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Graphene nano-ink biosensor arrays on a microfluidic paper for multiplexed detection of metabolites

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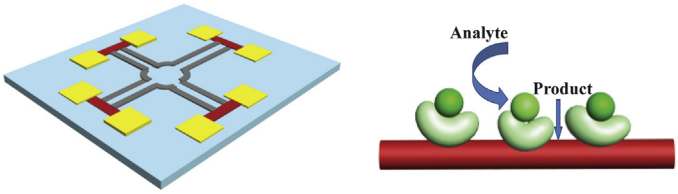
HIGHLIGHTS

- We report graphene-ink biosensor arrays on a microfluidic paper for metabolites.
- The device is able to detect multiple metabolites sensitively and rapidly.
- The device fabrication process is simple and inexpensive.

GRAPHICAL ABSTRACT



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ABSTRACT

The development of a miniaturized and low-cost platform for the highly sensitive, selective and rapid detection of multiplexed metabolites is of great interest for healthcare, pharmaceuticals, food science, and environmental monitoring. Graphene is a delicate single-layer, two-dimensional network of carbon atoms with extraordinary electrical sensing capability. Microfluidic paper with printing technique is a low cost matrix. Here, we demonstrated the development of graphene-ink based biosensor arrays on a microfluidic paper for the multiplexed detection of different metabolites, such as glucose, lactate, xanthine and cholesterol. Our results show that the graphene biosensor arrays can detect multiple metabolites on a microfluidic paper sensitively, rapidly and simultaneously. The device exhibits a fast measuring time of less than 2 min, a low detection limit of 0.3 μM, and a dynamic detection range of 0.3–15 μM. The process is simple and inexpensive to operate and requires a low consumption of sample volume. We anticipate that these results could open exciting opportunities for a variety of applications.

1. Introduction

Metabolites are the products of biological reactions inside cells, and are regulators which control the pace of metabolism. Metabolites have found wide applications in healthcare, pharmaceuticals, food science, and environmental monitoring. For example, glucose, lactate, xanthine and cholesterol, as metabolites, are important biomarkers for many diseases, such as diabetes, cardiovascular diseases, liver diseases, metabolic disorders, hyperuricemia, xanthinuria, and renal failure [1–4]. Despite these advances in the development of a variety of bioanalytical devices for metabolites, the ability to achieve rapid, sensitive, and low cost electrical detection of multiple metabolites remains unexplored. Paper is a low cost, easy-to-handle matrix which has recently attracted great recent interest as a platform for developing a variety of electronic [5,6], optical [7,8], sensing [9–11], and microfluidic devices [11–14]. A miniaturized biosensing platform on paper for multiplexed detection of metabolites is highly desired.

Graphene is a single-atom-thick, sp^2 carbon-based material that has attracted significant recent interest due to its remarkable electrical [15], optical [16], mechanical [17], sensing [18–21], and thermal [22] properties. Due to its single-atomic-layer structure, isolation of graphene has only recently been achieved, via epitaxial growth [23], chemical vapor deposition [24], sputtering [25], chemical exfoliation [26], and mechanical exfoliation [15,19]. Graphene-based ink is of particular interest due to its advantages of being processed in solution which facilitates the low-cost development of electronic and optical devices. Graphene sensors and biosensors have been developed for highly sensitive detection of a variety of analytes, including nitric oxide [20], ammonia [19],

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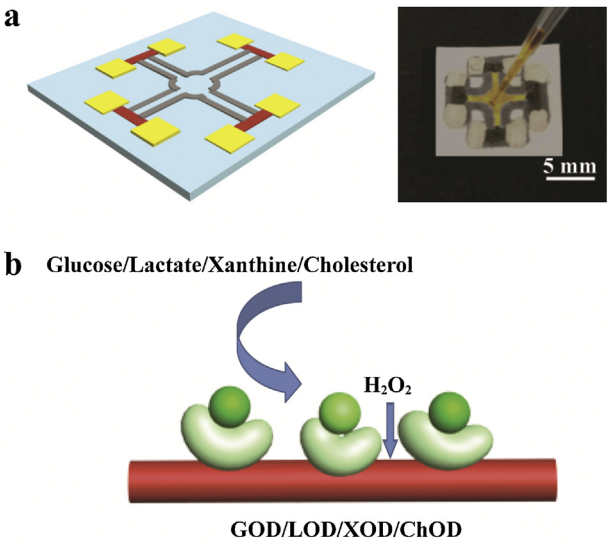

Fig. 1. (a) Schematic illustration (left) and camera image (right) of the multiplexed graphene-based electrical biosensor arrays on a microfluidic paper, and (b) biosensor detection principle. In (a) left and (b): red is graphene; yellow is silver paste; blue is paper; grey is microfluidic channel; light green is enzyme; dark green is analyte.

hydrogen [27], glucose [28], and glutamate [29]. Recently, we have also shown that graphene can be constructed as sensitive and selective biosensors for TNT, lactate, and 5-aminosalicylic acid [11,30–32].

Here, we present the development of graphene-based electrical biosensor arrays on a microfluidic paper for the multiplexed detection of metabolites. As shown in Fig. 1, graphene ink, silver paste and solid ink are printed on regular paper. Each individual channel is connected to its own enzyme-graphene electrode, which is connected to the two silver paste terminals, and all the channels share a common inlet. The solution containing the target metabolite is injected into the inlet, and flows into all the channels. When it is recognized by its own enzyme-graphene electrode, it changes the electrical signal on graphene electrode to generate a signal response. As an example, glucose, lactate, xanthine and cholesterol, which are typical important metabolites, are used as the sensor analytes. The device can also be constructed for the detection of a variety of other metabolites. The sensing principle is based on the oxidation of analyte by oxidase, e.g. glucose by glucose oxidase (GOD), lactate by lactate oxidase (LOD), xanthine by xanthine oxidase (XOD), and cholesterol by cholesterol oxidase (ChOD). The product of the enzymatic reaction, hydrogen peroxide, results in a detectable signal on graphene. Fig. 1 illustrates the basic design of the multiplexed channel structure. It shows the four graphene ink electrodes, each of them functionalized with different enzymes, namely GOD, LOD, XOD, and ChOD. The device can detect multiple metabolites simultaneously and requires a small sample volume due to the use of printed microfluidic channels, shows a high sensitivity and a fast measuring time due to the use of graphene-based electrical sensor, and has a low cost due to the use of paper-based substrate.

2. Material and methods

2.1. Apparatus and chemicals

The electrical measurement was done by an Autolab potentiostat with NOVA software (Metrohm USA, Riverview, FL) or a VSP-300 potentiostat with EC-lab software (Bio-logic USA, Knoxville, TN). A solid ink color printer, ColorQube 8570, was purchased from Xerox Corporation (Norwalk, CT) for printing the microfluidic channels. The optical microscope was purchased from Microscopes.com (Northbrook, IL). Graphene Nano Platelets (Grade 4 COOH rich, <4 layers) were from Cheap Tubes Inc. (Brattleboro, VT). PELCO Conductive Silver 187 used to form the terminals on the graphene electrode was purchased from Ted Pella. Inc. (Redding, CA). Lactate oxidase (LOD), xanthine oxidase (XOD) and cholesterol oxidase (ChOD) were purchased from Toyobo Co., Ltd. (Osaka, Japan). Glucose oxidase (GOD) was purchased from MP Biomedicals LLC (Solon, OH). Glucose and xanthine were purchased from Acros Organics (Pittsburgh, PA). Lactic acid, *N*-ethyl-*N*0-(3-dimethylaminopropyl) carbodiimide (EDC), and *N*-hydroxy succinimide (NHS) were purchased from Sigma–Aldrich (St. Louis, MO). Cholesterol was purchased from Alfa Aesar (Ward Hill, MA). Potassium phosphate monobasic and potassium phosphate dibasic were purchased from Fisher Scientific (Pittsburgh, PA). Human blood was purchased from Zen-Bio Inc. (Research triangle park, NC). All the solutions were prepared in deionized water obtained from Barnstead NANOpure® DIamond™ Ultrapure Water Systems (Thermo Scientific, Asheville, NC).

2.2. Biofunctionalization of graphene ink

The enzymes were immobilized covalently to graphene nano platelets using EDC–NHS chemistry. First, free and unbound—COOH groups of graphene nano platelets were activated by immersing them into phosphate buffer solution (PBS, 100 mM, pH 7.5) containing EDC (10 mM) and NHS (10 mM) for 6 h, then excesses of EDC and NHS were removed by washing with buffer. Finally, 6 mg of EDC–NHS treated graphene platelets was incubated with GOD (20 U), LOD (20 U), XOD (20 U), ChOD (20 U) individually at 4 °C overnight, then washed to remove the unbound enzymes. Enzyme-graphene ink concentrations from 0.05 to 0.6 mg μL^{-1} were investigated to obtain the optimal ink concentration.

2.3. Device fabrication and integration

Microfluidic channels were printed on paper with a solid color printer, and then baked at 110 °C for 5 min to melt the wax so that it penetrated the full thickness of the paper. One end of each channel (2.5 mm × 0.6 mm) was connected to a graphene sensor, and the other end was connected to the inlet of the device. A volume of 5 μL of the enzyme-graphene ink was printed on a paper sized 5 mm × 1 mm to form each graphene biosensor, positioned perpendicular to the direction of the sample flow. Highly conductive silver paste was printed as the positive and negative terminals to connect to the graphene electrode.

2.4. Sensing measurements

All measurements were carried out at room temperature by applying 0.5 V with the potentiostat to the silver ink terminals of the graphene sensor. 2.0 μL of PBS buffer (100 mM, pH 7.5) was first added at the inlet of the microfluidic channel to wet the graphene electrodes. After achieving a steady background current while wet, the measurement was started by adding 2.0 μL of analyte into the inlet to flow to the different enzyme-graphene electrodes at the end of the channels. The concentration of analyte (glucose, lactate, xanthine, and cholesterol) was determined by the increase in H_2O_2 oxidation current. Until a stationary current was obtained, current difference was recorded for plotting a calibration curve. After measuring, a washing step was performed by injecting 2.0 μL of buffer solution to flush the analyte from the enzyme-graphene electrodes before starting a new measurement. Blood samples were diluted 800, 500, 400 and 200 folds with PBS buffer, and dropped at the intersection of the device for the measurement of glucose, lactate, and cholesterol. Xanthine concentration was very low, and thus for

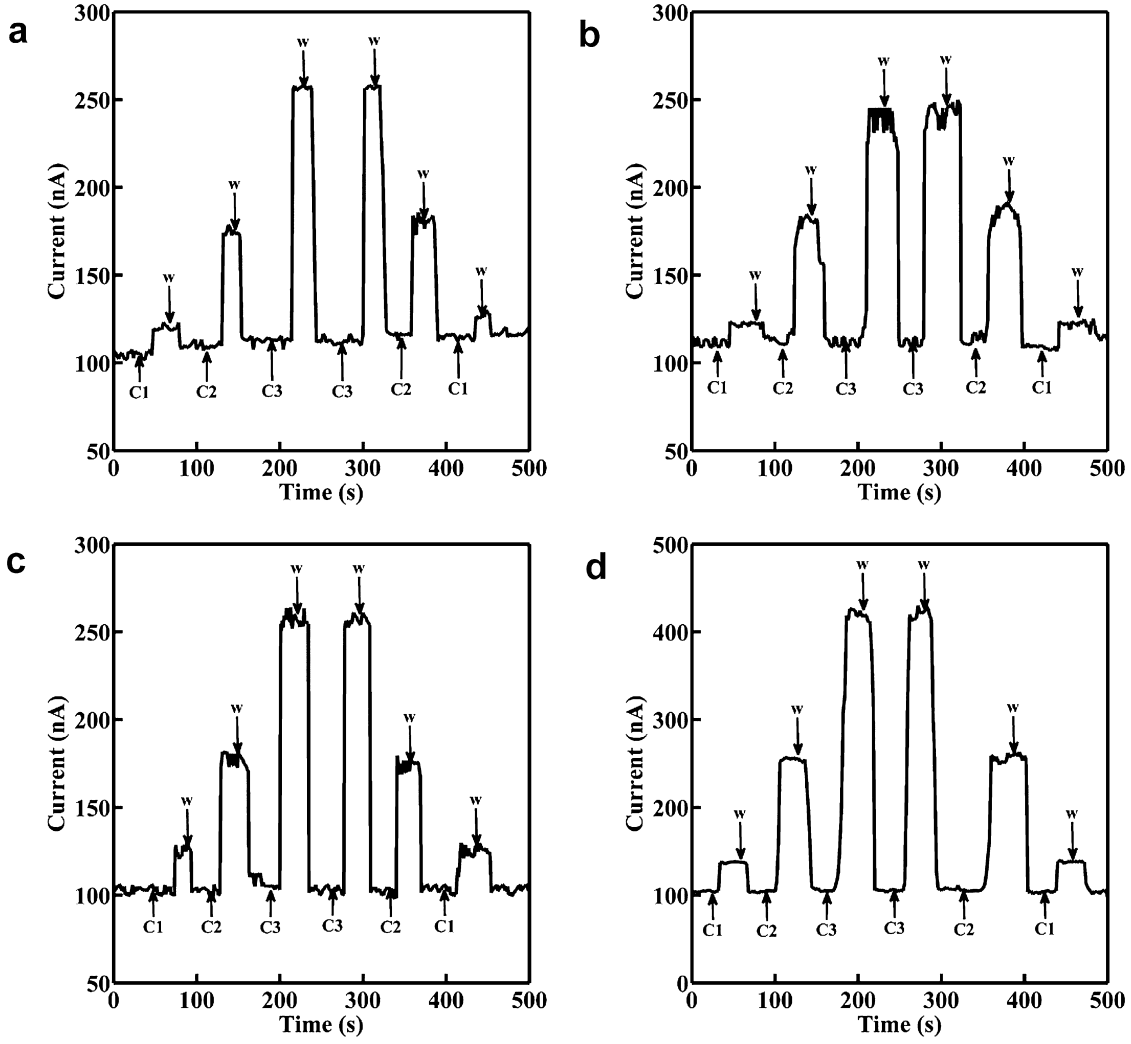

Fig. 2. Current-time curves of the graphene electrical biosensors to (a) glucose, (b) lactate, (c) xanthine, and (d) cholesterol. C1: 1 μM. C2: 5 μM. C3: 10 μM. W: washing step.

xanthine detection, blood sample was tested as it is and diluted 3, 2.5 and 2 folds and then tested using the sensor.

2.5. Screen print sensor for blood measurement

Screen-printed sensor based measurement for the determination of glucose, lactate, xanthine and cholesterol in human blood was performed to compare to that using the graphene-ink based device. The measurements were carried out at room temperature by applying 0.4 V. For glucose, lactate, and cholesterol, GOD (20 U), LOD (20 U) and ChOD (20 U) were immobilized on screen-printed electrodes. Blood samples were diluted 100, 50, 20 and 10 folds in PBS buffer for measurement. Due to the low concentration of xanthine in human blood, for xanthine measurement, 50 U of XOD was added into the blood sample to generate H_2O_2 in solution for the detection by screen-printed electrode. For each sensor, calibration curve was plotted to determine the analyte concentration.

3. Results and discussion

Optimization of the enzyme-graphene ink loading on paper was performed before the sensor characterization in order to improve its determination performance. As shown in Table 1, several paper-based biosensors with varied loadings of enzyme-graphene ink were investigated for the effect of ink loadings on

Table 1
Effect of graphene ink concentration on sensitivity and conductivity of the graphene sensor on paper ($n = 3$).

Ink concentration (mg μL^{-1})		Sensitivity (nA μM^{-1})	Conductivity (μA)
GOD-graphene	0.05	2.08	0.11
	0.1	4.85	2.47
	0.2	13.27	11.67
	0.4	13.27	23.71
	0.6	13.27	30.1
LOD-graphene	0.05	2.61	1.15
	0.1	5.20	4.21
	0.2	13.13	14.44
	0.4	13.13	17.94
	0.6	13.13	35.04
XOD-graphene	0.05	2.64	2.21
	0.1	3.17	9.11
	0.2	14.62	17.8
	0.4	14.62	45.01
	0.6	14.62	52.83
ChOD-graphene	0.05	0.97	0.54
	0.1	3.85	0.68
	0.2	29.34	13.35
	0.4	29.34	20.03
	0.6	29.34	21.46

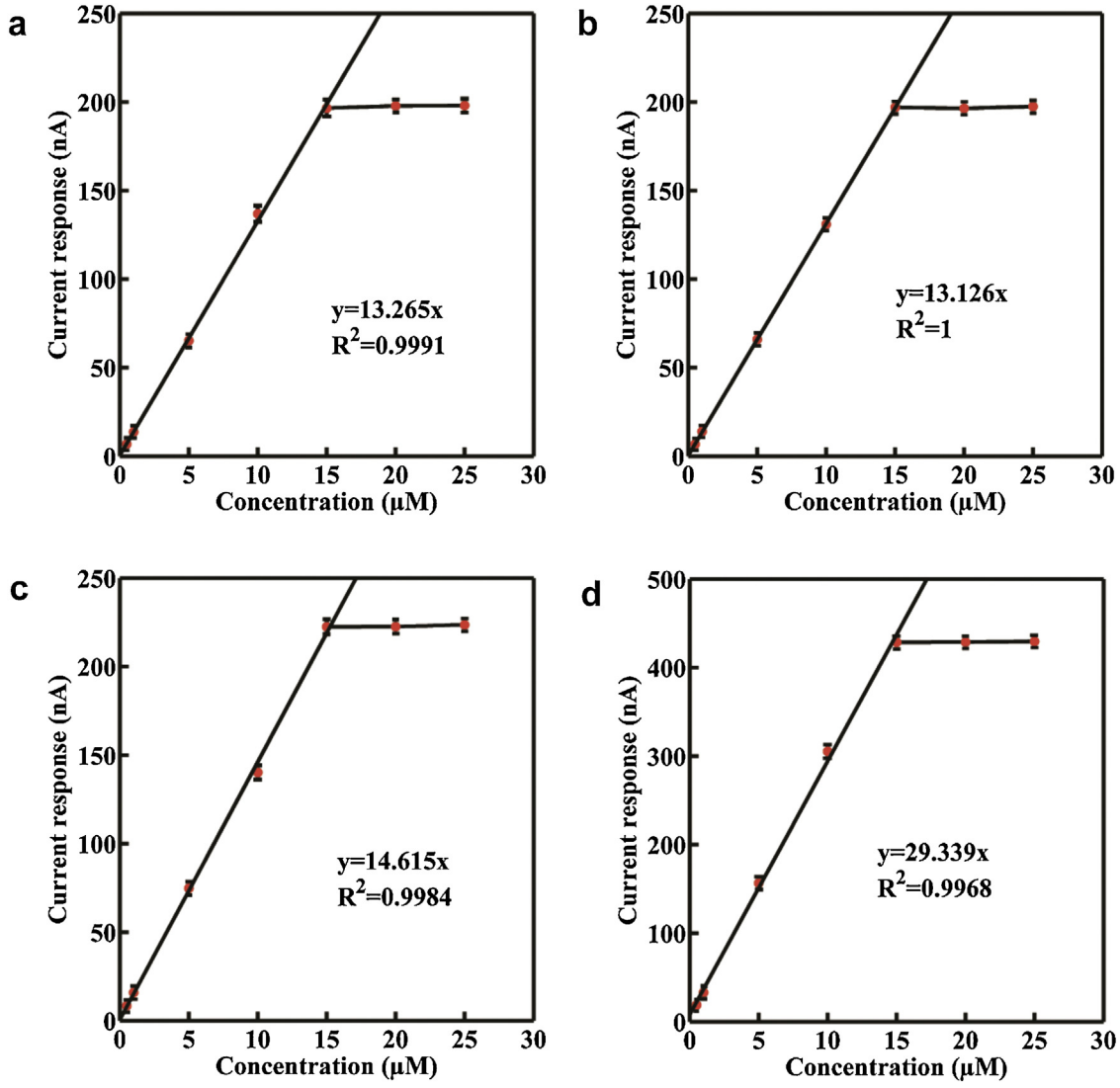

Fig. 3. Calibration curves for (a) glucose, (b) lactate, (c) xanthine, and (d) cholesterol with the multiplexed graphene-based electrical biosensor arrays on a microfluidic paper.

sensor performance. The enzyme-graphene ink was investigated with concentrations varying from 0.05 to 0.6 mg μL^{-1} for each of the four different enzyme functionalized biosensors. As the ink concentration increased from 0.05 to 0.2 mg μL^{-1} (4 times), sensitivity increased by 6.4 times for glucose, 5.0 times for lactate, 5.5 times for xanthine, and 30.2 times for cholesterol ($n = 3$). Doubling the enzyme-ink loading from 0.2 to 0.4 mg μL^{-1}, no increase was observed in the sensitivity of any of the four sensors. Maximum response was obtained in the system of 0.2 mg μL^{-1} and this was used as the standard ink loading for further experiments. The conductivities of all of the graphene electrodes increased significantly by 25–30 times as the ink loading increased from 0.05 to 0.4 mg μL^{-1}, which we attributed to the increase in electron carriers with increasing ink concentration. The change in sensitivity with different ink concentrations was due to the change in the rates of enzymatic reaction and electron transfer.

Our next step was to investigate the current response of the device towards various metabolites. Fig. 2 shows a current–time curve of the sensor to glucose, lactate, xanthine and cholesterol obtained by adding concentrations of 1 μM, 5 μM, and 10 μM of the target metabolite. The microfluidic channel was first injected with buffer solution to achieve a baseline current. After the injection of a given concentration of the analyte into the inlet of the microfluidic channel on the paper, the analyte flowed to the graphene sensor by capillary action through the channel in around 25–30 s. After the analyte reached the graphene sensor, the graphene current increased due to the oxidation of H_2O_2 generated from the enzymatic reaction, and the electron transfer took place on the graphene electrode. The steady-state background current increased and reached a stationary state in ~20 s. The increase of the graphene current was proportional to the concentration of analyte, with a high reproducibility. After a washing step to remove the analyte on the graphene sensor so that it could recover, the current decreased to the initial background current in ~20 s. The sensor demonstrated fast measuring times. The total measurement using the sensor took less than 2 min.

Fig. 3 shows the calibration curve for glucose, lactate, xanthine and cholesterol with the graphene ink nanosensor. A linear relationship was obtained between the current response and the concentration of the metabolite in the micro molar range: for glucose, a linear curve with a slope of 13.3 nA μM^{-1} ($R^2 = 0.9991$, $n = 3$); a slope of 13.1 nA μM^{-1} ($R^2 = 1.000$, $n = 3$) for lactate; a slope of 14.6 nA μM^{-1} ($R^2 = 0.9984$, $n = 3$) for xanthine; and a slope of 29.3 nA μM^{-1} ($R^2 = 0.9968$, $n = 3$) for cholesterol. The device was calculated to have a low detection limit of 0.3 μM (signal-to-noise = 3) for all the analytes. The comparative steeper slope

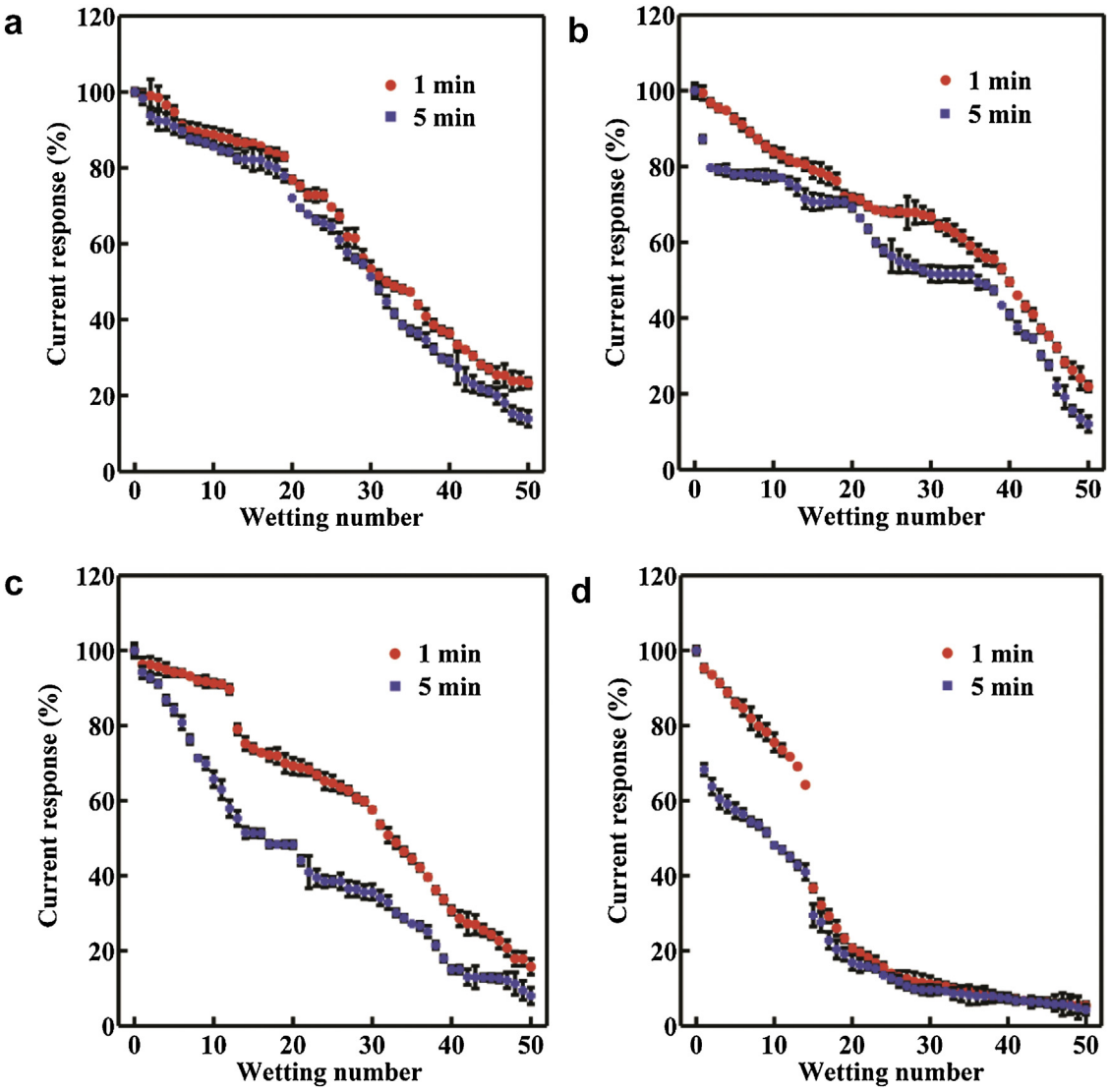

Fig. 4. Effect of wetting time-number on the current response of the multiplexed graphene based biosensor on a microfluidic paper for (a) glucose (10 μM), (b) lactate (10 μM), (c) xanthine (10 μM), (d) cholesterol (10 μM) ($n = 3$).

observed for cholesterol compared to the other metabolites could result from higher concentrations of H_2O_2 being generated during the enzymatic reaction.

The effect of repeated wetting on the biosensor's performance was also studied for a wetting period of 1 min and 5 min, as shown in Fig. 4. After each wetting cycle the sensor was dried for a period of 5 min. The activity of the enzyme electrode was investigated by adding 10 μM of the metabolite, e.g. glucose solution in PBS, and checking the current response by making electrical measurements, as shown in Fig. 4a. For all biosensors, it was observed that the current response decreased with increasing wetting repetitions, e.g. the glucose sensor reached a minimum at 24% after the 50th cycle of 1 min wetting time and a minimum of 14% after the 50th cycle of 5 min wetting time, and the biosensor retained around ≥50% of its initial activity until the 25th–30th wetting cycle. The change in current response after repeated wetting was caused because the graphene ink electrode layer was breaking down, resulting in reduced enzyme activity and graphene conductivity.

The surface morphology of the graphene ink electrode and the channel for analyte transport before and after wetting were characterized by an optical microscope. Fig. 5 shows the optical images of the enzyme immobilized graphene ink electrode and microfluidic channel (for lactate) before measurement and after wetting 50 cycles with 1 min wetting time, and the change in color and non-uniformity in the surface can be observed before and after wetting. It can be clearly seen that the ink layer was damaged due to repeated wetting, and therefore the response of the electrode was reduced. In addition, the morphology of the channel structure was disrupted, which resulted in analyte leakage during measurement.

The stability of enzyme electrodes was evaluated by measuring their performance every alternate day for 20 days at 4 °C and at room temperature for 20 days, as shown in Fig. 6. It can be seen in Fig. 6a that the glucose biosensors retain around 80% of their initial activities after 10 days. After 20 days, the glucose biosensors stored at room temperature were still active and retained around 50% of their initial response, while those stored at 4 °C retained up to 70% of their initial activity, indicating that enzyme functionalized graphene could efficiently retain its bioactivity. Repeated use of the electrode did not degrade its performance or reproducibility by a wide margin for at least 12–13 days, due to the stability of the enzymes and inks. Biosensors for other metabolites (lactate, xanthine, and cholesterol) show similar performances, and the stabilities for the biosensors stored at 4 °C are higher than those stored at room temperature. The performance reduction was probably due

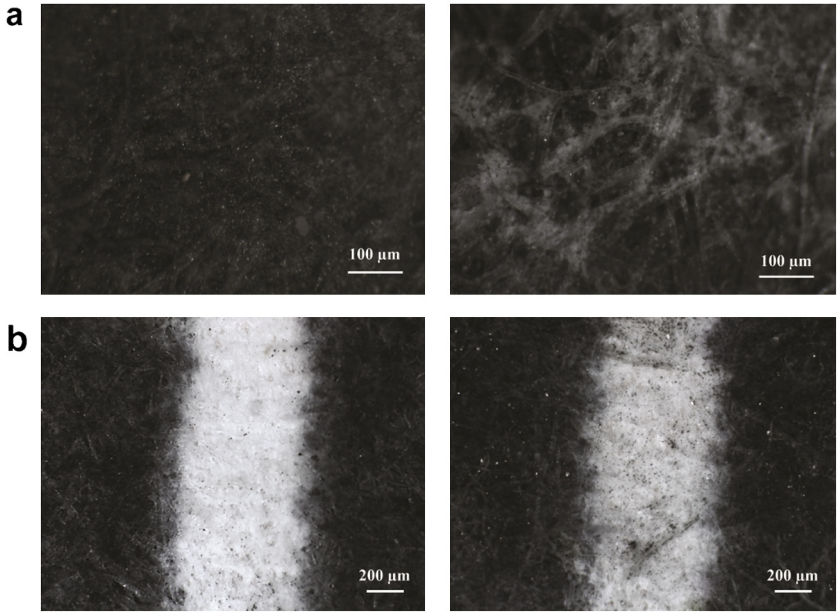

Fig. 5. Optical images of (a) graphene biosensor and (b) microfluidic channel (for lactate). Left: before use. Right: after wetting for 50 cycles with 1 min wetting time.

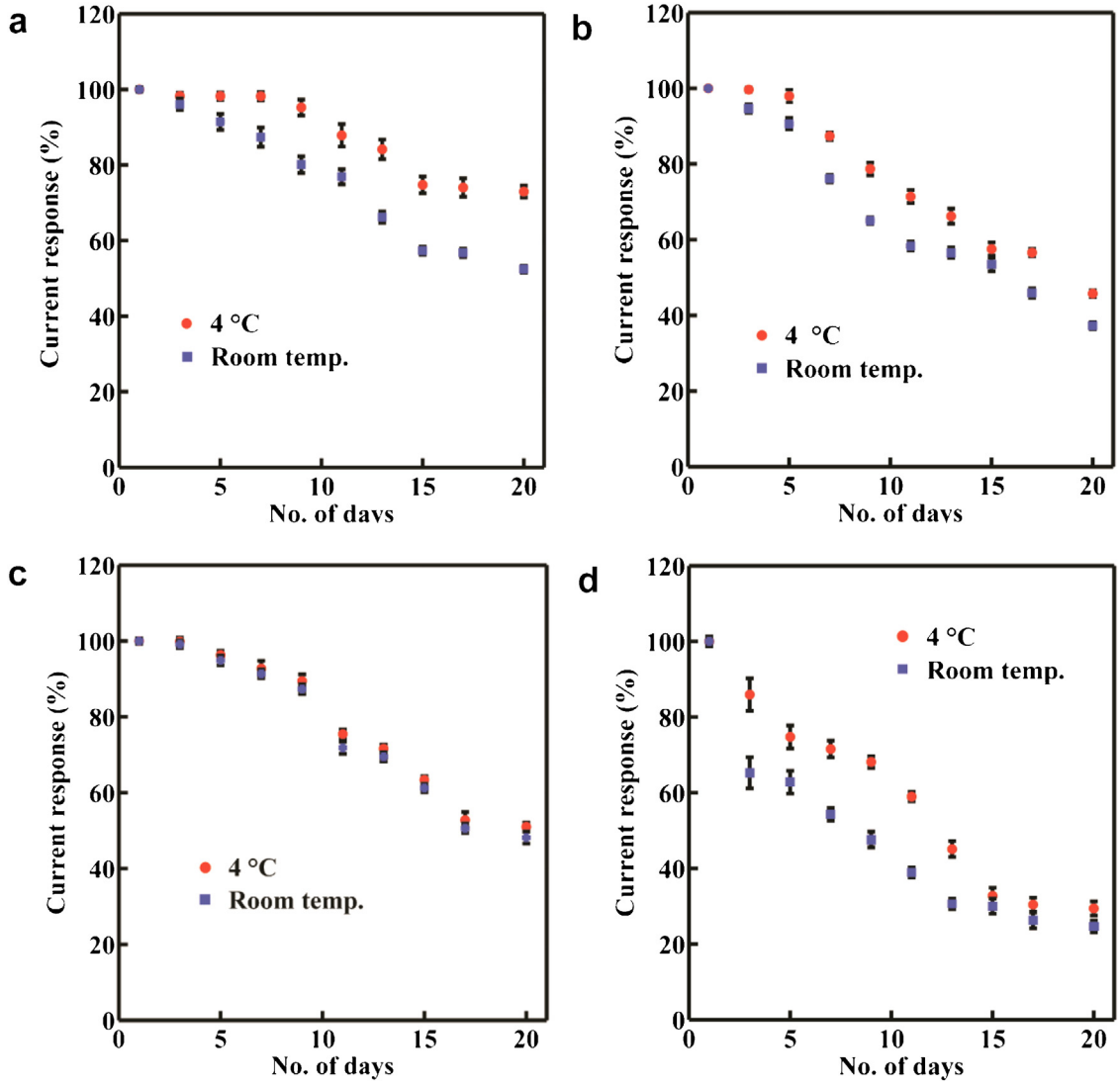

Fig. 6. Effect of time and temperature on the stability and response of the multiplexed graphene based biosensors for (a) glucose (10 μM), (b) lactate (10 μM), (c) xanthine (10 μM), and (d) cholesterol (10 μM) ($n = 3$).

Table 2
Comparison of graphene ink sensor and screen-printed sensor for detecting metabolites in human blood.

Metabolite	Graphene ink sensor (μM)	Screen-printed sensor (μM)
Glucose	4094.28	4106.18
Lactate	1041.22	1021.09
Cholesterol	5041.66	5051.88
Xanthine	1.12	1.02

to the lifetime of the enzyme being reduced during the storage of the biosensor. Although the results are promising, further studies are needed to improve the stabilities by investigating different immobilization methods.

For real sample analysis, the detection of multiple analytes in human blood was performed. Table 2 summarizes the result of the determinations of glucose, lactate, xanthine and cholesterol in human blood with the graphene-ink device and screen-printed sensors. As shown in the table, by using the graphene-ink device, the glucose concentration was determined to be ~4.09 mM, the lactate concentration was determined to be ~1.04 mM, the cholesterol concentration was determined to be ~5.04 mM, and the xanthine concentration was determined to be ~1.12 μM. The results were also compared with that by using screen-printed sensors, and the agreement was excellent between these two methods. The good agreement shows that the graphene-ink device is accurate for measuring metabolites in real blood samples. The results are also consistent with the reported metabolite concentrations, which show a range of 3.6–5.5 mM for glucose [33], ~1–1.5 mM for lactate [34], ~5–5.2 mM for cholesterol [35] and ~1–2 μM for xanthine [36].

4. Conclusions

In this work, we have demonstrated the construction of electrical graphene biosensor arrays on a microfluidic paper for the multiplexed detection of metabolites. The sensor arrays perform sensitive, specific, and rapid detection of different metabolites, including glucose, lactate, xanthine and cholesterol. The approach we describe here can also be used for the detection of other metabolites, and may open new avenues for a variety of applications in healthcare, pharmaceuticals, food science, and environmental monitoring.

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