Biomaterials 61 (2015) 1–9

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

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

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ARTICLE INFO

Article history:
Received 19 January 2015
Received in revised form
21 April 2015
Accepted 30 April 2015
Available online 15 May 2015

Keywords:
Bacteria
Whole-cell biosensor
Fluorescence
Genetic engineering
Membrane
Food allergies
Galactosaemia

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

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http://dx.doi.org/10.1016/j.biomaterials.2015.04.054
0142-9612/

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.

2.5. Detection limit

Serial dilutions of IPTG, lactose and galactose at concentrations of 10, 1, 0.1 and 0.01 mM were prepared in ultra-pure water. 1 μL and 10 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. Fluorescence measurements were performed after 5 h, 8 h and 20 h after induction. Light intensity was calculated using ImageJ [20] from inverted, 8-bit fluorescence trans-illuminescence images after optimizing contrast and brightness. The experiment was repeated twice.

2.6. Storability

The prepared living material was hermetically sealed using parafilm and then stored in the fridge at 2–6 °C for 1, 4, and 7 days. Serial dilutions of IPTG (500, 250, 125, 62.5 and 31.25 mM) were prepared in ultra-pure water. 5 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 experiment was repeated twice.

2.7. Measurement of lactose/galactose concentration in milk/lactose-free milk

Additionally to serial dilutions of pure lactose and galactose solutions at concentrations of 500, 250, 125, 62.5 and 31.25 mM, 1 μL droplets of milk (3.9% fat, Naturaplan, Coop Switzerland) or lactose-free milk (3.9% fat, FreeForm, Coop Switzerland) were placed on top of the cover membrane. The material was then incubated at room temperature (25 °C). Fluorescence measurements were performed after 7 and 19 h after induction. Lactose and Galactose concentrations in the standard concentrations and milk samples were validated by HPLC-MS analysis (Agilent 1100 Series LC/MSD, single Quadrupole, Electro Spray Ionization, flow injection (10 μL, dilution in water of 10^{-4}) with water/MeOH 20/80 isocratic with 0.1% formic acid) and a flourometric lactose/galactose enzymatic assay (MAK011, Sigma–Aldrich).

2.8. Cost analysis

Material costs for an assumed 1 dm^2 area, 10 mL agarose (1%) and 10 mL M9 medium (2×) were calculated. Following materials were included in the cost analysis: porous membrane, 2.33 $/$dm^2$ (Sterlitech, PCT0420030, 09.10.2014); agarose (1%), 0.07 $/10 mL (Apollo Scientific, BIA1176, 09.10.2014); M9 minimal medium (2×), 0.08 $/10 mL (Sigma–Aldrich, M6030).

3. Results

To create LiMBAS, we mixed pre-cultured *E. coli* containing the *emgfp* reporter gene under the control of the T7/lac promoter at a concentration of $3.2 \cdot 10^8$ colony forming units (cfu) per mL with selective nutrient medium and agar (0.5%) and applied the mixture on a transparent sheet of polystyrene. This resulted in an approximately 3 mm thick 'living layer' (Fig. 1). This layer was covered with a 30 μm thick porous pre-sterilized polycarbonate membrane with a pore size (⌀ 400 nm) small enough to retain the bacteria. The

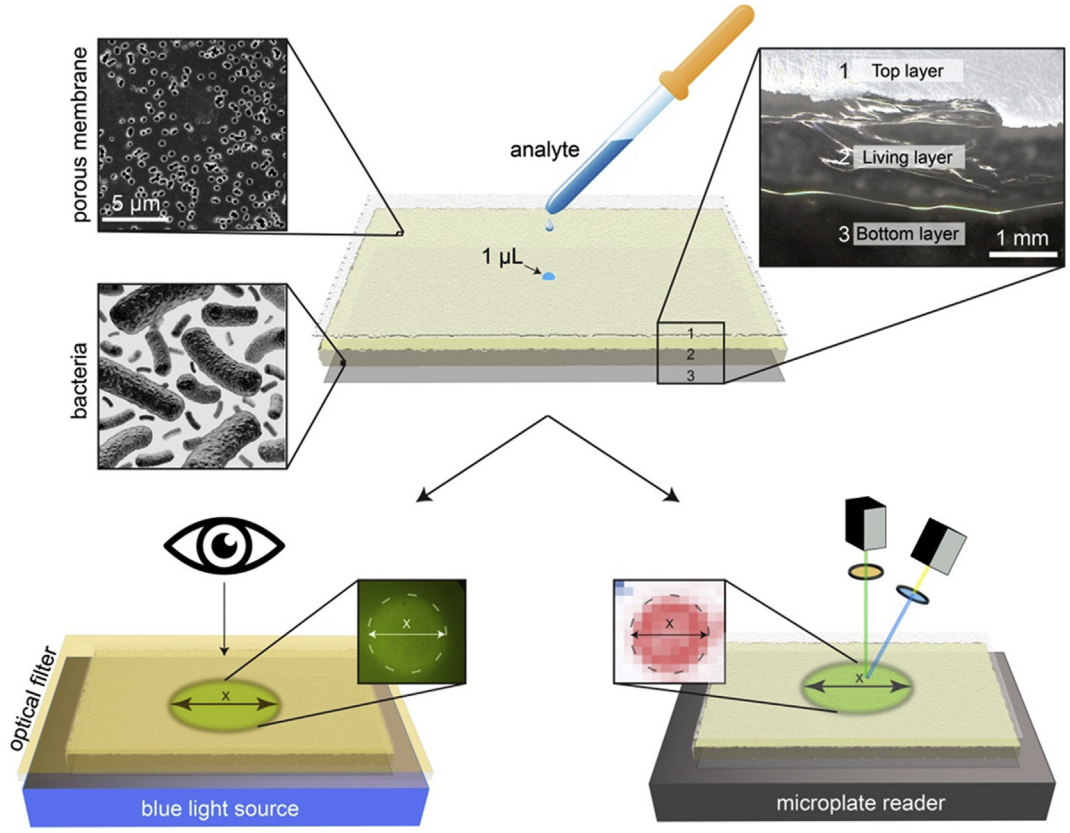

Fig. 1. Illustration of the quantification principle. A droplet of analyte (*e.g.* 1 μL) is applied on top of the porous membrane (1). In the upper left part a surface electron microscope image of the porous membrane is shown. The analyte diffuses radially through the living layer (2) inducing the bacteria to express fluorescent proteins. The extent *x* of the fluorescent zone is quantifiable by eye or camera using a blue light source and an optical filter, or alternatively by a fluorescence microplate reader.

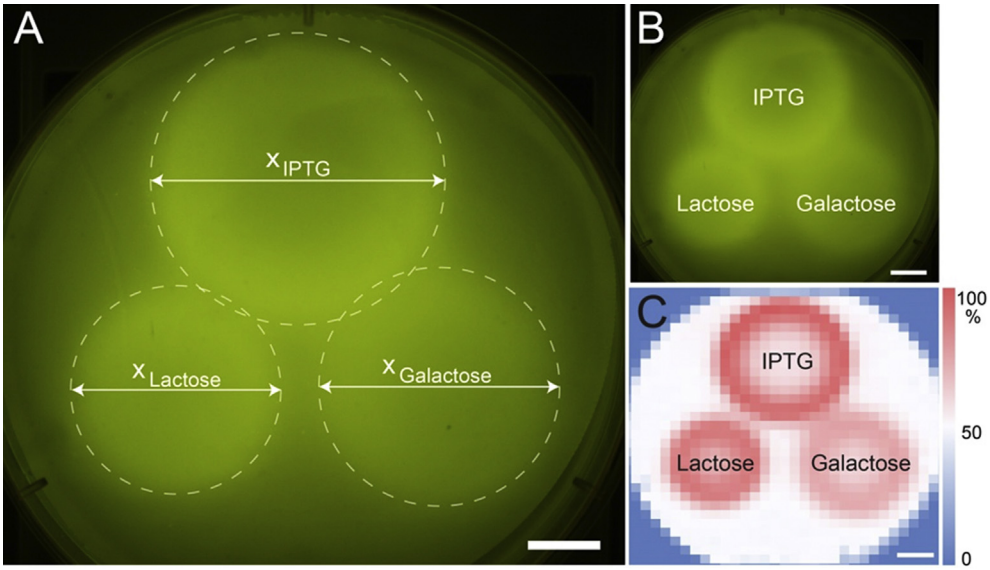

Fig. 2. Fluorescent diffusion zones caused by IPTG, lactose and galactose. (A) The extent of the zone (dashed line) was quantified by measuring the zone diameter *x*. The image was generated using a camera, a blue-light source (LED array) and blue-light filter. Panel (B) shows the same image as in (A) without lines. (C) Fluorescence heat map of the same assay generated with a fluorescence microplate reader (Ex/Em 485/510 nm). A volume of 1 μL at a concentration of 1 M IPTG, galactose and lactose was applied. The images were generated 13 h after induction. Scale bar, 1 cm.

composite material was incubated at 37 °C for 4–5 h. This is enough time for the bacteria to adapt to the new conditions and enter the growth phase during which reporter gene expression is optimal. After the initial incubation period, the biomaterial was ready to be used at ambient environmental conditions (25 °C, 50% humidity). To investigate whether the bacteria can be induced when incorporated into the living surface we applied 10 μL or 1 μL droplets of isopropyl β-D-1-thiogalactopyranoside (IPTG), at various concentrations on top of the living surface. IPTG is a well-known, non-hydrolyzable inducer for the T7/lac promoter. We initially used a standard microplate reader to visualize the developing fluorescence signals over time by conditionally formatting the obtained fluorescence values from each well with a color scale (Figure S1). But the fluorescence signal could also be directly made visible using a blue-light source and a blue-light optical filter (Fig. 1). Besides IPTG, the sugars lactose and galactose can also induce the expression of genes under the control of the T7/lac promoter, if the medium does not contain glucose as a major carbon source [21]. Therefore, we replaced glucose with glycerol for our experiments. The inducers diffused radially from the point of application causing a circular fluorescent zone. The border of the circular zone showed strong fluorescence intensities which enabled a clear discrimination of the zone from the background (Fig. 2).

When using the inducer IPTG, we observed a strong linear correlation between the zone diameter *x* and the logarithm of the initial inducer concentration *log*(*c*) for all time-points measured after the initial inducer application (Fig. 3). There was also a linear correlation between the zone area x^2 and *log*(*c*), but less pronounced as compared to *x* vs. *log*(*c*). The diffusion velocity described by plotting the zone area x^2 versus time behaves linearly in the experimental time range (0–540 min) (Fig. 3C).

After characterizing the material's response to IPTG, we compared the performance of the two other inducers galactose and lactose with IPTG. The results show that the slope of the linear correlation between diameter *x* and the logarithmic concentration *log*(*c*) depended much more on the time after induction then on the type of inducer (Fig. 4). In contrast to that, the *y*-intercept of the respective fitting functions, *i.e.* the zone extent at a given concentration, differed significantly depending on the type of inducer. After seven hours, the zone diameter for IPTG was on average 3.1 ± 0.3 mm larger than the one caused by galactose and 6.9 ± 0.3 mm larger than that of lactose. Besides the zone diameter, the development of fluorescence over time also differed between the three inducers. When measured in the center of the zone, the lag time of fluorescence development was 36 min for IPTG and thus much shorter than for lactose and galactose with 112 and 121 min, respectively (Fig. 4, Movie S1).

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2015.04.054.

Besides the time of fluorescence development, also the limits of detection differed between IPGT and the two other sugars based on a signal-to-noise ratio of 3:1. For IPTG, concentrations could be detected down to 0.1 mM using a volume of 10 μL (1 mM with 1 μL), which is equivalent to an absolute amount of 1 nmol. Lactose could be detected down to 1 mM (10 nmol) and galactose to 10 mM (100 nmol) (Fig. 5). For a future application of LiMBAS as a diagnostic tool, we tested the storability of the material at low temperatures. After freshly preparing the material, we stored it hermetically sealed in the fridge at 2–6 °C for up to 7 days before applying inducer at different concentrations again at ambient conditions. The material was storable for seven days at least and could be readily used after this period, although the background fluorescence increased over time (Fig. 6).

In a real-world application we used LiMBAS to determine the lactose or galactose content directly from food samples. Lactose was measured from commercially available pasteurized cow's milk. Galactose was measured in lactose-free milk. We used lactose-free milk derived from cow's milk that had been industrially treated with the enzyme lactase. Lactase is a glycosidase (EC 3.2.1.108) and converts the disaccharide lactose to the monosaccharides galactose and glucose. The content of lactose is negligibly small in lactose-free milk and vice-versa the content of free galactose in cow's milk is negligible compared to the amount of lactose [22]. Around the same concentration of galactose in lactose-free milk as lactose in the original cow's milk can thus be expected. We derived the concentrations of lactose in milk or galactose in lactose-free milk by

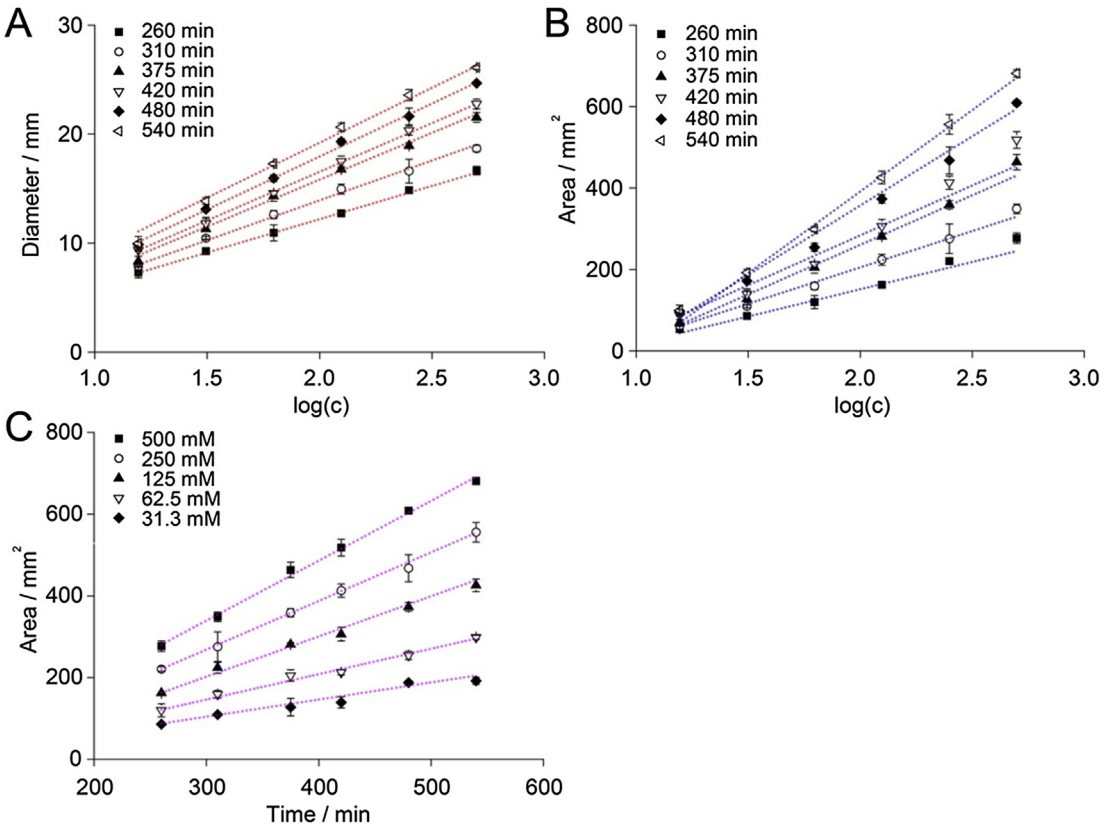

Fig. 3. Zone development in response to different concentrations of the inducer IPTG. The semi-logarithmic plots of (A) the fluorescent zone diameter x and (B) area x^2 against the initial concentration c_0 (15.1, 31.3, 62.5, 125, 250 and 500 mM) of IPTG are shown. The data points were fitted to a linear function (red and blue dotted lines), (A) $R^2 > 99.1\%$, (B) $R^2 > 97.1\%$. (C) Linear plot of the fluorescent zone area x^2 against the time after start of the measurement. The data points were fitted to a linear function (magenta dotted line), $R^2 > 97.7\%$.The applied inducer volume was 1 μL. Mean values and standard deviations are displayed (n = 3). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

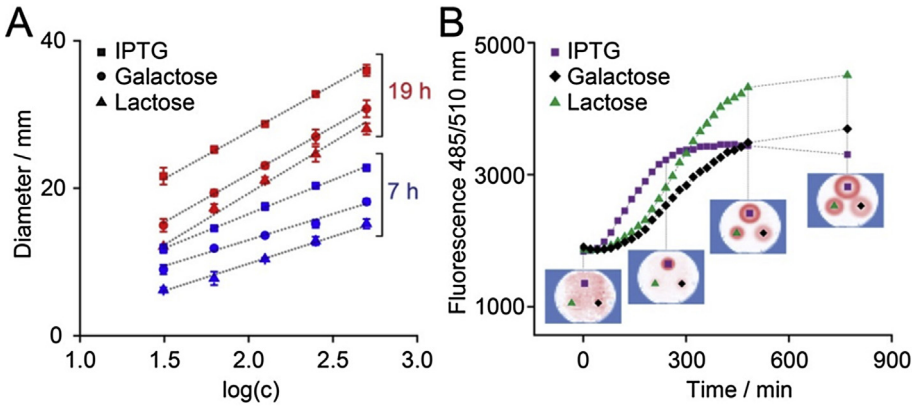

Fig. 4. Fluorescence development for lactose, galactose and IPTG. (A) Semi-logarithmic plot of the fluorescent zone diameter x against the initial concentration c_0 of IPTG, lactose and galactose (31.3, 62.5, 125, 250 and 500 mM) after 7 h and 19 h. The data points were fitted to a linear function (dotted line), $R^2 > 98.0\%$. The applied inducer volume was 1 μL. (B) Fluorescence intensity of zone centers for zones induced by IPTG, lactose or galactose at a concentration of 1 M (Vol = 1 μL). Fluorescence readings (arbitrary units, Ex 485 nm, Em 510 nm) measured by microplate reader are given (t = 0, 4, 8 and 13 h after induction). Mean values and standard deviations are displayed (n = 3).

applying a liquid food sample together with five standard solutions of the respective sugars prepared in water at concentrations ranging between 31 and 500 mM onto the sensing material. The incubation time after addition of the milk sample (7 or 19 h) did not have a significant effect on the final concentration (Fig. 7, see also Fig. 4). The results obtained from LiMBAS were compared to standard measurement methods for lactose and galactose concentration in milk, high pressure liquid chromatography (HPLC) coupled to a mass spectrometer (MS) and fluorometric enzymatic assays using the same food and standard samples.

4. Discussion

We showed that the diffusion behavior of a chemical substance can be visualized by a gel-matrix embedded whole-cell microbial reporter system and used to quantify the concentration of the

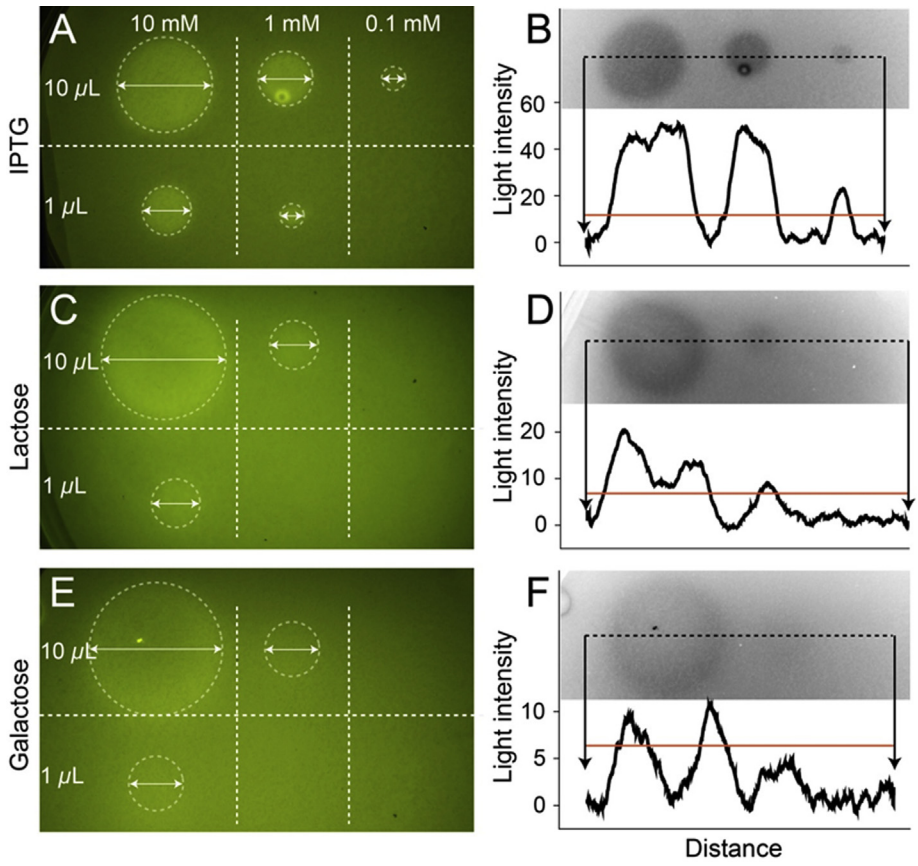

Fig. 5. Determination of the limit of detection for IPTG, lactose and galactose. The applied inducer volume was 10 or 1 µL, the applied concentration 10, 1 and 0.1 mM. LiMBAS was measured by blue-light trans-luminescence. (A, C, E) represent non-modified images. Arrows indicate the zone radius. (B, D, F) depict the light intensity of fluorescence zones calculated from an inverted gray-scale image of the upper 10 µL row of the respective left-side image. The horizontal red line represents a signal-to-noise ratio of 3:1. Pictures were taken after (A, B) 5 h, (C, D) 15.5 h and (E, F) 15.5 h post induction. The bright spot in panels A and B in the diffusion zone of 0.1 mM is caused by an air bubble entrapped in the agar layer.

investigated substance. As expected from previous diffusion studies with matrix-embedded micro-organisms (Equation (1)), the response of the material to the diffusing inducer chemical suggested a semi-logarithmic dependence between the diffusion zone extent and the initial inducer concentration (Fig. 3). However, in contrast to Equation (1), we observed that the correlation between the zone diameter x and the initial concentration c_0 is slightly better ($R^2 > 99.1\%$) than for the area x^2 and c_0 ($R^2 > 97.1\%$). This observation can be explained by the fact that the diffusing layer of our material is around three millimeters thick, thus not completely two-dimensional and additionally covered by a porous membrane, which is deviating from the two-dimensional diffusion behavior approximated by Equation (1). Concerning the diffusion velocity during the first few hours after induction, not x but x^2 was dependent on time, which can be expected for a two-dimensional diffusion (Fig. 3).

LiMBAS with *E. coli* works at ambient temperatures (~25 °C) which reduces the required equipment but slows down the production of reporter proteins by the bacteria, which have an optimal growth temperature at 37 °C [23]. The necessary time for signal development could thus be optimized by increasing the incubation temperature which has to be addressed in future studies. Nevertheless, using a blue-light transilluminator, the development of clear distinguishable fluorescent zones took between 2 h for IPTG to 5 h for lactose or galactose. The duration to develop a detectable response signal after exposure to an inducer differed for the tested substances. The lag time for fluorescence development was around three times higher for galactose and lactose than for IPTG. This difference is based on the fact that IPTG is capable to directly and irreversibly deactivate the lac repressor. The sugars lactose and galactose need to undergo further transformation steps to the IPTG-analog allolactose which naturally and reversibly inhibits the lac repressor [24].

Analogous to the time of development, the necessary amount of inducer to give a detectable fluorescence signal with an S/N ratio $\geq$3 was one order of magnitude lower for IPTG compared to lactose or galactose (Fig. 5). For higher concentrations of IPTG, lactose or galactose, the S/N ratio increased further (data not shown).

In a realistic application, we tested LiMBAS for its ability to detect lactose directly from cow's milk and galactose from lactose-free milk - without previous dilution, which is necessary for methods such as HPLC-MS or enzymatic assays. We chose milk for our proof-of-principle experiment since it is a widely used food product and a source of several common food disorders including lactose intolerance, allergies as well as galactosaemia, a genetic disorder of the human metabolism of galactose with potentially lethal outcome if untreated [25]. We observed that the time point of relative zone extent measurement (either 7 h or 19 h) does not have an influence on the measured analyte concentration. Thus, analysis could be done over a range of several hours after sample application.

We compared the results of our material to measurements obtained using a commercially available fluorometric enzymatic assay and HPLC-MS. The standard deviation of our assay was around one

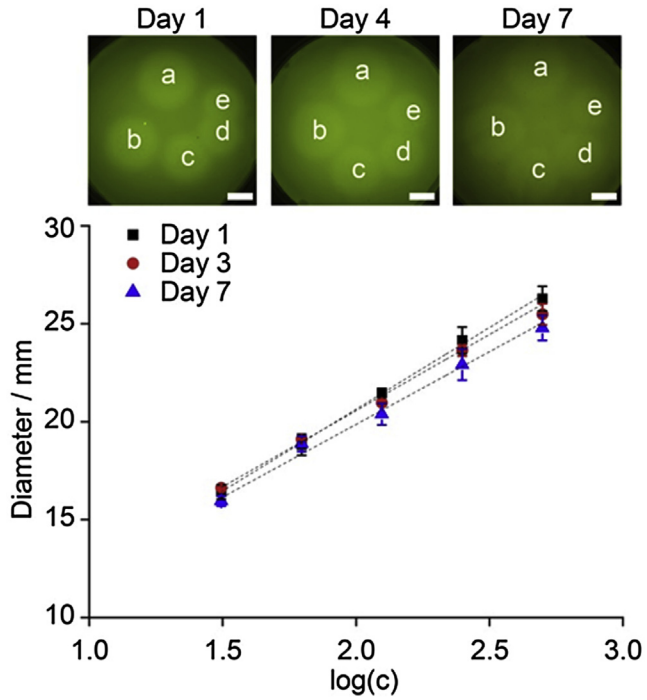

Fig. 6. Storability over time. The living material was stored for 1, 4 and 7 days in the fridge at 2–6 °C. The applied inducer volume was 5 μL, the applied concentration (a) 500, (b) 250, (c) 125, (d) 62.5 and (e) 31.25 mM. Semi-logarithmic plot of the fluorescent zone diameter x against the applied concentration c_0 of IPTG after $t = 6$ h. The data points were fitted to a linear function (dotted line), $R^2 > 98.6\%$. Scale bar = 1 cm. Mean values and standard deviations are displayed (n = 2).

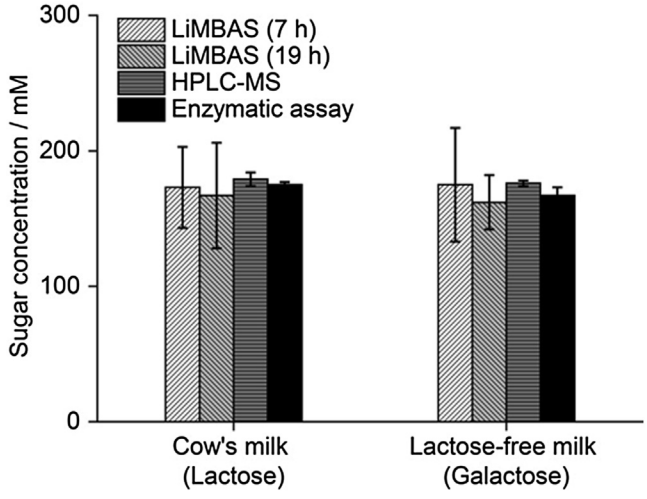

Fig. 7. Measured concentration (mM) of lactose in (galactose-free) cow's milk or galactose in lactose-free milk. Measurement was done with LiMBAS after 7 h and 19 h of analyte application (Vol. = 1 μL) and with HPLC-MS and an enzymatic assay. Mean values and standard deviations are displayed (n = 3).

order of magnitude higher in comparison to the other methods with the same number of replicates (n = 3). One possible factor contributing to a higher variation is the semi-logarithmic relationship of zone extent and concentration. Both HPLC-MS (signal intensity) and enzymatic assay (light/fluorescence intensity) are based on a linear correlation.

However, our material has several advantages compared to established diagnostic techniques: firstly, the handling of our diagnostic assay requires little technical knowledge and does not need complex and expensive devices. The user only needs to apply the liquid containing the analyte and standard solutions onto the membrane. After incubation at ambient conditions over a time course of a few hours, the living material has to be placed above a blue-light source and covered by a blue-light filter. As blue-light source an array of blue-light LED lamps is already enough to serve as a blue-light source.

Analysis can be done for instance by taking a picture with the camera of a smartphone, which could then automatically be analyzed by a smartphone application programmed to measure the zone extent and to calculate the resulting analyte concentration from a standard curve (Fig. 8). In contrast to enzymatic assays, there is no need for a colori- or fluorometric quantitative analysis using a spectrophoto- or fluorometer.

Secondly, our material has a size-selective porous top membrane, which prevents contamination of the environment and the living layer by bacteria and decreases dehydration of the living layer. For our experiments we used a top membrane with an average pore size of 400 nm, but also smaller pores could be used to retain smaller bacteria and to lower the risk of contamination with bacteriophages. Genetically modified micro-organisms could be used outside of a protected laboratory environment. In any case, these organisms should not be capable of surviving outside of LiMBAS. Mandell et al. (2014) recently demonstrated that genetically modified *E. coli* bacteria can be made incapable of surviving in or adapting to a natural environment by rendering the micro-organisms auxotrophic for certain artificially modified metabolites such as amino acids [26]. Such bacteria could be also used in LiMBAS. Nevertheless, the material's environmental safety and in particular its safe disposal need more in-depth investigation and testing.

Furthermore, our material is storable at fridge temperatures (2–8 °C) in a ready-to-use fashion without losing its functionality prior to a potential 'home' application. From literature it is known that a temperature of 5 °C reduces the growth and metabolism of *E. coli* to almost zero [27]. We showed that the prepared material is usable for at least seven days when stored in the fridge. However, for a final application as a real-world product, the time to manufacture, ship and purchase the material would have to be considered and the storage time would have to be prolonged to around at least one month. This could be done by optimizing the material preparation procedure, storing temperature and chemical

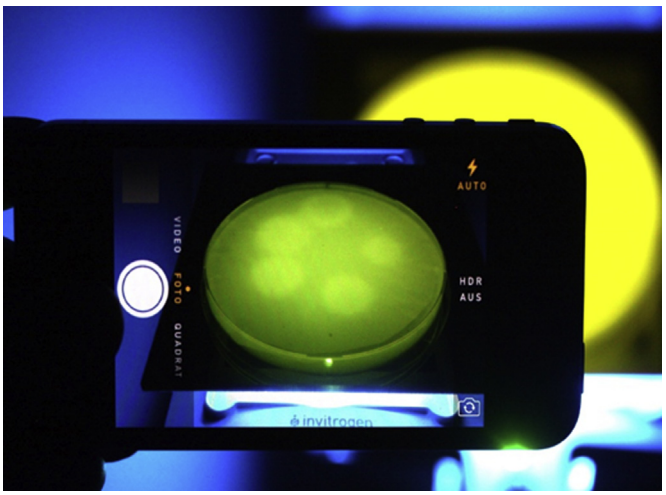

Fig. 8. Taking an image of LiMBAS using a smartphone and a blue-light source with optical filter.

Table 1
Examples of substances detectable by micro-organisms.

Micro-organism	Chemical	Reporter system	References
Escherichia coli	Chromated copper arsenate	lux	[29]
	Nitrate	gfp, ice	[30]
	Diverse toxins	lux	[31]
Pseudomonas spp.	Naphtalene	lux	[32]
	Benzene,Toluene, Ethylbenzene, Xylene	lux	[33]
Ralstonia eutropha	Polychlorinated biphenyls	lux	[34]

composition of the living layer medium.

Considering the costs, the raw materials sum up to around 2.50 $ per biosensor unit (assuming an area of 1 dm^2 per unit), of which more than 90% can be attributed to the porous top membrane. Moreover, micro-organisms such as *E. coli* can easily be propagated and numerous tools are available for genetic modification.

For this proof-of-principle study we programmed our bacterial reporter system by combining the well-established lac promoter with a highly fluorescent version of the traditional reporter gene for the green fluorescent protein. Many bacterial reporter systems with various promoters and reporter genes have been developed in the past years. Besides that, the technique to introduce new reporter systems *via* promoter-less reporter vectors is already well-established and commercially available [28]. Table 1 gives a selection of various chemical compounds that can be quantitatively reported by genetically engineered micro-organisms. Besides others, the bioluminescence reporter systems such as the lux reporter would also be applicable with LiMBAS and, compared to fluorescence reporter systems not even need an external light source.

Furthermore, the sensitivity of the living surface can be enhanced by using multiple promoter systems – each coupled to different fluorescence or bioluminescence reporter genes. Consequently, different substances could be detected and quantified in a simple or multiplex approach.

5. Conclusion

Overall, taking the mentioned advantages such as easy handling and analysis, use of well-established materials, potential out-of-lab application, low costs and high flexibility of adaptation into consideration, LiMBAS can clearly be advantageous compared to other diagnostic techniques such as HPLC-MS, enzymatic assays or also other whole-cell based assays. LiMBAS as biomaterial offers the possibility to safely use engineered micro-organisms outside of a laboratory environment without the need for culturing or complicated analysis. The quantification principle of combining diffusion behavior with matrix-embedded microbial whole cell reporter systems could be applied to develop a variety of diagnosis tools for the analysis of food, blood or environmental constituents, which can be used in domestic or outdoor applications.

Acknowledgments

We thank Gediminas Mikutis and Dr. Robert Grass for the helpful discussions. This work was financially supported by ETH Zurich, Switzerland.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2015.04.054

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