universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

Contributions to the development of novel nanotechnological sensor chips for the detection of meat deterioration in special consideration of optimising the sensitivity with regard to chemical modification of the sensitive polymer layer and the sensor layout

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag. rer.nat.)

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There is something fascinating about science. One gets such wholesale returns of conjecture out of such a trifling investment of fact.

(Mark Twain)

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Aims of my studies

The use of sensors for the solution of chemical questions has a long tradition [1] and also the application of biological recognition sites, e.g. enzymes [2], antibodies [3] or aptamers [4], is quite popular since their first appearance with Clark's oxygen-electrode [5]. In the field of food quality control and analysis, biosensors are frequently used.

Former members of the research group of Prof. Fritz Pittner at the Institute of Biochemistry and Cell Biology at the University of Vienna developed a freshness sensor for the real time detection of meat degradation on the surface of packed fresh meat [6, 7]. The proof of principle was shown, but afterwards, further investigations had to be done to realise a commercial product which can be used in food packaging to show the customer the freshness of the meat. During my diploma thesis, I focused on contributions for the development of the sensor properties especially with focus of the sensor sensitivity, the properties of the polymeric interlayer and their correlation to actually accredited standard microbiological tests for the quality control of meat.

I.) Introduction

Biosensors

General definition

There are many different and slightly inhomogeneous definitions of a biosensor. The amount of definitions of biosensors is as manifold as the biosensors themselves. The IUPAC defines a biosensor as a device that uses specific biochemical reactions mediated by isolated enzymes, immune systems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals [8].

Maybe the most common definition stems from one of the pioneers of biosensors, A.P.F Turner, who defined a biosensor as a chemical sensor in which the recognition system utilises a biochemical mechanism [9].

Biosensors can be characterised by their properties and the type of signal, which is produced or at least measured [10]. Transducers can be subdivided into electrochemical transducers (such as potentiometric, voltametric, conductometric biosensors, etc.), optical transducers (such as absorption spectroscopy, fluorescence spectroscopy, TIRF, SPR, light scattering, etc.), piezoelectric transducers (QCM, SAW) and thermal methods [11].

But biosensors may also be classified by their performance factors like selectivity, sensitivity range, accuracy, response time and recovery time. It is also possible, but maybe not very practicable, to use the area of application as a source of differentiation. Then, one has to distinguish pharmaceutical, health care, environmental monitoring, industrial processes or food biosensors.

Biosensor research became very popular in the last 20 years although it is under development for more than 40 years [1].

The biological recognition element, which provides the sensor with a high degree of selectivity, transforms the information from the biochemical domain into a physico-chemical output signal. The limit of detection, which is equivalent to the sensitivity, is desired to be very high.

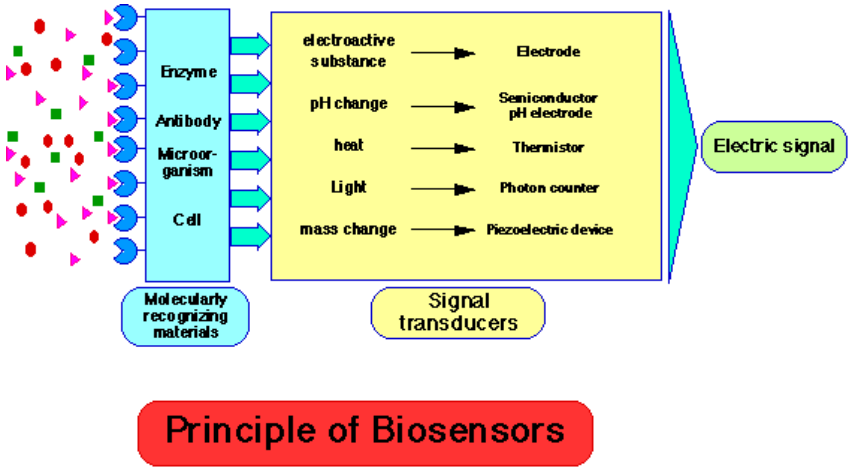


Figure 1: Typical biosensor setup with its 3 parts: recognition site – signal transducer – signal detection unit

The main criterion of a biosensor is that the biochemical recognition site is spatially close to the transducer (Figure 1). As sensors are often miniaturised devices of still known techniques or machines, an indication for a sensor is its typical smallness. Chemical sensors which contain non-biological receptors are not termed biosensors, even if they are used to detect biochemical analytes or monitor biological processes. The development of biosensors is currently driven by the tendency to carry out on-line monitoring. In recent years, numerous analytical tools have been developed for sensitive, fast, stable and low cost analyses.

In the subsequent pages, I want to give a short overview of the different types of biosensors and of the used techniques. After a short description of electrochemical, acoustic and piezoelectric biosensors, I will focus on optical sensors with respect to thin film optical sensors. Combined with an overview of the devices, which are currently used in the food area, the following part leads to the background and setup of our developed biosensor.

Electrochemical biosensors

It is not surprising that some definitions of biosensors include the transduction form a biochemical signal into an electrochemical signal. The majority of the biosensors is classified as electrochemical biosensors [12]. Electrochemical biosensors even stand at the very first beginning of biosensors evolution [13]. The IUPAC defines an electrochemical biosensor as a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor), which is retained in direct spatial contact with an electrochemical transduction element. [14].
Because of their ability to be repeatedly calibrated, it is recommended by IUPAC that a biosensor should be clearly distinguished from a bioanalytical system, which requires additional processing steps, such as reagent addition. A device which is both disposable after one measurement, i.e. single use, and unable to monitor the analyte concentration continuously or after rapid and reproducible regeneration should be designated a single-use biosensor. The differentiation between “real” biosensor and single-use biosensor disappeared almost completely in the scientific community.

There are different types of electrochemical biosensors which can be distinguished by the different electrochemical transducers.

Amperometric biosensors

Amperometric biosensors are designed to measure a current flow generated by an electrochemical reaction at constant voltage. There are only a few applications available for direct sensing, since most analytes are not intrinsically able to act as redox partners in an electrochemical reaction. Therefore electrochemically active labels are needed for the electrochemical reaction of the analyte at the sensing electrode. Oxygen and H_2O_2 electrodes are the most popular.

Electrodes for biosensors mainly consist of carbon (graphite, carbon black, carbon paste, etc.) or noble metals (Au, Ag, Pt). But also semi-conducting materials like indium tin oxide find their way into biosensor applications. These electrodes are mostly covered with a biochemical film, which builds up the biological recognition site. The first electrode of this type was proposed in 1956 by Clark and Lyon from the Children Hospital in Cincinnati. A thin layer of glucose oxidase (GOX) was entrapped over an O_2-electrode via a semi permeable dialysis membrane and the device monitored the O_2-consumption of the glucose oxidation reaction.

$$\textbf{D-Glucose} + H_2O + O_2 \rightarrow H_2O_2 + \textbf{D-Gluconate}$$

$$O_2 + 2e^- + 2\,H^+ \rightarrow H_2O_2$$

Figure 2: Chemical reaction of the Clark electrode

Here glucose reacts to gluconic acid and H_2O_2 with the catalytic help of the enzyme GOX.

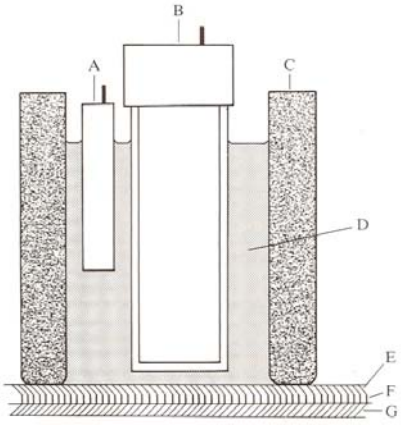


Figure 3: Amperometric biosensor – Clark electrode

Potentiometric biosensors

The Nernst equation [15] provides the fundamental principle of all potentiometic transducers. According to this equation, potential changes are logarithmically proportional to the specific ion activity. The potential difference between indicator and reference electrode is proportional to the logarithm of the ion activity of the analyte. The measurement of this potential difference is achieved by determining the difference between either both an indicator and a reference electrode or two reference electrodes, which are separated by a semipermeable membrane. Potentiometric transducer electrodes, capable of measuring surface potential alterations at near-zero current flow, are constructed by ion selective electrodes (ISE) or field effect transistors (FET) [16]. Also common pH-sensors rely on this principle. Massive discussion took place about the question whether an ion-selective-electrode (ISE) can be classified as a biosensor or not [10]. The specificity of a sensor depends on the membrane, so with a discriminating sensor multi-analyte detection is not possible.

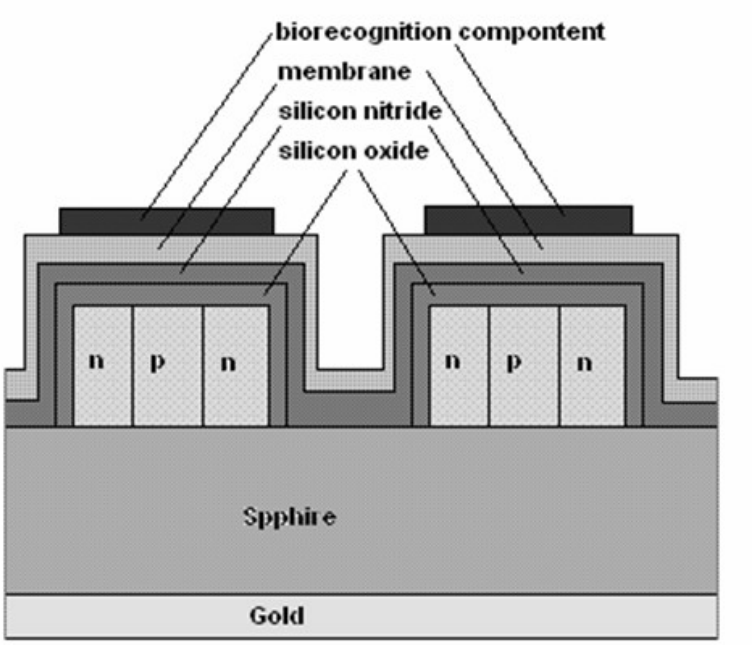


Figure 4: Potentiometic biosensor - Schematic drawing of a field effect transistor (n-p-n type) based biosensor

Microgravimetric biosensors

Mass dependent sensors are balances, which can detect very small mass differences by an electrically measurable frequency signal. Most of these sensors depend on the piezoelectric effect. It can be used as a biosensor, when a biochemical recognition element covers such a crystal. Preferred materials for such a piezo crystal are zinc oxide (ZnO) and the quartz crystal (SiO_2). These materials resonate mechanically at a specific ultrasonic frequency (in the order of tens of Megahertz) when excited in an oscillating electrical field. The resonant frequency is determined by the thickness of the quartz plate and the velocity of the acoustic wave within the quartz material [17]. The electromagnetic energy is converted into acoustic energy, whereby piezoelectricity is associated with the electrical polarisation of materials with anisotropic crystal structure. The microgravimetric sensor devices can be distinguished into quartz crystal microbalances (QCM) devices applying a thickness-shear mode (TSM), and devices applying a surface acoustic wave (SAW) [18]. In an often cited work [19], Sauerbrey showed for the gaseous phase that a weight change of the quart surface is precisely determined by a shift in the resonating frequency (Figure 6)

$$\Delta f = \frac{-2\Delta m f_0^2}{A\sqrt{\rho_q \mu_q}}$$

Figure 5: Sauerbrey equation

The equation can be interpreted that surface-added layers will extend the wavelength of the acoustic wave within the quartz device. If the quartz crystal is coated with a biorecognitive layer (Figure 7), the change of frequency can be correlated to the analyte concentration.

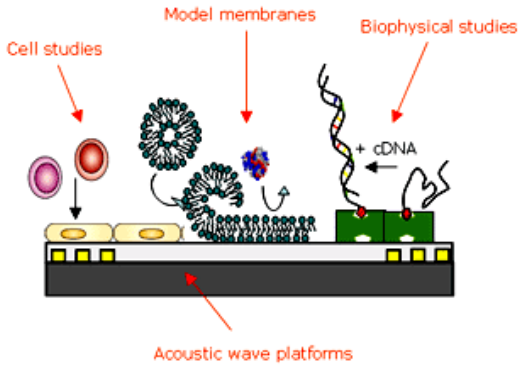


Figure 6: QCM - sensor

Thermometric and calorimetric sensors

Like many other chemical reactions, also biochemical reactions involving enzymes generate heat. The generated heat is proportional to the amount of substrate, which is used for measuring the rate of reaction and analyte concentration.
With a calorimetric device, thermal energy, which occurs because of a chemical bond or a catalytic conversion, is measured. The selectivity of the enzyme can be used for a selective biosensor. Such thermistors require a very sensitive electrical resistance thermometer.

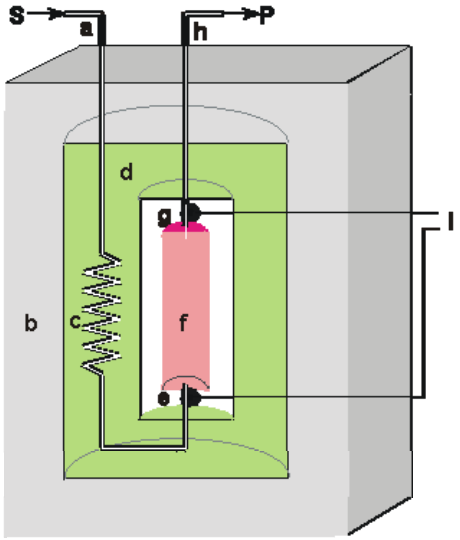


Figure 7: Schematic diagram of a calorimetric biosensor. The sample stream passes to the heat exchanger (c) within an insulating block (d) from where it flows past the reference thermistor (e) and into the packed bed bioreactor (f), where the reaction occurs. The change in temperature is determined by the thermistor (g) and the solution passed to waste (h). External electronics (l) determines the difference in the resistance, and hence temperature, between the thermistors.

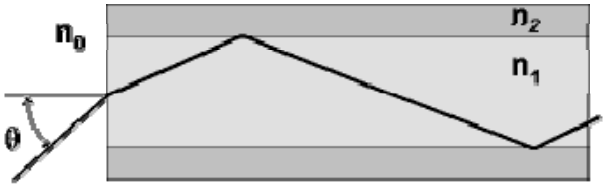
Three types of heat transfer between reaction vessel and the surroundings can be distinguished. Isoperibol calorimetry is the most common method. This method is featured by the recording of a temperature change within the reacting solution, whereas the immediate cell environment remains at constant temperature. Heat conduction calorimetry relies on a fast heat transfer between the chamber and the isothermal heat sink surrounding. This can be measured as a voltage output of a thermometric sensor. Isothermal calorimetry measures the required energy, which is needed for cooling or heating of a reaction chamber to keep the temperature inside on a constant level.

Optical biosensors

Optical biosensors are a branch, which has been growing in a very fast way in the recent years [20, 21]. This is due to the advantages of applying visible radiation compared to other transducer techniques, involving high speed and reproducibility of the measurement [22]. Additional benefits are the non-destructive operation mode as it often does not consume the analyte and the rapid signal generation and reading. In particular, the introduction of fibre optics (optrodes) as optical waveguides and sophisticated optoelectronics offers increased versatility of these analytical devices for bioanalytical applications [23].Their application has continued to progress and spread to many different areas like clinical, environmental and food applications [24]. By IUPAC definition, optical sensors are able to transform changes of optical phenomena, which are the result of an interaction of the analyte with the receptor part [25].

These types of sensors are based on excitation-response measurements of analytes in a sample, where electromagnetic waves leaking out of the guide interact with chemicals. Optical sensors can measure analytes that are not electro-active or have electro-active reactants or products as it is needed for electrochemical sensors [26]. Biomolecules are targeted with surface immobilised tags e.g. immunoglobines. Sensitive analytes must have functional optical groups, especially in the visible range, to form a unique sensor signal. Light, which is reflected at a reflective boundary is not reflected at all but also penetrates into the second medium. The existence of light beyond the sharp interfacial boundary is called evanescent.

The disadvantages of biosensors are often the high costs and limited long-term stability.



$$\sin\theta = \frac{\sqrt{n_1^2 - n_2^2}}{n_0}$$

Figure 8: Optical fibre and numerical aperture

Surface Plasmon Resonance

Surface Plasmon Resonance (SPR) is an optical technique that measures molecular binding events at a metal surface by detecting changes in the refractive index [27]. The benefits of SPR are its label free and time-dependent detection, which allows, in contrast to fluorescent methods, the measurement of kinetic and thermodynamic data. SPR sensors enable a wide range of real-time detection of biomolecular interaction. SPR is a technique, which is able to detect analytes of a wide range of molecular weights and binding affinities. It is a useful tool for studying interactions between biomolecules. Until date, SPR sensors offer only a small number of independent sensing channels, which is the main disadvantage of this technique. Surface Plasmon Resonance was developed from research works of Wood (excitation of surface plasma) and Kretschmann (attenuated total reflection).
Equivalent to all the other biosensors, an SPR optical sensor comprises an optical system, a transducer and an electronic data system. The optical system consists of a light source and an optical structure for the excitation of the surface plasmon wave.

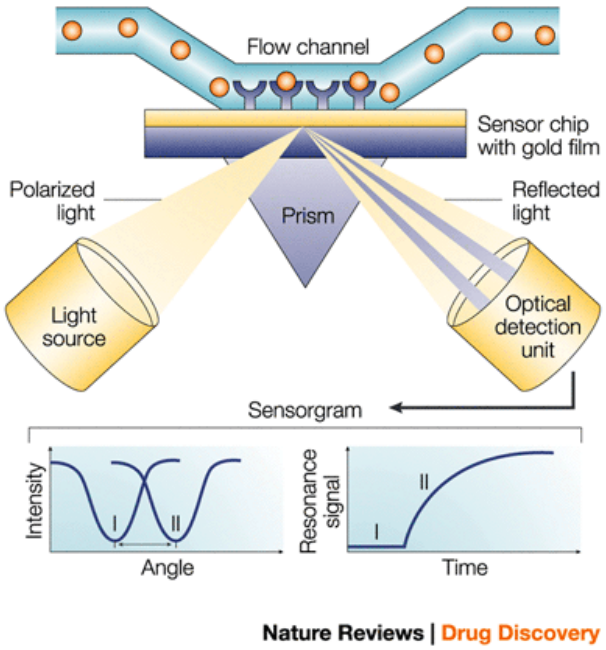


Figure 9: SPR sensor

Interference sensors

Interference describes the superposition of two or more waves, which leads to a new wave pattern. Full coherency of two different non-monochromatic waves is given if they both have exactly the same range of wavelengths and in addition the same phase differences at each of the constituent wavelengths.
Constructive interference is given if two peaks of different waves meat each other resulting in a higher amplitude. The phenomenon of interference is independent from the source of light, although monochromatic light, like laser, is the most frequently used source. A Mach-Zehnder interferometer is the most frequently used interferometer system. The light beam is split by a halfsilvered mirror and the resulting beams go separate ways. After reflection by a total reflecting mirror, the two beams pass a second halfsilvered mirror and reach the detector system were changes of the interference pattern can be observed.

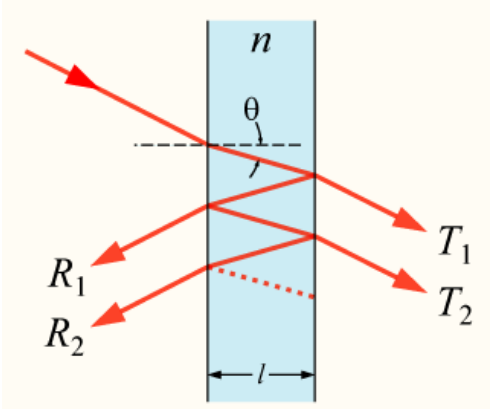


Figure 10: Fabry-Perrot Interferometer

A Fabry-Perot-Interferometer (named after Charles Fabry and Alfred Pérot) is made of a transparent plate with two reflecting surfaces (e.g. reflecting mirrors). Constructive interference occurs if the transmitted beams are in phase, and this corresponds to a high-transmission peak. In contrast, if the transmitted beams are out-of-phase, destructive interference occurs, which corresponds to a transmission minimum. Whether the multiple-reflected beams are in-phase or not, depends on the wavelength (λ) of the light, the angle the light travels through the interferometer (θ), the thickness (l) and the refractive index of the material between the reflecting surfaces (n).

Anomalous Absorption

Anomalous absorption is a strong, broad-band absorption in the visible range, which is referred to a special assembling involving a bulk metal film in near spatial separation from nanometric metallic particles. Nanoparticle clusters display optical and electrical properties that are different from their bulk properties [28]. Beside a weak metallic reflection, these cluster films show a strong colour. The partially-bound electrons in such nanoclusters react like resonators upon excitation, due to their spatial confinement [29]. If light is irradiated on a surface of an extended metal, unconfined electron movement is possible, which results in an unspecific reflectivity termed metallic glance.

A nanometric metal cluster film, which is spatial close to a smooth metal surface acts like a reflection interferometer and produces a spectral reflectivity minimum which depends on the interlayer thickness. A change in the thickness can be visualised by illumination with white-light by an altered colour impression of the reflected light. Fields, which are reflected by one of the mirrors, are in same phase at an appropriate distance of the absorbing layer. This sort of “feedback” mechanism is able to enhance the effective absorption coefficient of the absorbing layer in a very strong way.

A number of applications with the phenomenon of anomalous absorption are already patented. The so called MICSPOMS sensors use the swelling and shrinking effects of a polymer as a distance layer [30].

Meat spoilage

Freshness and spoilage of food

Freshness of food and the detection of changes in the freshness have always been important topics.
The spoilage of food is a complex process which is mainly due to microorganisms. Even in these days with the known preservation techniques tremendous amounts of foods get spoiled every day.

During decay several chemical compounds such as acids, bases, amines, aldehydes or mercaptones are released (by the food or by microorganisms). Because of the complex structure of decay, it is not easy to tell whether food is still fresh or already spoiled. A device which is able to do so is important as well in the grocery store as at home. In contrast to the disease-causing pathogenic bacteria (like *Brochotrix*, *Salmonella*, etc.), the spoilage bacteria (like *Acinetobacter*, *Lactobacillus*, etc.) are even faster in food deterioration due to their short reproduction times (down to 20 min).

During deterioration, bacteria grow and thereby release enzymes in order to get nutrients required for survival (amino acids, lipids, carbohydrates). The growth of bacteria usually follows the exponential growth curve. The growth at the beginning is rather slow (lag phase) but is followed by the log phase, in which the bacteria grow in a logarithmic scale. At a certain amount, the rapid growth comes to an end, which is called the stationary phase. The duration of each of these phases is determined by the environmental growth factors like nutrition, salts, humidity and heat. Very low temperatures (<4°C) inactivate the growth of most of the common food bacteria as well as very high temperatures (>50°C), which is the reason for food being either stored in a refrigerator or heated to increase the shelf life.
Meat is very sensitive towards spoilage, because it is an ideal medium for the growth of microorganisms due to its humidity and salt level. These microorganisms release lytic enzymes, which gradually degrade the meat tissue. The enzymes can have their

origin either from the meat cells themselves or from bacteria on the meat surface. To inhibit this process of deterioration, several methods of conservation are appropriate (e.g. heating, cooling, drying, salting or curing).
Meat spoilage takes place as well under aerobic conditions as under anaerobic conditions or also modified atmospheres. Under aerobic conditions, Gram-negative bacteria like *Pseudomonas sp.* mainly cause the degradation [31, 32]. They have a short reproducing time (about 8 h at 2°C) and are resistant to low pH levels caused by organic acids (like lactic acid). Under anaerobic conditions, like for vacuum-packed meat, psychotophic bacteria like *Lactobacillus sp.* and *Leuconostoc sp*. find their ideal growth conditions. Modified atmospheres (80% O_2, 20% CO_2) inside the package should inhibit colour deterioration. Their shelf life is usually between air-packaged and vacuum-packaged meat.
As the spoilage of meat is quite inhomogeneous, one can measure spoilage at one locus while another place on the meat is still unimpaired.

The quality of food is regulated within the European Union (EU) and its member states by several regimentations [33]. The “COMMISSION REGULATION” (EC) No 2073/2005 of 15th November 2005 regulates in article 2 that “foodstuffs should not contain microorganisms or their toxins or metabolites in quantities that present an unacceptable risk for human health.” As many other legal phrases, it is a quite rough formulation without any further definitions. These are the responsibility of each member state of the EU. Meat is defined as fresh in Austria if a total bacterial count is lower than 5 Mio colony-forming units per gram.

Established methods of bacterial detection

Established methods of bacterial detection mainly depend on microbiological standard methods. In the food section, microbiological tests for pathogenic bacteria are widespread. In the United States, approximately 5 million analytical Salmonella tests are performed per year [34]. These methods have to be sensitive and rapid to detect even low numbers of bacteria in complex biological matrices (e.g. food).

Standard analytical methods for microorganisms are based on CFU-count and require common cultural techniques. It is the oldest bacterial detection method, but

still remains the most used one. This method is very time-consuming, which is obviously too long for many applications in the food sector. To distinguish different species of bacteria, selective media are used, which contain different carbon sources and inhibitors.
Recent developments try to overcome the time-consuming steps of the traditional techniques and strive to enable on-line monitoring.

Food sensor market

According to the literature, approximately 76 million food-borne illnesses occur each year in the US and account for 325 000 hospitalizations and 5000 deaths [35]. So it is obviously that there is an urgent need for methods to destroy spoilage microorganisms during food processing. As this is almost impossible, it is necessary to develop devices for measuring the bacteria in food. These needs drive the development of the food biosensor market, which grows rapidly and is besides the medical, environmental and military sector one of the four important biosensor sectors. It can be subdivided into other sub-sectors as are described in Figure 11.

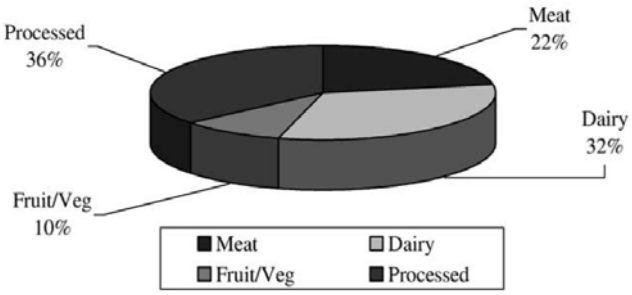


Figure 11: US food industry total microbial tests per sector

Established sensors for food

Healthy food and its safe production are urgent needs in our time. But there is a lack of scientific data on food quality changes, which is caused by the lack of appropriate on-line instruments. The applicability of so far developed optical on-line methods is often limited by the presence of interfering compounds. Until date, there are no sensors or tests on the market, which are able to detect the number of meat

deterioration causing enzymes. For other types of meat, there exist some electrochemical biosensors (e.g. fish [36]). There are several other tests with the power to indicate other important environmental parameters, which can lead to the decay of meat. These tests are described in more detail in the following.

Time-temperature sensors

As mentioned above, one of the most important factors of meat spoilage is the temperature at which the meat is stored. Small devices show a time temperature dependent irreversible change that mimics the changes of a target quality parameter undergoing the same variable temperature exposure. When temperature changes are only considered with respect to time, these devices are called time-temperature integrators (TTI). TTIs are able to measure changes of microbial, enzymatic, chemical or physical products.

pH Indicator

Another important indicator for spoilage is the pH of the meat. The pH changes during the deterioration process because acidic substances (e.g. lactic-acid) are released, then followed by basic amines. One already existing pH indicator is based on the release of basic volatile amines of spoiled fish [37]. This pH sensor detects amines with pH sensitive dye integrated into a polymer.
The main disadvantage of this sensor is the fact, that it detects only a variable symptom of the meat spoilage rather than the background of these effects.

Figure 12: pH indicator from FQSI international

Recent developments

Novel setup and new approach

Packed fresh meat, which can be widespread found in supermarkets all over the world, is labelled with a “best-use-before” date, which indicated the beginning of a deterioration state which could be risky for the consumer. This is nowadays the only way for the consumer to check the quality. But this fact deals with many problems: The meat inside the package is often kept under a protecting gas so it is not wise to harm the transparent foil. But this would be the only way for the consumer to check whether the meat is PSE (pale-soft-extruded) or DFD (dry-firm-dark). The tight packages also avoid an olfactory test. The fleece in the packages prevents the consumer from noticing high amounts of meat juice, which is also a classical indicator for the bad quality of the meat. The “best use before” date is also very problematic as it is only correct as long as the cooling of the meat is not interrupted. This often happens if meat bought at the supermarket is not stored immediately afterwards in a cooling bag.

Figure 13: Packaged fresh meat of a common Austrian grocery store

Nowadays, the detection of bacterial food deterioration is a very time-consuming, elaborate and expensive procedure, which relies on conventional culturing techniques and is therefore not suitable for on site monitoring as a sterile microbiology laboratory is needed.

The recently developed biomimetic biosensor has the goal to give the end consumer the possibility to check the quality of the meat s/he is intended to buy and reassure himself/herself of this quality even later just by looking at the sensor integrated in the package [38, 39]. The freshness sensor for food (developed by Bauer, Putz, Pittner et. al.) is an optical thin film sensor with an integrated biopolymer, which is degraded by the same enzymes at the same rate like food (e.g. meat). The enzymatic activity of the bacterial enzymes found in the spoiled meat is utilised to provoke a colour shift on the sensor. The sensor chip is able to show the decay of food through a specific colour shift.

The sensor's setup shows a characteristic spectral reflection behaviour, which depends on the one hand on the thickness of the biopolymer interlayer [40] and on the other hand on its optical density. The degradation of the biopolymer results in thickness reduction, thus generating the above mentioned colour shift. The optical property of metal island films, necessary for the 3-layer-sensor-device, is the so called "anomalous absorption". This is a strong, broad-band absorption in the visible, which is due to the confinement of the conducting electron plasma in nanometric particles and which has been used for numerous other sensor applications by other research groups before [41]. The major benefit of this sensor is the fact that as the sensor is integrated into the meat package to get into contact with the meat – and thus eventually with bacteria - real time measurement of the freshness of the meat is made possible. The setup is immediately activated and needs no further pre-treatment. In contrast to electrochemical biosensors, this setup does not need any electrical device or detection system. Another advantage is the simple fixation of the biological recognition layer – the biopolymer – onto the supporting foil via adhesion, not needing any sophisticated immobilisation technique.

Metal layer

The metal layer (which forms the support of the whole setup) has to be a smooth mirror surface necessary for the interaction of the distance layer and the nanoparticle layer which results in a strong colour. The metals of the mirror layer and the nanoparticle layer can be different. It has to be taken into account that for a commercial, a high grade of stability (e.g. against other ionic liquids like buffers) of each of these layers has to be assured. The whole sensor has to be stable when in

direct contact with the food over a few days up to a few weeks, depending on the type of meat product. The setup of the 3-layer-sensor (Figure 14) is as followed: metal mirror layer – sensitive distance layer – nanoparticle layer.

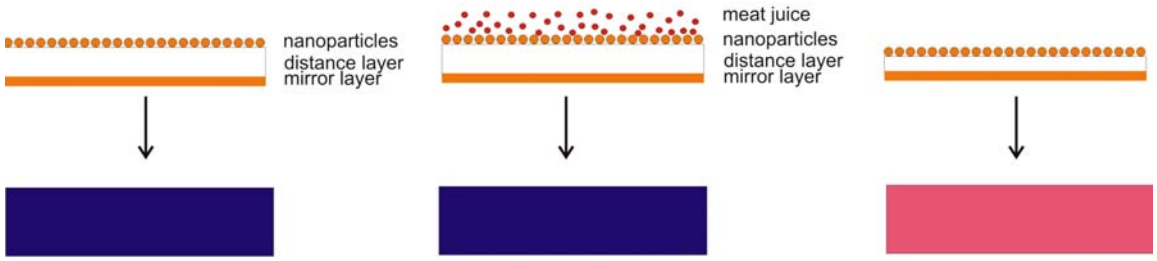


Figure 14: 3-layer-sensor setup

The sensor indicates the point of deterioration, if a change of thickness of the sensitive layer caused by degradation through enzymes results in a specific colour change.

Sensitive layer

A biodegradable polymer is the layer of the sensor, which is sensitive to the analyte – the lytic enzymes of the microorganisms on the meat surface. It is an optical transparent biopolymer, which can be degraded by lytic enzymes. The polymer is synthesized directly in the laboratory by stirring and heating it in a solvent (TFE) together with a linker.

One of the most important biomimetic polymers is polylactic acid (PLA). Besides its frequent use for many biomedical purposes, also different applications in the packaging of food exist [42]. Due to its biodegradability, it is also used in compostable bags, bio plastics and disposable devices [43]. In the scientific literature, many groups presented their results of their investigation on the enzymatic degradation of PLA. The degradable effects of pronase, proteinase K and bromelain [44] on poly(L-lactide) could be shown as well as the effects of proteinase K on poly(D,L-lactide) [45]. But also other esterase-type enzymes like peptidases and lipases can degrade the biopolymer of PLA [46]. The proof of principle of this effect on a substrate was shown in former works of our group [6].

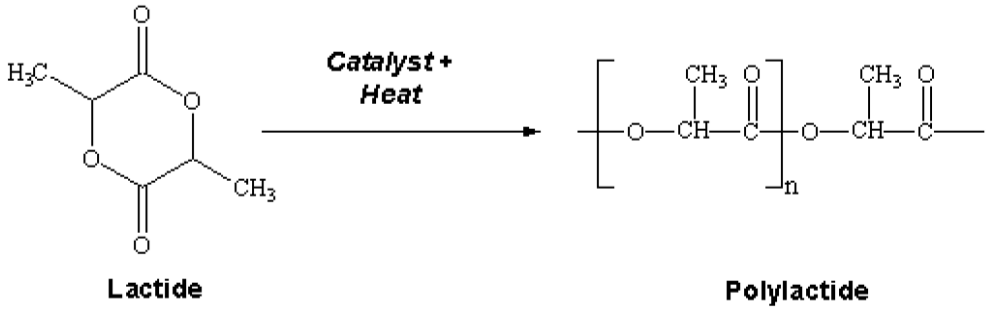


Figure 15: Polymerisation of lactide to polylactide (ring opening)

Another polymer, which I used during my thesis is the PLGA (poly(lactic-co-glycolic) acid). PLGA is a copolyester consisting of lactic and glycolic acid (Figure 16). It is soluble in many common hydrophobic solvents (e.g. dichloromethane, acetone, ethyl acetate, toluene, DMSO) but not in highly hydrophilc ones like water, methanol or ethanol. The polymer is not toxic and neither are its water soluble monomers. Degradation of the polymer is due to the hydrolytic cleavage of the ester bonds [47]. PLGA is a biomimetic polymer with a long tradition in medical methods and devices (e.g. as nanoparticles in the drug delivery [48] and artificial tissues [49, 50]).

Biomaterials like PLGA do not harm the mammal organism and do not evoke immune response. The monomeric acids, which are released by the degradation of the polymer, are metabolized in the body via the citric acid cycle resulting in CO_2 and H_2O. The use of PLGA in the food sector was approved by the FDA (Food and Drug Administration) in the year 2000 [51].

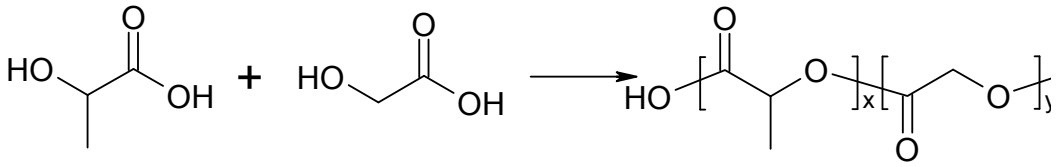


Figure 16: Lactic acid and glycol acid form PLGA

The glycolic/lactic ratio is directly proportional to the hydrophilic properties of the copolymer, which results in a higher water absorption. PLGA consists of molar equivalent parts of lactic and glycolic as RESOMER® RG 503 H, which was used during my diploma thesis.

Further alternatives to PLA and PLGA might be other biodegradable polymers like Polycaprolacton [52] or Polyvinyllactam.

Linker

During this thesis I linked the polymer with Desmodur® 2460 M ($C_{15}H_{10}N_2O_2$; 250.25 g/mol) purchased from Bayer. Bayer's Desmodur® polyisocyanates are in heavy use for the formulation of many polyurethane coatings and adhesives. Desmodur 2460 M is a monomeric diphenylmethane diisocyante with high 2,4'- isomer content. Because of its two isocyanate groups Desmodur 2460 M is a bifunctional linker (Figure 17).

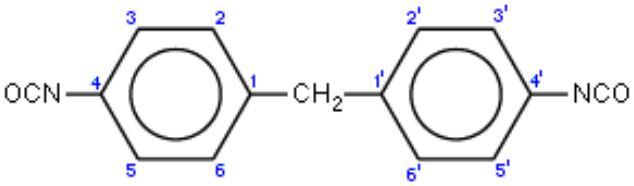


Figure 17: 4,4'-diphenylmethan-diisocyanate

The reactivity of the molecule is influenced by the position of the isocyanate groups. 4,4'-MDI is a symmetrical molecule with two equal reactive groups. Apart from this, 2,4'-MDI is an asymmetrical molecule with the OCN-group at position 4 being approximately four times more reactive than the group at the position 2.

In the following chapters, I focus on the most important analytical parameters that I investigated during my work. However a much higher number of tests have been carried out in order to relate these results to previous findings.

II.) Materials

II.1) Buffers, media and solutions

Tris/HCl-buffer (1 M)

- 121 g $C_4H_{11}NO_3$
- 1 L ddH_2O
- adjust to pH 8.2 (with HCl) at 25°C
- 0.1 M Tris/HCl-buffer is prepared by diluting 1:10 with ddH_2O

Peptone water

- 10.0 g/L Peptone
- 5 g/L NaCl
- 1.5 g/L KH_2PO_4
- 9.0 g/L K_2HPO_4
- adjust to pH 7.2 at 25°C
- treatment by autoclave is necessary (15 minutes at 121°C)

Ringer's-Solution

- 8.60 g/L NaCl
- 0.30 g/L KCl
- 0.33 g/L $CaCl_2$

20 x SSC (3 M sodium chloride, 0.3 M sodium citrate)

- 175.3 g NaCl
- 88.2 g $C_6H_5O_7Na_3 \cdot 2\ H_2O$
- 1 L ddH_2O
- adjust to pH 7 (with NaOH) at 25°C

20% PLA stock solution

- 10 g PLA is dissolved in 50 mL TFE
- Solution is stored in a 100 mL flask with a Teflon® screw cap surrounded by Parafilm®

Protease-Mix (Prot-Mix)

- 20 mg/mL Proteinase K
- 20 mg/mL Trypsin
- 20 mg/mL α-Chymotrypsin
- Enzyme solutions prepared in Ringer solution and mixed in equivalent amounts
- Dilution steps are done with Ringer solution

II.2) Solvents, polymers and chemicals

Empirical Formula	**Name**	**Provider**
C_4H_8O	2-Butanone	Fluka
$CHCl_3$	Trichlormethane	Fluka
$C_2H_3F_3O$	2,2,2-Trifluoroethanol	Sigma Aldrich
$C_4H_8O_2$	Ethyl acetate	Sigma Aldrich
$[C_3H_6O_3]_n$	Polylactic acid	Biomer
$[C_3H_6O_3]_n$ $[C_2H_4O_3]_m$	Poly(lactic-co-glycolic) acid RESOMER RG 503 H®	Böhringer Ingelheim
$C_{15}H_{10}N_2O_2$	Desmodur® 2460M, 4',4-Diphenylmethyl-diisocyanate	Bayer
$C_{15}H_{10}N_2O_2$	Diphenylmethane-4',4-di-/triisocyanate	Merck
$C_4H_{11}NO_3$	TRIS, 2-amino-2-(hydroxyl-methyl)propane-1,3-diol	Fluka
NaCl	Sodium chloride	Sigma Aldrich
NaOH	Sodium hydroxide	Fluka
HCl	Hydrochloric acid	Fluka
$C_6H_5Na_3O_7$	Sodium citrate	Sigma Aldrich
$(C_{22}H_{21}O_{13})_3Al$	E 120 Carmin	Brauns-Heitmann
$C_{15}H_{26}O_6$	Glycerol-tributyrate 98%	Sigma Aldrich

II.3) Microbiological reagents

Material	**Provider**
Enzymes:	
Esterase from horse	Sigma Aldrich
Proteinase K	Sigma Aldrich
α-Chymotrypsin	Sigma Aldrich
Trypsin from pig pancreas	Sigma Aldrich
Bacteria:	
Pseudomonas	isolated from spoiled meat in the lab
Enterobacter	isolated from spoiled meat in the lab
E.Coli	isolated from spoiled meat in the lab
Salmonella	isolated from spoiled meat in the lab
Serratia liquefaciens	DSMZ
Lactobacillus curvatus	DSMZ
Lactobacillus sakei	DSMZ
Leuconostoc mesenteroids	DSMZ
Brochothrix thermosphacta	DSMZ
Acinetobacter lwoffii	DSMZ
Pseudomonas ludensis	DSMZ
Pseudomonas fragi	DSMZ
Pseudomonas fluorescens	DSMZ
Plate Count Agar	Oxoid
Glycerine	Fluka
Peptone from meat, peptic digest	Fluka

III.) Methods

III.1) Table of devices

In the following section all used devices and gadgets are listed. It is obvious that these apparatuses are not equipped for large-scale production, but it was sufficient for research on the sensor on laboratory scale.

Device	**Provider**
Printer	Erichsen RK K Printing Proofer
Forepump	Vacuubrad RC4
Turbo pump	Varian Turbo-V 70
Sputter Coater	Agar Sputter Coater
Stomacher	Colworth Stomacher 400
Flatbed scanner	HP Scanjet 4890
Oven (adjustable up to 160°C)	Haereus

For survey of the devices and tools that I used during my work, photos with descriptions are shown on the following pages.

Printer: Erichsen RK Printing Proofer 628

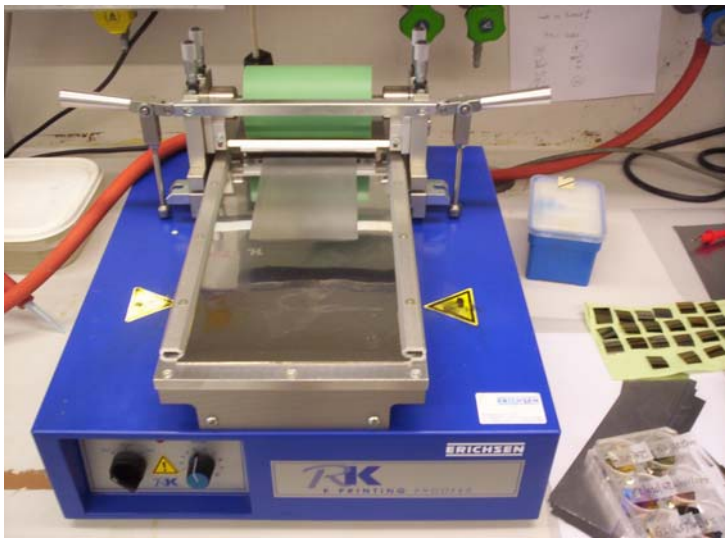


Figure 18: Gravure printing setup in the laboratory

The polymer solution is transferred from an electronically engraved printing plate directly onto the substrate film, which is fixed with a tape on the rubber covered impression roller. Doctor blade and impression roller settings are adjusted via micrometer-gauges which allow reproducible settings.

Agar Sputter Coater

Figure 19: Agar top sputter coater for electron microscopy

Like many other surface technologies, sputter coating derives from the semiconductor industry, which developed such thin film technologies first to industrial standards. According to the fact that electrochemical biosensors always formed the main part of this field of science in former years (see chapter “I.) Introduction”), it is not surprising that these techniques had, have and will have profound impact in chemical sciences too. As described above I used a simple Plasma Sputter Coater from Agar. This Agar Sputter Coater (Figure 19) can deposit metals like gold, palladium, silver, copper, tin and similar ones with rather low evaporation temperatures. These metals are deposited as atoms, which assemble into islands on the surface. The sputter process is carried out with argon plasma. A disc of one of the metals above is placed in the target holder and the sensor chip is fixed on the platform inside the vacuum chamber of the Agar Sputter Coater. The chamber is evacuated to at least 0.04 mbar and after flushing the chamber with argon at a pressure of 1 mbar, the pressure is adjusted to 0.08 mbar. By applying an electrical

field of 5 kV, plasma is started. The metal is deposited on top of the chips positioned on the platform.

Spectrometer

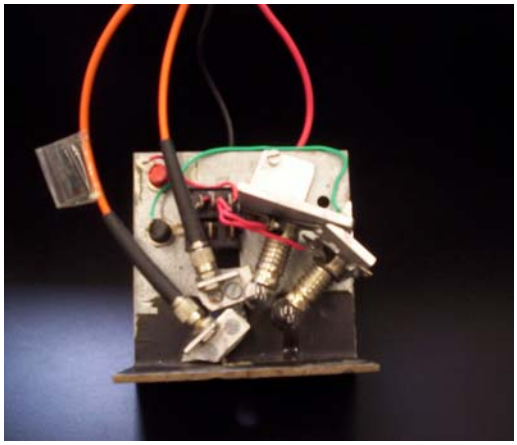


Figure 20: Simple spectrometer of the Pittner Lab for absorption measurement of the coated surfaces

This self made spectrometer (Figure 20) is used for the absorption measurement of the sputtered sensors to detect differences in the absorption spectrum according to the optical density.

Stomacher apparatus

Figure 21: Stomacher® 400 for homogenization of meat; (www.carl-roth.de)

The quality of microbiological analysis results depends on the accuracy of the sample preparation. For analysis of solid probes, crushing and homogenisation with a stomacher (Figure 21) is needed. This technique uses a polyethylene bag filled with

the solid probe and an appropriate dilution solvent. After closing the bag it is inserted into the Stomacher and homogenised by the pulsing movements of the two paddles. The probe in the bag is dissolved within short time (15 sec up to 3 min). The required time of homogenisation depends on the consistency of the probe.

III.2) Protocols for sensor fabrication

All the following protocols described were carried out using the devices and gadgets listed above.

Synthesising protocol

The polymer was solved in TFE in a 15 mL reagent vessel closed with a screw cap. In case of PLGA, the solid polymer was directly dissolved in the reaction vessel, while PLA was dissolved in TFE by preparing a 20% PLA stock solution. The solubilization process was accelerated by stirring the solution with a magnetic stirrer on a heating plate. All PLA containing polymers need to be heated to 50°C for 10 min. Then Desmodur is added to the polymer solution. Afterwards the polymer-linker mixture has to be stirred for another 15 min before printing.

Printing protocol

The automatic gravure printer is cleaned before and after every printing process with MEK, chloroform, MEK. While doctor blade and printing plate can be cleaned with chloroform, the impression roller is only treated with MEK in fact of the strong ability of chloroform to deform the rubber coated surface of the impression roller. The impression roller and the doctor blade are adjusted with micrometer gauges.
A substrate film with 12 x 22 cm or 6 x 22 cm is fixed with tape on the impression roller. The resulting print area of the larger film is 9.5 x 15 cm. Because of the fact that the lacquers have to be printed on top of the PET film with an Inconnel mirror, it has to be ensured that the side of PET foil is in direct contact with the surface of the impression roller and not the metal side. This is done by measuring the conductivity of the film, to ascertain which side is the metal and which is the polymer.

Washing and drying protocol

Immediately after the printing process, the PET film with the lacquer is dried at 80°C for 10 min in the Haereus oven to be ready for the sputter process. Alternatively, after this drying step, an additional washing step in a basket with ddH_2O on the shaker for another 10 min is done, which is followed by a second drying step at 80°C for 10 min in the oven.

Sputtering protocol

Sensor chips with the maximum size of 4 x 4 cm (usually 4 sensor chips with 2 x 2 cm area) are fixed onto the platform of the sputter coater with double sided adhesive tape. The lid is closed tightly and vacuum is generated by the forepump. When the vacuum reaches the 10 mbar boarder, the turbo pump is started in addition to create a vacuum of 0.04 mbar. Meanwhile, the chamber is flushed with Ar gas for 10 seconds at 1 mbar. After reaching the accurate vacuum, the desired operating pressure of 0.08 mbar is adjusted. By applying an electrical field of 5 kV the plasma is started for the required time (in my case usually 10 to 45 seconds). The metal is deposited on top of the chips positioned on the platform. After finishing of the sputter process itself, the turbo pump is switched off and after the turbo pump comes to a complete standstill, also the forepump is stopped and the chamber is flushed with air.

Spectroscopic protocol

Spectroscopic measurements of the surface are performed by a simple lab-made spectrometer (see Figure 20). Before each measurement, a dark reference (on a black pad) and a null reference (on a mirror) have to be taken. The detection time of each spectrum is 160 ms.

Incubation/Testing protocol

The sensors are incubated with different microbiological mixtures at different temperatures (4°C, rt (approximately 25°C) and 37°C) in a humidity chamber for a specific time period. Quick measurements are done within a time period of 4 h or 6 h while long time experiments are done over night (o/n) (approximately 16 h). The different temperatures should simulate common environment for meat inside the refrigerator (4°C), outside the refrigerator at room temperature (rt) or at the optimal growth temperature for the microorganisms (37°C). Normal storage should be at 4°C but also short periods at room temperature of high probability e.g. while the transport from the shop to the next freezer/refrigerator. A wet chamber is built by using a plastic Petri dish with wet paper towels to simulate the conditions inside a meat package. Here it has to be mentioned that it was not possible to create environments similar to those in a vacuum-packed meat package or with protective gas.
If no other pipetting scheme is presented beneath the sensor scans in the chapter "IV.) Results and discussions", the pipetting scheme as shown in Figure 22 was used.

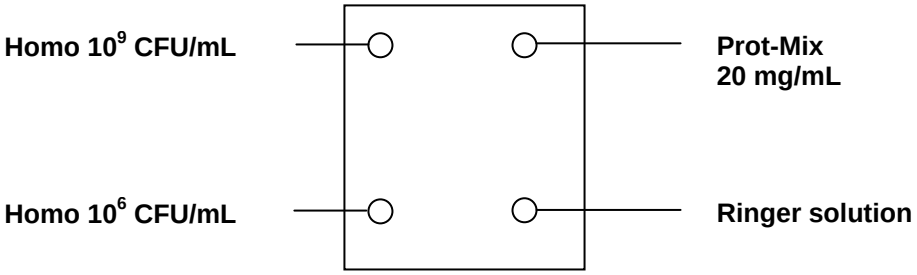


Figure 22: Pipetting scheme

Cleaning protocol

After incubation, sensor chips are washed with ddH_2O and dried under a strong air stream.

Documentation protocol

For documentations, all sensors are scanned with a flatbed scanner. To avoid strong reflectance during the scanning process, the sensors are placed on stripes of Parafilm® on the scanning area, which works like an optical interference filter to ensure a high quality of the pictures.

Stability testing protocol

To test the stability of the setup, a 2 x 2 cm sensor chip is incubated in 5 mL 3 x SSC for 4 h at 45°C in a lockable vessel. After incubation, the sensor chip is washed with ddH_2O and dried in an air stream.

Due to the fact that all the optimisations are carried out at a common laboratory level it has to be kept in mind that an upscaling for commercialisation needs slightly different methods.

III.3) Microbiology

During my diploma thesis, further investigations have been done in the group of Prof. Pittner on the improvement of the microbiological standard meat juice. For the functionality tests I was supplied with several mixtures of microorganisms which had been cultivated, mixed and produced by other members of the Pittner group [53]. Four different types of solutions were used to test the functionality of each produced sensor.

As a positive control, I used a Protease-Mixture (Prot-mix) of three enzymes – proteinase K, trypsin and α-chymotrypsin. It is known from literature [54, 55, 56] that these small enzymes show a high degradation rate of PLA. The use of this Protease-Mixture allowed me to check whether the metallic mirror-layer is too thick or the PLA-polymer is too tight. In these cases, no colour shift was visible.

As a simulation of the situation on the meat surface, I was equipped with two different mixtures. One microorganism mixture (bacteria cocktail) is made by dilution of inoculated bacterial growth media with a microorganism concentration of 10^6 CFU per bacterial strain. The mixture consists of four bacterial strains which have been detected and isolated from packed fresh meat in the research group of Prof. Pittner.

The second mixture is made by infection of fresh meat. Pork, which has been bought by an Austrian retailer (Billa), is cut into stripes (approximately 1 cm diameter), placed in a sterile glass Petri dish and then infected by pipetting 100 µL of nine different bacteria strains, with a concentration of 10^3 CFU. This infected meat is incubated for 4 d at 37°C to guarantee a high grade of deterioration. This spoiled meat is homogenized with 100 mL Ringer solution per 50 g meat in a stomacher apparatus (Colworth Stomacher® 400). Afterwards, the homogenized mixture (Homo) is portioned in Eppendorf tubes and stored in the freezer at -80°C.

As a negative control, I used Ringer solution, which is also used for all dilution steps.

Bacteria count detection

The mixtures of microorganisms are controlled by the detection of the bacteria count. According to the detected number, the aliquots are diluted with Ringer solution in the

appropriate rate to get the desired count. Therefore, a sub aliquot is diluted in Ringer solution to achieve dilutions of 10^4-10^{10}. These dilutions are distributed evenly with a spreader over separate plate count agar plate. The plates are incubated at 30°C. After 3 d, the number of colony forming units (CFU) is counted (Figure 23). Multiplied with the dilution rate, one gets the original count of bacteria cells in the solution.

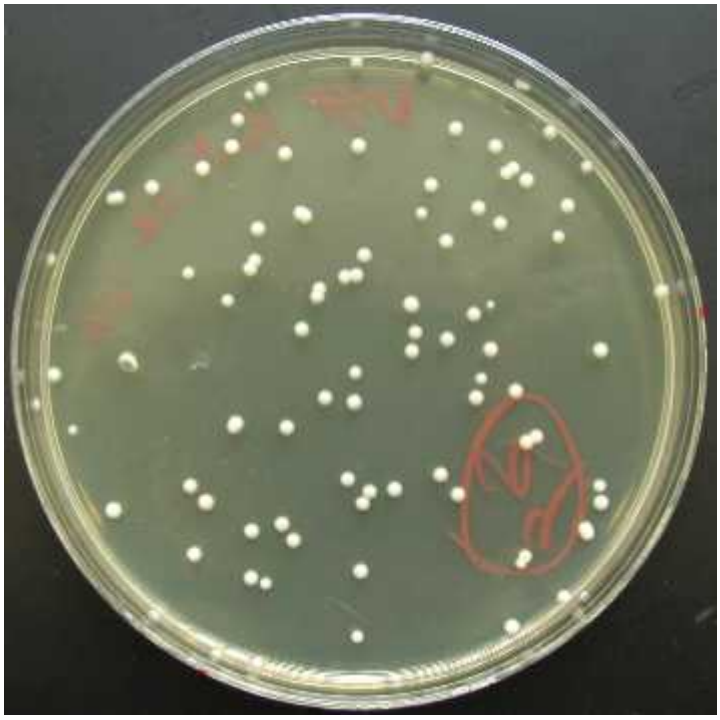


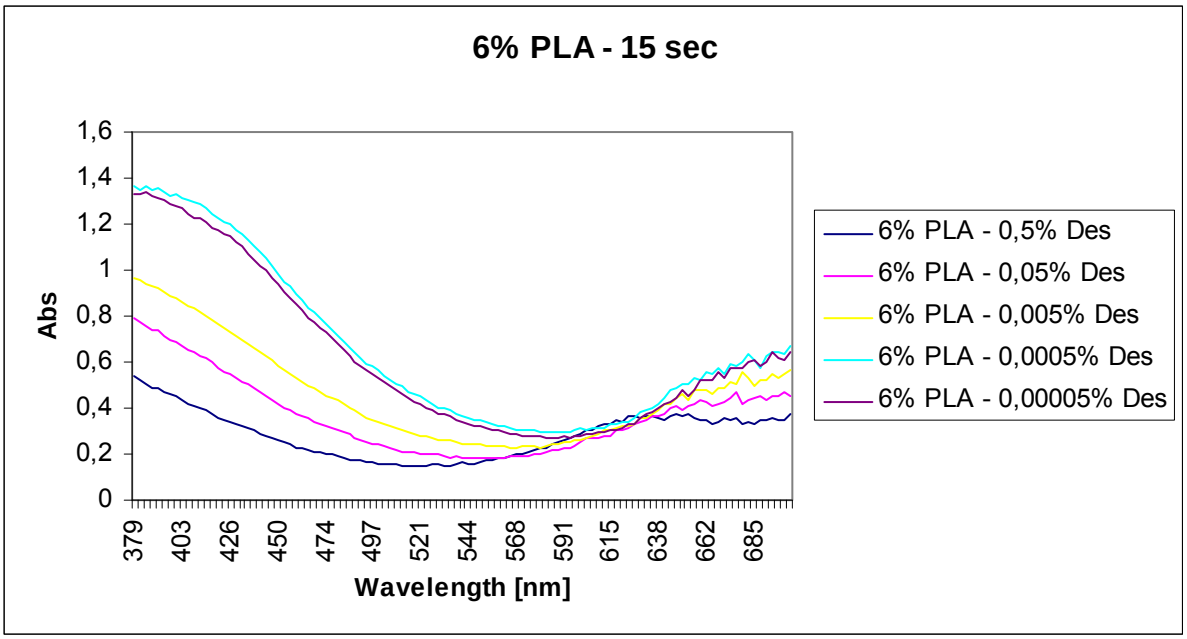
Figure 23: Plate Count Agar Plate

IV.) Results and discussions

As the development and optimisation of a biosensor is not a project in which the result delivers a simple “yes-or-no” answer but mainly little steps to an ameliorated product, I will try to guide through the phylogenesis of the developed devices on the following pages. This contains of course also some impasses but one has to bear in mind that the absence of an outcome is also a result. All the results are discussed immediately to make the thoughts, which directed me to the next steps and experiments obvious for the reader.

IV.1) Inconnel - Polymer - Au

A lot of work has been done in the research group of Prof. Fritz Pittner at the Department of Biochemistry and Cell Biology at the Max F. Perutz Laboratories on the topic of biosensors for the detection of food degradation [6, 7]. The “state of art sensor” was produced with the protocols mentioned in chapter “III.) Methods”. The sensor was built by a PLA polymer linked with Desmodur®, which was printed onto an Inconnel foil. The third layer was added through sputtering of Au for a period of time. A broad range of different sensors has been produced with the goal to detect maybe linearity or at least correlation between the tested parameters and the colour shift of the sensor surface which is equivalent to the sensitivity of the whole sensor setup. The necessity of his huge tested areas (Desmodur® concentration has a range of 10 orders of magnitude) can be observed in Spectrum 1 as there is a tremendous colour shift depending on the concentration of the linker Desmodur®.



Spectrum 1: Colour shift of the sensors with equivalent amount of PLA and different amount of linker Desmodur®

The following tables give an overview of the sensors with several variations of the parameters PLA concentration (w/v), Desmodur® concentration (v/v) and sputter-time (seconds). The limits of variations refer to the experimental data of the former works [6, 7]. All experiments have been carried out more often to assure that the effect is reproducible. I had no possibility to measure the deepness of the cavities produced by the enzymatic degradation of the microorganisms, which would have been an appropriate tool to qualify the results listed above in a more objective way. Due to the missing of such a tool, I tried to classify the results in a subjective classification. Four types of marks (good g, middle m, bad b, destroyed d) were given for every colour change. On the left side of each column, the results are presented which were gained after an incubation time of 4 h. The results of the right side of the column represent the output of the o/n-experiments. In order from left to right, the results for the specific sensors at 4°C (left), room temperature (middle) and 37°C (right) are shown. A minus (-) indicates, that at this specific temperature, no experiments have been done. This is also indicated through a free space in a cell.

Table 1: PLA overview - 10 seconds

10 sec	3% PLA	3,5% PLA	3,75% PLA	3,85% PLA	4% PLA
5*10^{-1}% Des	bbm	mmm mgm	bb- bb-		
5*10^{-2}% Des	bmm		bb- bm-		
5*10^{-3}% Des		mgm bbg			
5*10^{-4}% Des			bm- bm-		
5*10^{-5}% Des					
5*10^{-6}% Des		mbg ggg	bb- bm-		
5*10^{-7}% Des				bb- bb-	bm- bm-
5*10^{-8}% Des				bb- bm-	mm- bm-
5*10^{-9}% Des			bb- bm-		
5*10^{-10}% Des					
10 sec	**4,5% PLA**	**5% PLA**	**5,5% PLA**	**6% PLA**	**8% PLA**
5*10^{-1}% Des	bbm bmg	bb- bg-	bb- bm-	bg- bb-	
5*10^{-2}% Des		bd- dd-	bd- bd-	bd- mm-	
5*10^{-3}% Des		bd- bd-	bb- dd-	md- bd-	
5*10^{-4}% Des	bdd mdd	md- dd-	bb- bd-	bd- bd-	bd- bd-
5*10^{-5}% Des	bdd bdd	bb- md-	bb- bd-	bd- bd-	bd- bd-
5*10^{-6}% Des	gbd mdd	bd- md-	bd- bd-	bd- dd-	bb- bd-
5*10^{-7}% Des	bbd bdd	bd- bd-		bd- dd-	
5*10^{-8}% Des	bbd bdd	bd- dd-			
5*10^{-9}% Des					
5*10^{-10}% Des					

Table 2: PLA overview - 15 seconds

15 sec	3% PLA	3,5% PLA	3,75% PLA	3,85% PLA	4% PLA
5*10^{-1}% Des	bb- bgg	mmg ggg	bm- bg-		
5*10^{-2}% Des	bb- bmm	bb- mm-	md- mm-		
5*10^{-3}% Des	bmm bmm	bmg mgg	bd- md-		
5*10^{-4}% Des	bbm bmm	bb- mm-	md- bd-		
5*10^{-5}% Des			bd- bd-		
5*10^{-6}% Des		bmg mgg	bmd gdd	bbd bdm	bdd mmd
5*10^{-7}% Des			bdd bbd	bd- mm-	bm- mg-
5*10^{-8}% Des			bbd bdd	bm- mm-	bm- bdd
5*10^{-9}% Des			mm- bdd	dd- bd-	mdd ddd
5*10^{-10}% Des			bd- bd-	bd- bb-	
15 sec	**4,5% PLA**	**5% PLA**	**5,5% PLA**	**6% PLA**	**8% PLA**
5*10^{-1}% Des	bbg bmg	bb- bg-	bb- bm-	bg- bm-	
5*10^{-2}% Des		md- dd-	bb- bd-	bg- bm-	
5*10^{-3}% Des		bd- bd-	bd- bd-	md- bg-	
5*10^{-4}% Des	bdd bbd	bb- bd-	bb- dd-	bd- bd-	bd- bd-
5*10^{-5}% Des	bdd bdg	bb- bd-	bb- bb-	bd- dd-	bd- bd-
5*10^{-6}% Des	bmd bmd	bb- bd-	bb- bm-	bd- dd-	bd- bd-
5*10^{-7}% Des	bmd bmd	bd- bd-		bd- dd-	
5*10^{-8}% Des	bdd bdd	bd- bd-			
5*10^{-9}% Des					

Table 3: PLA overview - 20 seconds

20 sec	3% PLA	3,5% PLA	3,75% PLA	3,85% PLA	4% PLA
5*10^{-1}% Des	bmm bmm	mmg mgg	bb- bg-		
5*10^{-2}% Des	bbm bmm	bb- mm-	bb- bd-		
5*10^{-3}% Des	bbm bmm	mmg mgg	bb- bd-		
5*10^{-4}% Des	bbm bmm	bb- mm-	mg-		
5*10^{-5}% Des			bd-		
5*10^{-6}% Des		mgg ggg	bdb bdd	bbm bdd	bbb bdd
5*10^{-7}% Des			bbb bbd	bm- mg-	mm- mg-
5*10^{-8}% Des			bbb bdd	bd- mg-	bm- bm-
5*10^{-9}% Des			mm- mg-	bd- bb-	bdd bdd
5*10^{-10}% Des			bd- bd-	bd- bd-	
20 sec	**4,5% PLA**	**5% PLA**	**5,5% PLA**	**6% PLA**	**8% PLA**
5*10^{-1}% Des	bbg bmg	bb- bg-	bb- bg-	bg- bb-	
5*10^{-2}% Des		bb- bd-	bb- bb-	bd bm-	bbb bbm
5*10^{-3}% Des		bb- bb-	bb- bd-	bb- bm-	bbm mbm
5*10^{-4}% Des	bdd bbm	bb- bd-	bb- bd-	bd- dd-	bd- bd-
5*10^{-5}% Des	bdd bdd	bd- bd-	bb- bb-	bd- dd-	mm- bm-
5*10^{-6}% Des	bmd mdd	bb- dd-	bb- bb-	bd- dd-	bd- bd-
5*10^{-7}% Des	bbd bdd	bd- bd-		bd- dd-	
5*10^{-8}% Des	bdd bdd	bd- bd-			
5*10^{-9}% Des					
5*10^{-10}% Des					

Table 4: PLA overview - 30 seconds

30 sec	3% PLA	3,5% PLA	3,75% PLA	3,85% PLA	4% PLA
5*10^{-1}% Des	bbb bmm	bb- bm-	bm- bg-		
5*10^{-2}% Des	bbm bbm	bb- mm-	bb- bd-		
5*10^{-3}% Des	bbb bmm	bb- bm-	bb- bd-		
5*10^{-4}% Des	bbm bmm	bb- bm-	bd-		
5*10^{-5}% Des			bd-		
5*10^{-6}% Des			bb- bm-	bd-	bb- bm-
5*10^{-7}% Des				bd- mb-	bb- bb-
5*10^{-8}% Des				bb- bb-	
5*10^{-9}% Des				bb- bb-	
5*10^{-10}% Des			bb- bd-	bb- bd-	
30 sec	**4,5% PLA**	**5% PLA**	**5,5% PLA**	**6% PLA**	**8% PLA**
5*10^{-1}% Des		bb- bg-	bm- bg-	bm- bb-	
5*10^{-2}% Des		bb- bb-	bb- bm-	bg- bb-	bbm bmm
5*10^{-3}% Des		bb- bb-	bb- bm-	bb- bm-	dbb mbd
5*10^{-4}% Des		bb- bb-	bb- bd-	bd- bd-	bd- bd-
5*10^{-5}% Des		bb- bm-	bb- bb-	db- dd-	bd- bm-
5*10^{-6}% Des	bb- bb-	bd- bb-	bb- bb-	bd- dd-	bb- bd-
5*10^{-7}% Des	bb- bb-	bd- bd-		bd- dd-	
5*10^{-8}% Des		bm- bd-			
5*10^{-9}% Des					

Table 5: PLA overview - 45 seconds

45 sec	3% PLA	3,5% PLA	3,75% PLA	3,85% PLA	4% PLA
5*10^{-1}% Des	bbb bbm		bb- bb-		bbb bmg
5*10^{-2}% Des	bbm bbm		bb- bb-		bdb bdd
5*10^{-3}% Des	bbm bbm				bdb bdd
5*10^{-4}% Des	bbm bbm				bdd bdd
5*10^{-5}% Des	bbb bbm				bdd bdd
5*10^{-6}% Des			bb- bb-	bd-	
5*10^{-7}% Des				bd- bd-	bb- bb-
5*10^{-8}% Des					bb- bb-
5*10^{-9}% Des			bb- bb-		
5*10^{-10}% Des					
45 sec	**4,5% PLA**	**5% PLA**	**5,5% PLA**	**6% PLA**	**8% PLA**
5*10^{-1}% Des		bm- bg-	bb- bg-	bg- bb-	
5*10^{-2}% Des		bb- bd-	bb- bd-	bm- bb-	bbm bbm
5*10^{-3}% Des		bb- bd-	bb- md-	bb- bd-	dbm bmg
5*10^{-4}% Des		bb- bd-	mm- dd-	bb- bd-	bd- bd-
5*10^{-5}% Des		bb- bd-	bb- bb-	bd- bd-	bd- bd-
5*10^{-6}% Des	bb- bb-	md- bd-	mm- bm-	bd- md-	bb- bd-
5*10^{-7}% Des	bb- bb-	bd- dd-		bd- dd-	
5*10^{-8}% Des		bd- bd-			
5*10^{-9}% Des					

Some sensors are presented (Figure 24 - 26) to show, which type of colour change was classified with g, m or b. If the layers seemed to be destroyed, it is marked with a d (Figure 27).

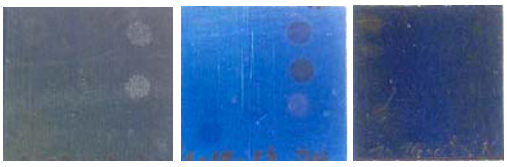


Figure 24: good results (g)

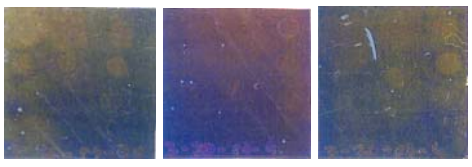


Figure 25: middle results (m)

Figure 26: bad results (b)

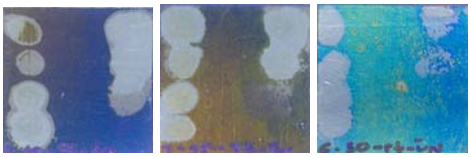
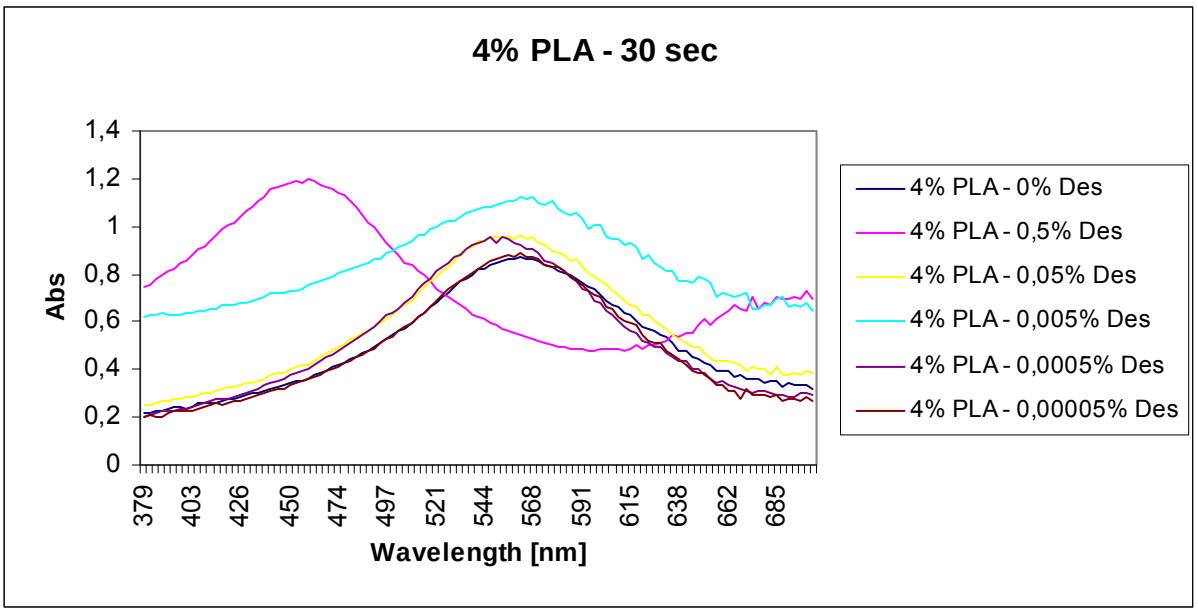
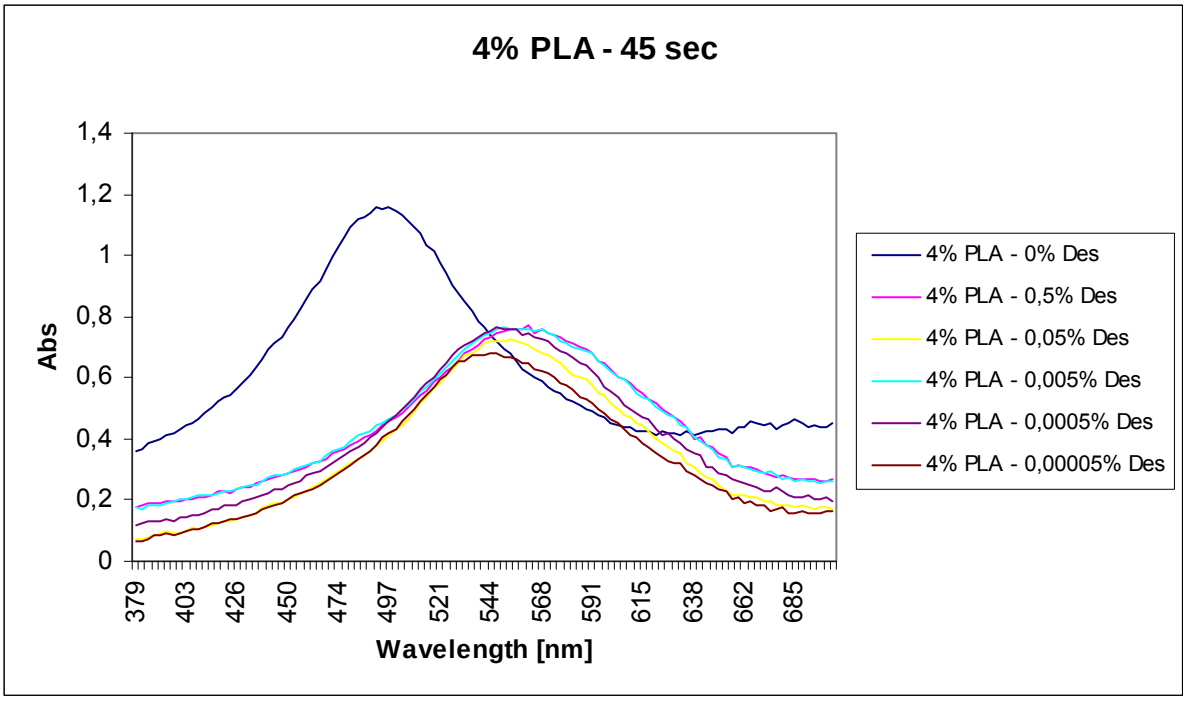


Figure 27: "destroyed" sensors (d)

The majority of positive results (at least one g at one of the three temperatures) could be detected at 3.5% w/v PLA (17), followed by 4.5% w/v and 5% w/v. At a Desmodur® concentration of 0.5% v/v, 27 positive results occurred. 17 positive results are generated at sputter periods of 15 respectively 20 seconds. This is also according to our information gained of the absorption spectra as there are no significant colour shifts at longer sputter periods.



Spectrum 2: 30 seconds; different Desmodur® concentration



Spectrum 3: 45 seconds; different Desmodur concentration

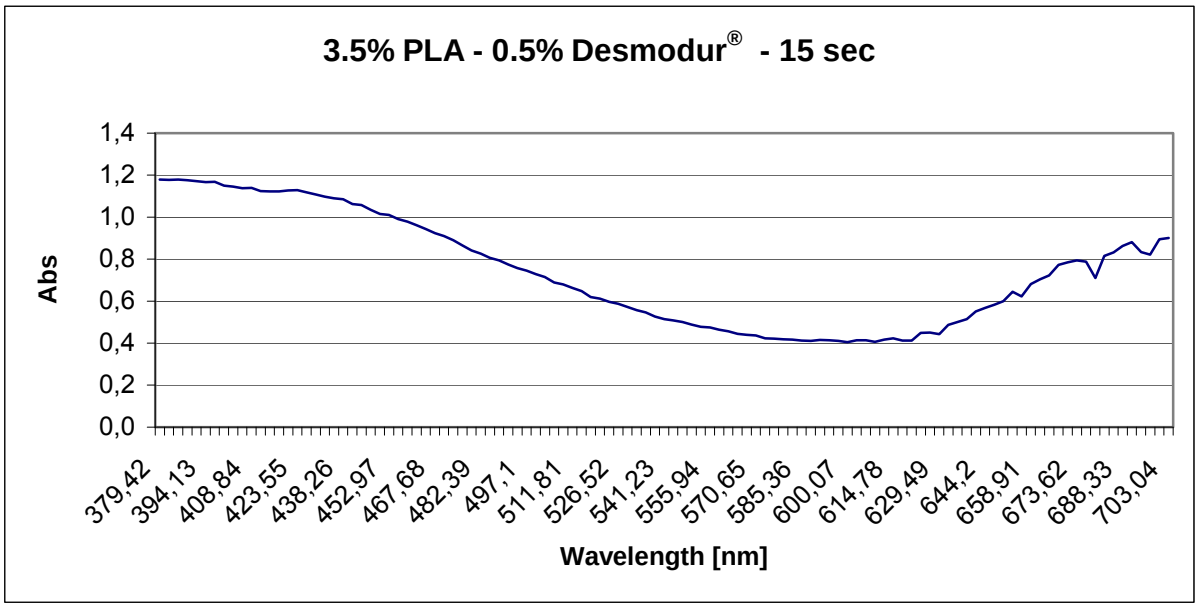
These results suggest that concentrations of 3.5% w/v, 4.5% w/v and 5% w/v PLA are "best" in term of best functionality (Table 6). These sensors have been further investigated:

Table 6: Composition of the "Best-of-PLA"-sensors

PLA concentration	Desmodur® concentration	Sputter time
3.5% w/v PLA	0.5% v/v Desmodur®	15 seconds
3.5% w/v PLA	0.005% v/v Desmodur®	15 seconds
4.5% w/v PLA	0.5% v/v Desmodur®	15 seconds
5.0% w/v PLA	0.5% v/v Desmodur®	15 seconds

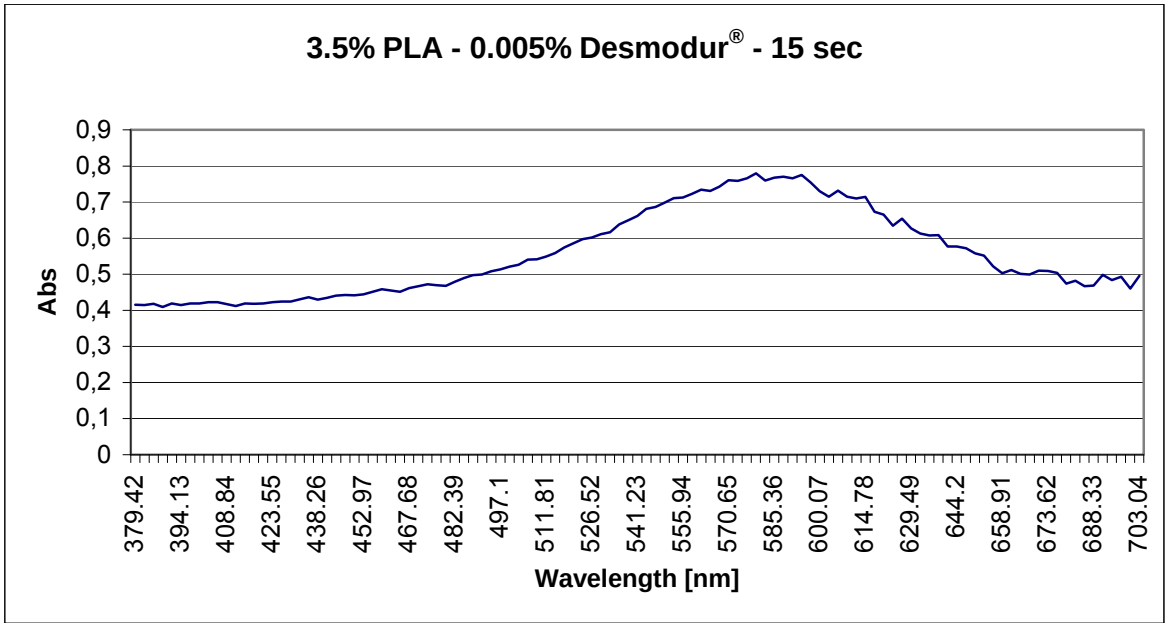
“Best-of-PLA”-sensor with the setup: Inconnel - Polymer - Au

Out of the whole amount of tested sensors, those sensors with the most definite colour changes have been selected for further experiments. These “best-of-sensors” should reduce the amount of sensors I had to produce to detect differences in the variation of additional parameters. The limiting step in the laborious sensor production was the sputtering step due to the quick heating of the vacuum pumps. In Spectrum 4 - 7, the absorption spectra of the four “best-of-sensors” are presented as well as typical figures of the sensors itself.



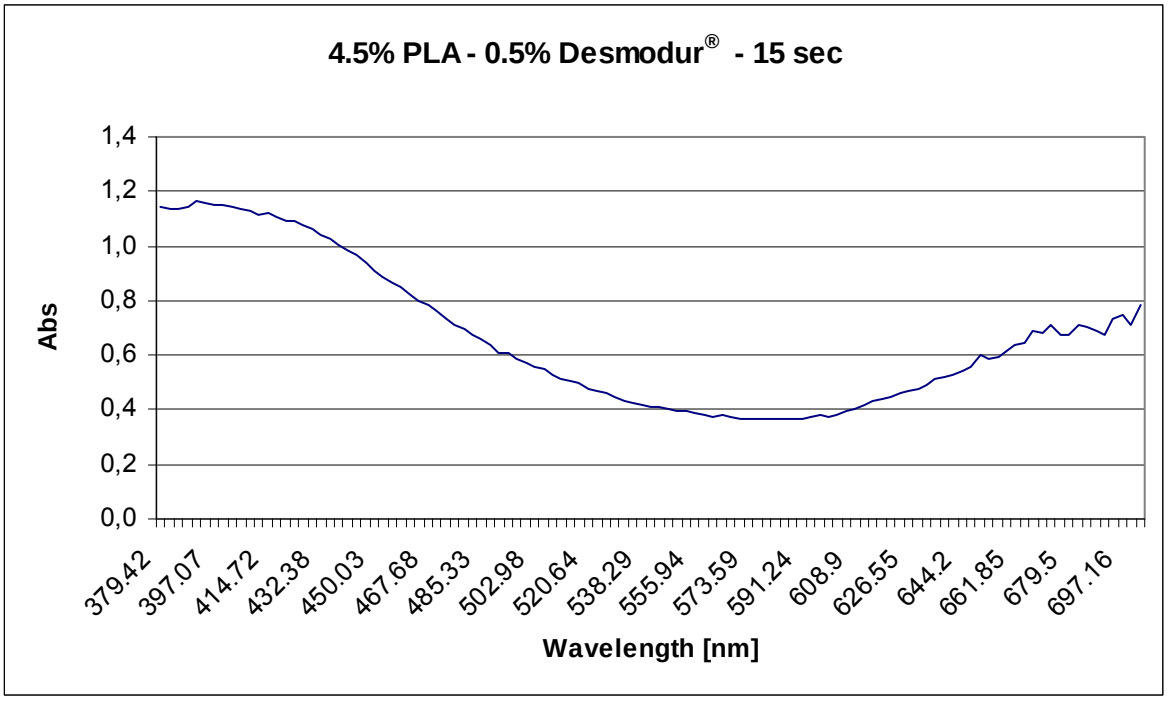
Spectrum 4: 3.5% PLA w/v – 0.5% Desmodur® v/v – 15 seconds

Figure 28: Sensor scan: 3.5% w/v PLA – 0.5% v/v Desmodur® – 15 seconds



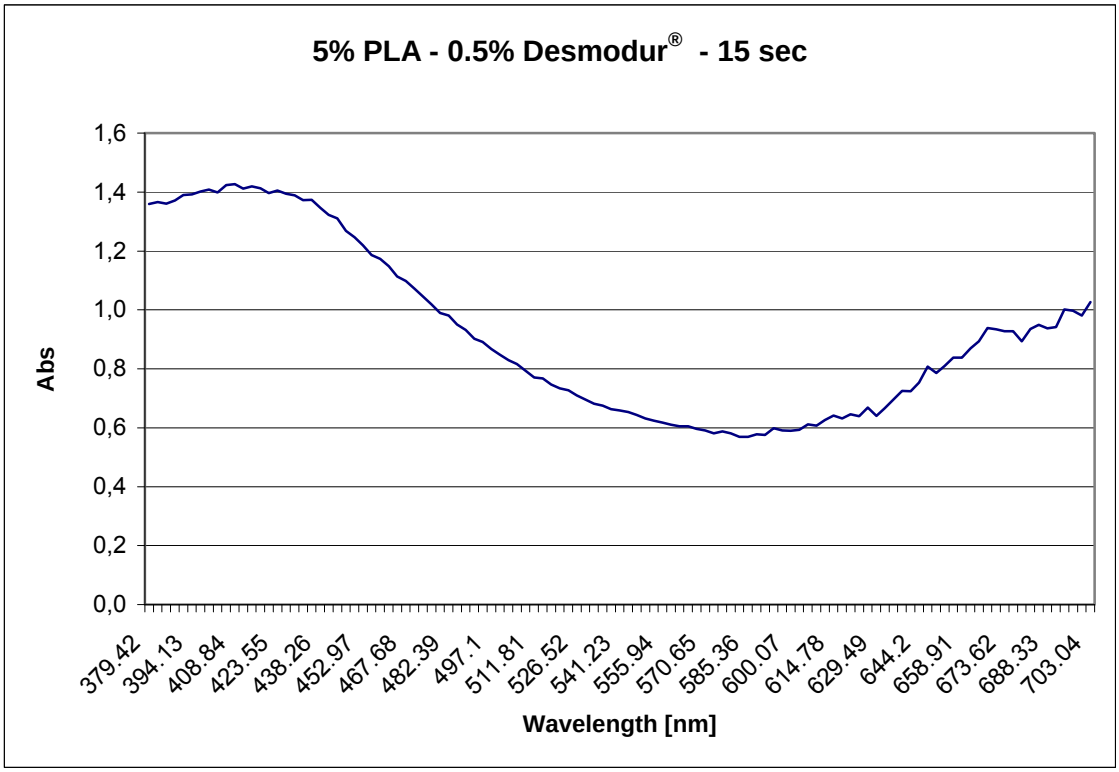
Spectrum 5: 3.5% w/v PLA – 0.005% v/v Desmodur® – 15 seconds

Figure 29: Sensor scan: 3.5% w/v PLA – 0.005% v/v Desmodur® – 15 seconds



Spectrum 6: Sensor scan: 4.5% w/v PLA – 0.5% v/v Desmodur® – 15 seconds

Figure 30: Sensor scan: 4.5% w/v PLA – 0.5% v/v Desmodur® – 15 seconds



Spectrum 7: 5.0% w/v PLA – 0.5% v/v Desmodur® – 15 seconds

Figure 31: Sensor scan: 5.0% w/v PLA – 0.5% v/v Desmodur® – 15 seconds

PLA setup: Stability tests

Besides the functionality of the sensor, the stability properties are very important for reproducibility. Therefore, the sensors were incubated at 3x SSC at 45°C for 4 h to test the influence of a salty solution. The results of these tests are shown below (Figure 32 - 35).

Figure 32: Stability test: 3.5% w/v PLA – 0.5% v/v Desmodur®

Figure 33: Stability test: 3.5% w/v PLA – 0.005% v/v Desmodur®

Figure 34: Stability test: 4.5% w/v PLA – 0.5% v/v Desmodur®

Figure 35: Stability test: 5.0% w/v PLA – 0.5% v/v Desmodur®

The stability of these sensors differs very strongly in their stability towards 3x SSC. All sensors can be classified as stable with exception of the 4.5% w/v PLA sensor.

Response time: 4 h vs. 6 h

As described in chapter “I. Introduction”, microorganisms show a lag phase before exponential growth is observed. Due to this fact, one has to consider that also the microorganism mixtures, which are stored at -80°C, need some time to display their full activity [53]. According to this, the short incubation time of 4 h was enlarged up to 6 hours which correlates to the time period the bacteria need to reach the log-phase. The “Best-of-PLA” sensors were incubated at 4°C, rt and 37°C for 4 h, 6 h and o/n to

compare the different periods and to examine, if the results after 6 h are more equivalent to those after 4 h or o/n.

Figure 36: 3.5% w/v PLA – 0.005% v/v Desmodur®, 10 sec; after 4 h incubation at 37°C

Figure 37: 3.5% w/v PLA – 0.005% v/v Desmodur®, 20 sec; after 4 h incubation at 37°C

Figure 38: 3.5% w/v PLA – 0.005% v/v Desmodur®, 10 sec; after 6 h incubation at 37°C

Figure 39: 3.5% w/v PLA – 0.005% Desmodur®, 20 sec; after 6 h incubation at 37°C

Figure 40: 3.5% w/v PLA – 0.005% Desmodur®, 10 sec; after o/n incubation at 37°C

Figure 41: 3.5% w/v PLA – 0.005% v/v Desmodur®, 20 sec; after o/n incubation at 37°C

After 4 h of incubation with homogenized standard putrid-meat juice (Homo), most of the sensors do not show good results – often there is not even one appropriate result to count. Significantly more colour shifts are observed after 6 h, which represented almost completely the best possible results after incubation for 16 h (o/n).

Other performance factors, which are at least worthwhile to be discussed, are the selectivity, nature of solution the recovery time and the working lifetime. As the sensor is very cheap in its production, it is designed as a disposable device, which means that there is no need for a recovery of the sensor. There is no need for a pre-treatment of the spoiled meat juice as it is possible to detect the change of colour

with the crude meat juice. The sensitive layer is built by a simple polymer with peptide and ester bonds. There is no use for the detection of specific enzymes, respectively the specific enzymatic reactions, as all the enzymes participate in the deterioration process. Some sensors were first tested 6 months after their fabrication and the results are similar to those, which were tested directly after production. The time span of working life seems to be accurate, though it is necessary to do further experiments on this topic.

IV.2) Au - PLA - Au

Inconnel is not appropriate for use in packaging materials because of the possible harmful properties of Nickel. As the effect of anomalous absorption is independent of the used mirror, other metals were tested as substrates. One was a gold film which is chemically inert and therefore practicable for the usage in packaging materials, but for a commercial use much too expensive, even if huge amounts of sensors would be produced. But it was useful to test it on a laboratory scale and to learn which effects the metal could have on the stability of the setup.

Figure 42: Au - 3,5% w/v PLA – 0.1% v/v Desmodur®; 15 seconds

IV.3) Inconnel – PLA + Trigger

The sensitive polymer layer is used as an artificial meat surrogate because of its ester bonds as seen in fat and comparable in reactivity to amides in the proteins of the meat. But until now, no data exist, whether degradation of the polymer or the meat itself is preferred by the lytic enzymes. As the release of enzymes is an evolutionary optimised phenomenon, it is not risky to postulate that the enzymes prefer the amide-connected amino acids rather than the linked PLA, although it could be inverted as well. The goal of a competing polymer in the sensor is to reach the

attractiveness towards the enzymes as the real meat sample has. These considerations initiated me to trigger the polymer with nutrient sources which could increase the attractiveness of the sensitive polymer. Several sources of carbohydrates, amino acids and lipids were added to the polymer. The major problem was the low solubility of these compounds in the polymer solvent TFE. Only small amounts (up to 20 mg/mL) of glucose, sun flower oil or glycerol-tributyrin could be added without precipitation.

Figure 43: 3.5% PLA – 0.5% Desmodur® + 7.5% v/v glycerol-tributyrat; 20 sec, 37°C, 6 h

Figure 44: 3.5% PLA – 0.005% Desmodur® + 7.5% v/v glycerol-tributyrat, 10 sec, 37°C, o/n

First results showed a slight increase of the intensity of the colour shift. Also the stability of these triggered sensors could be classified as being as stable as the ones without the trigger. According to the large number of possible triggers, further investigations have to be made on this aspect in order to find the most effective one.

IV.4) Inconnel – PLA

It is known from literature that lytic enzymes like esterase, phospholipase or aminopeptidase are able to cut a PLA polymer network [46-48]. We wanted to reproduce such type of degradation in order to get an impression of the intensity and power of this method. Therefore, directly after printing the polymer the unsputtered sensor was incubated with a mixture of different commercially available proteolytic enzymes (Prot-Mix) for 4 h and o/n. On the spots where the enzymes were dappled the degradation of the polymer network obviously took place and could be easily observed by the naked eye, although the colour shifted only from dark grey to light grey.

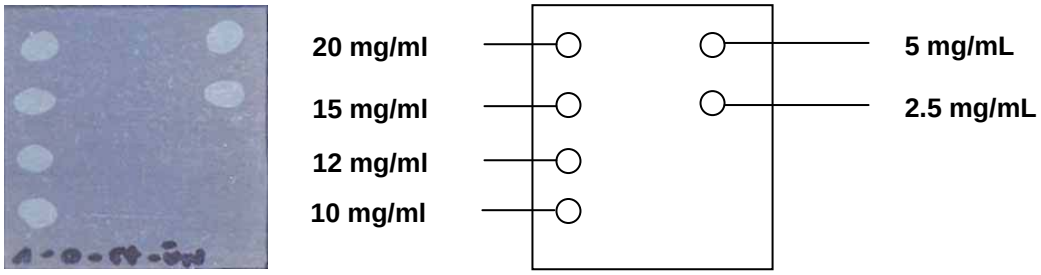


Figure 45: Inconnel - 3.85% w/v PLA – 0.000005% v/v Desmodur®; incubated o/n with enzyme solution

These results led us to the assumption that it might be helpful for our further investigations to get an overview of the effects of the lytic enzymes on the untreated printed polymer surfaces of the Inconnel foil. So the table of the results of the incubation of 0 seconds sputtered sensors has been established.

Table 7: PLA overview - 0 seconds

0 sec	3% PLA	3,5% PLA	3,75% PLA	3,85% PLA	4% PLA
5*10^{-1}% Des	bbb bmm	bbb bmm	bb- bb-		
5*10^{-2}% Des	bmm bmm	bb- bb-	bb- bb-		
5*10^{-3}% Des	bmm bmm	bbm bbm			
5*10^{-4}% Des	bmm bmm	bb- bb-	bb- bb-		
5*10^{-5}% Des					
5*10^{-6}% Des		bbb bbb	bb- bb-	bm- bm-	
5*10^{-7}% Des				bm- mm-	bb- bb-
5*10^{-8}% Des				bb- bb-	bb- bb-
5*10^{-9}% Des			bb- bb-		
5*10^{-10}% Des					
0 sec	**4,5% PLA**	**5% PLA**	**5,5% PLA**	**6% PLA**	**8% PLA**
5*10^{-1}% Des		bb- bm-	bb- bg-	bg- bb-	
5*10^{-2}% Des		mg- gm-	bm- mm-	mg- mg-	
5*10^{-3}% Des		mg- gm-	bm- gm-	bm- gm-	
5*10^{-4}% Des		bm- mm-	bm- gm-	mg- gm-	mm- gb-
5*10^{-5}% Des		bm- mm-	bm- bm-	mg- gm-	mm- gm-
5*10^{-6}% Des	bb- bb-	bm- mm-	bm- bm-	bm- mm-	bb- gb-
5*10^{-7}% Des	bb- bb-	bm- gm-		mm- mm-	
5*10^{-8}% Des		mm- gm-			
5*10^{-9}% Des					
5*10^{-10}% Des					

The classification of the results has been made in the same way as described above. It has to be mentioned that a good result was termed so, if there was a clearly visible change in the optic perception even if it was just a strong change in the intensity of the grey colour.

IV.4) New polymer - PLGA

The so far gained results show obviously a lack of sensitivity of the polymeric interlayer. Therefore, a new composition of the polymer was the worthwhile aim of my investigations during my diploma thesis.
The polymer, which was to be chosen, had to combine several properties. It had to be a biodegradable polymer and be approved by FDA. This is important because the polymer of the sensor is in contact to the tested food (in our case pork). Any contamination of the meat with harmful substances released from the biosensor has to be strictly avoided. To increase the sensitivity of the polymer towards the lytic enzymes of the microorganisms, the chemical bonds of the monomers need to be cleaved easily. Most of the enzymes are unspecific so there is a broad area of possible chemical bonds. The search ended with the decision for PLGA, which is quite similar to the so far used PLA (see chapter “I.) Introduction”). PLGA was chosen because of its well known properties from the pharmaceutical and medical chemical area [49, 50], its degradation behaviour and its biocompatibility.

Optimisation of the PLGA concentration

First of all it was important to get an overview of the possible range of polymer concentration, which is suitable to be printed onto the surface of the metallic PET foil. Therefore a large range of polymer concentrations was printed [57]. It could be identified, that the ideal range of printing is from 20% w/v PLGA up to a maximum of 35% w/v PLGA. If PLGA is in a lower concentration, the viscosity is very low and the quality of the printed films is bad and irreproducible.

Figure 46: 10% w/v PLGA with 0.1% v/v Desmodur® printed on Inconnel film

Figure 47: 40% w/v PLGA printed on Inconnel film

IV.5) Inconnel - PLGA - Au

The PLGA was used in the same manner as PLA was used before. The PLGA was solubilized in TFE and this solution was printed on the Inconnel film as described in the protocols in chapter “III.) Methods”. The following figures show typical PLGA sensors with different times of sputtering after incubation with homogenized standard putrid-meat juice (Homo).

Figure 48: Inconnel – 20% w/v PLGA – 0.1% v/v Desmodur® - 20 seconds; after 4 h incubation at 37°C

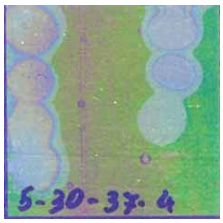


Figure 49: Inconnel – 20 % w/v PLGA – 0.1% v/v Desmodur® - 30 seconds; after 4 h incubation at 37°C

Figure 50: Inconnel – 20% w/v PLGA – 0.1% v/v Desmodur® - 45 seconds; after 4 h incubation at 37°C

A destroyed sensor surface could be observed with this type of setup. The negative control (Tris/HCl, pH 8.2) did not show any colour change but all the protease mixtures did. Unfortunately these strong changes are not very sensitive to the enzyme concentration as the sensor reacts similar to a concentration of 20 mg/mL as to 2.5 mg/mL.

IV.6) Inconnel – PLGA

The unwanted results of the Inconnel-PLGA-Au setup encouraged me to gain more experience on the fundamental degradation efficiency of PLGA. To prove the degradation efficiency of the new polymer, functionality tests with PLGA and Inconnel but without the sputtered Au nano-cluster layer were performed. Unsputtered and untreated PLGA on Inconnel has a slight green or red colour, which depends on the optical density of the polymer layer and the thickness of the layer itself. The difference between green and red areas on films with the same polymer concentration is caused by the thickness of the layer. These stripes are the result of a heavy used printer with old printing plate, old doctor blade and slightly uneven impression roller.

Surprisingly, the sensors showed clear colour shifts after incubation with the homogenized standard putrid-meat juice (Homo).

Figure 51: Inconnel – 20% w/v PLGA – 0.1% v/v Desmodur®; after 4 h incubation at rt

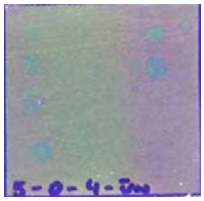


Figure 52: Inconnel – 20% w/v PLGA – 0.1% v/v Desmodur®; after o/n incubation at 4°C

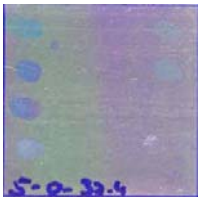


Figure 53: Inconnel – 20% w/v PLGA – 0.1% v/v Desmodur®; after 4 h incubation at 37°C

This discovery opened up many new ways and possibilities which lead to a patent application [58].

Inconnel - PLGA (without Desmodur®)

The brilliant colour shift, described in the chapter before, occurs even at small concentrations of the linker Desmodur®. The next step was to find out whether there is any need for the linker or not. If the linker is not essential, this would accelerate the production process and reduce the overall costs.

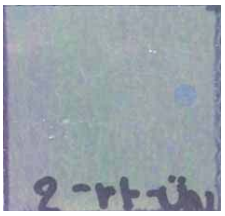


Figure 54: PLGA without the linker Desmodur®

Unfortunately the incubation of a PLGA 2-layer sensor without Desmodur® does not show clear colour changes. This could be similar to the PLA 3-layer sensor, where there is also an urgent need of Desmodur®. Otherwise, the polymer network is very tight and inhomogeneous, which results in the lack of a colour shift.

Optimisation of the linker concentration in the PLGA setup

Since the PLGA experiments, as well as the ones with PLA, show a strong dependency of the concentration of the linker Desmodur®, I had to gain an overview of the effect of the linker in the PLGA polymer as well. In the following step, I modified the concentration of the linking agent Desmodur® at constant PLGA concentration. The lacquers were printed onto the metallic top of the Inconnel mirror.
It has to be mentioned again that all the prints were done on the same printing proofer (Erichson) with a constant velocity of 5 and a constant pressure (adjustments see printing protocol chapter “III.) Methods”).

Figure 55: “Best-of” PLGA sensor; 20% w/v PLGA – 0.5% v/v Desmodur®

The sensors show similar results in a range from 20% w/v to 25% w/v PLGA. At a constant PLGA concentration, the sensors with a Desmodur® concentration of 0.5% v/v showed the clearest colour changes after incubation with protease mix respectively homogenized standard putrid-meat juice (Homo) (Figure 55).

Stability of the Inconnel – PLGA setup

A series of stability tests has been performed to find out a range of concentrations exists, which results in optimal properties with respect to the stability of the whole sensor. Over the range from 20 – 35% w/v PLGA and from 0.5 – $5 \cdot 10^{-6}$% v/v Desmodur®, the sensors showed a low stability level, which is shown with a typical picture of such an incubated sensor.

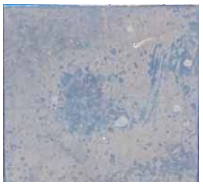


Figure 56: Inconnel - 25% w/v PLGA – 0.000005% v/v Desmodur®

Inconnel - PLGA + dye

Besides its low stability, one major problem of this new two-layer-sensor was the fact, that the intensity of the colour of the sensor is very low. This will not be applicable for commercial use where all customers should be able to easily detect by themselves whether the meat is fresh or not. I focused on possibilities to intensify the colour of the polymer before it is degraded. Therefore I tried to change the composition of the polymer by adding dyes of the same colour as the film. I tried to integrate commercially available green and red food colouring into the polymer. This could create an intensive green (or red) starting colour, which would fit best to the demands of the users as they are used from every day life that green signalises “positive” and red indicates “negative” (e.g. traffic lights). In addition, it would not be harmful if it is released by the sensor and taken up orally via the meat.

Figure 57: Inconnel – 25% w/v PLGA – 1% v/v Desmodur® + 0.25% w/v Carmin red; before incubation

The sensor did not show any enhanced results or even a change of the starting colour. One explanation is probably the weak solubility of the green food colouring in the solvent TFE. Until date, no solvent mixture has been found, which combines the hydrophilic properties of the polymer and hydrophobic properties of the dye. Further investigations in the solvent sector as well as a variation of possible harmless dyes will bring the favoured results.

Stability of the Au – PLGA setup

The same stability test with 3x SSC as in the chapter before was done with the Au – PLGA 2-layer setup.

Figure 58: Stability test: Au - 25% w/v PLGA – 0.00005% v/v Desmodur®

Obviously, the change of the substrate from Inconnel to Au did not increase the stability of the sensor towards 3x SSC (Figure 58). Like in all experiments, the PLA shows higher adhesion properties than PLGA, independent of the mirror substrate.

IV.7) Mix of PLGA and PLA

With respect to the fact, that PLGA differs from PLA only because of a certain amount of glycolic-acid, it was obvious that a mixture of these two polymers, which leads in the long run to a polymer with a lactic-acid to glycolic-acid ratio higher 1:1, could be a source of combining the positive effects of the 2-layer-PLGA-sensor with the benefits of the 3-layer-PLA-sensor.
The following combinations of PLA and PLGA were printed onto Inconnel foil:

Table 8: PLA/PLGA mixtures

PLA concentration	PLGA concentration	Desmodur® concentration	L:G ratio
21 % PLA	4.5 % PLGA	0.50 % Desmodur®	L:G = 58:42
10.5 % PLA	2.25 % PLGA	0.25 % Desmodur®	L:G = 58:42
12 % PLA	6.0 % PLGA	0.50 % Desmodur®	L:G = 67:33
6.0 % PLA	3.0 % PLGA	0.25 % Desmodur®	L:G = 67:33
10 % PLA	10 % PLGA	0.50 % Desmodur®	L:G = 75:25
5.0 % PLA	5.0 % PLGA	0.25 % Desmodur®	L:G = 75:25
5.0 % PLA	11.6 % PLGA	0.50 % Desmodur®	L:G = 85:15
2.5 % PLA	5.8 % PLGA	0.25 % Desmodur®	L:G = 85:15
10 % PLA	9.3 % PLGA	0.50 % Desmodur®	L:G = 65:35
5.0 % PLA	4.65% PLGA	0.25 % Desmodur®	L:G = 65:35

These sensors were sputtered for 0, 10 and 15 seconds to investigate, whether the stability of the sensors increased during incubation or not.

Figure 59: Inconnel - 5% w/v PLA + 11.6% w/v PLGA – 0.5% v/v Desmodur®; 0 sec; before incubation

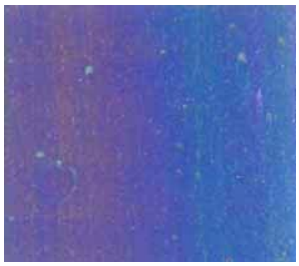


Figure 60: Inconnel – 5% w/v PLA + 11.6% w/v PLGA – 0.5% v/v Desmodur®; 10 sec, before incubation

Figure 61: Inconnel - 5% w/v PLA + 11.6% w/v PLGA – 0.5% v/v Desmodur®; 10 sec, after o/n incubation at rt

The brilliant colours, which were gained already in the 3-layer-PLA-setup could be repeated even with an amount of glycolic-acid in the polymer layer. The main benefit, which could be observed, was increased sensitivity due to the high sensitive properties of PLGA. In contrast to the 2-layer-PLGA-setup, it is possible to sputter a third layer without loosing sensitivity because of instability during incubation (Figure 62 - 63).

Figure 62: Stability test: 10% w/v PLGA, 9.3% w/v PLA, 0.5% v/v Desmodur®

Figure 63: Stability test: 10.5% w/v PLGA, 2.25% w/v PLA, 0.25% v/v Desmodur®

IV.8) Optimisation of the linker type

The linker Desmodur® 2460 M has some disadvantages. It contains only two isocyanate groups as reactive groups for interactions with OH-groups or COOH-groups [59]. This only leads to an increase of the chain length of the polymer because of the fact that the polymer (PLA or PLGA) contains also only two reactive side chains (see chapter “I. Introduction”). For a real cross-linking - in three dimensions - it would be necessary to introduce at least a third reactive group. We decided to try another linker form Merck, which is a mixture of diphenyl-diisocyanates and diphenyl-triisocyanates [60], which brings us to the wanted direction.
The incubated sensors (Figure 64 – 67) show, that the substitution of Desmodur® with the tri-/diisocyanate mix results in a more brilliant colour change.

Figure 64: 4.5% w/v PLA – 0.5% v/v di-/triisocyanat mix from Merck; 20 sec, o/n, 37°C

Figure 65: 5.0% w/v PLA – 0.5% v/v di-/triisocyanat mix from Merck; 20 sec, o/n, 37°C

Figure 66: 25% w/v PLGA – 0.5% v/v di-/triisocyanat mix from Merck; rt, o/n

Figure 67: 26% w/v PLGA – 0.5% v/v di-/triisocyanat mix from Merck; rt, o/n

Stability tests were carried out according to the protocols in chapter “III.) Methods”.

Figure 68: Stability test: Inconnel - 20% w/v PLGA – 0.1% v/v di-/triisocyanat

Figure 69: Stability test: Inconnel – 3.5% w/v PLA – 0.5% v/v di-/triisocyanat in EtOAc

As can be seen from Figures 68 and 69, the stability of the setup increases significantly but this new linker by now lacks admission of the governmental authorities for use in the food section. Thus at the moment, the food sector is no application field for a sensor with a polymer containing this linker, but this sensor type could be used in the cosmetics or health sector.

IV.9) Optimisation of sensor fabrication - additional washing step

Generally spoken, identically produced sensors show lack of reproducibility concerning sensitivity, intensity of the colour change or in the correctness. Sometimes, false-positive results could be detected although only autoclaved Ringer solution without any microorganisms was incubated. This led to the consideration, that there might be swelling and shrinking effects due to the humidity and ionic concentration of the Ringer solution. This was illustrated by a surprising colour effect.

The sensor with the setup Inconnel-PLA-Au showed a certain colour before the incubation process. After incubation a clear colour change was visible to the naked eye, even after washing the sensor with ddH_2O. These wet sensors were dried in a strong air stream as described in chapter “III.) Methods”. During the drying process, the colour-change showed reversibility. Experiments showed correlation with sensor’s dryness – independent of the method of drying. The effect occurred also while drying at room temperature without an air stream or at the oven at 80°C. The effect was reproducible after drying under an air stream. We tried to prepare the metal coated polymer surface in a way, that no modification happens at the contact with the incubation mixture respectively the meat surface.

Therefore, the protocol for the sensor production was adapted. After printing the polymer, the film was dried for 10 min at 80°C. Then, the film was washed for 10 min in ddH_2O and then dried again at 80°C. The results are shown in Figures 70 to 73.

Figure 70: 5.0% w/v PLA – 0.5% v/v Desmodur®; without additional washing step; 15 sec, rt, 6 h

Figure 71: 5.0% w/v PLA – 0.5% v/v Desmodur®; with additional washing step; 15 sec, rt, 6 h

Figure 72: 5.0% w/v PLA – 0.5% v/v Desmodur®; without additional washing step; 15 sec, 37, o/n

Figure 73: 5.0% w/v PLA – 0.5% v/v Desmodur®; with additional washing step; 15 sec, 37, o/n

The additional washing step did not show the desired results. No significant change in the clearness of the colour was observed. It seems that the quality decreases with the Inconnel-PLA-Au setup through the washing step. It is also possible that the visible colour change of the whole sensor is due to the inhomogeneous printing process.

IV.10) Optimisation of the sensor's performance concerning microbiology and correlation with standard test systems

One of the most important steps towards a well working sensor set up is the correlation with the existing and governmental accredited standard tests which are mainly of microbiological nature to detect the end point of freshness as is defined by law.

The correlation with the established standard microbial methods was carried out as follows:

At the beginning of my work, I used a system guaranteeing a certain amount of enzymes in the solution for testing the fabricated sensors. Therefore, a mixture of enzymes with hydrolytic and esterase-like properties was used. It was originally developed by Margit Barth and Mag.[a] Nadira Ibrišimović. Different concentrations were dappled onto each sensor to detect the minimal limit of enzymes at which a colour change is visible. This gained expertise would have been useful for further optimisation. The results were regrettably as manifold as the sensors so it was not possible for us to find a specific limiting concentration.

In the next step, nine strains of bacteria, causing typical severe food spoilage, were separately inoculated in LB-media and incubated at 37°C to reach a higher amount of bacteria. These highly concentrated bacterial solutions were used (separately and in a mix of all nine strains) as test solutions for the sensor. No results could be obtained with these mixtures, mainly because the release of enzymes was to low in this full medium.
In the next steps, microorganisms were isolated directly from fresh meat and were used for infecting other fresh meat to generate homogenised meat (Homo) with a reproducible ratio of meat spoilage microorganisms (Microbiological protocols see chapter "III. Methods").

This stage of microbiological correlation is sufficient for detection of the general functionality of the sensor. For simulation of the really occurring processes on the meat surface, this is far away from an optimum. Further highly successful

investigations have been done by Mirza and Nadira Ibrišimović [53, 61] in the research group of Prof. Fritz Pittner.

V. Conclusion and future aspects

Within my diploma thesis I got the possibility to develop a biosensor prototype for the detection of food decay by monitoring the degradation of a biomimetic polymer layer. The aim of my work was the optimisation of different parameters e.g. the type of polymer, the polymer concentration, the concentration of the linker, the time of sputtering and the individual steps of the producing protocols. In parallel, improvements in the reproducible standard testing of this sensor system via a homogenized standard putrid-meat juice (Homo) have been found out.
I could show a completely new setup of this type of sensor by reducing the 3-layer-setup to a 2-layer-setup [61]. Besides the fact that the intensity of the colour of the sensor is weaker than in the 3-layer-setup, it has many benefits like a faster, easier and cheaper production.

Further steps in the development must go hand in hand with the demands of the users. Especially the design can be modified in different ways (e.g. combining the sensor with a tampon to create a “flow-through-sensor”). The optimal setup has not been found so far and the ways of variation are multiple.
The field of sensor development is still a strongly interdisciplinary one – needing also knowledge of packaging industry and process engineering. It has to be mentioned that there are numerous other methods to improve this type of sensor besides the common experimental and analytical methods. Measurements of the absorption spectra could build a source for multivariant data analysis or other mathematical techniques, which would lead to a better understanding of the correlation between the degradation process of the polymer and the originated colour.

Besides the technical aspects of such a research and development topic, also chemical investigations have to be done. Improvements could be created by using various types of linkers or even combinations of them. For instance, triisocyanate linkers - as they are presented shortly in this thesis - could be an advantage because of their three reactive groups, allowing spatial cross linking. This leads to higher stability of the polymer network even at lower amounts of PLA, which would be adequate in order to reduce the costs of production.

Another suggestion might be the establishment of a sensor-setup following to an “all-or-nothing’” principle. This could either be produced with help of a coloured substrate film or by the use of a coloured polymer [62].

A variation in the amount of lactic acid and glycolic acid could possibly lead to different results of stability or degradation. This field is not exhausted completely and offers space for following researchers.

Other chemicals could be mixed with the polymer to guarantee a faster and more selective release of lytic enzymes. One trigger, which could induce the lytic enzymes would be phosphatidylcholin or phosphatidylserine [63], which was too expensive for us to use it in our first experiments. But also other nutrient sources like carbohydrates, peptides or other lipids might lead to a tuning of the colour shift.

For an increase of the stability of the setup, it could be wise to change the setup and rebuild it in a way, in which the metallic PET substrate is not used with the metallic mirror upwards. This inverted Inconnel film would lead to an additional layer (the PET itself) with its appropriate optical density, which could vary the colour effect of the sensor. But one has to bear in mind, that the adhesive properties are different when applied directly onto the PET layer without the metallic mirror in between. One should not forget that also other coloured metal mirrors could act as a substrate for the sensor, especially if an “all-or-nothing” setup is favoured.

It might be an improvement to engineer a mircofluidic setup on the surface to assure a continuous dispersion over the sensor surface. With such a setup it would be possible to measure the degradation of meat even if the sensor is not in contact with the meat itself.

From the microbiological point of view, a faster test tool for creating a standard meat juice would be in advance. For the development of such a homogenized standard putrid-meat juice (Homo) it would be helpful to save the time consuming determination of the microorganism but use instead more sophisticated techniques like the IR-microscopy to determine the ratio of the different decay producing

microorganisms [64, 65, 66, 67]. By testing the directly released amounts of enzymes, one would get higher reproducibility of the tested results.

Further investigations in this field of biosensors have to be carried out preferably with a focus of broadening the areas of applications. There are lots of other applications for this sensor, not only in other branches of the food sector (other types of meat e.g. fowls, beef and fish), but also in pharmaceutical and cosmetic industry (in the cartridge of sterile medical devices and even disposables). It has to be taken into consideration that many biological natural cosmetics lack commonly used preserving agents, so the risk of an increase of diseases connected to spoiled cosmetics is obvious [68].

The interdisciplinary field of biosensor research has a great future and the steady advancement of this research area will hopefully last for a long time.

Abstract

Standard methods for the detection and identification of microbiological spoilage of food are time-consuming as they use conventional culturing techniques or monitor environmental conditions like the increase of temperature or pH. These parameters do not reflect the real quality of the meat to be tested.

Aim of this study was to make contributions to the development of a novel nanotechnological sensor chip for the detection of meat deterioration, in special consideration of optimising the sensitivity with regard to chemical modification of the sensitive polymer and the sensor layout. This biomimetic sensor utilises the effect of anormolous absorption and is able to show the consumer in the grocery store the rate of deterioration in real time. Numerous developmental approaches for several parameters, e.g. polymer concentration, linker concentration and thickness of the metallic layer, were examined.

Even the sensor setup itself was modified within these studies. The result of this development was a novel 2-layer-biosensor with no need of a third metallic layer, which indicates the point of decay with a clearly visible colour shift. This sensor is superior to the original 3-layer-setup with regard of production process, costs as well as health aspects.

Zusammenfassung

Herkömmliche Nachweisverfahren für die Bestimmung und die Identifikation von mikrobiologischem Lebensmittelverderb sind zeitaufwändig, da sie auf üblichen Kultivierungstechniken beruhen oder die Veränderung von Umwelteinflüsse wie etwa die Temperatur oder den pH-Wert detektieren. Diese Parameter spiegeln jedoch nicht die reale Qualität des getesteten Fleisches wieder.

Ziele dieser Arbeit waren Beiträge zur Entwicklung eines neuartigen nanotechnologischen Sensorchips zur Detektion von Fleischverderb unter besonderer Berücksichtigung von Sensitivitätsoptimierungen im Hinblick auf chemische Modifizierung der sensitiven Polymerschicht und Sensor Layout. Dieser biomimetrische Sensor nützt die Effekte der anormalen Absorption aus und kann den/die Konsumenten/Konsumentinnen im Supermarkt in Echtzeit den Frischegrad anzeigen. Weitreichende Verbesserungsansätze wurden auf der Ebene mehrerer Parameter, u.a. der Polymerkonzentration, der Vernetzerkonzentration und der Dicke der metallischen Beschichtung, dargebracht. Auch das Setup an sich wurde bei diesen Untersuchungen modifiziert.

Ein Ergebnis dieser Entwicklung war ein neuartiger 2-Schicht-Biosensor, der keiner dritten metallischen Schicht bedarf und dennoch mit einem eindeutigen Farbumschlag den Zeitpunkt des Verderbs anzeigt. Dieser Sensor ist somit im Hinblick auf den Herstellungsprozess, die Fertigungskosten sowie gesundheitliche Aspekte dem ursprünglichen 3-Schicht-Setup klar überlegen.

Appendix

List of abbreviations

Abs	Absorption
AGES	Agency of Health and Nutrition Safety
Ar	Argon gas
Au	Gold
°C	degree Celsius
CFU	colony forming units
cm	centimeter
d	days
Des	Desmodur®
EtOAc	Ethyl acetate
EU	European Union
FDA	Food and Drug Administration
FET	field effect transistor
g	gram
GOX	glucose oxidase
h	hour
Homo	homogenised meat
HC	humidity chamber
ISE	ion selective electrode
kV	kilo volt
L	liter
LB	lysogeny broth
ISE	Ion-selective-electrode
IUPAC	International Union of Pure and Applied Chemistry
M	molar
mbar	millibar
MEK	Methylethylketone, 2-Butanone
MICSPOMS	Metal Island Coated Surface Plasmon Over Metal Surface
min	minute
Mio	million
µg	microgram

mL	milliliter
µL	micro liter
ms	milliseconds
nm	nanometer
o/n	over night
QCM	quartz crystal microbalance
PCA	plate count agar
PEG	Polyethylene glycol
PET	Polyethylene terephthalate
PLA	Polylactic acid
PLGA	poly(lactic-co-glycolic acid)
Prot-Mix	Protease-Mix
R	doctor blade
RT	room temperature
SAW	surface acoustic wave
sec	second
SPR	surface plasmon resonance
SSC	saline sodium citrate
t	time
TFE	2,2,2-trifluoroethanol
tFEtOH	2,2,2-trifluoroethanol
Tris	Tris(hydroxymethyl)-aminomethan
TTI	time-temperature integrators
ÜN	over night
W	printing roller

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Publication

Biomimetic sensor monitoring real-time food degradation: correlating chemical deterioration with microbiological status.

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Abstract

Conventional tests, currently in use for the detection and identification of food borne pathogens and also of microbial food deterioration are time consuming as they are based on conventional culturing techniques or monitoring environmental conditions like increase of the temperature or change in pH. These parameters do not reflect the real quality of the meat to be tested.

The aim of our novel approach was to create a simple and cheap sensor providing reasonable sensitivity and selectivity to indicate the “best use before” period combined with a memory effect that cannot easily be corrupted.

We have developed an optical thin film sensor chip able to detect the decay of food through a specific color change. The design of the sensor relates to the phenomenon of “anomalous absorption”, which can best be described as a thin film enhanced absorption. A metal cluster film positioned at a well defined distance to a smooth metal surface shows that the minimum of spectral reflectivity strongly depends on the thickness of the interlayer: This setup represents a special kind of reflection interference filter. In such a sensor setup we have integrated a biocompatible polymer degradable by the same enzymes as are excreted by microorganisms in food decay. Standard deterioration under controlled conditions is correlated to the amount of enzymes secreted by microorganisms and the bacterial number. Thus after incubation of the sensor setup with standard meat preparations the enzymes

released from decaying cell material change the thickness of the polymer layer and generate an easily visible colour change. This setup would be useful for integration into meat packaging.

Introduction:

Conventional methods for the detection and identification of food degradation show high accuracy and low detection limits, but are generally expensive, time-consuming, require the use of highly trained personnel and laboratory equipment. 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 with 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 [1].
Development and improvement of food processes is driven by the need for healthier, safer, more convenient, competitively superior and seasonally invariant foods and also more efficient processing plants with reduced waste. Online optical instruments such as refractometers, spectrophotometers, turbidity meters etc. that may be used to assess food composition have been developed. However, their applicability is often limited because of the complex composition of food interfering with the measurement and much stress has to be laid on correct sample preparation.

Available quick tests for food decay using parameters such as time, temperature or pH change are not really correlating to bacterial contamination. Monitoring of pH gives only indirect information and most tests are reversible, and therefore generally unapt to contribute to the safety of the meat supply chain. Microbiological tests concerning bacterial counts or stem are very time consuming as they rely on conventional culturing techniques and thus are far from real time monitoring. Especially when a consumer wants to get information on the quality of what he is buying in the shop a quick real time test integrated into the package would be of high interest, as well as also the producer can show the hygienic level of his production line by such a device.
In the last decades, the combination of knowledge in electrochemistry, biochemistry, physics and integrated circuit silicon technology made it possible to provide highly specific, sensitive, selective, accurate and reliable microsensors [2 – 6].

Results and Discussion:

For monitoring meat freshness during the interval between packaging and selling other methods are needed. The aim of our novel approach was to create a sensor that provides reasonable sensitivity and selectivity to indicate the “best use before” period combined with a memory effect that cannot easily be corrupted. The here presented freshness sensor for food is a biomimetic sensor, using the enzymatic decomposition of a biodegradable polymer in a sandwich setup between a mirror and a gold nanoparticle layer. It is irreversible and therefore suitable for single use only. It is not activated until it gets into contact with the meat.

Meat decay is caused mainly by microorganisms excreting lytic enzymes. Both, rate of bacterial growth and enzyme action are a function of time and temperature. Increasing temperature raises the amount of microorganisms and also the activity of the secreted enzymes, thus enhancing enzymatic deterioration of meat tremendously. (Fig. 1)

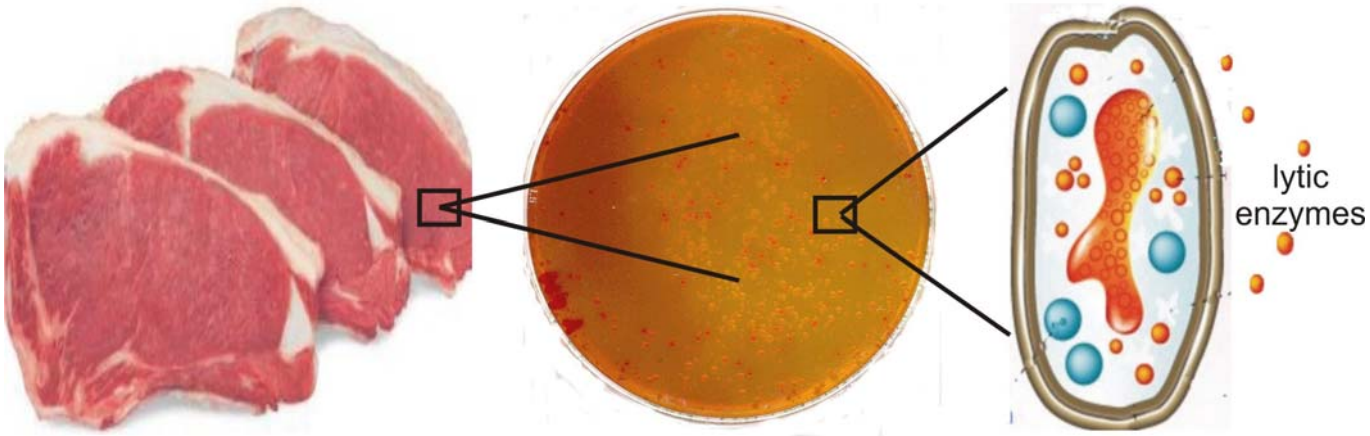

Figure 1: Bacterial growth on meat surface causes excretion of lytic enzymes

These cumulative actions are summed up by the gradual change of the polymer matrix, made visible by the sensor setup as shown in Figure 2

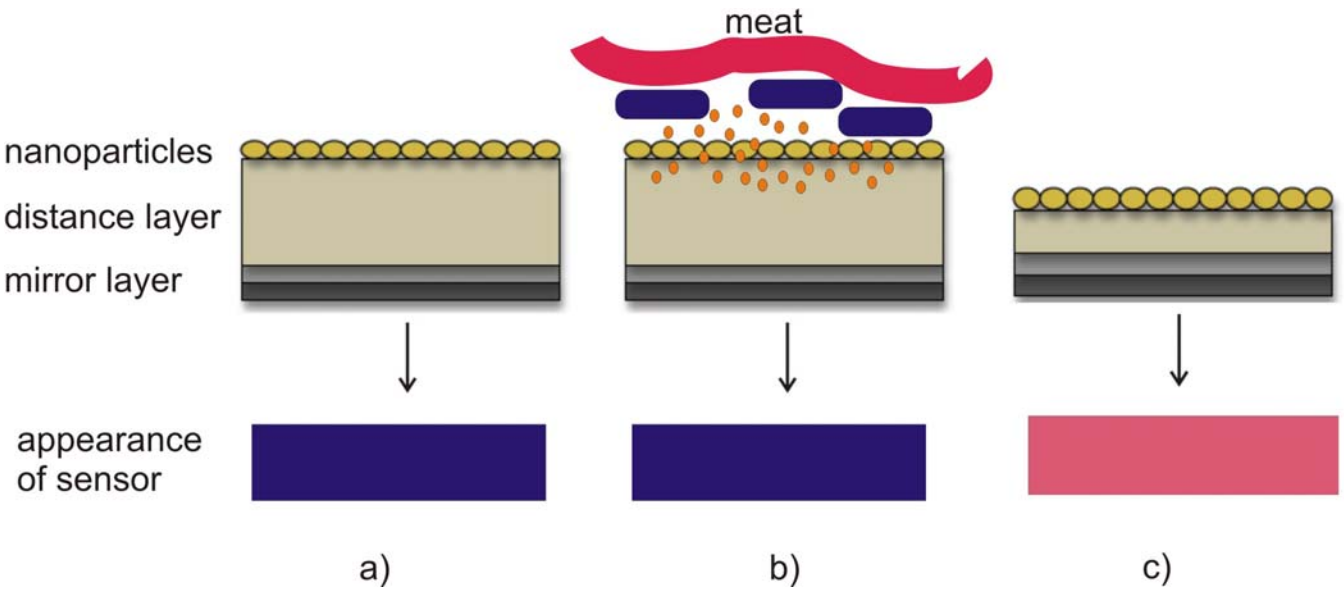

Figure 2: Setup and function of thin film sensor: a) sensor setup b) sensor in contact with meat and bacteria c) sensor after degradation of the biomimetic polymer. The colours shown in the lower level are only examples and are dependent on the conditions of production.

Due to the special optical behaviour of a metal island film and due to the thin film set up, this system shows characteristic spectral reflection behaviour, strongly dependent on the thickness of the transparent interlayer [7], in our case formed by the biomimetic polymer. The optical property of metal island films, necessary for our application, is the so called “Plasmon absorption”, a strong, broad-band absorption in the visible, due to the confinement of the conduction electron plasma in nanometric particles. This is in contrast to the unconfined electron movement in an extended metal, responsible for strong, unspecific reflectivity, well known as metallic glance. An absorbing thin film positioned at a defined distance to a metal mirror represents a special kind of reflection interference filter. At an appropriate distance of the absorbing layer to the mirror, fields reflected by the mirror have the same phase at the position of the absorbing layer as the incident fields and, thus, by this feedback mechanism the effective absorption coefficient of the absorbing layer is strongly enhanced. This combination of the two phenomena plasmon absorption and optical interference is generally called “anomalous absorption”.

Fig. 3 shows the visual impression obtained by observation of the reflected light upon diffuse white-light illumination of the layer system used in our sensor set up in the interlayer optical thickness range 0 – 490 nm (optical thickness = geometrical

thickness x effective refractive index) and some corresponding, measured reflection spectra.

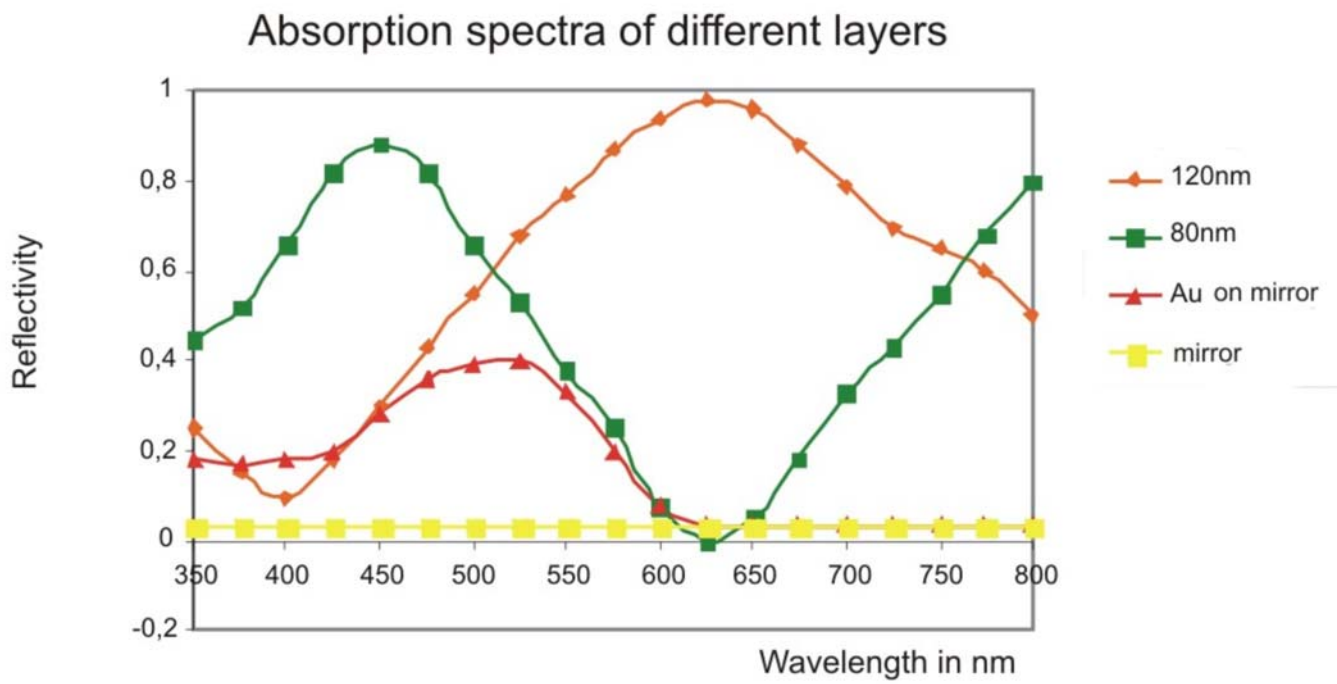

Fig 3: Colour of the sensor as a function of thickness of the distance layer

The thin film sensor presented here has to consist of a sensor layer within a nanometric range as can be deduced also from Figure 3 and thus shows very fast response. As the metal island film is highly permeable for the analyte the sensor layer is directly exposed to the analyte. The cleavage of links in the polymer chains causes instability to the degree that the sensor layer is destroyed upon immersion in aqueous solutions. For a tuning of interlayer thickness there is one important consideration: The visual perception of the sensor surface interference colour is strongly dependent on the distance layer thickness. Thus the visually observed colour can be used to determine the actual thickness of the polymer layer. Comparable to the decomposition of meat, one component of the sensor setup – a biodegradable polymer – can be cleaved by lytic enzymes, thus resulting in a distinct colour shift that is visible to the naked eye. Fig. 2 shows the basic function of the freshness sensor for food, which has to be in permanent physical contact with meat or meat juice so that the lytic enzymes are able to diffuse through the top layer of the sensor thus reaching the biodegradable distance layer.

The sensor monitoring meat decay is an optical thin film sensor with an integrated biomimetic polymer. It is able to detect the proper meat deterioration by responding to lytic enzymes, which on one hand can be produced by microorganisms and on the other hand emerge from the cell lysis that goes hand in hand with meat spoilage. Comparable to the decomposition of meat the biodegradable polymer is cleaved by lytic enzymes, thus resulting in a distinct colour shift that is visible to the naked eye. The colour changes shown in Fig. 4 are a result of enzymatic degradation of the sensor. This degradation is highly selective and very sensitive; negative controls showed no degradation at all. As can be seen with the naked eye, spoiled meat juice and dilutions shows the strongest signal at 4°C.

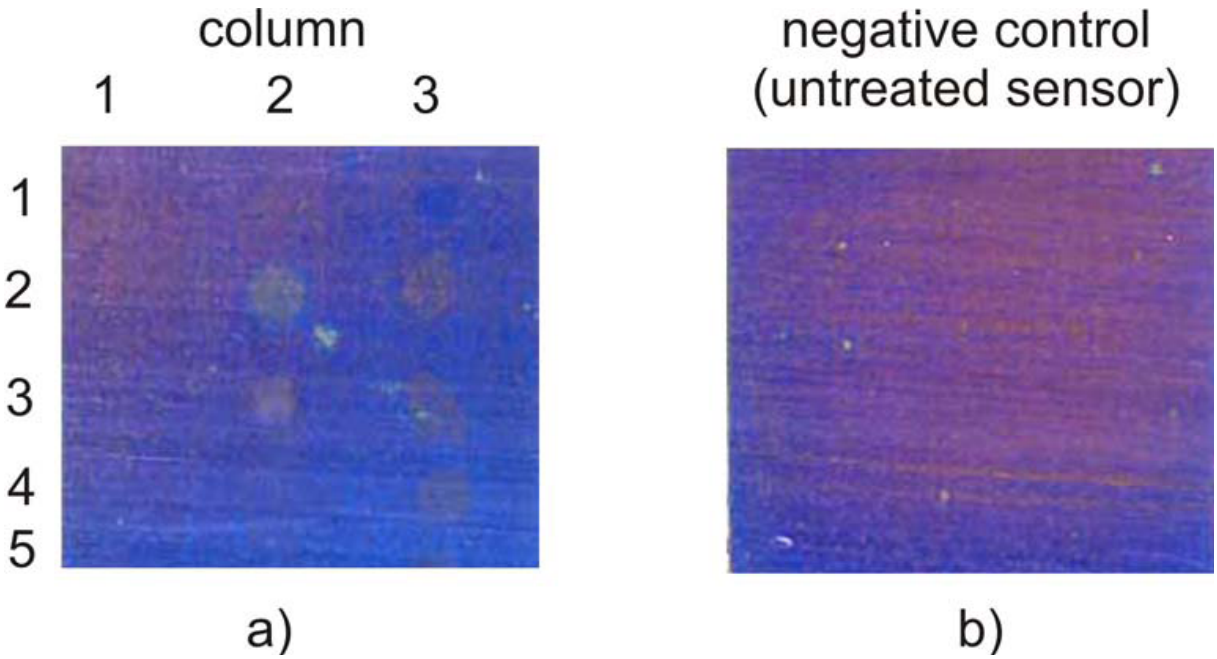

Figure 4: Sensor response after overnight incubation a) at 4°C b) at room temperature. The spots were applied according to the pipetting scheme as given in Table 2

Another big advantage of this new sensor system is the cost efficiency for its production. Every step of the sensor production has been carried out on industrial testing equipment and with coating procedures like physical vapour deposition and gravure printing. These production methods guarantee very good economies of scale and thus low cost per unit. [8]

Materials and Methods:

The sensor setup is performed according to [6].

Coating:

Gravure Printing, also known as Intaglio printing, is characteristically used for long run, high quality printing producing a sharp, fine image. (Figure 5) This is accomplished by cutting or engraving and etching various sizes or depths of minute cells (or wells) below the surface of a plate or cylinder. The depth and size of each cell determines the amount of liquid, in our case the biomimetic polymer solution that can be transferred to the print surface. The cells are flooded and loaded with this solution. The excess is scraped off the surface of the plate by a doctor blade, and what is left in the cells is transferred to the substrate. The nature of the process permits the formation of a layer with the desired thickness. A semi automatic gravure printer for laboratory use from Erichsen (Germany) was used. By this procedure the biopolymer of the distance layer was printed onto the mirror coated substrate.

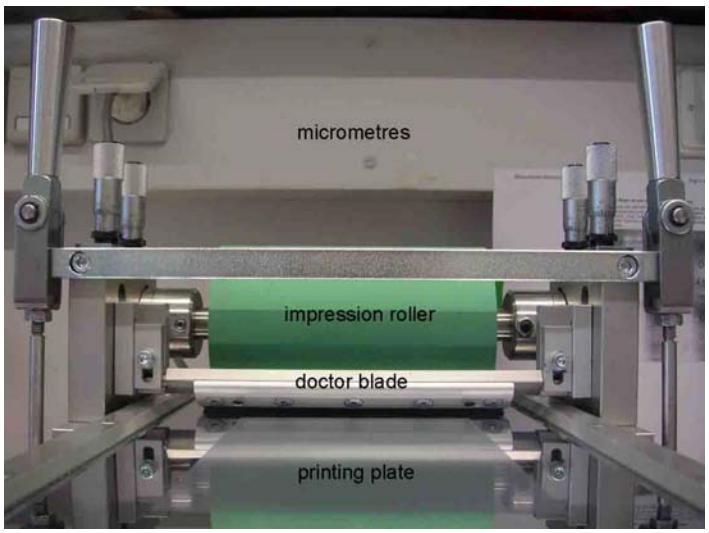

Figure 5: Gravure Printer (K Printing Proofer 628 by Erichsen)

Sputter Coating: Nanoparticles were applied by an Agar sputter coater as used for electron microscopy. In conventional SEM sputter coating techniques gold is bombarded with heavy gas atoms (Ar). Metal atoms ejected from the Au source by the ionized gas cross the plasma to deposit onto the sensor surface. Thus the

nanoparticle layer responsible for the anomalous absorption was generated. This allowed also for visualizing the homogeneity of the printed layers, as changes in thickness of the polymer layer by imperfect printing procedures result in inhomogeneous colours of the sensor.
The whole sensor setup is shown in Figure 2.

Sampling *procedure, strains isolation and growth conditions*
Gram-negative aerobic bacterial strains (Pseudomonas, Enterobacter, Proteus, Salmonella) were isolated from spoiled meat juice (incubated for 5 hours at 37°C) using Salmonella Agar acc. to ÖNÖZ (Merck).

Table 1: Characterisation of microorganism colonies

Microorganisms	**Appearance of colonies**
Pseudomonas	Glossy, dirty yellow to greenish; culture medium is yellow
Enterobacter	Large, mucoid, blueish or reddish, slight precipitation ring around the colonies
Proteus	Rust-coloured, culture medium surrounding the colonies of same colour, if growth is too dense, dark brown to black
Salmonella	Yellow, medium size; 1st day: black dots start to develop on the yellow colonies; 2nd day:clear black dot visible on the yellow colonies; culture medium surrounding the colonies is yellowish

A colony picked from the selective media was inoculated in 3 cm^3 pepton water (peptone 0.1%, NaCl 0.5%, $Na_2HPO_4.12H_2O$ 0.9% and KH_2PO_4 0.15% all in dd water) and allowed to grow overnight at 37°C. Then 2 cm^3 of the bacterial pre-culture was inoculated in 200 cm^3 pepton 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 onto Plate Count Agar (*Fluka*). The culture dilutions (10^{-1} to 10^{-9}) were prepared in Ringer Solution.

Preparation of homogenised infected meat:
Pork cutlets (100 g) were infected with nine different bacterial strains that can be found in spoiled meat (*Serratia liquefaciens, Lactobacillus sakei, Lactobacillus curvatus, Leuconostoc mesenteroides, Brochothrix thermosphacta, Acinetobacter lwoffii, Pseudomonas lundensis, Pseudomonas fragi, Pseudomonas fluorescens* from

DSMZ, the German Resource Center for Biological Materials), and incubated for 40 h at 25°C (homogenate I) or 11 days at 4°C (homogenate II). After these periods of incubation, the meat was homogenised in 200 cm^3 Ringer Solution using a homogenizer-mixer. With miracloth filtered bacterial cocktail was frozen by liquid nitrogen and stored at -80°C. Bacterial counts were performed, as described above, using the Plate Count Agar (*Fluka*).
For a functional assay different solutions were prepared as described in the scheme:

Table 2: Pipetting scheme:

Column 1	**Column 2**	**Column 3**
Bacteria-mix* (10^8 CFU/mL)	Homogenate II (10^8 CFU/mL)	Enzyme-mix** (20 mg/mL)
Bacteria-mix* (10^6 CFU/mL)	Homogenate II (10^6 CFU/mL)	Enzyme-mix** (15 mg/mL)
Homogenate I (10^8 CFU/mL)	Pseudomonas (10^7 CFU/mL)	Enzyme-mix** (10 mg/mL)
Homogenate I (10^6 CFU/mL)	Pseudomonas (10^6 CFU/mL)	Enzyme-mix** (5 mg/mL)
		Ringer Solution (neg. control)

* Equal amount of *Pseudomonas*, *Enterobacter*, *Proteus* and *Salmonella.*
**Equal amount of Proteinase K, Chymotrypsine and Trypsine.

The respective solutions (1 mm^3) were pipetted onto the sensor surface and then the sensors incubated in a humidity chamber (over night at 4°C and in parallel also at room temperature). After the incubation time the sensor surface was washed with ddH_2O and dried by an airstream.
Documentation of the results was done by scanning the sensors on a flat bed scanner (HP Scanjet 4890).

Conclusions:

In this work we could present a biomimetic sensor type for monitoring cumulative impacts caused by temperature, bacterial growth and lytic enzyme action. The deterioration of meat can be followed via colour change visible for the naked eye in real time.
This sensor prototype may be adapted for different potential commercial applications as eg facilitated quality control in store and also for shelf life, giving the consumer insight on the actual freshness of the respective product and also for the provider the

guarantee to having sold intact food and for the producer a proof for optimum hygienic conditions during packaging.

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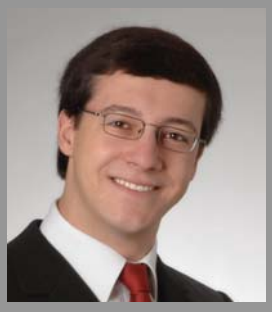

Education

Date	Location	Content
Apr 2008	University of Vienna	First diploma certificate Chemistry
Jan 2007	University of Vienna	Diploma in Technology law
Since Oct 2005	University of Vienna	Chemistry (diploma studies)
June 2005	University of Vienna	First diploma certificate Law
since Oct 2004	University of Vienna	Law (diploma studies)
1996 - 2004	BRG Wr. Neustadt	Matura (June 2004)

Civil service

2004 – 2005	Caritas	Social station / Mobile Services

Employment

Mar-Jun 2009	University of Vienna	Study assistant for Biochemistry
Sept 2008 – Jun 2009	University of Vienna	Diploma thesis at the MFPL Supervisor: Prof. Fritz Pittner
Oct 2008	Science-Communications	Coworker at the "1.Wiener Forschungsfest"
Aug 2008	Puchberger & Partner	Assistant (Patent attorney)
Mar -Jun 2008	University of Vienna	Study assistant for Organic Chemistry
Aug 2007	Austrian Patent Office	
Jul 2006	University of Vienna	Scientific Coworker at the Institute for Material chemistry

Scholarships and grants

2009	e-fellows-Stipendium	private support network
2008 - 2009	Students4excellence	private support network
2008	TOP-Stipendium	Lower Austrian Government
2008 – 2010	ProScientia	Austrian Bishops Conference
2007	Leistungsstipendium	University Foundation

Publication

Ibrišimović, N., Barth, M.*, Bohrn, U.*, Ibrišimović, M.*, Pittner, F., Special Edition of Chemical Monthly, 2009, ***Biomimetic sensor monitoring real-time food degradation: correlating chemical deterioration with microbiological status***

* contributed equally; invited paper;

Extracurricular activities

2007 – 2009	ÖH Uni Wien	Chairman of the students faculty representation
2006 – 2009	AG-NaWi	Austrian Students Representatives Coworker at the student paper „AGent NaWi"
1999 – 2004	High school	Chemistry Olympiad 2 times bronze medal winner