Article

Quantitative Assessment of Trout Fish Spoilage with a Single Nanowire Gas Sensor in a Thermal Gradient

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Abstract: The response of a single tin oxide nanowire was collected at different temperatures to create a virtual array of sensors working as a nano-electronic nose. The single nanowire, acting as a chemiresistor, was first tested with pure ammonia and then used to determine the freshness status of trout fish (\textit{Oncorhynchus mykiss}) in a rapid and non-invasive way. The gas sensor reacts to total volatile basic nitrogen, detecting the freshness status of the fish samples in less than 30 seconds. The sensor response at different temperatures correlates well with the total viable count (TVC), demonstrating that it is a good (albeit indirect) way of measuring the bacterial population in the sample. The nano-electronic nose is able to classify the samples according to their degree of freshness, but also to quantitatively estimate the concentration of microorganisms present. The system was tested with samples stored at different temperatures, managing to classify them perfectly (100%) and estimating their \textit{log(TVC)} with an error lower than 5%.

Keywords: metal oxide; gas sensor; resistive sensor; single nanowire; fish spoilage; food freshness

1. Introduction

Microbial growth is important in foods as it reduces their shelf life and increases the risk of foodborne illness. Fresh foods are even more susceptible to this problem as they deteriorate rapidly and this affects not only the food industry, but also the health of consumers, with social and health costs \cite{1,2}. Production chains and distribution networks have expanded and complicated and this has increased the time after which food reaches the consumer \cite{3}.

Fish is a health food that is increasingly consumed around the world, often fresh or thawed \cite{4}. Fish and fish products are considered "health food products" as they contain a large amount of high-grade proteins (including all vital amino acids). Rainbow trout is a sustainable fish labeled a "best choice" by the EPA and FDA for its healthiness and low mercury content. Its consumption is widespread also thanks to the fact that it is a fish that lives in both fresh and marine water \cite{5}. Rainbow trout production has grown exponentially since the 1950s, as reported by FAO statistics \cite{6}.

The quality of fresh fish is therefore a major concern for both industry and consumers \cite{7}. Initially groups of human experts used to assess the appearance, smell and texture of the fish \cite{8,9}, but this procedure was laborious and time-consuming, and therefore sensors capable of doing it automatically and objectively are being studied. Different methods have been used to evaluate the degree of freshness of the fish \cite{Error! Bookmark not defined,10}. It is important that the sensor is small (portable), cheap (to deploy many along the production and distribution chain, or integrate one into the packaging) and fast (to measure in real time).
After the death of the fish, the microorganisms on its surface multiply and gradually spread to various tissues [11]. The proliferation of microbes is a major cause of fish spoilage. In fact, the total viable counts (TVC) is commonly used as a reference and definitive index [12]. During this process, microbes degrade trimethylamine N-oxide (TMAO) into trimethylamine (TMA) and ammonia [13]. At the same time bacteria decompose urea and amino acids and produce NH₃ (ammonia) [14]. For these reasons, gas sensors usually measure total volatile basic nitrogen (TVB-N), consisting of ammonia, TMA and dimethylamine (DMA), which is commonly used as a freshness criterion for fish [15].

The most precise and accurate method to analyze volatile compounds is to extract the volatiles and then identify them by separation with chromatographic techniques [5]. Unfortunately, this takes a lot of time, trained personnel, and expensive equipment that is only accessible in a laboratory. This type of analysis can therefore be done only on a sample basis, and guarantees the freshness of the products only in a statistical way. Monitoring the agri-food chain in a widespread manner requires the creation of sensors that are small, cheap and fast.

Gas sensors are less invasive than other types of sensors, and resistive devices are usually simpler and cheaper. Metal oxide chemoresistors are ideal candidates for this purpose: their size is a few microns, and they are cheap because they are very simple. After thick and then thin films [16], the latest generation uses nanostructures, i.e. structures in which at least one dimension is of the order of nanometers [17]. The most commonly used nanostructures are nanowires (quasi-one-dimensional structures). The tiny diameter of the nanowires (NWs) causes the interaction on their surface to affect a large part of the wire section [18]. This way the response is much higher, and the limit of detection (LoD) is in parts per billion (ppb). Nanowires are commonly used as a porous thin film on which metal electrodes are deposited[19,20], but they can also be grown directly from the electrodes [21,22] or even contacted individually [23]. A single nanowire has already been used to measure the freshness of mackerel samples, but used in a traditional way, as a simple chemiresistor [24].

In this work a single tin oxide nanowire was used, made to work at three different temperatures. The responses were combined in a virtual array which, working as an electronic nose, was able to evaluate the freshness of the tested fish. The response of the gas detection system to the TVB-N was compared with the total life count, proving capable of measuring the freshness of the rainbow trout quickly and precisely.

2. Materials and Methods

2.1. Synthesis of SnO₂ nanowires

A forest of tin oxide (SnO₂) nanowires was grown by chemical vapor deposition. An alumina boat filled with pure tin monoxide was used as the evaporation source, placed in the center of a horizontal quartz tube inside an oven (Lindberg Blue M), at its maximum temperature. A piece of silicon wafer (about 1x3 cm²) was deposited with a thin gold film (about 5 nm) and placed 1 cm from the alumina boat. Silicon and gold respectively act as substrate and catalyst for the growth of nanowires. The quartz tube was pumped to 10⁻² mbar and purged with high purity argon (99.999%) three times and then the system was pumped up to its limit pressure. The temperature was raised from room temperature to 850°C with a ramp of 25°C per minute and the oven was left at 850°C for five minutes. Then a flow of 0.35 standard cubic centimeters of oxygen was flowed through the system, starting the process. The growth process lasted 30 minutes and finally the system was shut down and allowed to cool. At the end of the process, the samples were covered with a soft and homogeneous white layer, composed of SnO₂ nanowires.

2.2. Material Characterization

The CVD-grown tin oxide nanowire forest was characterized by X-ray diffraction (XRD) using a Philips Xpert Pro operating at 40kV with CuKα radiation. Secondary
electron microscopy (SEM) and transmission electron microscopy (TEM) images were acquired with a Hitachi S-4800 and a JEM-100CX, respectively.

2.3. Fabrication of the sensor

A square of the substrate with the forest of nanowires (approximately 1x1 cm2) was sonicated in dimethylformamide for two seconds and the resulting dispersion was drop cast onto a Si/SiO₂ wafer by spinning it at 6000 rpm. An array of Ti/Pt (10/250 nm) electrodes was deposited on top of the dispersed nanowires using UV lithography. Using resistance measurement and optical microscopy, pairs of adjacent electrodes connected by nanowires were found. These electrode pairs were characterized by SEM to find cases where a single nanowire was connecting the metal pads.

2.4. Gas sensor measurements

The single nanowire sensor was tested in a system consisting of a measuring chamber with a heatable holder and microprobes. The measuring chamber is connected to high purity gas cylinders through mass flow controllers. The microprobes are connected to a multimeter (Keithley 2410) interfaced with a data acquisition program (LabView, National Instruments). Initially the device was kept at 500°C in nitrogen for 4 hours while it was powered at 1 V in order to stabilize the nanostructures and their intrinsic resistance. This procedure serves to stabilize the electrical properties of the nanostructures so that they do not change over time [17]. The electrical contact of the semiconductor nanowires with the metal electrodes was studied by analyzing the I-V curves. The good linear behavior found proves a good ohmic contact.

The sensor worked under a voltage of 1 V, at three different temperature values (200, 250, 300°C) towards low concentrations of ammonia (0.1 - 5 parts per million, ppm), with a total gas flow maintained at 400 sccm. The sensor response was calculated with the standard definition $S = \frac{R_{\text{air}}}{R_{\text{gas}}}$, where $R_{\text{gas}}$ and $R_{\text{air}}$ are respectively the resistance of the sensor in the presence of ammonia and in air. The response time of the device is also calculated in the standard way, as the time it takes to reach 90% of the maximum response. Similarly, recovery time is calculated as the time to reach 90% of complete recovery. The limit of detection (LoD) was calculated as $3 \cdot \text{SD}_{\text{noise}} / \text{sensitivity}$, where $\text{SD}_{\text{noise}}$ is the standard deviation of the sensor signal and sensitivity is the derivative of the sensor response as a function of gas concentration [25].

2.5. Trout spoilage measurements

The fresh rainbow trout fish was purchased from a fish farm in Verona (Italy) and was kept on ice for less than 1 hour upon arrival in the laboratory. Several pieces of trout weighing 20g were cut from fresh fish using disposable gloves and autoclaved tools. Each piece was stored in a separate vessel until measurement with the gas sensor. Some samples were stored at room temperature (25°C) and some in the refrigerator (4°C). A sample was placed in the sensing chamber initially every hour, then every three hours and finally every six hours to measure the emitted TVB-N. Immediately after measuring with the gas sensor, the sample was subjected to microbial analysis, in order to compare the two measurements. The total viable count (TVC) was performed using a spread plate technique [16] on a plate count agar and agar base (Oxoid CM0463 and 0055). The plates were counted after an incubation time of 48 h at 30°C.

2.6. Multivariate statistics and data mining

Principal component analysis (PCA) was applied to the response values of the gas sensor at three different temperatures combined together. In this case the PCA does not reduce the dimensionality, but only serves to visualize the spatial relationships between the points in a more evident way. The same three-dimensional points are used to quantitatively estimate the TVC value of the fish samples by means of a linear kernel support vector machine [26] used as a regressor. The points measured in double blind were randomly divided into two sets: train (32 points) and test (18 points), in order to calibrate the system and then evaluate its quantification performance.
3. Results and discussion

3.1. Nanowires characterization

The morphology of the spaghetti-like SnO$_2$ nanowires obtained by CVD was studied by scanning electron microscopy. An SEM image of the nanowire layer is shown in Figure 1a.

![SEM image of SnO$_2$ nanowires](image1.png)

**Figure 1.** a) SEM image of the SnO$_2$ nanowires grown by CVD; b) SEM image of the sensor: a single SnO$_2$ nanowire bridging the metallic electrodes (on the sides); c) TEM image of two crossing nanowires.

Fig. 1a shows long and thin nanowires with a constant diameter whose average value is 40-80 nm. The SEM image in Fig. 1b shows the single nanowire which was used as a sensor by connecting the two electrodes to the sides. The nanowire forks in the center of the space between the two electrodes. The diameter of the nanowire is approximately 57 nm on the left side and 33 nm on the right side. The thin diameter of the right side and the probable potential barrier in the center contribute to improve the sensor performance. Fig. 1c shows a TEM image of two crossing nanowires. The interplanar fringes of 0.267 nm correspond to the crystalline planes (101) of the tetragonal SnO$_2$ structure. The image confirms that the nanowires are monocrystalline with no amorphous layers.

The composition and structure of the SnO$_2$ nanowires are also confirmed by the X-ray diffraction pattern shown in Figure 2.

![XRD pattern of SnO$_2$ nanowires](image2.png)

**Figure 2.** XRD pattern of SnO$_2$ nanowires grown on the substrate (one of which was used as a single nanowire sensor). The tetragonal SnO$_2$ reference pattern (JCPDS 77-0450) is shown below (red in line).

All the diffraction peaks present in the pattern can be easily indexed to the tetragonal phase of SnO$_2$ with lattice parameters of $a = b = 4.742$ Å and $c = 3.186$ Å, and therefore agree well with the standard values (JCPDS n. 77-0450). The absence of amorphous contributions, impurity peaks, or other SnO$_2$ phases, confirms the high purity of the nanowires.
3.2. Ammonia sensing performance

Sensor performance was initially tested with low ammonia concentrations (0.1 to 5 ppm). The dynamic resistance of the sensor was tested at three different temperatures (200, 250 and 300°C). The three answers obtained will then compose the 3D signal processed by the machine learning algorithms. Dynamic resistance plots at different temperatures are shown in Figure 3a.

![Figure 3](image)

**Figure 3.** a) Dynamic resistance at three temperature values, during the injection of different concentrations of ammonia, b) sensor response as a function of ammonia concentration for different working temperatures, c) response and recovery times as a function of sensor working temperature.

The resistance of the nanosensor is constant in air, and at any working temperature it drops sharply when ammonia gas is flushed into the chamber. When the ammonia flow is stopped and pure air is returned into the system, the resistance returns to its original value. This behavior is typical of n-type semiconductors [17], in response to reducing gases like ammonia [18]. The detection mechanism is known in the literature: when the nanowire is exposed to air, oxygen is adsorbed in the form of O- and O2-, draining electrons from its interior to form chemical bonds on the surface. Decreasing the number of charge carriers increases sensor resistance. When ammonia molecules land on the surface of the nanostructure, they react with the adsorbed oxygen atoms breaking their chemical bond and releasing electrons into the nanowire. The increase in the number of charge carriers decreases the resistance of the sensor. The three graphs in Fig. 1a show that the resistance variation is proportional to the ammonia concentration. It can also be seen how, as the temperature increases, the air resistance of the sensor decreases, and the response and recovery become faster. Fig. 1b shows the sensor response (calculated as explained in section 2.4) as a function of the gas concentration for the three temperatures tested. The response increases with concentration almost linearly, and is greater for higher working temperatures. The speed of the sensor is quantified in Fig. 1c where the response time and recovery time are shown, as defined in section 2.4. Both times decrease according to the working temperature. The response time is higher than the recovery time at 200°C, but at higher temperatures it becomes shorter or comparable. In general, response and recovery times are very fast: at the lowest temperature they are respectively 24 and 15.5 s, while at higher temperatures they are always less than 13 s. The limit of detection (calculated as specified in section 2.4) is very low at all temperatures tested: 13.4, 4.9 and 1.8 ppb at 200, 250 and 300°C, respectively [25].
3.3. Trout fish spoilage measurements

The sensor was then used to measure the freshness of rainbow trout samples stored at 25°C. Since the sensor measures the volatiles emitted by the fish sample (mainly TVB-N i.e. ammonia, dimethylamine and trimethylamine), it is not possible to compare the response with a known concentration. For this reason, together with the sensor response, Fig. 4 also shows the microbial count, used as a reference.

![Figure 4. Sensor response (solid symbols, left scale) and bacterial population (green open circles, right scale) in fresh trout fish kept at room temperature (25°C) over a period of 60 hours.](image)

The responses of the gas sensor are read on the left scale, while the total viable count on the right. The response of the sensor increases as the working temperature increases, as in the case of ammonia. At all temperatures the response increases over time, slowly over the first six hours and then faster. The TVC increases similarly, starting at a value of 4.2 (note that the log of the TVC is plotted), reaching the maximum slope around 20 hours and exceeding a value of 10 after 60 hours. The response of the single nanowire resistive sensor can be considered a good indirect measure of the microbial count, and therefore of the freshness of the fish. The dashed horizontal green line identifies the threshold considered as the end of the shelf life of the fish both in literature [27,28] and for the authorities [29,30]. The threshold was exceeded approximately after 22 hours and 40 minutes of storage at room temperature (25°C).

The responses of the gas sensor are read on the left scale, while the total viable count on the right. The sensor was also tested with rainbow trout samples stored at 4°C for 84 hours. Fig. 5 shows the sensor response at 200, 250 and 300°C and the microbial count detected on the samples over time. During the first 12 hours the microbial count remains more or less constant around a value of 4.2, then begins to rise almost linearly, to reach a value of 7.84 after 84 hours. The edibility threshold in this case is reached after approximately 64 hours. The response of the gas sensor behaves in a similar way at all temperatures.
The trend of the response of the gas sensor at the various temperatures in Fig. 4 and 5 is very similar to that of the microbial count. This can be explained by the fact that TVB-N is the metabolic product of the microbes responsible for the degradation of fish [31] and meat [32].

To evaluate how good the response of the single nanowire gas sensor could be as a measure of fish freshness, a series of samples stored at different temperatures were measured in double blind. For each sample the response of the gas sensor was first measured and immediately afterwards the microbial analysis was carried out, in order to have the comparison under the same conditions. The measurements are shown in Fig. 6, where the response is reported as a function of the logarithm of the total viable count. The response is linear at all working temperatures, with Pearson’s correlation coefficients r greater than 0.99 in all cases. The error decreases as the temperature rises, but is always less than 10%. This demonstrates that sensor response can be considered a good indirect measure of TVC.
The three responses of the single nanowire gas sensor were combined to obtain a sort of virtual electronic nose, following the approach already tested previously [33]. The 3D points obtained also include the correlations between the various answers, and are therefore much more informative than a single response, and can be processed with machine learning algorithms. The three-dimensional points were initially processed with principal component analysis (PCA) in order to visually assess how they are spatially related.

The PCA graph in Fig. 7 shows the points divided by color according to the TVC value. Each group of points is colored according to a unit interval of the logarithm of TVC, as shown in Fig. 6. It can be seen that the points are arranged in a zigzag line that goes down to a log value (TVC) of 6-7, then goes up to 8, goes down to 10 and then goes up again. Each group of points is well separated from the others, with a possible small overlap only with the immediately preceding or following group.

This overlap was expected, as the measurement of the microbial count is continuous and therefore the points along the zigzag line should also be continuous. This is evident in the point clouds of the intervals 7-8, 8-9 and 9-10, which concatenate well along the imaginary zigzag line.

Fig. 7 demonstrates that the single nanowire sensor is very sensitive and accurate, since it not only distinguishes spoiled fish samples (over the threshold of $10^7$ cfu/g), but also the various stages of the degradation process, as measured by the total viable count.

To obtain an automatic quantitative estimate by the nanosensor, a support vector machine was used as a regressor [23]. In this way, the three responses of the gas sensor were automatically transformed into an estimate of the TVC and therefore of the degree of freshness of the fish. Fig. 8 shows the regressor estimates against the “true” values (obtained from TVC measurements). Clearly, the diagonal represents an estimate identical to the TVC value and therefore a perfect functioning of the nanosensor.

Figure 7. PCA plot of random samples of rainbow trout. The color indicates the log(TVC) with the same scale of the X-axis in Fig. 6.

The points in Fig. 8 are all very close to the diagonal, indicating a very good estimate of the freshness of the fish. The average error obtained on all points is less than 5%, demonstrating that the single nanowire gas sensor approximates the microbial count measurement very well.
Figure 8. Estimates of TVC value versus actual measured TVC values for random rainbow trout samples. The green color indicates the area in which the microbial load is compatible with consumption, while the red one indicates that the fish has deteriorated.

The points are collected in the two colored areas. The green zone indicates a TVC value that allows the consumption of fish, while the red zone indicates that the fish has deteriorated. This again means that the sensor is in perfect agreement with the microbial count and can be used as a tool to ascertain the freshness of rainbow trout samples. It should be noted that the results are obtained under laboratory conditions with samples collected from only one fish. It is reasonable to expect a larger error when working in the field on different fish.

The single nanowire gas sensor is in fact tiny and portable (while TVC can only be done in a laboratory equipped by trained personnel) and takes less than half a minute (while TVC usually takes days). For this reason we think that the proposed sensor could be ideal to assess the freshness of the fish during its shelf life.

5. Conclusions

A SnO$_2$ single nanowire gas sensor was used to assess the deterioration of rainbow trout fish. Performance was initially tested by measuring ammonia concentrations from 0.1 to 5 ppm at three different operating temperatures. The sensor responds quickly even at very low concentrations. The responses of the nanosensor at different temperatures were then used to monitor trout spoilage over time. The gas sensor response proves to be a good approximation of the total viable count at all working temperatures. The use of machine learning algorithms allows to determine the spoilage stage of the fish and to estimate its total viable count. Considering the tiny size (tenths of a millimeter), economy, ease of use and speed, the single nanowire gas sensor would be ideal as a non-invasive tool for monitoring the freshness of the rainbow trout along its production and distribution chain.

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