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Diagnosing disease by nanomechanical olfactory sensors – system design and clinical validation

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Abstract

The medical art of recognizing disease by its odor is known at least since Hippocrates wrote in 400 BC in his Book of Prognostics^[4] that " smells which are somewhat putrid and fetid, are bad...". Mimicking this approach by nanoscience methods, we report the design and clinical validation of a nanomechanical cantilever array - based olfactory system coupled with an artificial neural network for fast detection of disease-specific breath patterns. Arrays of silicon cantilevers produced by microfabrication were differentially surface functionalized with polymers that exhibit different swelling properties upon exposure to various vapor mixtures. The resulting complex, nonlinear bending pattern of the cantilever array was analyzed by pattern recognition techniques and allowed in cross-validation experiments the noninvasive, fast, label-free, sensitive and specific detection of disease states in clinical samples from normal individual and patients with kidney failure and with respiratory failure in an intensive care unit setting. This demonstrates the applicability of nanomechanics-based systems for noninvasive medical diagnosis.

Hunziker P. Eur J Nanomed 2008 1:44-47

Introduction

In medicine, the smell of a patient can give the doctor diagnostic hints to underlying disease, e.g. the "green apple" flavor in diabetic ketoacidosis or the characteristic odors in renal or liver failure. However, this approach is neither objective nor quantitative, and the human nose is not as sensitive as that of certain animals, which can also been trained to recognize certain diseases (2). Thus, this approach is limited to few detectable disease classes, is performed in a subjective and qualitative manner, and is often only clearcut at an advanced stage of disease, so that olfactory diagnosis has largely been abandoned in clinical practice and has been replaced by biochemical examination of blood samples and other body fluids, although that latter approach is intrinsically more invasive, timeconsuming, labor-intensive, and thus costly. Nevertheless, reports using gas chromatography (3) and electronic gas sensors (4) have hinted at a diagnostic potential of exhaled air in certain disease states using more objective methods for gas analysis.

The development of atomic force microscopy has led to highly sensitive, force sensing cantilevers built from silicon that are characterized by a very large length to thickness aspect ratio. When combined with very accurate optical deflection measurement at subnanometer resolution at the cantilever end, these sensors become extremely sensitive for force measurement down to the pico-Newton range (5). This extreme sensitivity permits their use as transducers of physicochemical signals, in particular when the cantilever surface is coated on one side with a material that changes its conformation and thus induces a surface stress on the cantilever upon interaction with analytes in solution or in the vapor phase (6).

We have exploited these features of functionalized cantilevers to design and evaluate an array sensor for medical disease diagnosis from the breath of healthy individuals and patients. In contrast to the situation in body fluids, where functionalized cantilevers can be used for sequence-specific recognition of DNA strands (7) and for specific detection of proteins (8), macromolecules like DNA and proteins are unlikely constituents of the vapor phase and are therefore not suited as diagnostic markers on a breath analyzing diagnostic device. The physiology of olfaction in vertebrates is characterized by large number of olfactory cells – sensors – that react in a parallel, but differential fashion to certain odorous substance mixtures, whereby a given odor is typically composed of numerous small, volatile molecules like alcanes, alcohols, aldehydes, carboxylic acids dimers, benzene derivatives and etheric oils. The complex pattern of activation of the array of olfactory cells is then analyzed in the neural "network" of the olfactory cortex in the forebrain, although the details of processing in the brain are not completely

understood.

We chose to apply a similar approach for disease detection by utilizing an array of cantilevers of which each is functionalized in a different way, although this functionalization is not specific for certain ligand/receptor pairs. Thus, disease classification is not based on the response of a single sensor that is highly specific for a particular marker of a specific disease as done in conventional biochemical testing. Classification is done instead through pattern recognition/classification techniques that take the overall response pattern of the cantilevers induced by the interaction of the complex mixture of volatile gas into account. Both, linear discriminant analysis, and a nonlinear approach based on an artificial neural network were applied, because nonlinear behaviour of the system was expected in advance and is evident from the sensor data, with nonlinear analysis proving to be advantageous.

Methods

The measurement chamber consisted of arrays of 8 cantilevers with a length of 500 μ m, a width of 100 μ m and a thickness of 1 μ m produced from silicon

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by microfabrication and coated on one side with an individual polymer for each cantilever. Polymers used were polyurethane, polyvinylchloride, polystyrole, Araldit-epoxy-red, Aralditepoxy-blue, polyvinylpyrrolidone, polyvinylacrylate, and polymethylmethacrylate. A schematic diagram of the cantilever array with differentially functionalized indi-vidual cantilevers is shown in figure 1. A photograph of



Fig. 1: An array of 8 cantilevers that are differentially polymer-coated on one surface. Asymmetric changes in surface tension lead to bending of the cantilever. Bending is measure by a laser integrated into the system so that it becomes a highly sensitive force sensor.

the system by a motorized syringe pump during 300 seconds at 0.03ml/sec followed by flushing with N2 at the same flow rate. System temperature was maintained by operating the system in steady state conditions at fixed environment temperature of 20 degrees Celsius, and injectate temperature was maintained at 20 degrees Celsius through sample tubing immersion in a constant temperature water bath.

Data were transferred to a PC using a LabView Interface. Approximately 12000 sensor data points were digitized for a single experiment. Data noise measured at baseline was 0.7% of peak deflection and standard error of the mean was 3.6% at peak deflection on repeat measurement. Intraday variation within a patient was 14%. Using custom software, sensor data were time-aligned, baseline-corrected and subsampled to yield 112 characteristic averaged datapoints per experiment (7 during injection and 7 during flushing for each cantilever).

A 3-layer, feed-forward, error backpropagation artificial neural network was built that featured hidden nodes with sigmoid response function of the hidden layer; a number of 24 hidden nodes was chosen according to the ability of the

the silicon chip containing carrying the cantilevers and a view of the whole setup is shown in figure 2.

Illumination and readout were done using vertical cavity surface-emitting lasers as described elsewhereⁱ in detail; measurement of reflected light with a position-sensitive photodetector allowes deflection measurement down to the 0.1nm range. Exhaled air was sampled into plastic bags and was injected into



Fig. 2: Photograph of the chip carrying the sensor array (upper left) and the entire measurement and analysis system (lower panel)

network to represent a test dataset; to avoid overfitting, the training cycle number was chosen by observing the time course of fitting in a separate dataset not used for training. Training involved 3000 training rounds at a learning rate of 0.005. Crossvalidated results from "leave-oneout classification" are given, i.e. an actual observation was classified from a model that excluded that sample for network training.

Linear discriminant analysis was performed on SPSS software version 12.0. Predictive linear models were built using stepwise variable selection using Wilk's lambda as selection parameter. Crossvalidation results are given here, too.

Results

After preliminary testing to assess the response of the system to changes of simple constituents and physicochemical properties of a gas mixtures and documentation of the expected complex sensor signal, we performed 81 experiments of samples from 7 normal individuals (28 samples), from 7 patients with advanced renal failure (13 samples), and 7 patients with respiratory failure (40 samples).



Fig. 3: Bending of the differentially coated cantilevers upon injection of a gaseous analyte.



Fig. 4: Differential response of the 8 cantilever sensors (from left to right) upon injection of different samples (each sample coded by one color)

A typical cantilever deflection graph showing the response of all 8 cantilevers upon injection of test sample is shown in figure 3.

Figure 4 shows the response of the 8 cantilevers upon injection of multiple samples.

Linear discriminant analysis was performed and principal components of the sensor response plotted as shown in figure 5. Shown are the two main principal components; when more than two disease classes were to be classified, discrimination ability of the algorithm strongly degraded due to overlap of clusters (data not shown). Due to the



Fig 5: Principal component analysis of multiple samples. The two largest principal components are plotted. Each dot represents a sample.

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Table 1: Diagnostic performance ofdisease states using an cantileversensors and artificial neural networkDetection of "normals"

Detection of "normals"	
sensitivity	86%
specificity	87%
positive predictive value	77%
negative predictive value	92%
diagnostic accuracy	86%
Diagnosis of "renal failure"	
sensitivity	100%
specificity	99%
positive predictive value	93%
negative predictive value	100%
diagnostic accuracy	99%
Diagnosis of "respiratory failure"	
sensitivity	83%
specificity	95%
positive predictive value	94%
negative predictive value	85%
diagnostic accuracy	89%

nonlinear characteristics of the data, nonlinear data analysis in the form of artificial neural networks was then performed.

Disease classification was most successful using nonlinear analysis by the artificial neural network. Table gives classification results on patient data.

Cantilever deflection could be observed within seconds and the apparent time constants of deflection were generally in the range of minutes, varying as a function of individual cantilever coating and gas components.

Interaction of a single molecule class in the vapor phase with a surface-bound polymer and the resulting change in polymer properties leading to surface stress are a physically complex phenomenon: among many factors and beyond the geometric, static and dynamic properties of the polymer, the diffusion of molecules in the gas phase, the interaction of the gas molecule with the polymer molecules and the diffusion of the molecule into, within, and out of the polymer will play a role. In our case of multiple vapor components, the situation becomes even more complex. An important question is therefore, how many individual parameters can be represented by a given cantilever array and used for classification. In the simple case that assumes binding and eluting kinetics of the type

 $C_{\text{binding}} = a_i^* 1/(1-e^{-bt}) \text{ and } C_{\text{elution}} = a_i^* (1-1/(1-e^{-ct}))$

there are already 24 degrees of freedom for an 8 cantilever system, sufficient to represent a very large number of different patterns. Experimentally, it was evident that certain cantilevers showed a more complex deflection pattern than others. On the other hand, certain cantilevers might respond in a collinear fashion, tending to reduce the number of degrees of freedom of the system. We estimated the actual number of degrees of freedom in our experiments by performing principal component analysis of the raw sensor data from 81 experiments and found that the largest 16 principal components were needed to represent 99% of the sensor information in linear analysis, and that in nonlinear analysis, optimal description of the dataset was achieved with a neural network of 24 hidden nodes.

Discussion

This study shows that nanomechanical olfactory sensors coupled to an artificial neural network can be used for medical diagnosis with good diagnostic accuracy. This setup mirrors the design of the human olfactory system, where mechanotransduction in olfactory cells is coupled to the biologic neural network, i.e. the brain. The old medical art of olfactory disease diagnosis, which is limited by observer dependence, lack of quantitative analysis, and the limited sensitivity of the human nose thus finds its correlation in nanomedicine, where nanomechanical olfactory sensors allow quantitative and objective analysis of disease odors for diagnosis.

In conventional biomedical testing, highly specific measurement of individual analytes is a broadly adopted strategy, exemplified in the biochemical diagnosis of myocardial infarction where generations of biomarkers with increasing specificity for cardiomyocyte damage have replaced each other, ranging from nonspecific transaminase assays to more specific creatin kinase measurement which have recently been replaced by troponin assays. The troponins, although highly specific for cardiomyocyte damage, may still be elevated in a large spectrum of diseases including sepsis, pulmonary embolism and pericarditis, highlighting the fact that specificity for a certain cell does not necessarily translate in equal specificity for a certain disease. In this work, we abandoned the quest for optimally specific single-parameter measurement in favor of a pattern recognition approach

of an array of less specific measurements. This paradigm change was motivated by design as well as by practical reasons: First, the body is a complex nonlinear system, and clinical disease is therefore frequently a malfunction of multiple biological processes. This is implicitly mirrored in clinical decision making, whereby parameters like patient and family history, symptoms and signs, ECG measurements and laboratory analyses are taken together tomake a diagnosis and supports the use of pattern recognition and nonlinear analysis for diagnostic purposes. Replacing single marker measurement by pattern recognition for breath analysis has a second important reason: Highly specific biochemical analysis is much more difficult to achieve for a mix of small volatile molecules than for conventional biochemical serum markers because the most important techniques based on antibody-antigen interactions and nucleic acid strand-strand interactions are difficult to apply to small molecules and are usually performed in aqueous solutions.

Diagnostic testing using the nanomechanical olfactory sensor can be performed in a label-free manner. Label-free testing is a desirable strategy in laboratory medicine because it may reduce the known problems associated with test reagents including storage and transport problems in different climates, batch-to-batch variations of reagents, and consumable costs. Manufacture of the sensor uses established silicon chip technology, thereby limiting device production costs, and the ability to reuse the system for multiple tests in a number of patients also has the potential to keep costs down, what renders this approach not only attractive to high-tech hospitals, but also in third-world situations.

An acceptably short testing time is prime requirement for tests to be applicable to clinical testing. Conventional sampling of body fluids necessitates a variable combination of steps that typically include venipuncture, sample centrifugation, sample transfer to a lab, and actual testing, each of which typically requires several minutes, so that in a clinical setting, the time from "decision to measure" to actual reporting of results is often about 1 hour, with a limited number of rapid bedside tests that are available within 10-15 minutes. We found that nanomechanical breath analysis could be performed without patient preparation

just by sampling an expiratory breath, immediately followed by injection of the sampled air into the measurement device during 5 minutes, flushing of the system during another 5 minutes, and concomitant online analysis of the sensor data; the resulting overall testing time of approximately 10-15 minutes compares thus well to current practice, although the minimal necessary sampling time for parameter estimation could probably be further optimized.

Nanomechanical olfactory diagnosis is similar to other pattern recognition techniques in that it requires training before classification can be performed. To work in a robust manner, especially nonlinear classification algorithms depend on large, representative training samples. In our context, this points to the need for a large "smell library" of a wide variety of diseases for optimal use of this device, a quite unique requirement. However, because training samples are easily and noninvasively available in the case of breath analysis, this should not present a fundamental obstacle to nanomechanical olfactory diagnosis.

To summarize, nanomechanical olfactory sensors coupled to an artificial neural network can be used for medical diagnosis with good diagnostic accuracy. This setup mirrors the design of the human olfactory system, where mechanotransduction in olfactory cells is coupled to the biologic neural network, i.e. the brain. The old medical art of diagnosing disease by it's odor, limited by observer dependence and lack of quantitative analysis and the limited sensitivity of the human nose, thus finds its correlation in nanomedicine, where nanomechanical olfactory sensors allow quantitative and objective analysis of disease odors for diagnosis.

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