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Sensing by Smell. Nanoparticle-Enzyme Sensors for Rapid and Sensitive Detection of Bacteria with Olfactory Output

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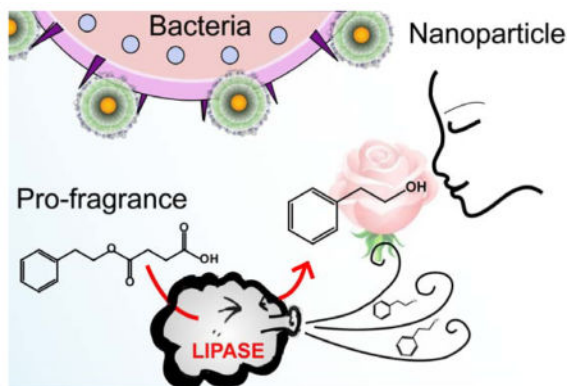
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Abstract

We present here a highly efficient sensor for bacteria that provides an olfactory output, allowing detection without the use of instrumentation, and with a modality that does not require visual identification. The sensor platform uses nanoparticles to reversibly complex and inhibits lipase. These complexes are disrupted in the presence of bacteria, restoring enzyme activity and generating scent from odorless pro-fragrance substrate molecules. This system provides rapid (15 min) sensing and very high sensitivity (10^2 cfu/mL) detection of bacteria using the human sense of smell as an output.

Graphical Abstract



Keywords

nanoparticles; human olfaction; enzymes; self-assembly; bacteria

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Additional experiments including (1) colorimetric assay for bacterial detection, (2) NMR of profragrance, (3) GC calibration curve, and (4) serial dilutions of the rose fragrance.

The human olfactory system has evolved to detect extremely low concentrations of volatile organic compounds present in complex environments.¹ Humans can discriminate more than 1 trillion olfactory stimuli, several orders of magnitude greater than their capability in visual discrimination.² This sensitivity and versatility makes olfaction a promising platform for biotechnological applications,³ however there have been few examples of the application of translation of sensor responses to olfactory outputs.⁴⁻⁷

Nanotechnology provides new opportunities to redefine the bounds of human perception.⁸ There have been a wide variety of examples where the intrinsic properties of nanomaterials have been used to generate visual output,^{9, 10} with additional examples of nanomaterials modulating other colorimetric processes.¹¹⁻¹³ Engineered nanomaterials have also been shown to influence the behavior of fragrance molecules.¹⁴ In a recent study, Weder *et al.* demonstrated cellulose nanocrystals functionalized with pro-fragrance molecules that could be used to control the production of volatile compounds.⁴ These covalently bound complexes remain odorless until functional groups are cleaved in response to specific external stimuli, generating pungent aroma molecules.⁵ Taken together, we hypothesized that pro-fragrances in combination with surface-engineered nanomaterials could provide reactive constructs to transduce molecular interactions into outputs that could be 'read out' through our sense of smell, providing a useful sensor modality for detection of bacteria that provides a potential strategy for combatting the threat of bacterial drinking water contamination that contributes to over 1.5 million deaths worldwide a year.^{15,16}

We use a supramolecular-based approach to generate an effective smell-based sensor platform for bacteria. The system is comprised of three tunable components: 1) surface functionalized nanoparticles, 2) pro-fragrance molecules, and 3) enzymes to cleave the pro-fragrances to generate the olfactory output (Figure 1). In this sensor, the surface moieties of the nanoparticles behave as both selective recognition elements for analytes present in solution and to reversibly inhibit the complexed enzymes.¹⁷ The pro-fragrance molecules¹⁸ provide a 'turn-on' response for the sensor system, going from odorless to strongly odiferous upon cleavage by the enzyme. Finally, the enzyme provides a strategy for amplifying the output, generating multiple fragrance molecules per recognition event.¹⁷ Bringing these components together provides a sensitive sensor system for bacteria, allowing human subjects to rapidly detect bacteria in solution at levels as low as 10² cfu/mL, a relevant limit of detection for overall bacterial load in drinking water, and consistent with other recently published sensor systems.^{19,20,21,22}

Results and Discussion

Our sensor design uses nanoparticles to both recognize the bacteria and to inhibit the fragrance-generating enzyme. We chose AuNPs possessing ligands with terminal benzyl headgroups, as these nanoparticles have been shown to interact strongly with the anionic cell surface of bacteria.^{23,24} We used the robust and industrially used *Candida Rugosa* lipase as the enzymatic amplifier,²⁵ relying on the negative charge of the protein to provide electrostatic complementarity with the cationic nanoparticle, and hence inhibiting catalysis.^{11,17,26,27} Given the ability of human olfaction to discern an enormous variety of scents, we had a wide range of pro-fragrance options to choose from. We ultimately chose the succinic

acid ester of phenylethyl alcohol (SAEPE) as our substrate/pro-fragrance, due to the low odor threshold of phenylethyl alcohol,²⁸ coupled with the orthogonality of the pleasant rose scent with odors commonly found in contaminated drinking water.

We initially performed a colorimetric assay to optimize the AuNP:lipase ratio required for inhibition. These studies were performed using p-nitrophenylbutyrate (pNPB) in sodium phosphate buffer solution (5 mM, pH 7.4). As shown in Figure 2, an approximately 3:1 AuNP to lipase ratio provided essentially complete inhibition of the lipase. This AuNP:lipase ratio was used to generate the nanozyme complex for all further studies. This colorimetric assay was able to detect both Gram positive and negative bacteria, including: *E. coli*, *B. subtilis*, *M. luteus*, and *P. aeruginosa* (Figure S1), indicating the generality of the enzyme activation process.

We next turned to bacterial sensing using the enzyme platform, beginning with an instrument-based analytical strategy. These studies used *E. coli* as a non-pathogenic “safe” bacteria strain to minimize health concerns in both the instrumental and human studies.²⁹ Solutions of the sensor elements were incubated for 30 minutes prior to the addition of the pro-fragrance. We then used headspace gas chromatography to quantify the production of scent generated by our bacterial sensor.³⁰ The concentration of the volatile product present in the headspace of the sample vial was quantified according to an external calibration curve (see Supporting Information). As shown in Figure 3, the uninhibited lipase cleaves significantly more pro-fragrance than the nanoparticle-enzyme complex and controls. Significantly, no signal was observed using the substrate alone and bacteria, indicating that the bacteria do not hydrolyze the pro-fragrance in the timeframe studied. As expected, the sensor system generated measurable and distinctly different signals in the presence of 10^4 and 10^6 cfu/mL of *E. coli*.

Having established the generation of fragrance output, we next determined the ability of humans to serve as “detectors”. Ten volunteers were asked to smell glass vials at two time points: 1 minute as a control, and 15 minutes for sensing, with the interval chosen to ensure olfactory clearance.³¹ They ranked the samples in order from least/no smell (1) to strongest smell (5), and the raw ranking order data were analyzed using the Kruskal-Wallis H-test. Initial studies focused on the sensitivity of the scent response. As expected, participants were able to detect the phenylethyl alcohol at both time points (Figure 4a). In contrast, the enzyme-substrate pair was not detected at 1 minute, but readily discerned at 15 minutes. In our sensing studies, no significant difference in response was observed across conditions at 1 minute under any conditions; however after 15 minutes participants were successfully able to detect *E. coli* concentrations at both 10^2 and 10^4 cfu/mL (Figure 4b) with high significance relative to the controls. Interestingly, 10^2 cfu/mL of *E. coli* did not produce a detectable signal using gas chromatography, demonstrating that human olfaction was more sensitive than the chromatographic method.

Conclusions

In summary, we report here the development of a supramolecular-based sensor that uses the human olfactory system to read out the response. This sensor was able to detect bacteria

with high sensitivity. These studies demonstrate that by controlling the behavior of responsive nanomaterials at the molecular level, we can alter how human beings observe their surroundings in a manner that is otherwise impossible. We believe this responsive strategy can be broadly applied to other surface functionalized nanoparticles and enzymes to provide sensing of a wide variety of analytes, with the availability of an almost limitless number of aroma profiles providing versatility unavailable with other transduction strategies.

Materials and Methods

All reagents/materials were purchased from Fisher Scientific and used as received. Benzyl functionalized AuNPs were synthesized according to previous reports.³²

Bacteria Growth Conditions

Bacteria were cultured in LB medium at 37 °C and 275 rpm until stationary phase. The cultures were then harvested by centrifugation and washed with 0.85 % sodium chloride solution for three times. Concentrations of resuspended bacterial solution were determined by optical density measured at 600 nm. 5 mM sodium phosphate buffer was used to make dilutions of bacterial solutions.

Plate Reader Assay

Lipase inhibition assay was done at 25 °C with the final concentrations in Costar clear 96 well plate of 15 nM lipase, 0.6 mM pNPB, and 20, 40, 60, 80, 100 nM benzyl AuNP. Lipase and benzyl AuNP were first incubated for 30 minutes in 96 well plate to insure their interaction reaches equilibrium, then 10 μ L of substrate p-NPB was added into the well. The activity of lipase was monitored every 30 seconds for a total of 40 minutes time frame at the absorbance of 405 nm.

Human Trial Assays

Olfactory detection of lipase activity

Four different solutions were made in 20 mL glass vials with a final volume of 1 mL each. The volume of 1 mL was chosen to maintain the easy-to-use format of the sensor for eventual on-site detection use. 5 mM sodium phosphate buffer and 4 mM SAEPE were used as the negative controls and the rose scent (2-Phenylethyl alcohol) was used as the positive control, a strong standard. The activity of lipase was assessed by incubating 100 nM of lipase with 4 mM of SAEPE for 20 minutes. The participants were asked to smell these samples and rank them in the order from 1 to 5 with 1 has the lightest smell and 5 has the strongest smell.

Olfactory detection of *E. coli*

The same procedure was followed as above for buffer and sensor samples. For the *E. coli*-containing vials, 100 nM lipase was incubated with 300 nM benzyl AuNP for 30 minutes, and then 10 μ L of *E. coli* was added into each vial so that the final concentrations of *E. coli* in each vial are 10^2 and 10^4 cfu/mL.

Gas Chromatography Head-Space Analysis

Headspace phenylethyl alcohol was measured using a gas chromatography (model GC-17A, Shimadzu Co., Tokyo, Japan) equipped with a solid-phase microextraction (SPME) auto injector (model AOC-5000, Shimadzu Co., Tokyo, Japan). Samples (1 mL) in 20 mL glass vials capped with aluminum caps with polytetrafluoroethylene (PTFE)/silicone septa. Samples were prepared using 500 nM lipase, 1.5 μ M benzyl AuNP, and 4 mM of SAEPE. A 50/3 μ m divinylbenzene (DVB)/carboxen/polydimethylsiloxane (PDMS) stable flex (SPME) fiber (Supelco Co., Bellefonte, PA) was then inserted into the vial headspace for 2 min to absorb volatiles. The fiber was transferred to the GC injector port (250 °C) for 3 min. The injection port was operated in split mode, and the split ratio was set at 20:1. Volatiles were separated on a fused-silica capillary Equity-1 Supelco column (30 \times 0.25 mm inner diameter \times 25 μ m) coated with 100% PDMS at an initial oven temperature of 70 °C to final temperature of 220 °C over 10 min (step rate 15 °C/min). A flame ionization detector was used at a temperature of 250 °C. Phenylethyl alcohol concentrations were determined from peak areas using a standard curve made from dilutions of phenylethyl alcohol in 5 mM sodium phosphate buffer. Each measurement was performed in triplicate and results were expressed as mean values \pm standard deviation.

Kruskal-Wallis H-test

Kruskal-Wallis test is a non-parametric version of one-way ANOVA which is applied when the assumption of normal (Gaussian) distribution is not met. This test can compare the medians of multiple samples to determine if they come from the same population or not. This methodology uses ranks of the data to compare the test statistics. To do so, the results from all groups are pooled and arranged in rank order from smallest to largest. The numeric index of this ordering is then used to evaluate the null hypothesis (sample are coming from the same distribution) using chi-square statistics. MATLAB software (MATLAB and Statistics Toolbox Release 2012b, The MathWorks, Inc., Natick, Massachusetts, United States) was used to perform Kruskal-Wallis test.^{33,34}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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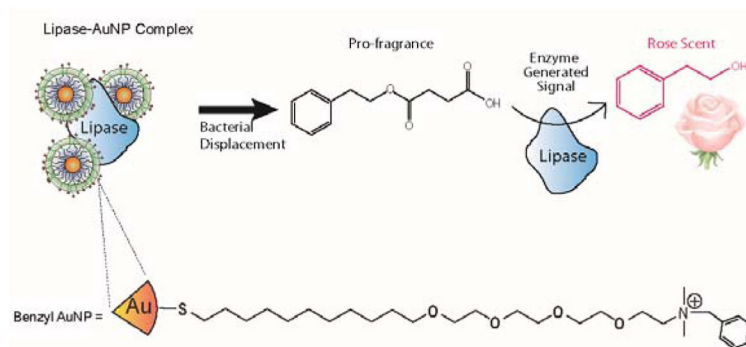


Figure 1. Schematic representation of sensor elements used in this study. Cationic AuNPs bind with the anionic enzyme inhibiting the catalysis of the pro-fragrance into scent. Bacteria present in solution compete for the AuNP surface and displace the enzyme inducing the production of the rose fragrance.

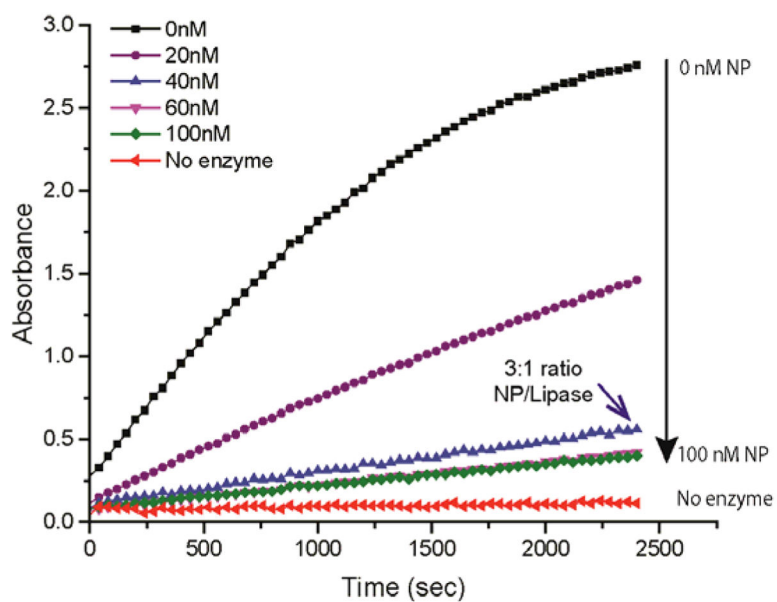


Figure 2. Lipase inhibition assay in the presence of benzyl AuNP. Lipase (15 nM) was incubated with a series of benzyl AuNP concentrations before adding the colorimetric substrate p-NPB (0.6 mM).

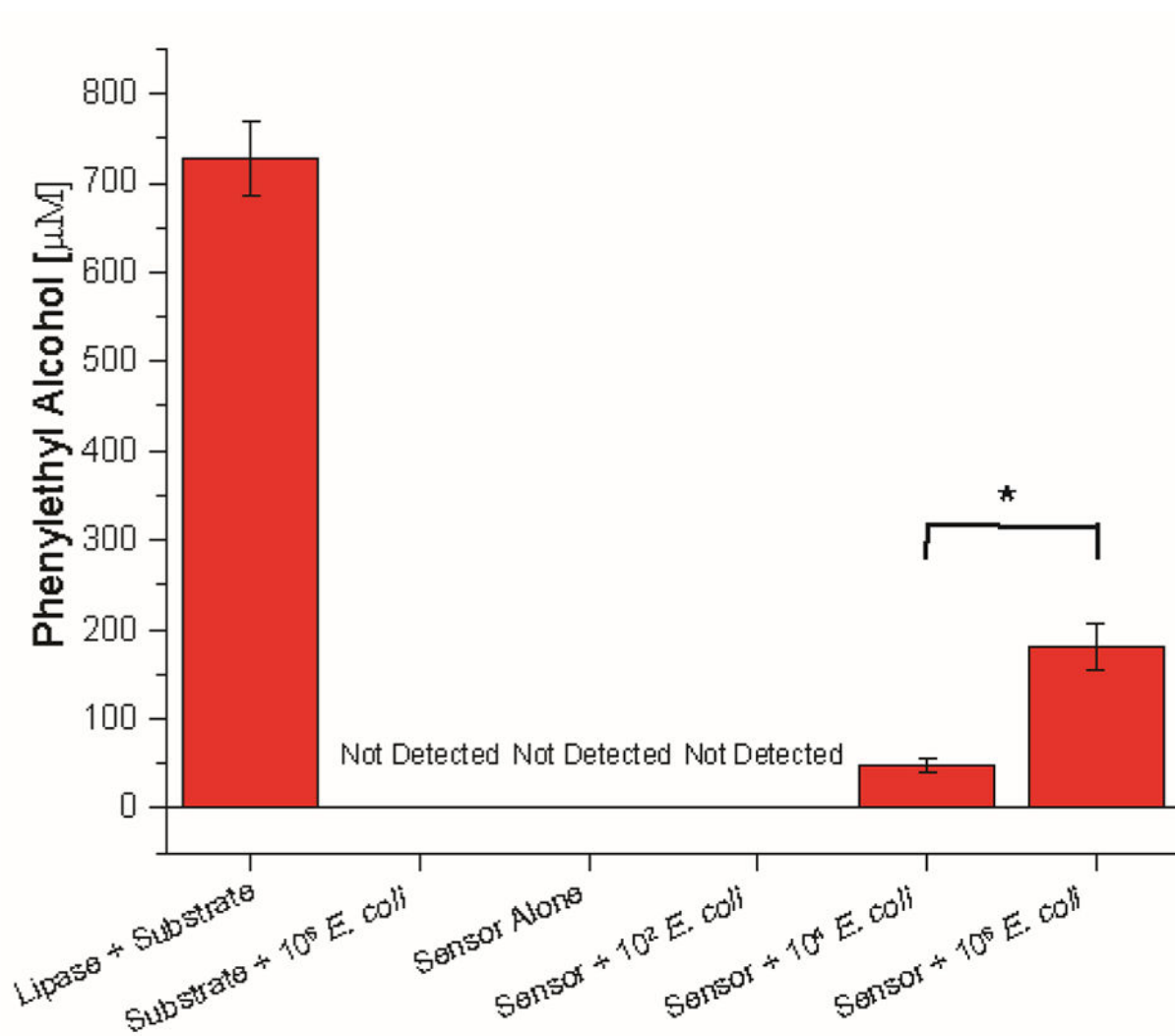
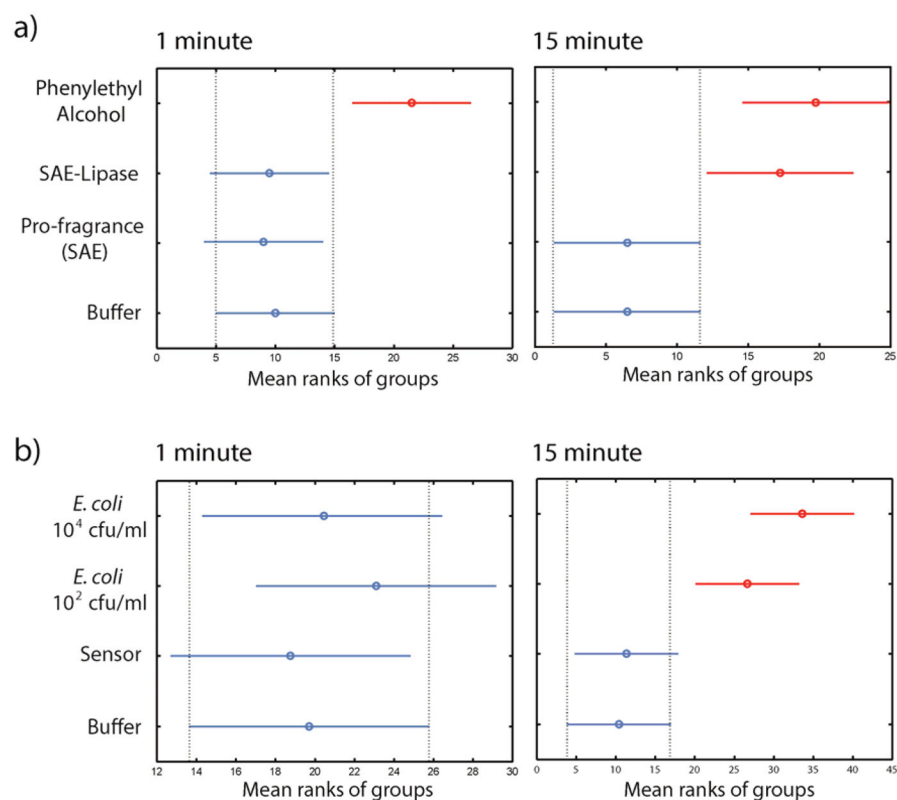


Figure 3.

Headspace gas chromatography analysis of sensor response to increasing concentrations of bacteria. Samples were prepared in triplicate. Error bars represent standard deviations of the measurements. * = $p < 0.05$, *** = $p < 0.001$.

**Figure 4.**

Human olfactory detection studies. (A) Lipase activity test in the presence of the pro-fragrance SAEPE was carried out with six participants. SAEPE only and 5 mM Phosphate buffer were used as the negative control. The hydrolyzed form of SAEPE was used as the positive control (strong standard). Hydrolyzed SAEPE and SAEPE in the presence of uninhibited lipase are significantly different from the negative controls SAEPE alone ($p < 0.01$ and $p < 0.01$, respectively) after 15 minutes. (B) With ten participants, olfactory detection of *E. coli* at 10^2 and 10^4 cfu/mL were compared to the controls of just buffer and sensor only after 15 minutes. The olfactory signals from the vials which contained 10^2 and 10^4 cfu/mL of *E. coli* are significantly different from the signal from the sensor-only vial ($p < 0.001$ and $p < 0.0001$, respectively).