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Meat and fish freshness evaluation by functionalized cantilever-based biosensors

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Abstract

Meat spoilage is a result of an increase in the number of microorganisms and increased levels of biogenic amines (e.g. cadaverine). Destruction of non-spoiled meat products results in substantial waste of resources each year. Currently, the expiration date of meat-products is determined by either subjective sensory and/or expensive and time-consuming microbiological analysis. Cadaverine levels have been demonstrated to be related to the product freshness; however current sensing methods require bulky and expensive chromatography techniques. In this work, the cadaverine binding to a functionalization layer of cyclam (1,4,8,11 – tetraazacyclotetradecane) is demonstrated by a systematic study of cyclam/solvent solution influence on morphology and binding. The degradation of the functionalization layer due to storage conditions has also been investigated, and the optimum solvent for the functionalization solution is found to be ethoxyethanol. Functionalized surfaces and cantilevers have been exposed to different types of meat (beef, fish, chicken or pork) and the cadaverine binding has been demonstrated, either by morphology changes (surfaces) or by changes on cantilever resonance frequency due to mass increase (cantilever). The results show a higher cadaverine emission rate for fish, followed by chicken, beef and finally pork.

Introduction

Food waste has significant environmental and economic impact. In terms of greenhouse gas emissions, the overall impact of food waste corresponds to 20-30% of total EU emissions [1] and the carbon footprint can be reduced by approximately 1.7 kg carbon emissions for each kg of food which is not wasted [2]. Through poor shelf-life estimation, the meat and fish industries unfortunately contribute in a major way to waste in retail and private households. The waste is in specific terms related to extreme but understandable caution in the industries re mitigating the potential risk it could cause to the consumers in case of product spoilage: a simple consequence of a reliance on existing imprecise designation methods and models.

Thus, there is a clear need to implement a technology/methodology which is better at estimating the potential for spoilage such that the level of caution can be reduced and the dates set more accurately, extending shelf life use and sell-by estimates, allowing longer times for sale and thus ensuring a sharp reduction in products being reduced in price and/or withdrawn from the shelves and designated as unfit for consumption long before necessary in the market place.

Microbial spoilage of the food is a result of unconstrained bacterial growth once an animal's/fish's immune and circulatory system is terminated at the time of slaughter/catch. Direct detection of bacteria is the industry standard method for determining product use-by dates [3], however it is expensive, time consuming and ultimately imprecise. The sell-by date or best before date (coming before the use-by date) is usually estimated by inaccurate non-objective sensory analysis methods and/or based on a set number of days before the defined use-by date. Both these dates (use-by and sell-by) are ultra-conservative and have a

margin of error included to ensure product safety as the bench mark. In terms of alternative methods with clear future potential, decarboxylation of amino acids by enzymes originating from microorganisms in and on meat might be successfully exploited as pronounced spoilage markers, since the concentration of these correlate very well with the relative numbers of spoilage bacterial [4-7].

We see e.g. cadaverine, as a core candidate for predictive assessment as a clear biogenic amine with potential for precise quantitation as produced during putrefaction of animal tissues. The level of biogenic amines indicates meat/fish freshness for both raw and lightly processed products [8]. Its level increases significantly over meat aging both for red and white meat and it has already been used for determining a quality index of freshness in meat, by measurements of cadaverine levels in meat at different ages, through invasive techniques [9-10]. Existing methods for detecting cadaverine are usually expensive and slow. Every method employs two different procedures, including the extraction of the amines and their quantitative determination. The extraction process is critical and can negatively influence the analytical results [9]. A common method is to use Intramolecular Excimer Fluorescence [11], which is an invasive method not convenient or applicable for a wide use. Other methods applied for biogenic amines determination are thin layer, gas and high-performance liquid chromatography [12], which give a good precision, but are very large and time consuming. We have performed investigations on nano-functionalization chemistry for sensory surfaces which are high selectively binding to cadaverine [13-14]. This functionalization layer is applied into micro-cantilever sensors.

Methodology

Silicon substrates were coated with a titanium adhesion layer (3nm), followed by gold deposition (50 nm) by electron beam physical vapor deposition. Cyclam (1,4,8,11 – tetraazacyclotetradecane) solutions were prepared using either chlorobenzene, 2-ethoxyethanol or chloroform with a concentration of 0.03 mg/ml. The solutions were drop-casted on the gold-coated silicon substrates without any post-annealing treatment. Samples containing cyclam layers from different solvent solutions were exposed to different types of meat samples (beef, fish, chicken or pork). Cadaverine gas emission from meat causes cadaverine molecules to bind to cyclam. The morphology of the functionalized samples before and after cadaverine exposure was analyzed on a Hitachi S-4800 Scanning Electron Microscope (SEM) and by bright-field microscopy. Cadaverine binding was quantified via SEM image processing techniques by thresholding a grayscale image into a binary analysis algorithm, and the error of using of this method has been measured to be 0.193 ± 0.229 %. Cyclam layers degradation mechanisms were studied by keeping substrates functionalized with the different solvent solutions in normal conditions of light, air and temperature. The morphology of the functionalized samples after 3 days was examined as in the previous step.

Silicon nitride cantilevers containing piezoelectric layers - [Si-Oxide(1 μ m)/Poly-Si(14 μ m)/Si-Oxide(1 μ m); AlN(50 nm)/Mo(100 nm); AlN(2 μ m)/Mo(150 nm); Si-Nitride (1 μ m);] (width = 1000 μ m, length = 1500 μ m) were functionalized by drop-casting, based on the previous functionalization results. The resonance frequencies of the commercial cantilevers before and after cadaverine exposure were measured by optical beam deflection, by using an optical sensing system where a laser beam is reflected by the cantilever and detected by a photodiode, as described in [13]. The increase in mass on the cantilever due to cadaverine binding was calculated based on the changes of resonance frequency and on the cantilever parameters.

Results and discussion

The key factor for sensing of cadaverine molecules is to find the correct required functionalization surface morphology for target binding. We have previously demonstrated the binding of cadaverine to cyclam layers [10], however surface morphology optimization has the potential to result in higher resolution and should

be further investigated. Functionalization solutions of cyclam on different solvents result in significant morphological changes and are presented here. Figure 1 shows the surface morphology of functionalization layers produced by drop-casting of solutions based on chloroform, chlorobenzene and ethoxyethanol, before and after cadaverine exposure (5 minutes exposure to pork meat – 48 hours out of fridge). As it can be seen in the figures, the morphology is changing, as well as the cadaverine binding. Recrystallized cyclam obtained from a chloroform solution seems to result in the smallest structure type, while chlorobenzene result in the largest crystal dimensions (the average dimensions are found in table 1).

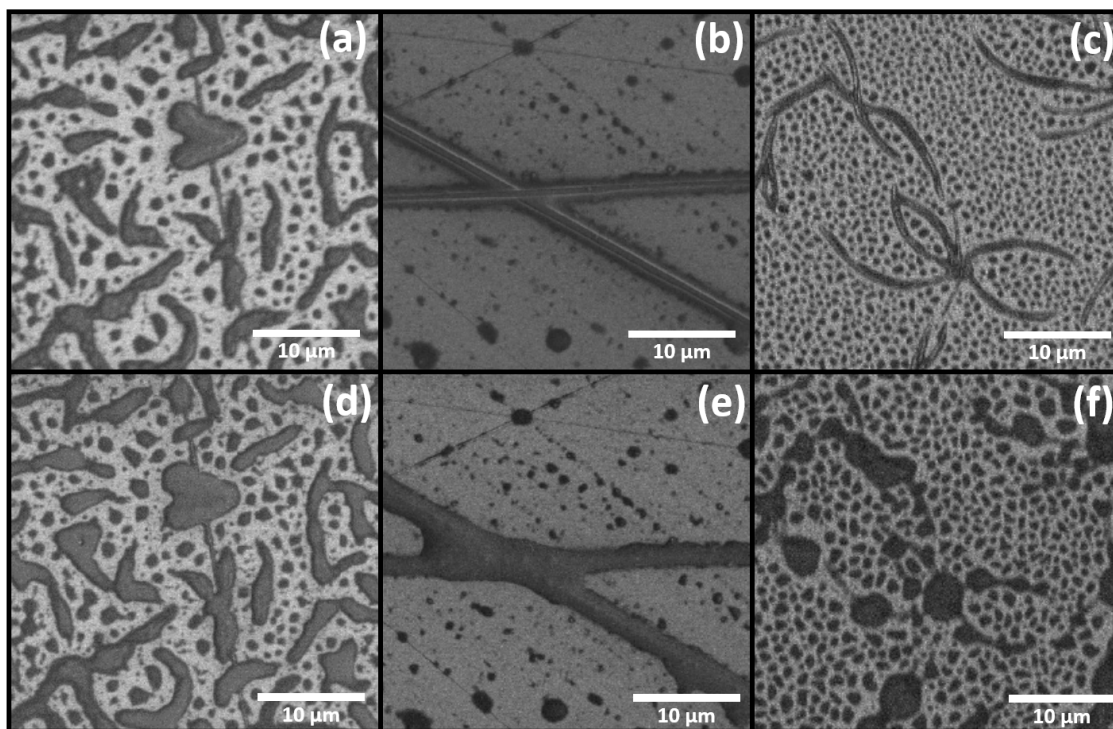


Figure 1: SEM images of cyclam functionalization layers before cadaverine exposure based on (a) chloroform, (b) chlorobenzene and (c) ethoxyethanol. Images after exposure can be respectively found in (d-f).

Regarding cadaverine binding, images of the crystals before and after exposure clearly show cyclam-cadaverine binding for the crystals obtained with all different solvents. Quantitative results based on image processing analysis can be found in table 1 and indicate a better binding performance for the crystals based on ethoxyethanol solvent solutions.

Solvent	Chloroform	Chlorobenzene	Ethoxyethanol
Cyclam crystal size (μm)	6.08 ± 3.64	357.09 ± 85.58	17.29 ± 5.55
Measured difference after cadaverine exposure (%)	14.72 ± 1.84	1.05 ± 7.42	21.96 ± 0.72

Table 1: Morphology and cadaverine binding parameters for different solvents, based on image analysis

The reliability of a cantilever sensor based on the presented functionalization layer depends on the stability of the cyclam layer. Therefore, degradation studies were performed, where the samples were kept at controlled conditions. Figure 2 shows how the morphology of cyclam layers change after 3 days under ambient conditions. A slight change on morphology has been observed, change that need to be considered in practice for sensor reproducibility. The degradation process has been quantified by using image processing analysis and a degradation of 2.9 % has been observed for crystals from ethoxyethanol as a solvent, 8.17 %

for chloroform and 10.56 % for chlorobenzene, indicating ethoxyethanol to be the most stable solvent for cyclam crystal formation.

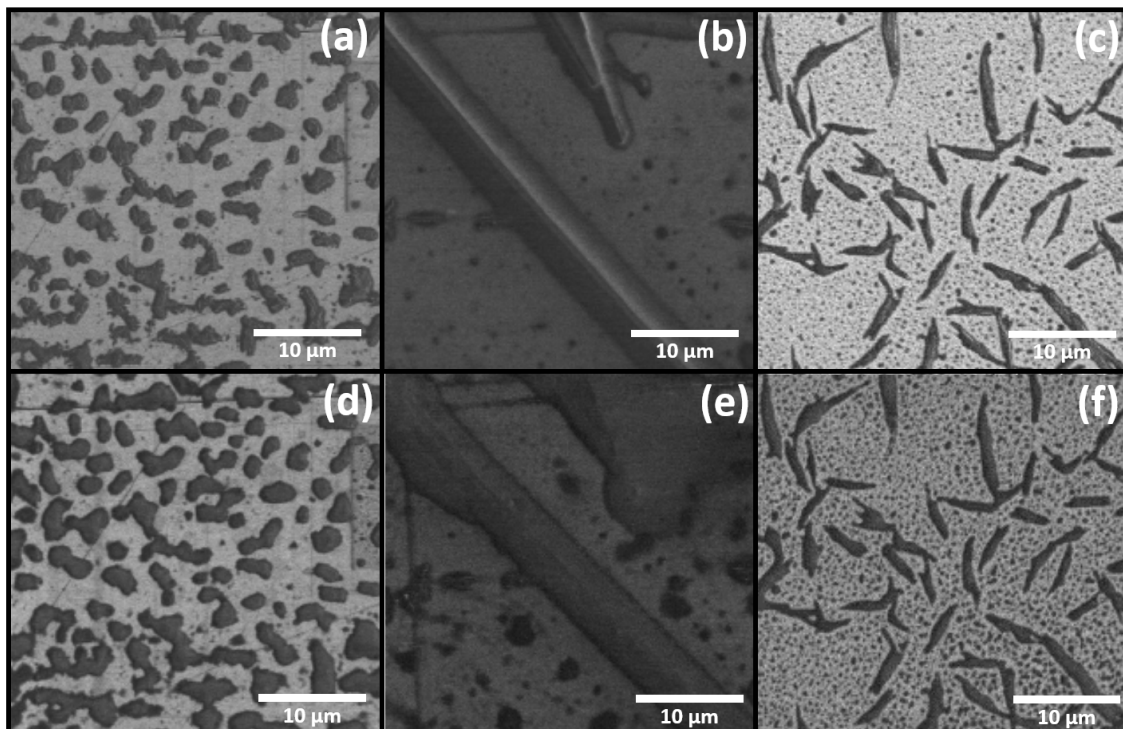


Figure 2: SEM images of cyclam functionalization layers based on (a) chloroform, (b) chlorobenzene and (c) ethoxyethanol just after drop-casting, and after 3 days under ambient condition (d), (e) and (f).

Cadaverine binding rate for different types of meat has been investigated as well. Figure 3 shows the cadaverine binding rate as a function of days for different types of meat. The results were obtained by image analysis of morphological changes after exposure. The image shows that fish has the fastest cadaverine production, while pork has the slowest. Since cadaverine is a biogenic amine resulting of protein oxidization and its concentration increases proportionally to the days past since slaughter, samples with higher protein content produce cadaverine at higher rates than samples with high fat content. The data obtained in this study show exactly this trend, since fish has a higher amount of protein than pork, for example.

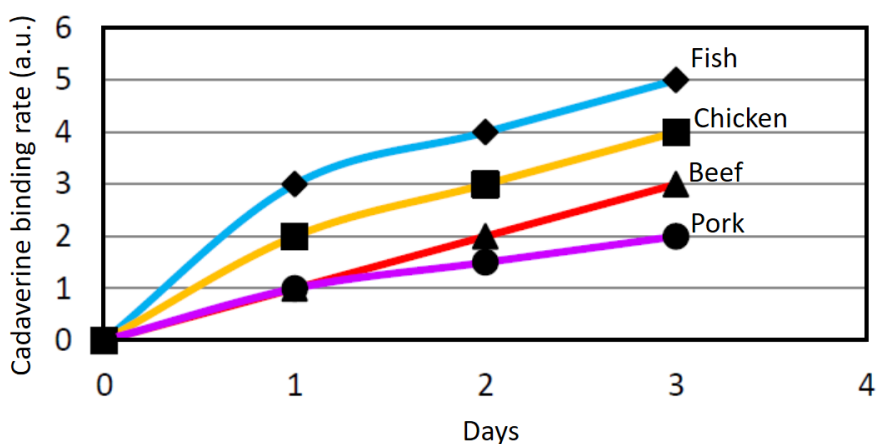


Figure 3: Cadaverine binding rate on chloroform based cyclam solutions for different types of meat.

To demonstrate the cadaverine binding on cantilever-based sensors, a cantilever containing a piezoelectric layer was functionalized with the chloroform based cyclam solution. The cantilever was exposed to chicken (stored at 5°C after each measurement) for 5 consecutive days, and the changes on cantilever resonance frequency due to mass increase (cadaverine binding) was measured daily, by optical beam reflection. The mass of bound cadaverine can be calculated by the following equation [13]:

$$\Delta m = 4\pi\Delta f \sqrt{\frac{m^3}{k}} = 8 \times 10^{-9} \Delta f (g)$$

The cumulative mass increase over the 5 days when the cantilever is exposed to meat, as it is illustrated in figure 4. As it can be seen on the graph, the cadaverine emission is increased proportionally to the days since slaughter and fits well to the model based on morphological change analysis (figure 3). The model also fits previous monitoring of cadaverine concentration from literature [8]. On the other hand, if the cantilever is left at ambient conditions (exposed to air), there is a slight mass loss due to cyclam layer degradation, as shown in figure 4. The initial functionalization layer mass was measured to be 8.8 µg, by measuring the resonance frequency shift before and after functionalization. One can notice that the total functionalization layer weight loss due to degradation was 0.5 µm (5.7% of the initial mass), which is of the same order of magnitude of the degradation level measured by image processing techniques.

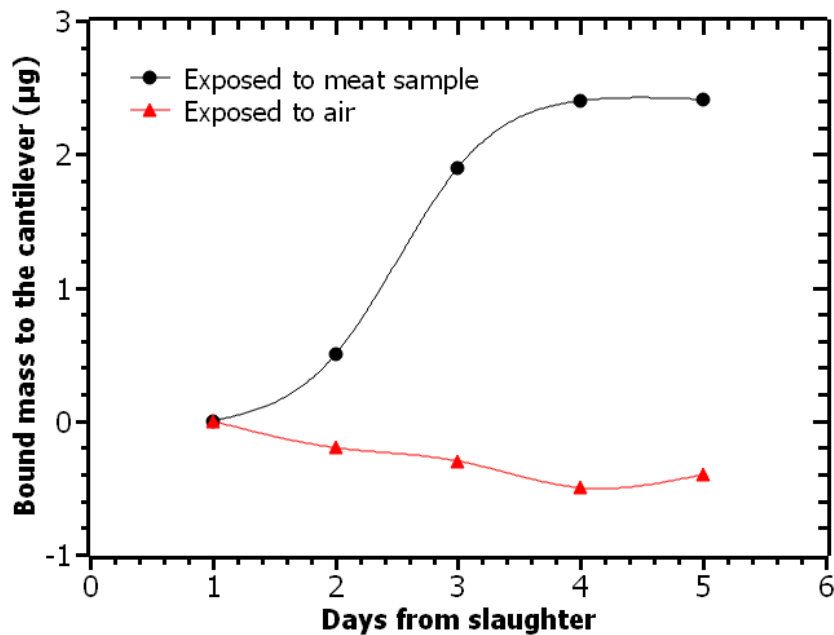


Figure 4: Cumulative cadaverine bound mass on a cantilever sensor as a function of meat age, plotted together with a negative control, where the cantilever is kept at ambient conditions and exposed to air.

Conclusions

We have demonstrated a method to detect cadaverine concentration, which will be useful on meat freshness assessments. A functionalization layer based on cyclam has been optimized both in terms of morphology, cadaverine binding efficiency and stability. The layer has been applied in surfaces for determination of cadaverine levels on different types of meat and the functionality of the layer on a cantilever-based sensor has been demonstrated.

In conclusion, using ethoxyethanol as a solvent for cyclam has resulted on the best cadaverine binding, best functionalization layer homogeneity and best functionalization layer stability, which are desired parameters to obtain good sensor reliability.

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