate the methylresorufin, which can be detected fluorometrically at $Ex/Em = 529/600$ nm. Lipase activity was measured after the incubation of 2.0×10^5 cells with 0.5% sodium azide for 2 and 4 h at 37 °C. As shown in Fig. 6a, the lipase activity in the supernatant of PaCa-2 cells was detectable after a 2 h treatment. In the case of P19 and HepG2 cells the lipase activity could be

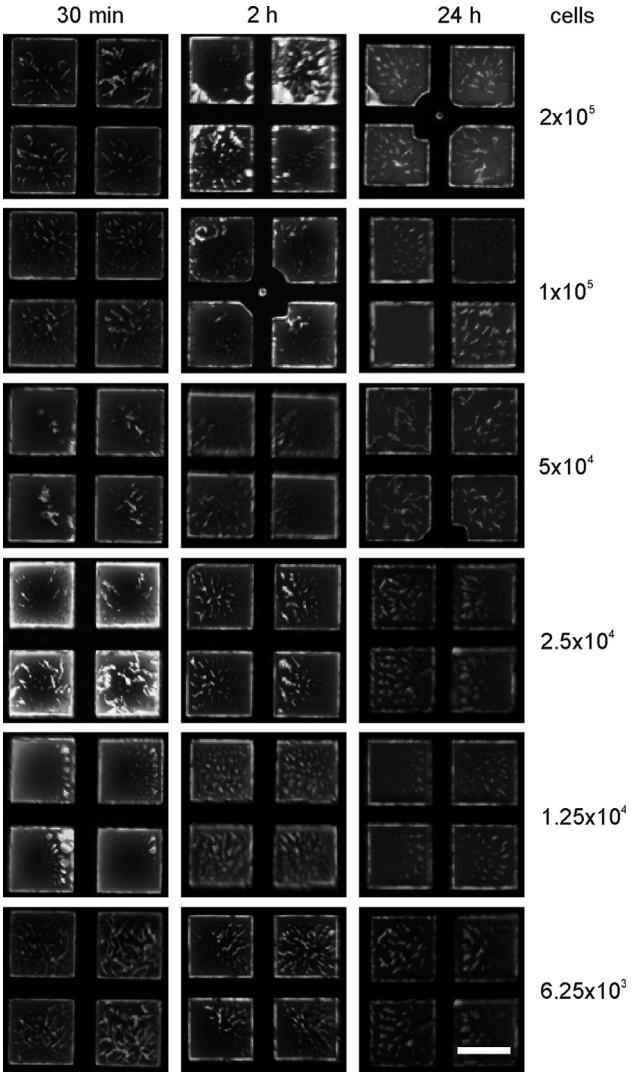

Fig. 5. Polarized light micrographs showing the interaction of cell supernatants of HepG2 on LC-based sensors after the formation of a DOPC lipid layer (0.1 mM in PBS). The incubation time of 30 min, 2 h and 24 h in 0.05% paracetamol containing culture medium are indicated in the first row as well as the cell seeding numbers per well used in these experiments. The scale bar represents 100 μm.

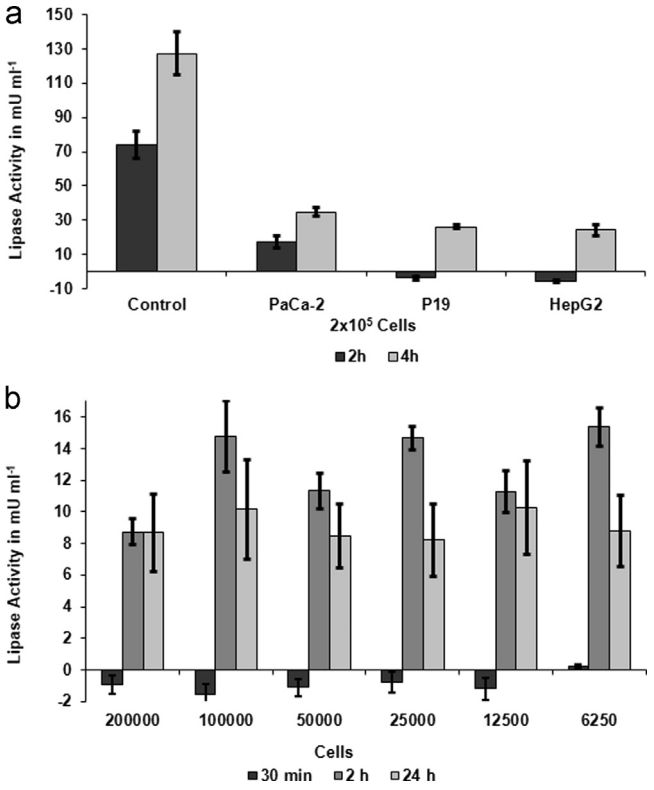

Fig. 6. Results of lipase activity fluorescence assays of cell supernatants obtained from (a) 2×10^5 cells of: PaCa-2, P19 and HepG2 after the treatment with 0.5% NaN_3 for 2 and 4 h, and (b) different cell numbers of HepG2 cells after treatment with 0.05% paracetamol for 30 min, 2 h and 24 h. The control sample represents in both cases the lipase positive sample. Error bars represent the standard deviation of six individual measurements performed in triplicate.

detected only after an incubation time of 4 h. On the other hand, using an LC-based sensor proved that an incubation time of 2 h was enough for the detection of lipases released from all cell types.

The higher sensitivity of LC-based sensors is more evident for experiments using a lower number of cells and paracetamol as cytotoxic agent. Using this method, it was possible to detect lipase activity in the supernatants of HepG2 cells even after 30 min of treatment down to ~6500 cells as is shown in Fig. 5. In contrast, using the commercial kit, the lipase activity could only be detected after 2 h in all supernatants. Additionally, a decrease of lipase activity was observed after 24 h (Fig. 6b). On the other hand, the LC-based method measured a steady increase of lipase activity even after 24 h of treatment.

4. Conclusion

This work describes an elegant detection approach for quantitative studies on cytotoxicity. The sensing system is based on the interaction of LC with phospholipid vesicles forming a lipid/LC interface in aqueous solutions. Such a layer can be disturbed and destroyed by lipases due to their hydrolyzing abilities towards esters. This mechanism allows a direct determination of lipase activity but restricts the LC-based sensor to single use, as the reaction products remain on the surface of the LC, modifying it.

Lipases are released in mammalian cells usually after their death. This principle was used to evaluate the presented detection system. For this reason, three different cell types (PaCa-2, P19 and HepG2) have been chosen as models to observe the lipase release. Cell death was initiated by a physical method using freeze-thaw cycles and by chemical methods using the well-known cell toxins NaN_3 and paracetamol. Generally, lipase activity could be detected regardless of the release method. In the case of physical release the application of two freeze thaw cycles was found to result in a maximal lipase release. The results of the chemical release with NaN_3 showed that differences in lipase activity from different cell lines could be detected. Particularly, lipases from PaCa-2 cells showed a stronger interaction with the LC sensor compared to the others. This result could be confirmed by a commercial fluorescence assay. Cytotoxic experiments with paracetamol revealed a high sensitivity of the detection system, recognizing lipases in a broad range of concentrations, from 2×10^5 to 6250 cells, for HepG2. Again, a fluorescence assay was used to confirm the results. Both methods indicated a maximal activity of lipase after 2 h incubations and a more or less constant activity for all investigated cell numbers that decreased after 24 h. The effect of the enzyme, however, could be detected already after 30 min with the LC system which was not possible with the standard fluorescence assay.

Overall, the LC based sensor with a DOPC layer represents a general tool that can be used for the detection of lipases. In a direct comparison to a fluorescence assay, the LC detection was responding very fast towards shorter incubation times, indicating a higher sensitivity to detect minute traces of lipases. These advantages, together with the fast responding time of the LC-base sensor (within minutes) and a comparably simple handling prove the applicability of such sensors in the assessment of early stage lipase secretion, which may play a significant role in future developments of novel diagnostics.

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