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Programmable living material containing reporter micro-organisms permits quantitative detection of oligosaccharides

Carlos A. Mora, Antoine F. Herzog, Renzo A. Raso, Wendelin J. Stark*

Institute for Chemical- and Bioengineering, Department of Chemistry and Applied Biosciences, ETH Zurich, Vladimir-Prelog-Weg 1, 8093 Zurich, Switzerland

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ABSTRACT

The increasing molecular understanding of many diseases today permits the development of new diagnostic methods. However, few easy-to-handle and inexpensive tools exist for common diseases such as food disorders. Here we present a living material based analytical sensor (LiMBAS) containing genetically modified bacteria (*Escherichia coli*) immobilized and protected in a thin layer between a nanoporous and support polymer membrane for a facile quantification of disease-relevant oligosaccharides. The bacteria were engineered to fluoresce in response to the analyte to reveal its diffusion behavior when using a blue-light source and optical filter. We demonstrated that the diffusion zone diameter was related semi-logarithmically to the analyte concentration. LiMBAS could accurately quantify lactose or galactose in undiluted food samples and was able to measure food intolerance relevant concentrations in the range of 1–1000 mM requiring a sample volume of 1–10 μL. LiMBAS was storable for at least seven days without losing functionality at 4 °C. A wide range of genetic tools for *E. coli* are readily available thus allowing the reprogramming of the material to serve as biosensor for other molecules. In combination with smartphones, an automated diagnostic analysis becomes feasible which would also allow untrained people to use LiMBAS.

1. Introduction

In the past few years the spectrum of applications for stimuli-responsive materials has broadened significantly ranging from materials that heal themselves, regulate environmental conditions, defend themselves against micro-organisms or protect from vandalism to biomedical devices including drug delivery, tissue replacement or diagnostics [1–6]. Particularly in biomedicine, the increasing understanding of many diseases on a molecular basis has led to a need for novel diagnostic tools that are preferably easy-to-use and inexpensive. For example, food intolerances and allergies affect up to 20% of the population [7,8]. Thus, from a consumer's perspective, it would be of great advantage to have tools that allow a facile analysis of diet constituents in order to avoid adverse food reactions.

Recently, stimuli-responsive materials containing living organisms have been reported [9,10]. By enclosing micro-organisms into a sandwich consisting of a solid bottom and a porous top polymer sheet, the organisms could not escape but were still able to interact with the outer environment by absorbing nutrients or by producing antibiotics. Many micro-organisms offer a broad range of possibilities for sensor-related applications, since they can be relatively easily genetically manipulated, cultivated and flexibly adapted to produce own or foreign proteins under programmable, external conditions [11,12]. Another benefit of using living organisms is their capability to perform several complex tasks at once, such as detection, amplification and response to an external stimulus. Additionally, expensive and laborious material synthesis procedures can be avoided due to the fact that living organisms can reproduce themselves. Several microbial whole-cell sensing systems have been developed in the past years that employ different reporting systems (*e.g.* growth rate, oxygen respiration, engineered fluorescence or bioluminescence reporter genes) and implemented in different applications and setups (*e.g.* biochip arrays) to evaluate the toxicity of chemicals or to measure the concentration of environmental pollutants and other chemicals [13–15].

However, when assessing these applications for their potential usage by non-scientifically trained people outside of a laboratory environment, many of these applications are not easy to handle and require relatively expensive scientific analysis equipment such as

* Corresponding author.
E-mail address: wendelin.stark@chem.ethz.ch (W.J. Stark).

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spectrophoto-, fluoro- or luminometers. Additionally, ways have to be found to keep living micro-organisms alive, functional and protected from contaminating agents over a relevant timespan. According to bio-safety regulations, it must be also assured that genetically modified organisms cannot escape from the sensor device.

Diffusion, a substance-specific physical property, has been used among others in the classical agar diffusion assay, and there in particular to analyze growth inhibiting substances - such as antibiotics [16,17] or signaling molecules involved in quorum sensing [18]. Another example of diffusion-based analytics is the quantification of antigens by immuno-diffusion [19]. The quantification in agar diffusion assays is based on the fact that the diffusion behavior of a substance correlates to its initial concentration. For instance, to quantify the activity of a bactericidal substance, the area of the clear, bacteria-free "inhibition" zone needs to be measured. It has been shown, that the diameter x of the clear zone depends on the initial (c_0) and critical (c_{crit}) inhibitory antibiotic concentration as well as on bacterial growth. This dependence can be described using the following formula which also includes the diffusion factor D and the critical time for zone development t_{crit} [17]:

$$x^2 \propto D \cdot t_{crit} \cdot \ln(c_0 - c_{crit}) \quad (1)$$

In this study, we report the development of a sensor material that combines the diffusion behavior of an analyte with an analyte-specific bacterial fluorescence reporter system. The analysis only requires a camera, a blue-light source and a blue-light filter. We incorporated the well-established model bacteria *Escherichia coli*, genetically modified with a fluorescent reporter system, in a matrix of different polymers to create a programmable living material-based analytical sensor (LiMBAS). This material shows an easily quantifiable and specific response when coming into contact with an analyte anywhere on its surface and works at ambient temperature and humidity conditions. We established methods to easily detect and analyze the diffusion behavior of chemical stimuli that have been applied as droplets onto the materials surface using well-known optical techniques such as fluorescence trans-illumination and fluorometry. We programmed the organisms in the material to recognize the sugars lactose, galactose and isopropyl 1-thiogalactopyranoside and evaluated its application as a sensor for measuring lactose and galactose in food products in comparison with other established quantification techniques. We further determined the detection limit and storability of the material.

2. Materials and methods

2.1. Organisms and bacterial culture

For overnight cultures the bacteria were grown in 10 mL Lysogeny broth (Difco LB Broth Miller, tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L) supplemented with the appropriate antibiotics in a 50 mL culture tube under constant mixing (250 rpm) at 37 °C (KBF–ICH, Binder). For the material preparation, M9 minimal agar medium was used. M9 minimal medium (1×) contained M9 salts (17.2 g/L $Na_2HPO_4 \cdot \cdot 12\ H_2O$, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl), 0.2% glycerol as main carbon source, 0.2% casamino acids (Amresco), 1 mM thiamine hydrochloride (Fisher Bioreagents), 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, filled up to 1 L with ultrapure water. Appropriate amounts of pre-dissolved sterile agarose (electrophoresis grade, Apollo Scientific) were added to the media to reach the desired agar content. All media components had been sterilized by autoclaving or filtering (0.2 μm, Filtropur S, Sarstedt). 25 μg/mL chloramphenicol (Cm, Fisher Bioreagents) and 100 μg/mL ampicillin (Amp, Fisher Bioreagents) were added to all media.

E. coli BL21(DE3)pLysS cells containing the pLysS plasmid (Cm^R) coding for T7 lysozyme, a natural inhibitor of the T7 RNA polymerase allowing for improved transcriptional control, were purchased from Sigma–Aldrich (chem.-competent, Cat No: B3310). The BL21 strain is deficient for two proteases and therefore degradation of heterologous proteins is reduced. The strain genome includes the λ(DE3) prophage containing an inducible T7 RNA polymerase under the control of the *lac*UV5 promoter. The bacteria were transformed with pRSET/EmGFP plasmid (Life Technologies, Amp^R, Cat No: V353-20) following the transformation protocol provided by the manufacturer. pRSET/EmGFP codes for emerald green fluorescent protein (EmGFP) under the control of the T7/lac promoter. A total number of 20 colonies were selected after transformation of pRSET/EmGFP. After culturing them in liquid LB medium at 37 °C overnight, they were re-suspended at an OD_{600} of 0.4 in a volume of 200 μL LB medium containing 0.5% agarose and filled into 96-well microplate. After incubation for 3 h @ 37 °C they were induced with 10 μL 1 M Isopropyl-beta-D-thiogalactopyranoside (IPTG, >99% dioxin-free, Apollo) and incubated at 20 °C overnight. The best fluorescing colony was then selected and used in all further experiments.

2.2. Preparation of living materials

The optical density of the bacterial culture grown overnight was measured at λ = 600 nm. The volume of cell suspension for $OD_{600} = 0.4$ in a volume of 20 mL was centrifuged at 11′000 × g for 1 min and re-suspended in 20 mL M9 minimal agar medium containing 0.5% agarose at 40–45 °C. The resulting suspension was poured into a sterile petri dish (polystyrene, 16 × 92 mm, Sarstedt) and let solidify. A nano-porous polycarbonate membrane (400 nm pore size, PCTE, thickness ⌀ 30 μm, Sterlitech) was placed on top of the agar layer. After incubating at 37 °C for 4–5 h, the material was ready for usage.

2.3. Fluorescence imaging and zone analysis

Microplate reader: a well plate of 41 x 32 wells at a diameter of 1 mm had been previously designed as template grid on the microplate reader (TECAN). emGFP fluorescence was measured at ex = 485 nm, em = 520 nm at 25 °C. The data from the microplate reader measurements were arranged in an Excel data sheet (Office 2010, Microsoft) so that the fluorescence values of a plate were ordered according to their measurement time point and spatial location. The differences in fluorescence were made visible by conditional formatting (lowest to highest value, blue – white – red) (see Figure S1). Camera: the biosensor material was placed on a blue-light transilluminator (E-Gel® Safe Imager™ Real-Time Transilluminator, Invitrogen) and covered with a blue-light filter to detect emGFP fluorescence. Images were made with a camera (450D, Canon) or a smartphone (iPhone 4, Apple). Image analysis was done with Photoshop CS5 (12.0.4, Adobe). The zone diameter was measured using the integrated ruler tool of Photoshop.

2.4. Diffusion experiments

Serial dilutions of IPTG, lactose (anhydrous, Fluka) and galactose (D-(+), >99%, Acros) at concentrations of 500, 250, 125, 62.5 and 31.25 mM were prepared in ultra-pure water (18.2 MΩ, Millipore). 1 μL droplets of each dilution were placed on top of the cover membrane so that all dilutions of a specific inducer fitted onto one plate. The material was then incubated at room temperature (25 °C). Fluorescence measurements were performed repeatedly up to 20 h after induction.