Supporting Information

Transparent paper-based platform for multiplexed bioassays by wavelength-dependent absorbance/transmittance

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Experimental section

Materials and instruments

Methyl orange, tetrabromophenol blue (TBPB), 4-aminoantipyrine (4-APP), cholesterol esterase (CE), cholesterol oxidase (CO), N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methoxyaniline, sodium salt, dihydrate (ADOS), and cholesterol were purchased by Shanghai Yuanye Biotechnology Co., Ltd. Trisodium citrate, citric acid, horseradish peroxidase (HRP), bovine serum albumin (BSA), BSA oxidase (GOx), potassium iodide, and ethanol were purchased from Nanjing Liangwei Biotechnology Co., Ltd. Polydimethylsiloxane (PDMS), sodium hydroxide, urea, sulfuric acid (H₂SO₄) and silicone elastomer curing agent were purchased from Nanjing Wanqing Chemistry Glass Instrument Co., Ltd.

The absorption spectra and the transmittance were recorded on 1750 UV-vis spectrophotometer (Shimadzu Corporation, Japan). The photograph was taken with a Canon camera (EOS 800D). SEM characterizations of transparent paper, chromatography paper and office paper were carried out on a JSM-7800F (Jeol Corp). AFM images of transparent paper were obtained using a XE-70 (Park Corp). The water contact angle of the transparent paper was measured by DSA1005 (Kruss Corp). The screen printer was manufactured by Daeyoung Technology Co., Ltd. Deionized water (18.4 MΩ/cm) used for all experiments was obtained from Milli-Q system (Millipore, Bedford, MA, USA). All color
measurements were performed with dry paper substrates. Human serum samples were purchased from Solarbio reagent Co. Ltd. (Beijing, China).

RGB method: the colour changes of reactions were captured by a camera (EOS 800D) with subsequent analysis of red, green, and blue (RGB) intensities at each image. The quantitative relationships between concentrations and the intensities of the red, green, and blue channels in the captured images were established by software (Adobe Photoshop CC) on the computer.

**Preparation of transparent paper**

The transparent paper was prepared by our group and the preparation process was as follows: 1) a solution containing NaOH/urea/H$_2$O with a weight ratio of 7:12:81 was precooled to $-12 \, ^\circ\text{C}$. Then 4.0 g cotton linter pulps were put into 100 mL of this precooled solution to form viscous solution under vigorous mechanical stirring (1000 rpm) for 2 min at room temperature. 2) The viscous solution was centrifuged at 10,000 rpm for 10 min and the clear upper solution was the nanocellulose solution for further use. 3) The nanocellulose solution was spread on a glass plate by rod coating method (~0.5 mm thick). The glass plate was immersed into a coagulation bath of 5 wt% H$_2$SO$_4$. A sheet of hydrogel was formed after 5 min, separated from the glass plate spontaneously. The hydrogel was thoroughly soaked in deionized water for 12 h. 4) The hydrogel was put on a substrate of polymethyl methacrylate (PMMA) to dry at room temperature for 48 h to form final transparent paper. During the drying process, the sides of the hydrogel were attached to the PMMA by adhesive tapes to prevent shrinkage$^2$. In this work, the employed paper is transparent due to fiber treatments, with no formation of fibrous
network structures compared to chromatography paper and office paper.²

Fabrication of the transparent paper-based analytical devices (tPADs)

The transparent paper used was made by our group. In this study, we used screen-printing technology for developing hydrophilic and hydrophobic channels on transparent paper.³ The screen printer was manufactured by Daeyoung Technology Co., Ltd. The screen-printing process is simple and easy, thereby allowing the fabrication of large quantities. We designed and applied a template to the transparent paper, after which a mixture of PDMS and silicone elastomer curing agent (Mass ratio is 10:1) was screen printed, using a squeegee. The curing process for screen-printing technology can be performed via various methods such as the use of a well-ventilated oven, dryer curing, and thermal sintering.⁴,⁵ In this study, we used vacuum drying oven. The curing was performed in an oven at 80 °C for 12h.

Proof-of-feasibility experiment

Methyl orange and TBPB assays were selected to demonstrate the feasibility of the proposed devices. Experiments were carried out at room temperature. Samples of methyl orange and TBPB were prepared at concentrations from 0.025–1 mg/mL and 0.01–0.5 mg/mL, respectively. Both solutions of different concentrations were added to the detection zones of tPADs and chromatography paper. After drying, the color change was obtained using a camera and the absorbance and transmittance of methyl orange and TBPB on tPADs were separately measured by UV-vis spectrophotometer. The absorbance and transmittance of methyl orange and TBPB were also measured simultaneously when two layers of tPADs were put together (one with methyl orange and the other with TBPB) with the detection zones completely aligned.
Bioassays of BSA and cholesterol in standard solution and in serum

The BSA assay is based on the color change of TBPB when it ionizes and binds to BSA (Fig. S1a). A solution of 125 mM citrate buffer at pH = 1.8 was mixed in a 10:1 ratio in a solution of 9 mM TBPB in 95% ethanol and 5% water. Solutions of bovine serum albumin (BSA) at concentrations ranging from 0 μM to 120 μM in standard solution and 0 μM to 120 μM in serum, respectively were prepared. A drop of 5 μL of a mixture of citrate buffer and TBPB was added to the detection area of the transparent paper-based analytical devices. Then, 5 μL of the BSA sample was placed over this solution and allowed to react. Finally, after drying at room temperature, the colour change was obtained using a camera and the absorbance of BSA on tPADs were measured.

For the cholesterol assay, the detection zones were prepared by adding of 8 μL of solution containing CE (14.1 U), CO (10 mg/mL), 4-APP (10 mM), HRP (1 mg/mL) and ADOS (1 mM), followed by adding 2 μL of various concentrations (0 mM–5 mM in standard solution and 0 mM–5 mM in serum) of cholesterol solution on tPADs (Fig. S1b). After drying at room temperature, the color change was obtained using a camera and the absorbance of cholesterol on tPADs were measured. The absorbance of BSA and cholesterol was also measured simultaneously when two layers of tPADs were put together with the detection zones completely aligned.

Transmittance properties of tPADs

For bioassays that measure absorbance on tPADs, it is very important to have high optical performance of transmittance. As shown in Fig. S5, the optical transmittance for different concentrations of methyl orange and TBPB on tPADs were measured by UV-vis spectrophotometer,
separately. The absorbance and transmittance of methyl orange and TBPB were also measured simultaneously when two layers of tPADs were put together (one with methyl orange and the other with TBPB) with the detection zones completely aligned. Scanning the tPADs without any samples as a blank sample set the baseline of the spectroscopy before test. As can be seen from the separate measurements in Fig. S5a and Fig. S5b, as the concentrations of methyl orange and TBPB increase, the transmittance gradually decreases and is higher than 70% from 400 nm to 800 nm. Methyl orange and TBPB showed two peaks at 450 nm and 625 nm, respectively, which are due to the appearance of the color of methyl orange and TBPB on tPADs. As shown in Fig. S5c, when we simultaneously measured methyl orange and TBPB, as the concentrations of the two increase, the transmittance also gradually decreases and the transmittance of both is higher than 60%. This indicates that tPADs still showed excellent transmission when two layers of tPADs were put together with the detection zones completely aligned for simultaneous detection of methyl orange and TBPB.
Fig. S1 SEM images of (a) transparent paper, (b) chromatography paper, and (c) office paper. Corresponding enlarged SEM images of (d) transparent paper, (e) chromatography paper, and (f) office paper. AFM images of (g) transparent paper, (h) chromatography paper, and (i) office paper. (j) The contact angle measurement with water of the transparent paper.
Fig. S2 Thickness of transparent paper obtained from SEM images of different positions and different batches of transparent paper.
Fig. S3 Analysis of TBPB by absorption spectroscopy and RGB method. (a) UV-vis absorption spectra of different concentrations of TBPB on tPADs (0.005 mg/mL, 0.01 mg/mL, 0.025 mg/mL, 0.05 mg/mL, 0.1 mg/mL, 0.25 mg/mL and 0.5 mg/mL). (b) Calibration curve of the logarithm of different concentrations of TBPB by absorption spectroscopy on tPADs. (c) Calibration curve of the logarithm of different concentrations of TBPB by RGB method on tPADs. (d) Calibration curve of the logarithm of different concentrations of TBPB by RGB method on chromatography paper. Each data point is the mean of five analyses.
Fig. S4 (a) The comparison of colorimetric results of different concentrations of methyl orange on tPADs and chromatography paper. (b) The comparison of colorimetric results of different concentrations of TBPB on tPADs and chromatography paper. (c) UV-vis absorption spectra of different concentrations of methyl orange on tPADs (0.025 mg/mL, 0.05 mg/mL, 0.1 mg/mL, 0.25 mg/mL, 0.5 mg/mL and 1.0 mg/mL). (d) UV-vis absorption spectra of the logarithm of different concentrations of TBPB on tPADs (0.01 mg/mL, 0.025 mg/mL, 0.05 mg/mL, 0.1 mg/mL, 0.25 mg/mL and 0.5 mg/mL). Calibration curve for simultaneous detection of the logarithm of different concentrations of (e) methyl orange and (f) TBPB on tPADs. Each data point is the mean of five analyses.
Fig. S5 Transmittance of detection of multiple analytes on tPADs. (a) Transmittance of detection of methyl orange with different concentrations (0.025 mg/mL, 0.05 mg/mL, 0.1 mg/mL, 0.25 mg/mL, 0.5 mg/mL and 1.0 mg/mL). (b) Transmittance of detection of TBPB with different concentrations (0.01 mg/mL, 0.025 mg/mL, 0.1 mg/mL, 0.25 mg/mL and 0.5 mg/mL).
**Fig. S6** (a) The colorimetric results of different concentrations of BSA and the corresponding UV-vis absorption spectra (0.1 μM, 5.0 μM, 25 μM, 50 μM, 100 μM and 120 μM). (b) Calibration curve of different concentrations of BSA in standard aqueous solution. (c) UV-vis absorption spectra for simultaneous detection of BSA and cholesterol with different concentrations.
Fig. S7 (a) UV-vis absorption spectra for simultaneous detection of different concentrations of BSA (0.1 μM, 0.5 μM, 2.5 μM, 15 μM, 30 μM and 120 μM) and cholesterol (0.1 mM, 0.25 mM, 0.5 mM, 1.0 mM, 2.5 mM and 5.0 mM) in serum. Calibration curve for simultaneous detection of different concentrations of (b) BSA and (c) cholesterol on tPADs. Each data point is the mean of five analyses.
References


