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A New Microfabricated Nanocalorimeter: Design, Characterization, and Chemical Calibration

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Abstract

A microfabricated titration calorimeter having nW sensitivity is presented. The device is achieved by modifying a commercial, suspended-membrane, thin-film thermopile infrared sensor. Chemical reactions are studied by placing a 50.0 nL droplet of one reagent directly on the sensor and injecting nL droplets of a second reagent through a micropipette by means of a pressure-driven droplet injector with 1% reliability in volume delivery. External thermal noise is minimized by a two-layer thermal shielding system. Evaporation is prevented by positioning the micropipette through a tiny hole in a cover glass, sealed by a drop of oil. The device is calibrated using two acid-base reactions: H_2SO_4 + HEPES buffer, and NaOH + HCl . The measured power sensitivity is 2.90(4) V/W, giving a detection limit of 22 nW. The 1/e time constant for a single injection is 1.1 s. The day-to-day power sensitivity is reproducible to ~2%. A computational model of the sensor reproduces the power sensitivity within 10% and the time constant within 20%. For a 50 nL sample and 0.8 to 1.5 nL titrant injection volumes, the heat uncertainty of 44 nJ corresponds to a 3 σ detection limit of 132 nJ, or the binding energy associated with 2.9 pM of IgG-protein A complex.

Microcalorimetry is widely used to measure enthalpy changes in chemical reactions, biochemical processes, and phase transitions, with typical detection limits of μW or μJ .¹⁻⁷ In the last decade, micromachining techniques^{8,9} have been used to produce calorimetric devices with greatly reduced sample volumes and detection capabilities in the nW range.¹⁰⁻¹³ There is a growing realization that these advances could enlarge the role of calorimetry in drug discovery,¹⁴ optimization of yeast metabolism¹⁵ and the study of

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bioprocess dynamics¹⁶. Low-cost enthalpy arrays are particularly attractive.^{17;18}. In general, microfabricated nanocalorimeters possess such high sensitivity that they work at essentially constant temperature and hence qualify as isothermal calorimeters.¹⁹ One such device is designed with a microfluidic reaction chamber and two inlet and one outlet channels,²⁰ while others are flow-through types built on microfabricated silicon chips.²¹⁻²⁷ Electrical calibration is routinely used for both microcalorimeters and for this recent generation of nanocalorimeters,²⁸ primarily because it is convenient and more precise than the calorimetric signal with which it is compared.²⁹ However, the spatiotemporal profile for the heat from an electrical heater may not be representative of that for the heat involved in the process being studied, so it is desirable to use calibration procedures that more closely replicate the conditions of the experiments. For this purpose, a number of standardization processes have been employed.^{19;29-31}

Here we describe a microfabricated commercial thermopile infrared (IR) sensor (S25 from Dexter Research Center, Inc.), which we have modified into a nanocalorimeter suitable for biological and chemical calorimetric measurements. The S25 is a thin-membrane, silicon-based thermopile sensor, with sensing junctions at the center of the membrane. The planned experiments for this device involve small numbers of metabolically active cells occupying a volume of ~50 nL, so the present calibration experiments employ droplets of this volume (base droplets) placed at the center of the membrane (the sensitive area). Reactant droplets (~1 nL) are injected into the base droplet through a micropipette by a pressure-driven droplet injector (PicoSpritzer II, Parker Hannifin). The power sensitivity of the device is calibrated using two chemical reactions: 0.01 M H₂SO₄ with 0.2 M 7.5 pH Hepes buffer, and 0.01 M NaOH with 1M HCl. These experiments yield a calibrated power sensitivity of 2.90(4) V/W. For its response time of ~1 s, the detection noise is 21 nV, which translates into a detection limit (three standard deviations above background) of 22 nW. This is comparable to the sensitivity of 13 nW reported by Johannessen *et al.*¹³ A computational model designed to simulate the response of the sensor predicts a sensitivity of 3.05 V/W, which is in good agreement with our experimental results.

Experimental Section

Implementation of the Calorimeter

The S25 silicon-based thermopile sensor (Dexter Research Center, Inc., Dexter, MI) is shown in Figure 1. It has a twenty-junction thermopile with a Seebeck coefficient of 24 μ V/K per junction. The thermopile is assembled under a thin (~1.5 μ m) membrane of SiO₂/Si₃N₄. The metal tracks are directly below the membrane, and the sensing junctions are clustered at the center. Surrounding the free-standing membrane (the square shown in Figure 1D) is the aluminum heat sink that is attached to the porcelain body of the S25. The 0.5 mm well is formed by the membrane/heat sink at the bottom and the porcelain body of the sensor surrounding it. This well is modified to serve as our reaction chamber.

The voltage signal generated by the S25 sensor is detected using an amplifier with a gain of 10⁵, then recorded by a PCI-6024E data acquisition board (National Instruments) in a desktop computer. A LabVIEW interface is used to control the experiment and display the readings.

Electrical Noise Characterization

The S25 has an intrinsic noise voltage of 19.4 nV/ Hz. We use a low-noise instrumentation amplifier and chopper-stabilized operational amplifier to minimize additional contributions to the intrinsic Johnson noise. In addition, a metal amplifier chassis and shielded cables are used to avoid interference from external electrical noise. The resulting output noise, as measured from the direct Fourier transform of a 600 second signal, is shown in Figure 2. Comparable frequency spectra were obtained with a spectrum analyzer (Hewlett-Packard 4562). A low-pass filter with 10-Hz cutoff frequency was used to produce Figure 2, so the noise voltage density towards the 10Hz end is reduced even below the sensor's stand-alone noise voltage. At 1 Hz, the system produces 20 nV/ Hz noise voltage density, which is only slightly above the sensor's intrinsic noise. In the experiments, however, a 1-Hz low-pass filter was used instead of the 10-Hz filter, giving a total noise of 21.4 nV. The S25's nanowatt sensitivity corresponds therefore to a temperature noise of 44 μ K in a 1 Hz bandwidth, qualifying this device as a heat-conduction isothermal nanocalorimeter.¹⁹

Thermal Shielding and Evaporation Prevention

All experiments were conducted at ambient temperature ($\sim 23^{\circ}\text{C}$), without active temperature control. However, the system was isolated thermally from the environment by a double-layer thermal shielding system. Such isolation is important, because for the $\sim \mu\text{J}$ reaction heats involved here, the measured temperature differences are $\lesssim 10$ mK. The system also has to be evaporation-free, since sample evaporation can produce a large, time-dependent signal.²⁸ Calorimetric experiments on such small volumes without evaporation protection have been done by Neugebauer *et al.*,³² however, their method cannot be used to study biological processes, which is a major goal for developing this system. In addition, a method for delivering reactant liquid must be included in the design of the system. Our system, illustrated in Figure 3, accommodates these needs.

The outer shield (Figure 3A) encloses the thermal sensor and incorporates the microscope objective. It is made from a Cu tube for good electrical and convection shielding. The lid of the outer shield is attached to the microscope objective. The Cu tube is attached to the upper surface of the amplifier box, and the sensor is inserted into a socket mounted on top of the box. When the objective is lowered for observation, the lid firmly closes and thus seals the sensor compartment. The microscope is only used to position the microinjectors and is turned off during the experiment to avoid any parasitic thermal power. An inner shield is created by placing a glass cover slide on the ceramic body of the S25 sensor (Figure 3B). The cover glass shields the sensor from external thermal noise and prevents droplet evaporation. A ~ 600 μm diameter hole drilled through the cover glass allows access to the reaction chamber via the micropipette for the injection of reagent. The hole is covered with a Mylar film that has a ~ 100 -200 μm three-corner perforation, and the chamber is hermetically sealed with a single oil droplet after insertion of the pipette. Surface tension prevents the oil from leaking into the reaction chamber.

Sub-nanoliter Sample Delivery

Liquid reactant is delivered through micropipettes pulled from 1.12-mm ID glass capillaries by electrically controlled pressure pulses from a commercial droplet injector (PicoSpritzer

II, Parker Hannifin). Figure 4 illustrates the injection process. The position of the micropipette is controlled by a micromanipulator (5171, Eppendorf). Initially, the micropipette is away from the sensor membrane. It is then moved close to the reaction volume on the surface of the membrane, where the nL injections are made. The micropipette is then retracted to its original position inside the chamber.

The calibration experiments employed 50 nL droplets placed at the center of the membrane, with subsequent titration injections of ~ 1 nL. The latter were done using micropipettes having tip openings of 2 - 4 μm , together with appropriate pressure (~ 30 PSI) and pulse duration (~ 20 - 50 ms) of the PicoSpritzer. The injection volume was determined by measuring the total volume dispensed over a large number (~ 104) of such injections. This total dispensed volume was calculated from the inner diameter of the glass capillary (measured to ± 2 μm with a microscope) and the length of liquid dispensed (~ 1 cm, measured to ± 0.1 mm with a caliper), giving an uncertainty in measured average droplet size of $\sim 1\%$. The drop-to-drop variation in volume from the PicoSpritzer could not be determined directly but has been demonstrated to be less than 3% under similar circumstances.³³ Experiments were typically started 10 minutes after deposition of the base droplet to allow all system components and reagents to reach thermal equilibrium. Because the tip region of the micropipette has a very large surface-to-volume ratio, is surrounded by the thermal enclosure, and is not subject to evaporation, and because the body of the micropipette is also thermally connected to the top of the shield by means of the oil drop, we concluded that the droplets would be isothermal with the sample immediately beneath them and hence thermal control of the micropipette was unnecessary. Control experiments injecting ~ 1 nL DI water droplets into the 50 nL DI water droplet on the sensor membrane resulted in no measurable temperature increase, demonstrating that the system is close to thermal equilibrium.

Calibration Experiments

Most of the calibration experiments employed 0.01 M H_2SO_4 injected into 0.2 M pH 7.5 HEPES solution ($H^\circ = -53.4$ kJ/mol H_2SO_4 at 23°C ¹⁷). The acid solution was prepared by dilution of 98% H_2SO_4 (Fisher) and was standardized by titration. The HEPES buffer was made from powder (Sigma-Aldrich), and its pH was adjusted to 7.5 by adding KOH. The solution was stored at 4°C . Ten constant-volume injections were made in a single experiment. The injection volumes for different experiments ranged from 0.65 to 1.54 nL. Each injection increases the volume by 2%, which is small compared with the total volume, and we assumed in our calibration that the total heat generated by dilution per droplet was constant for all injections.²⁹

Some experiments were also done with 0.01 M NaOH injected into 1M HCl solution. The NaOH solution was standardized by titration with potassium hydrogen phthalate (KHP), and the HCl was prepared by diluting 37% acid with DI water (Fisher). For the neutralization of NaOH with HCl, the heat of reaction at 25°C is -55.84 kJ/mol at infinite dilution; correction for finite concentrations and conversion to 23°C gives -58.2 kJ/mol.

Results and Discussion

Modeling the calorimeter

The calorimeter was modeled using a simplified geometry and a finite element method to solve the diffusion equation for heat. The sensor and the sample were approximated as a cylindrically symmetric system and thus modeled in cylindrical coordinates. Figure 5a provides details of the geometry for modeling. The heat diffusion equation in cylindrical coordinates is

$$\frac{\partial}{\partial r} \left(k \frac{\partial T}{\partial r} \right) + \frac{\partial}{\partial \theta} \left(\frac{k}{r^2} \frac{\partial T}{\partial \theta} \right) + \frac{\partial}{\partial z} \left(k \frac{\partial T}{\partial z} \right) + S = \rho C_p \frac{dT}{dt} \quad (1)$$

where k is the thermal conductivity, S is the heat source power density, ρ is the density and C_p the heat capacity. All parameters are based on the specific materials and the geometry as explained in Figure 5. The exact composition of the membrane is considered proprietary information by the manufacturer and hence is unavailable, so we assumed that the membrane is a two-layer structure consisting of 500 nm of silicon dioxide and 1000 nm of silicon nitride, based on the common structure of such sensors and the limited information provided by Dexter. The model was programmed in MATLABTM and was applied for two situations: for constant power supplied to the sensor and for an impulse of heat transferred onto the sensor.

The results of the constant power simulations for different reaction volumes are shown in Figure 5c. The depicted sensor temperature at the sensing junctions is linearly related to the sensor's output voltage by the Seebeck coefficient. The added sample volume thermally shorts the membrane and reduces the sensor's response. The magnitude of the change in the thermal signal with droplet size is determined not only by the difference in the heat capacity of the droplet in which the reaction is occurring, but also by the change in the thermal conductance between the edge of the droplet and the edge of the sensor membrane. This becomes particularly obvious when there is an additional oil cover filling the sensor enclosure, which completely shorts the thermal path from the sensing junctions on the membrane to the reference junctions on the sensor body. (An oil layer was previously used in the literature to prevent evaporation and provided the motivation for the modeling.)

The time response of the sensor also increased with increasing sample volume due to the additional thermal mass. In our experiments the sample volumes never exceeded 65 nL, which is well below the volume that would thermally short the membrane to the sensor body. Furthermore, variations in the time constant in this range of sample volumes are small compared with the experimental noise in determining the time constant. The expected power sensitivity and time constant for the four configurations are listed in Table 1. The simulation results for impulse heat input will be presented later in comparison with our calibration experimental data.

Calibrated Power Sensitivity

Figure 6 shows results obtained from one calibration experiment. Ten injections were made during the course of the experiment, each with an estimated 0.651 nL injection volume and expected 0.346 μ J heat production. By comparing this heat with the integrated sensor output above background for one injection (see Figure 7), we calculate the power sensitivity of the nanocalorimeter. Results of a number of such experiments are summarized in Table 2. The uncertainties given in the table are the standard deviation from the mean for the ten injections constituting an experimental run. The first four of these were in the nature of preliminary runs, using different reactions, injection volumes, and sensors. The last four were done with a single sensor to check day-to-day reproducibility. However, in general even the runs done with different sensors showed good overall agreement with the more carefully conducted runs: the last four alone give 2.90(4) V/W; a weighted average of all eight yields 2.91(3) V/W. Based on our model calculation, the sensitivity of the sensor decreases by 3% when the volume increases from 50 nL to 60 nL during the 10 injections used for a calibration. Since the baseline drift is of the same order of magnitude we did not correct for the increase in sample volume. However, corrections would be needed for more significant volume increases.

The uncertainties in Table 2 are larger for smaller injection droplets, suggesting that these results may be manifesting a constant heat uncertainty σ_q , related to the limited ability to estimate peak areas above the background in traces like those in Figure 7. When interpreted this way, these data yield $\sigma_q = 44$ nJ for a single heat

Modeled Impulse Response

The sensor's response to a single injection is its response to an impulse of heat, because the time required for diffusion and chemical reaction ($\sim \mu$ s) is far less than the ~ 1 -s time constant of the nanocalorimeter for a 50-nl sample. The experimental response to a single injection in Figure 7A is compared with the modeled response in Figure 7B. Taking the time constant as the $1/e$ time, the experimental value is 1.1 s, compared with 1.3 s resulting from the model for the same assumed heat impulse. This level of agreement is considered quite good.

It is worth noting that both the time response and the power sensitivity are specific results for our adopted base volume of 50 nL, which was chosen to be representative of intended biological experiments. Since the device responds directly to temperature changes, smaller base droplets would give larger power sensitivities and smaller σ_q . Thus, for example, the model shows that titration experiments could be done with 5-nl droplets at a power sensitivity two times greater (and σ_q eight times smaller due to the reduced time constant) than that obtained for 50-nl droplets. The magnitude of the change in the thermal signal with droplet size is determined not only by the difference in the heat capacity of the droplet in which the reaction is occurring, but also by the change in the thermal conductance between the edge of the droplet and the edge of the sensor membrane.

Conclusion

We have described the use of a microfabricated infrared-sensor-based nanocalorimeter with power sensitivity and power resolution comparable to the best previously described nanocalorimeters. Further, we incorporate specific features designed to facilitate biological measurements with sub-second response times. These include a novel system for delivering sub-nl droplets of reactants while avoiding evaporation. Chemical calibration experiments demonstrate that the device has $\sim 2\%$ day-to-day reproducibility in its power sensitivity. It can be operated with better than 1% reliability for delivery of specified volumes of reagents down to sub-nl levels. Furthermore, we successfully implemented a model capable of predicting the thermal response of the system, thereby providing us with a quantitative means for optimizing the performance of the entire system and measurement protocols and for quantitative analysis of the data.

It is instructive to compare our nanocalorimeter (nCal) with other techniques for measuring molecular interactions. A recent report³⁴ compared the Microcal isothermal titration calorimeter (ITC),³⁵ enthalpy arrays,¹⁸ and back-scattering interferometry (BSI).³⁴ In Table 3, we extend the comparison by selecting the IgG-protein A reaction observed with BSI as a benchmark and use the supplementary data from Ref. 34 to obtain the 45 kJ/mole heat of reaction. Our heat uncertainty of 44 nJ (for a 50 nL sample and 0.8 to 1.5 nL titrant injection volumes) corresponds to a 3σ detection limit of 132 nJ, or 2.9 pM of IgG-protein A complex. This is almost thirty times more sensitive than BSI. While the stated probe volume for BSI is 350 pL, this does not include the unused sample in the capillary outside the sensing volume. The nCal volume is 50 nL for our present measurements, but could be reduced substantially, limited only by the accuracy with which the droplet volume can be controlled. With nCal, all delivered sample is measured. Our injection technique could be adapted for high-throughput screening, including microfluidics. We conclude that nCal is significantly more sensitive than the other techniques. However, nCal does require a heat signature, so for processes with low heat generation, BSI may be better.

Our nCal system has the potential to replace standard titration microcalorimetry methods. Its high response speed immediately suggests its applicability to kinetics studies on processes 100-1000 times faster than those currently amenable to study with ITC. Extension to a multiple sensor format should be straightforward.¹⁸ With attention to temperature control and a more sophisticated droplet delivery system, this technique should yield high-throughput results as reliable as those currently available from ITC, with a factor of 10^4 reduction in reactant materials.

Acknowledgments

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Figure 1.

Photographs of S25 thermopile sensor with the original cover removed. A) Top view. The membrane is at the bottom of the 0.5-mm deep well in the center. B) Tilted view showing the package base. C) Blowup of the transparent sensor membrane with an added scale bar. The black dots in the center are the thermopile junctions on the underside of the membrane. D) View of the sensor membrane from the underside. The metal junctions form the center “X,” and the metal tracks extend to the reference junctions at the edge of the membrane on the Al heat sink (dark). (The reference junctions are shielded by the heat sink when viewed from the top.)

Figure 2.

Voltage noise spectral density S_v of our calorimetric system and a low-pass filter with a cutoff frequency of 10 Hz.

Figure 3. Integrated system for thermal shielding, evaporation control, and sample delivery. A) Overall system view. B) Detailed view of sensor seals (inner shield) and injecting micropipette.

Figure 4.

Injection of a 20-nl droplet onto the center of the sensor. A) Pipette tip away from the sensor membrane. B) Pipette tip at the center of the membrane. C) Droplet delivered and pipette tip retracted to its original position.

Figure 5.

Modeling of the square sensor as a cylindrically symmetric system. A) Schematic illustrations with a lateral section and a top view. B) Dimensions of the modeled device. C) Temperature response following the application of constant power. Four configurations are modeled: membrane free of sample; membrane with 5 nL and 50-nl water droplets; and membrane with 50 nL droplet covered by mineral oil.

Figure 6.

A) Thermal response of the nanocalorimeter from the injection of 9.7 mM H_2SO_4 into 0.2 M 7.5 pH HEPES. The HEPES volume is 50 nL, and each injected droplet is estimated to be 0.651 nL, giving a heat production of 0.336 μJ . B) Power sensitivity (in V/W) calibrated from individual shots, assuming every shot produced 0.336 μJ of heat. The mean of the calibrated power sensitivity is 2.91 V/W, with a standard deviation of 0.37

Figure 7.

Nanocalorimeter response to a single injection compared with modeled impulse response.

A) Experimental response when one droplet (0.651 nL) of 0.0097 M H_2SO_4 is injected into 0.2 M 7.5pH HEPES. B) Modeled temperature response of nanocalorimeter to a 0.346 μJ impulse, filtered by a digital low-pass filter with a 1-Hz cutoff frequency.

Table 1

Power sensitivities and time constants predicted by the model.

Situation	Power sensitivity (V/W)	Time constant (s)
Free membrane	9.43	0.009
5 nL sample	6.15	0.37
50 nL sample	3.05	1.23
50 nL sample with oil filled reaction chamber	0.48	0.26

Table 2

Results of calibration experiments with different titrant injection volumes

Reaction type	Results (V/W)	Volume (nl)	Heat per injection (μ J)
H ₂ SO ₄ with HEPES	2.85 $\pm$ 0.14	0.792	0.420
H ₂ SO ₄ with HEPES	2.91 $\pm$ 0.12	0.785	0.417
NaOH with HCl	3.05 $\pm$ 0.10	0.810	0.453
NaOH with HCl	2.89 $\pm$ 0.06	1.18	0.659
H ₂ SO ₄ with HEPES	2.94 $\pm$ 0.12	0.651	0.355
H ₂ SO ₄ with HEPES	2.89 $\pm$ 0.03	1.54	0.369
H ₂ SO ₄ with HEPES	2.92 $\pm$ 0.05	0.982	0.533
H ₂ SO ₄ with HEPES	2.88 $\pm$ 0.11	0.679	0.836

determination; this represents 5-12% of the respective heats.

Table 3

A comparison of free-solution, label-free molecular interaction assays (Based upon Ref. 34)

Technique	Minimum Sample Volume	Detection Limits	
Microcal ITC	1.3 mL	1.0×10^{-6} M	1.3 nmol
Enthalpy arrays	500 nL	5.0×10^{-5} M	25 pmol
BSI	350 pL	8.6×10^{-11} M	30 zmol
nCal	50 nL	2.9×10^{-12}	150 zmol