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Diagnosing lung cancer in exhaled breath using gold nanoparticles

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Conventional diagnostic methods for lung cancer[1,2] are unsuitable for widespread screening[2,3] because they are expensive and occasionally miss tumours. Gas chromatography/mass spectrometry studies have shown that several volatile organic compounds, which normally appear at levels of 1–20 ppb in healthy human breath, are elevated to levels between 10 and 100 ppb in lung cancer patients[4–6]. Here we show that an array of sensors based on gold nanoparticles can rapidly distinguish the breath of lung cancer patients from the breath of healthy individuals in an atmosphere of high humidity. In combination with solid-phase microextraction[7], gas chromatography/mass spectrometry was used to identify 42 volatile organic compounds that represent lung cancer biomarkers. Four of these were used to train and optimize the sensors, demonstrating good agreement between patient and simulated breath samples. Our results show that sensors based on gold nanoparticles could form the basis of an inexpensive and non-invasive diagnostic tool for lung cancer.

Lung cancer accounts for 28% of cancer-related deaths. Approximately 1.3 million people die worldwide every year[1,2]. Breath testing is a fast, non-invasive diagnostic method that links specific volatile organic compounds (VOCs) in exhaled breath to medical conditions[8,9]. Gas chromatography/mass spectrometry (GC-MS)[4,6], ion flow tube mass spectrometry[10], laser absorption spectrometry[11], infrared spectroscopy[12], polymer-coated surface acoustic wave sensors[5] and coated quartz crystal microbalance[13] sensors have been used for this purpose. However, these techniques are expensive, slow, require complex instruments and, furthermore, require pre-concentration of the biomarkers (that is, treating the biomarkers by a process to increase the relative concentration of the biomarkers to a level that can be detected by the specific technique) to improve detection.

Here, we report a simple, inexpensive, portable sensing technology to distinguish the breath of lung cancer patients from healthy subjects without the need to pre-treat the exhaled breath in any way (see also refs 14–16 for the diagnosis of lung cancer by sensing technology that is based on arrays of polymer/carbon black sensors). Our study consisted of four phases and included volunteers aged 28–60 years. Samples were collected from 56 healthy controls and 40 lung cancer patients after clinical diagnosis using conventional methods and before chemotherapy or other treatment. The clinical characteristics of the volunteers are listed in Supplementary Tables S1 and S2.

In the first phase, we collected exhaled alveolar breath of lung cancer patients and healthy subjects using an 'offline' method. This method was designed to avoid potential errors arising from the failure to distinguish endogenous compounds from exogenous ones in the breath and to exclude nasal entrainment of the gas (see Methods). Exogenous VOCs can be either directly absorbed through the lung via the inhaled breath or indirectly through the blood or skin[17]. Endogenous VOCs are generated by cellular biochemical processes in the body and may provide insight into the body's function[17,18].

In the second phase, we identified the VOCs that can serve as biomarkers for lung cancer in the breath samples and determined their relative compositions, using GC-MS in combination with solid-phase microextraction (SPME)[7,19]. GC-MS analysis identified over 300–400 different VOCs per breath sample, with >87% reproducibility for a specific volunteer examined multiple times over a period of six months. Forward stepwise discriminant analysis identified 33 common VOCs that appear in at least 83% of the patients but in fewer than 83% of the healthy subjects. Figure 1 shows the average abundance ratios of the 33 common VOCs for healthy subjects and lung cancer subjects. These compounds are mostly C_4–C_{11} straight[18] or monomethylated alkanes[18] as well as certain benzene derivatives[4,6]. The compounds that were observed in both healthy breath and lung cancer breath were presented not only at different concentrations but also in distinctively different mixture compositions. The higher concentration and lower concentration in lung cancer breath compared with healthy breath can be understood, respectively, in terms of the release of certain VOCs and the consumption of other VOCs by the lung cancer cells[20]. The observation of 33 lung cancer biomarker VOCs in our study, rather than 22 as reported earlier[6], could be attributed to different pre-concentration methods. Further forward stepwise discriminant analysis revealed nine uncommon VOCs that appear in at least 83% of the patients but not in the majority (83%) of healthy subjects. This additional class of VOCs has not been recognized in earlier GC-MS studies[4–6]. In spite of these advances in the GC-MS analysis, these data certainly do not account for all the VOCs present in the exhaled breath samples, because the pre-concentration technique can be thought of as a solid phase that extracts only part of the analytes present in the examined phase and, subsequently, releases only part of the extracted analytes. So, it is likely that the actual mixture of VOCs to which, for example, an array of gold nanoparticle sensors would be responding (see below) is different from that obtained by GC-MS.

In the third phase of this study we designed an array of nine cross-reactive chemiresistors, in which each sensor was widely responsive to a variety of odorants for the detection of lung cancer by means of breath testing (Fig. 2)[21]. We used chemiresistors based on assemblies of 5-nm gold nanoparticles[22–24] with different organic functionalities (dodecanethiol, decanethiol, 1-butanethiol, 2-ethylhexanethiol, hexanethiol, tert-dodecanethiol, 4-methoxy-toluenethiol, 2-mercaptobenzoxazole and 11-mercapto-1-undecanol). Chemiresistors based

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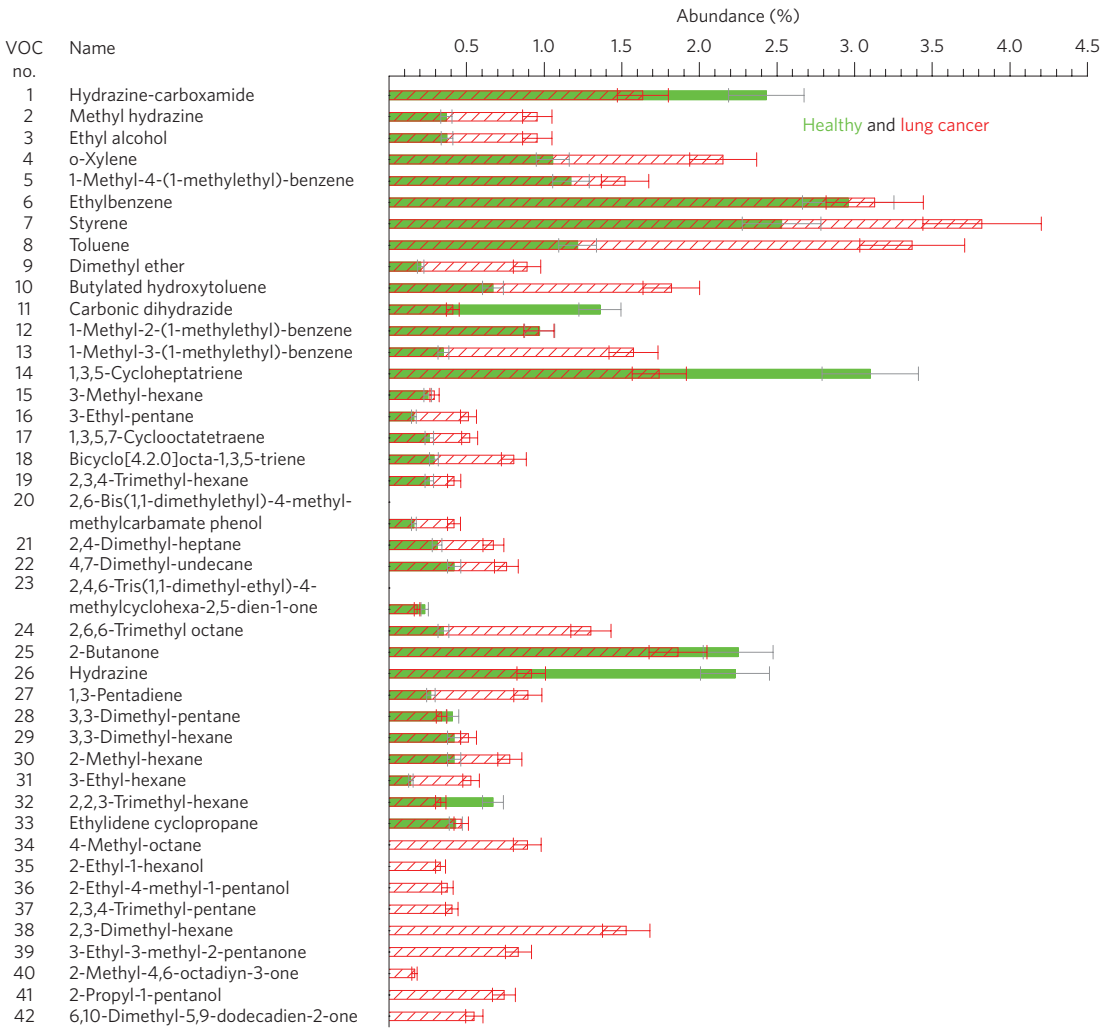

Figure 1 | Gas chromatography/mass spectrometry (GC-MS) analysis of healthy and lung cancer breath. Average abundance ratio of the 42 lung cancer biomarkers identified with solid-phase microextraction (SPME)-aided GC-MS. Thirty-three common VOCs and nine uncommon VOCs were found in at least 83% of the patients and in fewer than 83% of the healthy subjects, respectively. The variance of a specific VOC in either the healthy group or the lung cancer group was relatively small, most probably due to the relatively 'homogeneous' population included in this study. Note: setting a cutoff value of 100% (rather than ~83%, which considered a typical value in the literature) would give lower variances than those presented in the current figure. The variances are indicated by the error bars.

on functionalized gold nanoparticles combine the advantages of organic specificity with the robustness and processability of inorganic materials[22].

Before exposure to the breath samples, we examined the response of each sensor to several representative lung cancer biomarkers (see Supplementary Fig. S1). We found that the sensors responded rapidly and fully reversibly to a wide variety of concentrations. Similar characteristics were obtained when we exposed the sensors to real breath of healthy and lung cancer volunteers (see Fig. 3). Most of the sensors showed a detection limit of 1–5 parts per billion (ppb) (see Supplementary Fig. S2). Complementary experiments indicated (data not shown) that gold nanoparticles functionalized with 4-methoxy-toluenethiol, 2-mercaptobenzoxazole or 11-mercapto-1-undecanol have detection limits of 2–10 ppb on exposure to acetaldehyde (a promising VOC for lung cancer[10]) and formaldehyde (a promising VOC for breast cancer[25]), much below the concentration levels of these VOCs in the exhaled breath of cancerous patients.

The response of the nine-sensor array to both healthy and lung cancer breath samples was analysed using principal component analysis (PCA; see Methods). Figure 4 shows principal component 1 (PC1) and principal component 2 (PC2) for each subject, which accounted for >90% variance. It can be seen that there is no overlap of the lung cancer and healthy patterns. Note that clear discrimination was achieved without pre-concentration or dehumidification of the breath sample. This is a marked improvement over our recently demonstrated ten-sensor array based on organically functionalized carbon nanotubes, which required adequate pre-treatment of the simulated breath samples (that is, a mixture of representative VOCs that have a composition similar to those extracted by GC-MS for real exhaled breath samples) to achieve a clear distinction[26]. The PCA of the healthy control group revealed that the set of gold nanoparticles sensors was not influenced by characteristics such as gender, age or smoking habits (see Supplementary Fig. S3), thus strengthening the ability of the sensors to discriminate between healthy and cancerous breath. Experiments with a wider population of volunteers to thoroughly probe the influence of diet, alcohol consumption, metabolic state and genetics are under way and will be published elsewhere.

Undoubtedly, clinical trials are the best choice for validating the efficiency of sensor arrays in the diagnosis of lung cancer. However, clinical trials are time-consuming and expensive. Therefore simpler approaches are called for, such as using

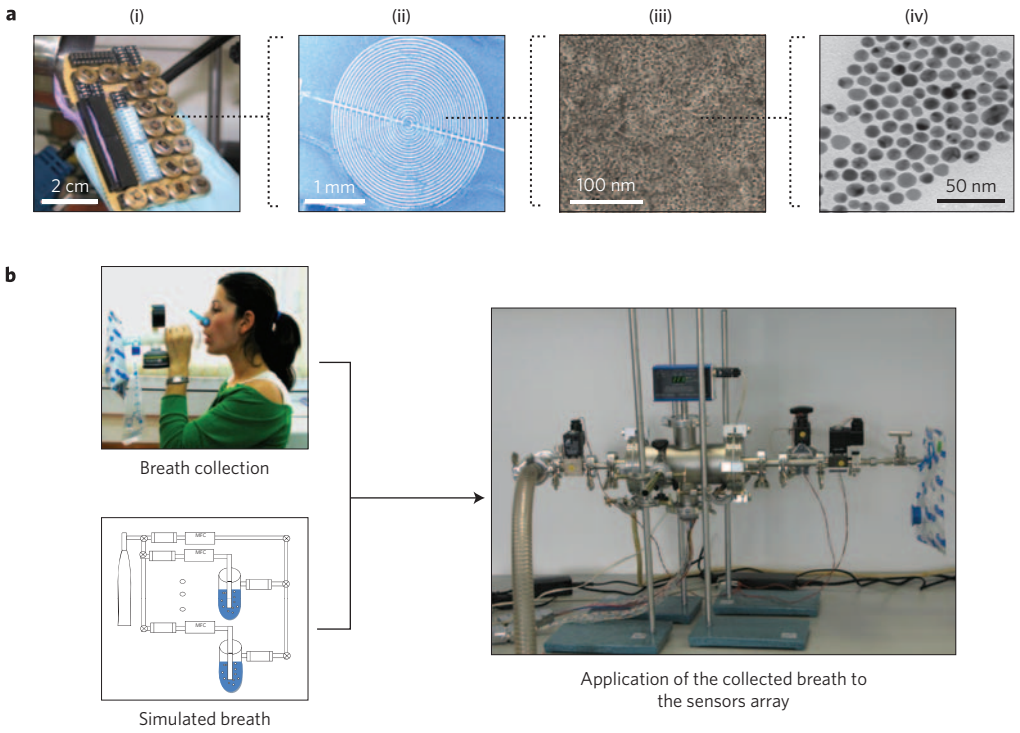

Figure 2 | Illustration of the diagnosis of lung cancer using breath testing. a, A photograph of the array of chemiresistors (**i**), a scanning electron microscopy image for the chemiresistor (**ii**), a scanning electron microscopy image of a gold nanoparticles film located between two adjacent electrodes (**iii**), and a transmission electron micrograph (TEM) of the monolayer-capped gold nanoparticles (**iv**). Note that in the TEM image, the gold nanoparticles appear as dark dots and the capping organic molecules appear as a bright medium between the adjacent dark dots. In these films, the metallic particles provide the electrical conductivity and the organic film component provides sites for the sorption of analyte molecules. **b**, Testing the exhaled breath (collected from patients) and simulated breath (that is, a mixture of representative VOCs at concentrations similar to those determined by GC-MS analysis of exhaled patient's breath) using the array of gold nanoparticle sensors.

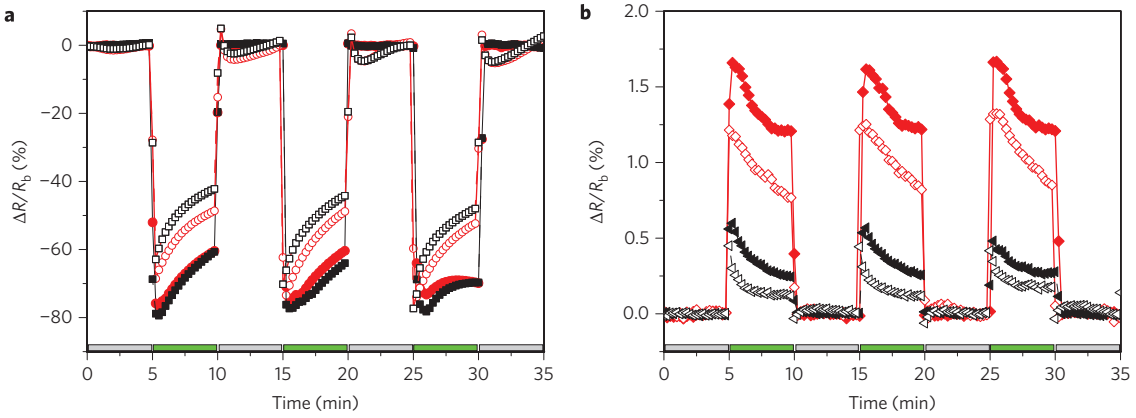

Figure 3 | Typical responses of the chemiresistors to real breath samples. a, Typical responses, $\Delta R/R_b$ (where R_b is the baseline resistance of the sensor in the absence of analyte and ΔR is the baseline-corrected steady-state resistance change upon exposure of the sensor to analyte), of 11-mercapto-1-undecanol–gold nanoparticles (red circles) and decanethiol–gold nanoparticles (black squares) upon exposure to healthy breath (filled symbols) and lung cancer breath (open symbols), as representative examples for sensors having positive responses. The sensors show a decrease in resistance, most likely due to increase in the permittivity of the organic matrix surrounding the metal cores[23,24]. **b**, Typical responses of 2-mercaptobenzoxazole–gold nanoparticles (red diamonds) and tert-dodecanethiol–gold nanoparticles (black triangles) upon exposure to headspace of healthy breath (filled symbols) and lung cancer breath (open symbols), as representative examples for sensors having negative responses. The sensors show an increase in resistance, most likely due to swelling that may increase the interparticle tunnel distance[23,24]. The grey bars on the *x*-axis indicate that the sensors are under vacuum. The green bars on the *x*-axis indicate that the sensors are exposed to either healthy or lung cancer breath.

'artificial' mixtures[26] of VOCs that simulate the cancerous and healthy breath, based on the GC-MS analysis of suitable breath samples[26,27]. This approach provides three main advantages over clinical studies in the development or adaptation of an array of sensors, because it can precisely determine[26,27] (i) the signature of each individual lung cancer biomarker on the sensor array, (ii) the correlation between the sensor's sensitivity and specificity for individual VOC biomarkers to its existence in a mixture of other compounds, and (iii) the necessary iterative feedback during sensor optimization without the intervention of

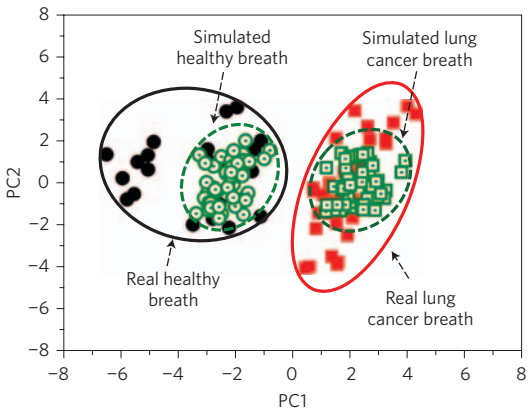

Figure 4 | Principal component analysis (PCA) of the dataset of real and simulated breath. Each data point corresponds to the multidimensional $\Delta R/R_b$ (where R_b is the baseline resistance of the sensor in the absence of analyte and ΔR is the baseline-corrected steady-state resistance change upon exposure of the sensor to analyte) of one breath sample and is the averaged response of 3–5 exposures. The results yield well-defined clusters for cancer states and healthy states, thus allowing fast, reliable and non-invasive lung cancer diagnosis. All data points were obtained using the same nine-sensor array described in the text and Methods section.

(disruptive) parameters, such as patients' diet, metabolic state, genetics and so on.

In the fourth phase of this study we prepared simulated breath patterns, based on the above-mentioned GC-MS analysis of lung cancer breath and healthy breath (see Methods). As observed in Fig. 4, the simulated clusters, obtained by the same nine-sensor array used earlier for the diagnosis of real breath, are well confined, with no overlaps occurring between the simulated healthy breath and lung cancer breath mixtures. Also, the simulated clusters are fully contained in the clusters resulting from actual breath testing. The perfect overlap between the clusters of simulated and actual breath samples indicates that the simulation approach is robust and serves our purpose of 'training' the array of sensors. The simulation experiments showed satisfying accuracy (>86%) and, furthermore, good reproducibility (>90%) when performed on different days. Also, it shows that our choice of the four most influential of 42 identified lung cancer biomarkers was justified. Our results also indicate that the sensitivity of our gold nanoparticle sensor array to the lung cancer biomarkers is hardly affected by the presence of water molecules (80% relative humidity in breath samples versus 10% relative humidity in simulated breath).

To summarize, we have demonstrated that an array of chemiresistors based on functionalized gold nanoparticles in combination with pattern recognition methods can distinguish between the breath of lung cancer patients and healthy controls, without the need for dehumidification or pre-concentration of the lung cancer biomarkers. Our results show great promise for fast, easy and cost-effective diagnosis and screening of lung cancer. The developed devices are expected to be relatively inexpensive, portable and amenable to use in widespread screening, making them potentially valuable in saving millions of lives every year. Given the impact of the rising incidence of cancer on health budgets worldwide, the proposed technology will be a significant saving for both private and public health expenditures. The potential exists for using the proposed technology to diagnose other conditions and diseases, which could mean additional cost reductions and enhanced opportunities to save lives.

Methods

Test population. Breath samples were taken from 62 non-smokers aged 28–60 years, who had not ingested coffee or alcohol for at least 1 h and 12 h, respectively (note that sampling 1 h after drinking coffee might not be enough to get rid of its mixture of odorants from the mouth). Forty of the subjects were patients with primary stage-3 and stage-4 lung cancer following clinical diagnosis at the Rambam Hospital (Haifa, Israel) by conventional diagnostic methods such as bronchoscope biopsy, computed tomography scan and pulmonary puncture. No breath collection was carried out in proximity (<4 days) to the biopsy. None of the patients had received chemotherapy or other cancer treatment before breath testing. The 56 healthy controls were chosen to match the lung cancer study group in age and lifestyle. The clinical characteristics of the study population of patients with lung cancer and healthy volunteers are listed in Supplementary Tables S1 and S2, respectively. All experiments were performed according to the guidelines of the Technion's committee for supervision of human experiments (Haifa, Israel) and only after each volunteer had signed a consent form.

Breath sampling. Exhaled breath was collected in a controlled way from individuals with lung cancer and from healthy subjects. The people tested cleared the inhaled air of ambient contaminants by repeatedly inhaling to total lung capacity for 5 min through a mouthpiece (Eco Medics) that contained a filter cartridge on the inspiratory port, thus removing more than 99.99% of the exogenous VOCs from the air during inspiration. Immediately after lung washout, the subjects exhaled through a separate exhalation port of the mouthpiece against 10–15 cm H_2O pressure to ensure closure of the vellum so that nasal entrainment of gas was excluded. Exhaled breath is a mixture of alveolar air and respiratory dead space air. In this study, subjects exhaled continuously into the breath collection kit, which, in turn, automatically filled the dead space air into a separate bag and the alveolar breath into a 750 ml Mylar sampling bag (Eco Medics). It should be emphasized that the described breath collection is a single-step process that does not require the volunteer to take care of changing between dead space and alveolar breath bags. The Mylar bags used in this study were made from polyvenyl fluoride, which is chemically inert with respect to most compounds in the breath. The Mylar bags were re-used and thoroughly cleaned before each use with flowing nitrogen (99.999% purity) gas for 5–8 min (note that GC-MS in combination with pre-concentration techniques has shown this purification process to eliminate >99% of the contaminants or VOCs from the Mylar bags). A minimum of five bags were collected from each person tested. Three of these bags were used for GC-MS analysis (one bag for each SPME fibre; see below), and the other two bags were used for analysis with an array of gold nanoparticle sensors (see below). All bags were analysed within two days from the time of breath collection, much earlier than the three-week storage period that was found in our control experiments to be the starting point for a correlation between the length of the storage period and the obtained results.

Chemical analysis of the breath samples. GC-MS (GC-6890N; MS-5975; Agilent Technologies) combined with SPME was used for chemical analysis of the tested breath. SPME was used for pre-concentrating the VOCs in the breath samples. A manual SPME holder with an extraction fibre was inserted into the Mylar bag. Three fibres with different coatings were used: (i) polydimethylsiloxane, (ii) polydimethylsiloxane-divinylbenzene and (iii) polydimethylsiloxane-carboxen (Sigma-Aldrich). Between 500 and 1,000 cm^3 of sampled breath was concentrated using the SPME method during an extraction period of 2 h and delivered to the GC-MS using a manual SPME holder. The extracted fibre in the manual SPME holder was inserted into the injector of the GC (splitless model). The oven temperature profile was: 60 °C, 2 min, 8 °C min^{-1} to 100 °C, 15 °C min^{-1} to 120 °C, 8 °C min^{-1} to 180 °C, 15 °C min^{-1} to 200 °C, 8 °C min^{-1} to 225 °C. Capillary column H5-5MS 5% phenyl methyl siloxane (30 m length, 0.25 mm i.d., 0.25 μm thickness) was used (Agilent Technologies). The column pressure was set to 8.22 psi and initial flow was 1.0 ml min^{-1}. The molecular structures of the VOCs were determined using the standard modular set. VOCs at concentrations below 0.08% were disregarded in the present analysis.

Synthesis of monolayer-capped gold nanoparticles. Monolayer capped 5-nm gold nanoparticles were fabricated by a modified two-phase method[28]. Dodecanethiol, decanethiol, 1-butanethiol, 2-ethylhexanethiol, hexanethiol, tert-dodecanethiol, 4-methoxy-toluenethiol, 2-mercaptobenzoxazole and 11-mercapto-1-undecanol were all purchased from Sigma-Aldrich and used as organic capping layers. $AuCl_4^-$ was first transferred from an aqueous $HAuCl_4 \cdot xH_2O$ solution (25 ml, 31.5 mM), purchased from Sigma-Aldrich, to a toluene solution by 80 ml of 34.3 mM phase-transfer reagent tetraoctylammonium bromide (Sigma-Aldrich). After the organic phase was isolated, excess thiols were added to the solution. The molar ratio of thiol:$HAuCl_4 \cdot xH_2O$ was varied between 1 : 1 and 10 : 1 depending on the kind of thiol to prepare a monodispersed solution of gold nanoparticles with an average size of 5 nm. For example, mole ratios of thiol:gold were 10 : 1 and 1 : 1 for dodecanethiol and butanethiol-capped gold nanoparticles, respectively, with a size of 5 nm. After vigorous stirring of the solution for 10 min, an aqueous solution of reducing agent $NaBH_4$ (Sigma-Aldrich) in large excess (25 ml, 0.4 M, ice-cooled) was added. The reaction occurred by stirring at room temperature for at least 3 h, producing a dark brown solution of the thiol-capped gold nanoparticles. The resulting solution was subjected to solvent removal in a rotary evaporator and followed by multiple washings using ethanol and toluene. Nanoparticles capped with 2-mercaptobenzoxazole were synthesized by the ligand-exchange method from pre-prepared hexanethiol-capped gold nanoparticles. In a typical reaction, excess

incoming thiol, 2-mercaptobenzoxazole (7 μg) was added to solution of hexanethiol-capped gold nanoparticles in toluene (3 mg ml^{-1}, 5 ml). The solution was stirred constantly for a few days to allow a fuller extent of ligand conversion. The nanoparticles were purified from free thiol ligands by repeated extractions.

Fabrication and design of the sensors array. An array of sensors was designed by combining nine different chemiresistors based on the gold nanoparticles described into a sensor array. Ten pairs of circular interdigitated gold electrodes were deposited by an electron-beam evaporator TFDS-870 (Vacuum Systems & Technologies) on a piece of device quality silicon wafer capped with 300 nm thermal oxide (Silicon Quest International). The outer diameter of the circular electrode area was 3,000 μm, and the gap between two adjacent electrodes and the width of each electrode both 20 μm. The functionalized gold nanoparticles were dispersed in chloroform by sonication and drop-cast onto the electrodes. While still coated with solution, the substrate was blown dry with nitrogen. This process was repeated several times to yield a desired resistance of about 1 MΩ. The device was dried for 2 h at ambient temperature and then baked at 50 °C in a vacuum oven overnight.

Breath testing with an array of sensors. Exhaled breath was analysed using the sensors array described above. The sensors were mounted into a custom polytetrafluoroethylene circuit board inside a stainless-steel test chamber with a volume of <100 cm^3. The sampling system delivered, in sequence, pulses of breath and ambient air to the sensors. Alternatively, the chamber could be evacuated. Each sensor of the array underwent a reversible change in electrical resistance when exposed to a vapour or analyte. The responses were unique because of the chemical diversity of the sensor materials. An Agilent multifunction switch 34980 was used to select the active sensor and measure the corresponding resistance at a given time. The entire system was computer controlled. Five analyses, at a minimum, were carried out on the exhaled breath of each sample.

In a typical experiment, signals from the sensor array elements were collected for 5 min in vacuum, followed by 5 min for breath that filled the chamber housing the array, followed by another 5 min of vacuum environment. The cycles were typically repeated 3–5 times to test reproducibility. Data analysis of the signals that were collected from all the sensors in the array was performed using standard PCA[21]. PCA is a statistical method to effectively reduce the multidimensional data space to its main components to allow convenient visualization of the differentiation ability of the sensor array. PCA determines the linear combinations of the sensor values so that the maximum variance between all data points can be obtained in mutually orthogonal dimensions.

Analysis of simulated breath by an array of sensors. A computer-controlled automated flow system was used to simulate 'healthy' breath and 'cancer' breath by supplying pulses of representative VOCs at a controlled fraction of their vapour pressures, based on the earlier GC-MS analysis, to the detectors[26]. As simulated lung cancer breath, we took a mixture of 145 ± 35 ppb ethylbenzene, 24 ± 4 ppb undecane, 67 ± 5 ppb 4-methyl-octane and 20 ± 5 ppb 2,3,4-trimethyl-hexane with 80 ± 1% relative humidity, 16 ± 1% O_2, 5 ± 1% CO_2 and 1.0 ± 0.2 ppm CO. As simulated healthy breath, we took 141 ± 28 ppb ethylbenzene, 51 ± 7 ppb undecane, 31 ± 5 ppb 2,6,6-trimethyl-octane and 10 ± 3 ppb 2,3,4-trimethyl-hexane with 80 ± 1% relative humidity, 16 ± 1% O_2, 5 ± 1% CO_2 and 1.0 ± 0.2 ppm CO. Dry purified air served as the carrier gas, and was obtained from a compressed air source. In a typical experiment, signals of sensor array elements were collected for 5 min of clean air, followed by 5 min of analyte vapour in air, and then followed by another 5 min of clean air to purge the system.

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Author contributions

H.H. conceived and designed the experiments. G.P. fabricated the sensors, performed the sensing experiments and analysed the pertinent data. O.A. conducted the synthesis and characterization of gold nanoparticles. M.H. conducted the GC-MS experiments. N.Sh. and U.B. collected the breath samples and investigated the effect of clinical characteristics of healthy volunteers. S.B., R.A.-B. and A.K. obtained the volunteers and diagnosed them clinically. U.T. and H.H. analysed the data and wrote the paper. All authors discussed the results and commented on the manuscript.

Additional information

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