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An optical sensor for the detection of human pancreatic lipase

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ABSTRACT

We present the development of a new heterogeneous immunoassay to determine the concentration of human pancreatic lipase (HPL) using reflectometric interference spectroscopy (RIfS) and total internal reflection fluorescence (TIRF). HPL is a digestion enzyme used as a biomarker to detect an acute inflammation of the pancreas. The assay is carried out using the binding inhibition assay format. RIfS is used for antibody characterization and assay development as with this method binding signals can be observed time-resolved. A good surface stability and reproducibility of the HPL biosensor can be realized. Using a suitable regeneration agent makes it possible to perform up to 50 measurements on each RIfS transducer. To achieve the required limit of detection, the immunoassay is transferred to the TIRF principle and is therefore the first HPL immunoassay based on fluorescence labeling of the antibody. A series of concentration measurements and their calibration show a sufficient working range of 0.068–3.849 mg/L HPL using TIRF. On each TIRF transducer, 20–30 measurements can be performed.

1. Introduction

Severe pancreatitis requires immediate medical treatment to reduce irreversible damage to the pancreas [1,2]. Pancreatitis is an acute inflammation of the pancreas accompanied with uncontrolled release of digestion enzymes, e.g. pancreatic lipase and α-amylase. Released into the tissue these enzymes start to digest tissue components in adjacent areas of the body [3]. Both enzymes can be used as biomarkers in medical diagnostics to detect pancreatitis [4–6]. For a long time, mainly α-amylase was used as biomarker since α-amylase activity is easier and cheaper to determine [7] than pancreatic lipase. However, in the last few decades several studies revealed that human pancreatic lipase (HPL) is more specific [5,7–10], and so the number of measurements of lipase activity increases in the daily routine of medical laboratories. Furthermore, HPL is less influenced by age and gender compared to α-amylase [11]. At present, HPL is determined measuring its enzymatic activity instead of its overall concentration in blood. Therefore, measuring the HPL concentration could further increase the significance of the determined values.

Up to now, the HPL test to determine the lipase activity level in blood is carried out in several steps. It is a colorimetric test [12] that requires several reagents and trained personnel. As a consequence, this test is only provided in fully equipped laboratories.

For the first time, we present an optical immunoassay based on fluorescent labeled antibodies to determine pancreatic lipase concentration. A correlation between concentration and activity level of HPL has already been verified [13,14]. Normal concentration levels for adults are usually in the range of 7–57 μg/L HPL. An acute pancreatitis increases the HPL level to several hundreds or even more than 1000 μg/L [11,15]. In the 1980s, approaches to develop an immunoassay detecting HPL were based on enzymes using peroxidase labeled antibodies and required incubation times of 2–4.5 h [11,13,16,17]. In contrast, the test format of the immunoassay developed in the present approach is a binding inhibition test that requires just approx. 20 min incubation time and 10 min assay time. The assay is developed and optimized using the label-free optical detection method RIfS. This label-free method does not reach the necessary limits of detection for the assay but is most appropriate to optimize the surface chemistry and assay conditions. Furthermore, antibody interactions and affinities can be characterized by RIfS, since it is a method for time-resolved observation of binding signals. To achieve the required limit of detection, the assay is transferred to a sensor setup based on TIRF. Both techniques were already used in former approaches to detect medically relevant biomarkers, like C-reactive protein (CRP) [18]. A novel TIRF setup is established (not discussed in this paper) that can be further miniaturized, making it suitable for Point-of-Care Testing (POCT) devices [19–21]. Now, combining this POCT device with the developed biosensor allows for fast, cost-effective and simple measurements that provide physicians at under-equipped places (e.g. doctor's surgeries and rural clinics) with a small, cheap

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and easy-to-use analytical instrument for pancreatic failure. In this article, we present a new HPL immunoassay and demonstrate the feasibility of determining HPL concentrations on a TIRF setup that can easily be further miniaturized down to a POCT device.

2. Materials

RIfS glass transducers having a 1 mm D263-glass substrate with a layer of 10 nm Ta_2O_5 covered with 330 nm SiO_2 on top are obtained from Schott AG, Mainz, Germany. TIRF optical glass slides are obtained from KROMBACH Optische Werkstätte KG, Wetzlar, Germany. Amino-functionalized dextran (AMD; 50% aminofunctions w/w, 20 kDa) is purchased from Innovent e.V. Jena, Germany.

Common chemicals of analytical grade are purchased from Sigma-Aldrich, Deisenhof, Germany and from Merck KGaA, Darmstadt, Germany. 3-Glycidyloxypropyl-trimethoxysilane (GOPTS) is purchased from Fluka, Neu-Ulm, Germany. The lyophilized protein powders Ovalbumin (OVA) and Bovine Serum Albumin (BSA) are purchased from Sigma-Aldrich, Deisenhof, Germany. Lyophilized human pancreatic lipase (HPL) is purchased from Cell Sciences Inc., Canton, USA. Monoclonal mouse antibodies to human pancreatic lipase are purchased from QED Bioscience Inc., San Diego, USA (A1), from Abcam plc, Cambridge, United Kingdom (A2, Clone 8.F.195) and a monoclonal antibody against testosterone is purchased from Acris Antibodies GmbH, Herford, Germany (Clone 7003). For fluorescence labeling of the antibodies A1 and A2, the Dyomics DY-652 Label Kit is used. For regeneration of the sensor surface, a solution of 0.5% SDS (pH 3.5) in milli-Q water is used. The buffer solution is phosphate-buffered saline (PBS) with pH 7.4 and contains 150 mM NaCl and 10 mM KH_2PO_4.

3. Methods

3.1. Preparation of the sensor surface

The transducers for RIfS and TIRF measurements are prepared in the same way. Using the sandwich technique, each transducer is covered with another transducer during the modification process to increase homogeneity and to reduce the amount of the required solutions. 3–8 μl of the respective reagents are used for RIfS, and 20–50 μl for the TIRF transducer. After each step the transducers are washed with the solvent used for modification and dried under nitrogen.

1. Initially, the glass surface is pre-cleaned with 6 M potassium hydroxide solution (1–2 min) and further cleaned and activated in a freshly prepared Piranha solution (H_2SO_4:H_2O_2 with the ratio 3:2) for 15 min using an ultrasonic bath. Afterwards, the transducers are washed thoroughly with MilliQ water.
2. The activated glass surface is modified with pure 3-glycidyloxypropyl-trimethoxysilane (GOPTS). After 1 h, the transducers are washed with dry acetone.
3. [AMD]: The biopolymer aminodextran is covalently attached using a solution of 1 mg AMD in 7 μl MilliQ water (7 μmol) that is applied to the glass substrate.
4. [AMD-GA]: The next day, the amino functions on the surface are converted into carboxylic functions with a solution of 2 mg glutaric anhydride (GA) in 1 μl dry DMF (~17.5 M). The reaction is carried out for at least 6 h.
5. For the covalent attachment of HPL, N-hydroxysuccinimide (NHS) and Diisopropylcarbodiimide (DIC) are dissolved in dry DMF with a ratio of 1:1.5 and a final DIC concentration of 1.5 M. The solution remains on the substrate for 4 h.
6. [AMD-GA-HPL]: The applied HPL is delivered lyophilized into a TRIS buffer and is resuspended to obtain a concentration of about 1.7 g/L. TRIS contains amino functions which necessitates a buffer exchange previous to the coupling using phosphate buffered saline (PBS, pH 7.4). Now, several microliters of the protein solved in PBS are applied to the activated surface. The transducers are stored in a chamber saturated with water vapor for at least one day.

3.2. Antibody labeling with a fluorescence dye

Monoclonal antibodies against human pancreatic lipase (QED Bioscience Inc.) are labeled with the Dyomics DY-652 Label Kit. In a first step, 10–20 μl of sodium bicarbonate buffer (1 M) are added to a solution containing 100 μg anti-lipase antibodies (conc. 0.73 g/L). Then, 10–20 μl of DY-652 dye in DMSO (2 mM) are added and mixed. After 10 min, the mixture is purified by size-exclusion chromatography using spin columns. They are centrifuged for two minutes at 1500 rpm. Protein concentrations are about 0.034–0.090 g/L in PBS. The degree of labeling is 0.80–0.93. To obtain a higher degree of labeling, 40 μl of the above-mentioned dye solution are used. The antibodies processed in this way show a degree of labeling of 1.37 and a protein concentration of 0.036 g/L.

3.3. RIfS setup for label-free detection

RIfS is a label-free method used for time-resolved observation of binding events onto a surface. Here it is used to characterize antibody/antigen interactions and to gain information about assay properties during the optimization process, e.g. binding kinetics, surface stability and reproducibility of the binding signals.

White light (halogen lamp, 20 W) is directed perpendicularly onto the back of the glass transducers prepared before. Due to the design of the glass slide with several thin layers (described in Section 2), the light beam is partially reflected and all partial beams superimpose, causing a characteristic interference spectrum that is detected by a diode array spectrometer (Spekol-1100, Analytik Jena, Germany). If binding events occur on the sensor surface covered with recognition elements, the reflectance spectrum shifts. This method is nearly temperature-independent due to opposing temperature effects of the involved variables [22]. A detailed description of the setup can be found in literature [23,24].

3.4. TIRF setup for detection based on fluorescence-labeled antibodies

The miniaturized TIRF setup (Fig. 1a) consists of the diode laser OBIS LX 640-40 (Coherent Europe B.V., Utrecht, The Netherlands) emitting a wavelength of 640 nm with a power level of 15 mW. The intensity of the emitted fluorescence (680 nm) is detected perpendicularly to the surface by the ESELog USB detector (Qiagen Lake Constance GmbH, Stockach, Germany). Buffer and sample solutions are pumped through the PMMA flow cell using the syringe pump PSD/3 mini together with an 8-way-valve MVPmini and a 500 μl piston syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland).

A laser beam is guided through a glass slide and reflected several times, if the conditions of total internal reflection are met (Fig. 1b). On each reflection spot an evanescent field is induced, and only dye molecules close to the transducer surface are excited (644–649 nm) and emit fluorescence (667–672 nm). Therefore, only those labeled antibodies that bind to the sensitive surface (Fig. 2) and are not washed away during the subsequent washing step can be excited. Further details about this measuring principle can be found in literature [25–27].