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ORIGINAL PAPER

Biomimetic PLGA sensor: proof of principle and application

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Abstract Spoilage of products can mainly be attributed to microorganisms which "live on the product", i.e. which are able to utilize and/or metabolize components and/or molecules of the product. The objective of this work was to develop and optimize sensor prototypes indicating the quality of a product "in real time", i.e. at the time the consumer is looking at the product. The design of the presented sensors relates to optical phenomena, for example anomalous absorption and remission of light. The crucial point of the sensor prototypes is a layer sensitive to the analyte, a polymer degradable by enzymes produced by the respective microorganisms. After incubation of the sensor setup with contaminated products, the lytic enzymes released from decaying cell material change the thickness of the polymer layer and generate a colour change visible by the naked eye. Production of the sensor prototypes is very simple and inexpensive and they might be successfully integrated into product packaging.

Keywords Biomimetic sensor chip · Biodegradable polymer · Decomposition of natural products · Lytic enzymes

Introduction

Conventional tests for detection and identification of pathogens and detection of microbial deterioration of products from the cosmetics and pharmaceutical industries give very reliable results. They do, however, need conventional culturing techniques and thus are time consuming, and need sophisticated laboratory equipment and well trained staff. Thus only testing of random samples is possible and contaminated samples might be detected too late.

The current tendency to carry out field monitoring has driven the development of biosensors as new analytical tools able to provide fast, reliable, and sensitive measurements at lower cost. These biosensors do not yet compete with official analytical methods, but they can be used both by regulatory authorities and by industry to provide enough information for routine testing and screening of samples [1].

The main area of biosensor application and potential application is in health care. Measurements of blood, gases, ions, and metabolites are often necessary to show the patients' metabolic state. Biosensors can also be used to monitor industrial processes in three ways: off-line in a laboratory, off-line but close to the process, or on-line in real time. Such monitoring could result in improved product quality, increased product yields, and increased health care and safety for consumers.

During this work it could be shown that a device comprising two layers only, a reflective layer and biodegradable polymer layer, can be used for analysis of the quality and/or age of products such as medical products, cosmetics, and nutritional supplements.

Typically, degradation of a polymer occurs by cleavage of covalent bonds within the material. Polymer degradation is caused by lytic enzymes secreted from either the product to be tested or from a microorganism associated with the product, or both. Examples for such enzymes are phospholipases, pronases, proteinases, hydrolases, lipases, or esterases. Thus, the process of degradation is irreversible,

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resulting in a definite "signal" of the device in the form of an irreversible colour change.

The colour of the device is defined by remission of incident light by the reflecting layer in combination with the properties of the polymer layer. Thus, the polymer layer may have different characteristics as long as it is biodegradable and optically transparent such that incident light is able to contact the reflecting layer and remitted light is able to pass the polymer layer (wherein the polymer layer does, however, affect the remission). The intensity or degree of colour change is, in this respect, proportional to the degree of change in the polymer layer. One major factor affecting the characteristics of the polymer layer is, obviously, its thickness; another factor is the refractive index of the layer, which characterizes its optical thickness.

Incident light is remitted by the reflecting layer as described above and ultimately leads, in combination with the optical properties of the polymer layer, to a specific colour of the device which may appear to the human eye as, e.g., red, white, blue, or green.

Most preferably, the biodegradable polymer comprises a biomimetic polymer, i.e. a polymer with characteristics mimicking a natural material, which has already been approved and extensively tested for its behaviour when exposed to a natural product and/or a living organism. Poly(lactic-co-glycolic acid) (PLGA), which is preferably used as a biodegradable polymer, is approved for in-vivo use. Other polymers which can be used in the sensor setup described above may be selected from the group of polymers comprising polylactic acid (PLA), poly-L-lactic acid (PLLA), polyhydroxybutyrate (PHB), and polyvinylcaprolactam (PVCL), or any other polymer which can be classified as degradable by biomolecules, as defined above. Preferably, biodegradable synthetic polymers can be used. These may also comprise synthetic polymers of gelatine, agarose, dextrose, lipids, cellulose, starch, chitin, chitosan, polyhydroxyalkanoates, poly(caprolactone) (PCL) or PCL-systems, poly(ethylene/butylenes) succinate, poly(ethylene/butylenes) adipate, or polynucleic acids. The biodegradable polymer layer may also comprise a cross-linking agent. This cross-linking agent may be a bifunctional agent, for example diisocyanate, glutardialdehyde, or Desmodur (Desmodur 2460M, Bayer) products based on diphenylmethane diisocyanate (MDI), toluene diisocyanate (TDI), hexamethylene diisocyanate (HDI), and isophorone diisocyanate (IPDI) may also be used.

The sensor setup is, in general, based on remission of light by a reflecting layer; thus, the colour of the device is mainly dependent on the reflecting layer used and thus the nature of the reflecting material. Furthermore, however, the polymer layer, which is positioned on the reflecting layer, also affects the process of remission. Thus, the colour of the device also depends on the presence of the polymer layer and the optical properties and/or characteristics of the polymer layer [2]. If, e.g., a PLGA layer 100 nm thick is positioned on a reflecting layer, the device will show a specific colour resulting from the cumulative effect of remission and the optical properties of the PLGA polymer. Obviously, the device displays a different colour if the PLGA layer is absent, because the effect is then dependent on the remission by the reflecting layer only. Thus, such a device consisting of a reflecting layer only displays a colour different from that of the device comprising polymer layer also.

General sensor setup

The current tendency to conduct field monitoring has driven the development of biosensors as new analytical tools able to provide fast, reliable, and sensitive measurements at lower cost. These biosensors for the moment do not compete with official analytical methods, but they can be used both by regulatory authorities and by industry to provide enough information for routine testing and screening of samples [3].

The objective of this work was to create a simple and inexpensive biomimetic sensor providing reasonable sensitivity and selectivity to indicate bacterial infection in real time monitoring combined with a memory effect that cannot easily be corrupted. In contrast with the sensor, whose design relates to the phenomenon of "anomalous absorption", which can best be described as thin film-enhanced absorption [4], the sensor presented in this work is simpler in its setup. The newly developed biosensor consists of two different layers (Fig. 1): a mirror layer (Ni–Cr composition called Inconnel) (i) and a biomimetic polymer PLGA (ii) which can be degraded by microorganisms (iii) excreting lytic enzymes (iv) leading to a colour change effect observable by the naked eye (v, vi).

PLGA distance layer

Because it is the task of the second layer (Fig. 1) to be degraded by microorganisms responsible for product spoilage, it must have the ability to react with various microbial and cellular lipolytic enzymes. For this purpose use of PLGA as the biodegradable polymer led to extraordinary results.

PLGA

PLGA is a co-polyester of lactic and glycolic acid, soluble in dichloromethane, chloroform, acetone, ethyl acetate, tetrahydrofuran, dioxane, DMSO, and toluene. PLGA is biodegradable and often used as a biomaterial for medical

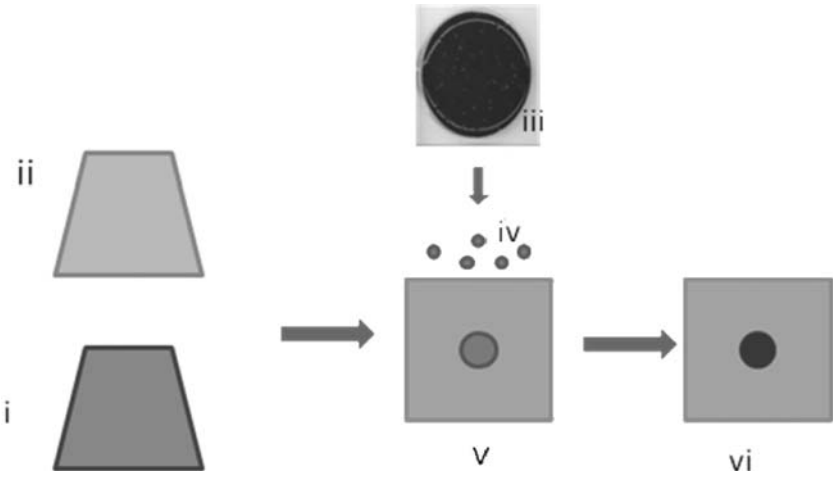

Fig. 1 Sensor setup with PLGA as a second layer. *i* Mirror layer (Ni–Cr composition called Inconnel), *ii* biomimetic polymer PLGA, *iii* microorganisms, *iv* lytic enzymes, *v*, *vi* effect of colour change observable by naked eye

and pharmaceutical applications. Hydrolysis of the polymer chain leads to PLGA degradation and results in lactic and glycolic acid, which can be metabolized in the Krebs-cycle to CO_2 and H_2O.

For this application we used Resomer RG 503 H from Boehringer Ingelheim with a viscosity of 0.38 dl/g and a molecular mass of 50 000 Da containing a large number of ester bonds. Resomer RG 503 H contains D,L-lactic acid and glycolic acid in equal amounts, an ideal ratio of both components for rapid degradation in the human body. PLGA was approved in 2000 by the FDA for application in drug targeting [5, 6].

In the sensor setup described above, the polylactide interlayer is decomposed by enzymes associated with food spoilage. These can be either lytic enzymes produced by the bacteria always present on the surface of biological materials or arise from cell lysis itself.

Crosslinker

The crosslinker used for the polymer is Desmodur (diphenylmethane diisocyanate or triphenylmethane-4,4′,4″-triisocyanate) from Bayer. The crosslinker strongly affects the viscosity and composition of the polymer solution and thus the thickness of the polymer layer.

Results and discussion

The two-layer sensor setup consists of a metal layer that is made up of Ni and Cr, which is then coated with a polymer layer. This polymer is dissolved in an organic solvent containing different amounts of Desmodur. Desmodur serves as a linker of the polymer molecules, affecting the composition, stability, and viscosity of the polymer. When lytic enzymes come into contact with the polymer layer, e.g. after secretion by bacteria, the polymer will be degraded, resulting in a colour change of the biomimetic sensor.

Degradation of PLGA and proof of principle

To describe the degradation behavior of PLGA, functionality tests with PLGA printed on Inconnel were prepared. PLGA sensors have a green or red colour which depends on the thickness and optical properties of the polymer layer. The different colour stripes on the sensor surface result from inhomogeneities during the printing process, because the gravure printer used cannot compete with industrially used devices. The first tests were carried out with protease mixture: proteinase K, trypsin, and α-chymotrypsin in 0.1 M Tris–HCl pH 8.2. The different concentrations of PLGA were printed on top of a metallized PET film. A dilution series of the protease mix in 0.1 M Tris–HCl pH 8.2 and buffer and double-distilled water (ddH_2O) as a negative control were applied to the sensor surface.

After the respective incubation times (2 h, 4 h, and overnight) the reaction was stopped by washing with ddH_2O and the sensor was then dried in a stream of air. The colour changes shown in Figs. 2 and 3 are the result of enzymatic degradation of the biomimetic polymer layer by lytic enzymes. The degradation is highly selective and clearly visible with the naked eye. The signal intensity correlates with increasing incubation time, and as temperature is one of the important factors affecting the catalytic activity of enzymes, the sensors incubated at room temperature (~25 °C) showed stronger signals as those incubated at 4 °C.

Application of PLGA sensors for analysis of natural products

PLGA two-layer sensors may also be used in order to analyse if the production steps and/or storage of medical

Fig. 2 Inconnel–21% PLGA with 0.5% Desmodur in ethyl acetate. Signal intensity after incubation for 2 h, 4 h, and overnight at 4 °C

Fig. 3 Inconnel–21% PLGA with 0.5% Desmodur in ethyl acetate. Signal intensity after incubation for 2 h, 4 h, and overnight at room temperature

products, cosmetics, or nutritional supplements have been performed correctly. Because most of the microorganisms responsible for spoilage proliferate at 37 °C, natural products are stored and transported at temperatures below 37 °C, preferably at 4 °C or even frozen at −20 °C to maintain an unfavourable temperature range for such microorganisms. Failure to comply with these conditions may result in contamination, which can be detected by use of this device.

The general procedure for preparing the devices was: Inconnel, Ni–Cr, alloy-coated PET foil was used as reflective layer. On to this layer, a polymer layer of PLGA with Desmodur in ethyl acetate was printed, followed by incubation for 10 min at 80 °C. The sensors were then incubated with a cocktail of bacteria, contaminated solution for contact lenses, and contaminated hand cream to test their ability to identify contamination and spoilage of natural products. The dots for proof of principle of PLGA sensor were applied according to Table 1. Finally, the sensors were incubated at 37 °C for 6 h and then scanned for documentation of the colour changes.

Phospholipase was used as a positive control and resulted in a colour change. The negative control Ringer solution did not show a signal. A concentration-dependent effect could be observed for the colour change upon incubation with bacterial cocktail and meat homogenate: a higher concentration seems to result in a more pronounced colour change, i.e. towards a brighter colour. The brightest colour could be observed upon incubation with contaminated hand cream; however, incubation with the contaminated solution for contact lenses also resulted in a clearly visible colour change.

Table 1 Pipetting scheme for proof of principle of PLGA sensors

	Column 1	Column 2
1	Protease-Mix[a] (20 mg/mm^3)	Protease-Mix[a] (5 mg/mm^3)
2	Protease-Mix[a] (15 mg/mm^3)	Protease-Mix[a] (2.5 mg/mm^3)
3	Protease-Mix[a] (12.5 mg/mm^3)	0.1 M Tris–HCl Buffer pH 8.2
4	Protease-Mix[a] (10 mg/mm^3)	ddH_2O

[a] Equal amounts of proteinase K, chymotrypsin, and trypsin

Materials and methods

Agars

Plate count agar (Fluka) is a nonselective microbiological growth medium for monitoring total bacterial counts from testing samples. ÖNÖZ agar (Merck) is a selective medium for isolation and identification of Salmonella and some other Enterobacteriaceae. GSP agar (Merck) is a *Pseudomonas/Aeromonas*-selective agar in accordance with Kielwein (base) for microbiology.

Liquid culture media

Concentrations of the ingredients are given in g/dm^3.

Peptone water Tryptone 10.0, sodium chloride 5.0, disodium hydrogenphosphate dihydrate 9.0, potassium dihydrogenphosphate 1.5.

LM medium Tryptone 10.0, yeast extract 10.0, disodium hydrogenphosphate dihydrate 0.4. LM medium was used for promotion of bacterial growth and induction of fermentation processes.

Ringer solution Sodium chloride 2.25, potassium chloride 0.105, calcium chloride hexahydrate 0.12, sodium hydrogencarbonate 0.05, pH 7.0 ± 0.2.

Ringer solution was used for dilutions of bacterial cultures for detection of bacterial counts.

Gravure printing

Printing settings: w (impression roller) = ±15, v (velocity) = 5. A printed foil was dried for 10 min at 80 °C, washed for 10 min in demineralized water and again dried at 80 °C for 10 min. Through these washing and drying steps all salt and solvent residues were removed.

Sensor sensitivity test

In order to test the potential of various sensors for changing their colour, they were investigated regarding their reaction to various dilutions of decomposed pharmaceutical products, cosmetic products, and bacterial cocktails. Approximately 2 mm^3 of the sample was pipetted on to the sensor surface and incubated in a humid chamber for 4, 6, or 16 h at 4 °C, room temperature (~25 °C), or 37 °C. After these incubation times, the sensors were washed with double-distilled water and then dried under an intense air stream. Because this sensor's application domain is observation of a colour change with the naked eye, each signal must be observed easily by just looking at it.

The examined sensors were checked optically and scanned. Scanning was somewhat difficult, because of a peculiarity of the sensors—their ability to shift the colour depending on the visual angle. The use of a scatter filter, in this case Parafilm, ensured reproducibility and enhanced presentation of the results.

Preparation of LM bacteria

Aerobic Gram-negative bacteria of the strains *Pseudomonas*, *Enterobacter*, *Proteus*, and *Salmonella* were cultured in LM medium (tryptone 0.1%, yeast extract 0.1%, disodium hydrogenphosphate dihydrate 0.004%). For the cocktail, 3 cm^3 selective liquid media was inoculated with these four strains and incubated at 37 °C for 24 h with shaking at 180 rpm. Following this incubation, 0.5 cm^3 of this culture was used to inoculate 100 cm^3 liquid media with phosphate (used to induce secretion of enzymes). The bacteria were then incubated at 30 °C for 16 h (180 rpm). Following this incubation, the bacterial cocktail was divided into 1 cm^3 portions which were frozen in liquid nitrogen and stored at −80 °C, representing LM bacteria.

Table 2 Pipetting scheme

	Column 1	Column 2
1	Acumed[a] (10^8 CFU/mm^3)	4 Bacteria-LM (10^8 CFU/mm^3)
2	Kamill[b] (10^6 CFU/mm^3)	4 Bacteria-LM (10^6 CFU/mm^3)
3		Phospholipase (10 mg/mm^3)
4		Ringer solution

[a] Acumed: solution for contact lenses

[b] Kamill: hand and nail cream

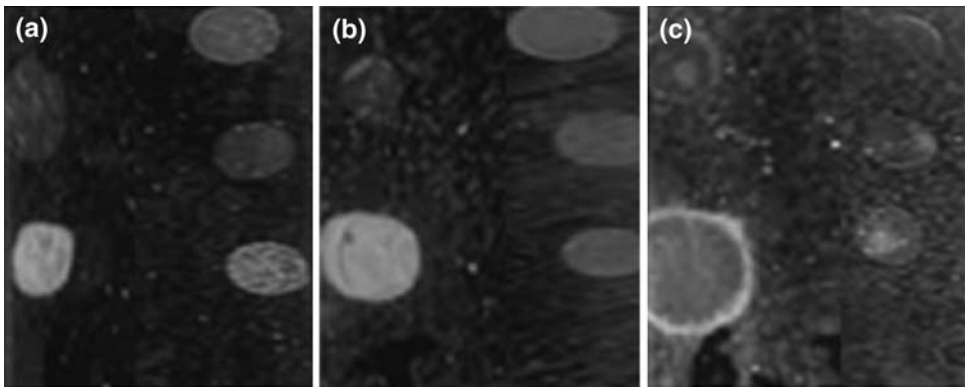

Fig. 4 Degradation of PLGA layer. **a** 21% PLGA, 0.5% Desmodur; **b** 21% PLGA, 1% Desmodur; **c** 23% PLGA, 5×10^{-8}% Desmodur

Preparation of bacterial cocktail

Gram-negative aerobic bacterial strains (*Pseudomonas*, *Enterobacter*, *Proteus*, *Salmonella*) were also used for bacterial cocktails preparation. The bacterial strains were isolated from spoiled pork meat and cultured in our laboratory using a different selective media, for example Salmonella agar according to ÖNÖZ and GSP agar. A colony picked from the selective media was used to inoculate 3 cm^3 peptone water (peptone 0.1%, NaCl 0.5%, $Na_2HPO_4 \cdot 12H_2O$ 0.9%, and KH_2PO_4 0.15%, all in ddH_2O) and allowed to grow overnight at 37 °C. Then 2 cm^3 of the bacterial pre-culture was used to inoculate 200 cm^3 peptone water and incubated at 37 °C for 24 h. Aliquots (1 cm^3) were frozen in liquid nitrogen and stored at −80 °C. After incubation (up to 3 days at 30 °C) bacterial counts were determined by plating 100 mm^3 bacterial cultures on to plate count agar. The culture dilutions (10^{-1} to 10^{-9}) were prepared in Ringer solution.

Preparation of the contaminated pharmaceutical product

Samples (50 mm^3) of both *Pseudomonas* and *Enterobacter* strains (grown separately in liquid media as mentioned above) were added to 50 cm^3 Acumed, a solution for contact lenses (4Care, Kiel, Germany). The resulting contaminated solution was incubated for 24 h at room temperature corresponding to the sample "contaminated solution for contact lenses" as mentioned above.

Preparation of the contaminated cosmetic product

"Hand and nail cream Kamill" (Burnus, Darmstadt, Germany; 10 g) was mixed with 50 cm^3 Ringer solution and thoroughly vortex mixed. To the resulting suspension, 50 mm^3 of both *Pseudomonas* and *Enterobacter* strains (grown separately in liquid media as mentioned above) were added. The resulting contaminated suspension was incubated for 24 h at room temperature corresponding to the sample "contaminated hand cream" as mentioned above (Table 2; Fig. 4).

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