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EARLY DETECTION OF FUNGAL PATHOGENS IN PATIENTS WITH IMMUNODEFICIENCY INVOLVING A NOVEL BIOSENSOR TECHNOLOGY

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ABSTRACT

Oportunistic fungi such as *Candida* spp. and *Aspergillus* spp. are responsible for development of invasive fungal diseases (IFDs) in cancer patients and patients undergoing stem cell or solid organ transplantation, and are one of the most often causes of mortality in immunodeficient patients. Early fungal pathogen detection is prerequisite for fast intervention and adequate therapy administration. Fast pathogen differentiation between fungi, bacteria or even viral infections is highly required. During our study we developed biosensor chip based on the polymer poly lactic-co-glycolic acid (PLGA), as a novel diagnostic tool for rapid and quantitative detection of fungal infections from plasma sample of the immunodeficient patients. This double-layer, biomimetic, biosensor chip detects fungal enzyme aspartyl proteinase with ability of parallel elimination of eventual bacterial co-infection, achieved due to modulation of the biosensor's matrix. The biosensor setup is consisting of a thin-metal layer called inconnel, which is covered by polymer layer that can be degraded by lytic enzymes such as enzyme aspartyl proteinase. Enzymatic activity of aspartyl proteinase that can be produced by low number of fungal cells is sufficient to cause reduction in thickness of the polymer layer of the biosensor that can be visible even with the naked eye, as change in surface color of the biosensor. Biosensor prototypes were produced in a limited number under lab conditions and revealed clear signal in patient's sample with fungal infection which strength correlated with amount of excreeted asparty proteinase. Modification and optimization of the biosensor matrix due to addition of specific stimulative agents, increased metabolic activity of present fungal pathogens and positively affected release of enzyme aspartyl proteinase. This simple in use and relatively cheep biosensor-based fungal detection test provides first and rapid screen for presence of IFDs from very small amount of a test sample, in this case serum or plasma. Presented biosensor tehnology detects presence of fungal cells within few hours only and is not time consuming as most of the available conventional microbiological methods, and enables a good and fast diagnostic basis in making decision about the treatment therapy.

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INTRODUCTION

Invasive fungal infections (IFDs) may be mild or can even cause a life-threating diseases. The clinical manifestation of the fungal infections is dependent on physiological condition and immunity of the host. A systemic infections are often caused by *Candida spp.* which can penetrate into the mucosa, or on the other hand, be responsibe for allergic symptoms such as in the case of infection by *Aspergillus spp*. (Badiee *et al*. 2014). An adequate treatment requires an early diagnostic that is not easy task, since the interpreting of results of the diagnostic test can be challenging as well. IFDs are identified as one of the main reasons for high morbidity in immunodeficient patients, in both adult and pediatric patients (Gullo, 2009). Also single-gene inborn errors of immunity may be associated with susceptibility to the fungal infections and

development of errors of INF-Y immunity or congenital neutropenia and leukocyte adhesion deficiency type I, as predisposition to invasive candidasis and aspergillosis (Lanternier *et al*., 2013). Early diagnosis faces variety of obstacles, including low sensitivity, hard time to differentiate between colonization and invasive infection and of high costs, requiering special equipment and trained users. Biosensors present a novel piece of technology that already found successful application in detection of bacterial infections (Rushworth *et al*., 2014 and Ibrišimović *et al*., 2010). However, there is still lack on development of biosensors for detection of fungal infections. The aim of our study was to present a novel, fast and not expensive biochip for quantitative detection of invasive fungal infections within few hours only. The biosensor setup is made of metal layer and polymer layer that attracts fungal invading cells which attach to it and enhance their metabolic activity. The matrix of biosensor contains a stimulating agents which act as triggers for excretion of fungal enzymes which degrade the polymer layer and exhibit the detection signal. The obtained results open the way for considering biosensor technology as potential novel diagnostic tool for IFDs with multiple benefit, not only for treatment of immunodeficient patient, but for reduction of healthcare costs as well.

MATERIALS AND METHODS

Sample preparation: The blood samples were collected from immunocompromised patients with invasive fungal infectionsand kindly provided by Labdia Labordiagnostik GmbH, Vienna, Austria. The plasma samples were separated from the whole blood and as such used for testing of biosensors chip prototypes, produced under laboratory conditions.

General biosensor setup: The biomimetic polymer solution of PLGA [poly(lactic-coglycolic acid)] (Sigma-Aldrich) is directly transferred onto the Inconnel layer via Gravure Printing. The polymer solution has to be applied onto the printing plate, after which the impression roller can be run over the plate resulting in a defined layer with the desired thickness over the mirror layer. Between printing procedures, the plate, impression roller and doctor blade have to be cleaned thoroughly with 2-butanol and trichloromethan. Additional 10 minutes long incubation at 80°C is necessary toprovide complete polymer stability. Polymer degradation is caused by lytic enzymes secreted from a fungi. The process of degradation is irreversible resulting in a definite "signal" of the device in form of a irreversible colour change.

Linker: Desmodur® 2460 M (Sigma-Aldrich) was preferably used, as a monomeric diphenylmethane diisocyanate with high 2,4'-isomer content, and also triphenylmethane- 4, 4', 4''-triisocyanate. The big advantage of this isomer is that the storage and handling are greatly simplified because at room temperature it is a liquid.

Biosensor's detection signal: The intensity/degree of color change is in this respect proportional to the degree of change in the polymer layer. One major factor influencing the characteristics of the polymer layer is obviously the thickness of the polymer layer; another factor relates to the refractive index of the polymer layer, which is characterizing the optical thickness of the layer. In order to test the biosensor´s potential for changing its colour in contact with fungal enzymes, it was exposed to plasma sample containing fungal cells in the count of 10 CFU/mm^3. The positive control was sterile human plasma.10 µl of the sample were pipetted onto the biosensor surface and incubated in a humid chamber for ca. 16 hours at 37 °C. Therafter, the biosensor was washed with double-distilled water and then dried under an intensive airstream. The color of the device is defined by remission of incident light by the reflecting layer in combination with the properties of the polymer layer.

RESULTS

Development and optimization of the sensor setup: The biosensor is based on topical degradation of a specific bio-layer by fungal lytic enzymes facilitating sensitive optical read-out. The selectivity and specificity of the sensor setup is achieved by careful material selection for the reflective layer (the mirror layer). We used Inconnel, which is a nickel-chromium alloy with good oxidation resistance and often used in food processing. The printing process of the sensor is based on gravure printing technique (Figure 1) that implies transfering of polymer in mixture with the linker and various additives (glucose, tryptone, inorganic compounds (salts)) to the printing plate. The polymer solution gets sandwiched between the impression roller and the printing plate and is evenly dispearsed from the recessed cells to the foil (Ni-Cr alloy). The impression roller applies force, pressing the foil sheet onto the gravure cylinder, ensuring even and maximum coverage of the printing solution. The polymer layer, also called distance layer, is made of biodegradable polymer, that has ability to react with fungi, which attach to it and use it as suitable media for reproduction and excretion of different enzymes such as phospholipase in case of *Candida* species, or aspartyl proteinase in *Candida* and *Aspergillus* species, which are the main detection target of the sensor.

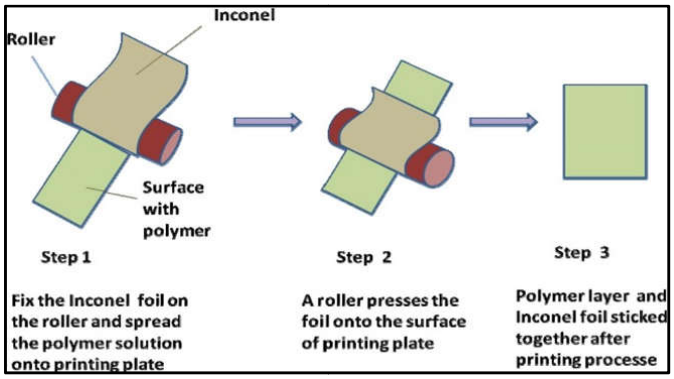

Figure 1. Schematic illustration of the gravure printing process

Enzymatic activity of fungal species was tested/confirmed in parallel with spectrophotometric assays for quallitative and quantitative analysis of fungal enzymes. The main problem in sensor setup was integration of various substrates within the matrix of the sensor (the polymer layer). The additives are important for stimulation of fungal enzyme excretion. However, in some cases some of additive compounds such as peptone, glucose or dextrose could not solve properly in polymer solution when applied in concentrations higher than 5 mg/ml. It was necessary to minimize concentration of additive substances in order to perserve the homogenity and stability of the polymer layer. This did not have any negative influence on sensitivity of the sensor, but simply ensured a better stability of the biosensor setup.

Changes in microbial metabolic rate directly influence the grade of biosensor's sensitivity: Aspartyl proteinase is an enzyme that acts as one of the crucial virulence determinants of *Candida albicans* and is involved in dissemination of infection. Due to stimulatory effects of drugs and other agents, excretion of microbial proteinases can be significantly enhanced. We could see that four members of analgesics (analgin, paracetamol, difen and brufen) as well as some corticosteroids (urbason) act as stimulatory agents for *C. albicans* excretion of aspartyl proteinase and increase its virulence and pathogenicity, increasing the sensitivity of the biosensor at the same time, when added to the test (Figure 2 and Figure 3).

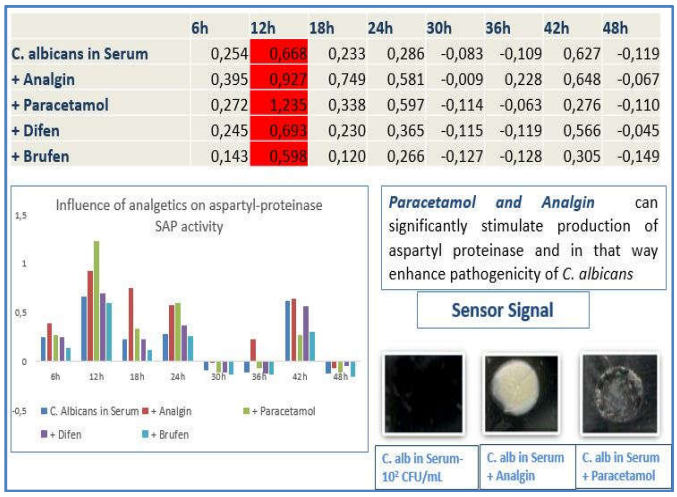

Figure 2. Influence of analgetics on fungal excretion of secretory aspartyl proteinase (SAP)

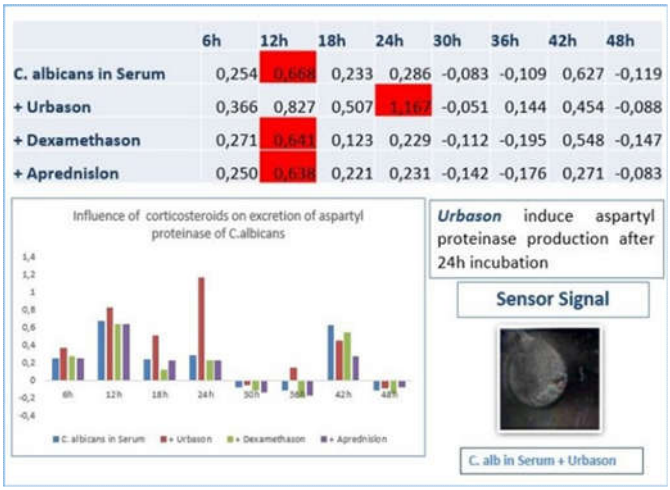

Figure 3. Influence of corticosteroids on fungal excretion of secretory aspartyl proteinase (SAP)

This discovery is suggesting that besides some of the nutritive compounds such as glucose that are commonly used by microbes, including the fungus, we can use a certain drugs such as analgesics or corticosteroids to stimulate metabolic activity of the fungal cells in a biological sample and to enable a stronger biosensor's detection signal.

Biosensor chips showed evident detection signal and high sensitivity due to presence of fungal cells in the patient's plasma samples: The biosensor prototypes of high stability and sensitivity, which were produced under laboratory conditions, were used for testing of plasma samples from three clinical patients suffering on invasive fungal infection. The blood samples were taken on three different days for each patients, and plasma sample was separated from the whole blood before it was applied on the surface of the biosensor in volume of 10 µl. The rafter, the biosensors were incubated at 37°C over night (16-24h). After incubation time the biosensors were washed with distilled water, dried and inspected for the signal. For positive control we used *C. albicans* in end concentraion of 1 CFU/mL and negative control was human serum. Parallel with validation of biosensor prototypes with clinical sample we monitored the key virulence determinants of *C. albicans* – amount of secreted aspartyl proteinase (SAP) at beginning and at the end of incubation which amount correlated with the strength of the detection signal (dada not shown).

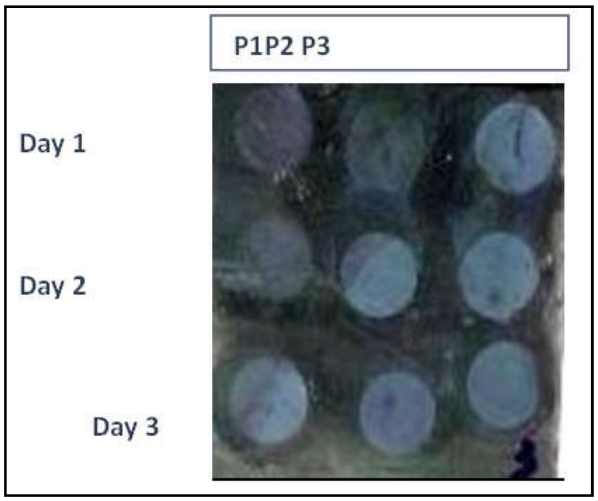

Figure 4. Biosensor chip prototype incubated with plasma samples from three different patients (P1, P2 and P3) suffering on invasive fungal infection (IFD), which were collected on three separate days, but day after day (Day 1, Day 2 and Day 3)

All negative controls showed no signal. All tested positive plasma samples showed detectable and with a naked eye visible signal via color change of the surface of the biosensors which was in contact with the fungal cells from the test sample (Figure 4.).

CONCLUSION

Our main goal was to develop a biosensor chip for detection of fungus-specific enzymatic activity, which would exhibit a strong detection signal, visible with a naked eye, if a test sample contains fungal cells. This approach is an additional rapid and inexpensive screening tool for broad detection of fungal pathogens in clinical specimens. The mechanism of action of the biosensor is based on the feature of microbial enzymes to degrade the polymer layer of the biosensor, which is prerequisite for manifestation of the signal (a color change of the biosensor). The biosensor prototypes which were tested with the clinical samples revealed a clear signal in all tested samples which correlated with amount of secretory aspartyl proteinase (SAP) as well. These findings suggest that this technology can be used as novel, fast and quantitative test for detection of fungal infections in plasma samples of patients with invasiva fungal infections, and reduce the time gap between diagnosis and making a treatment choice.

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