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Electrochemical immunoassay on a 3D microfluidic paper-based device†

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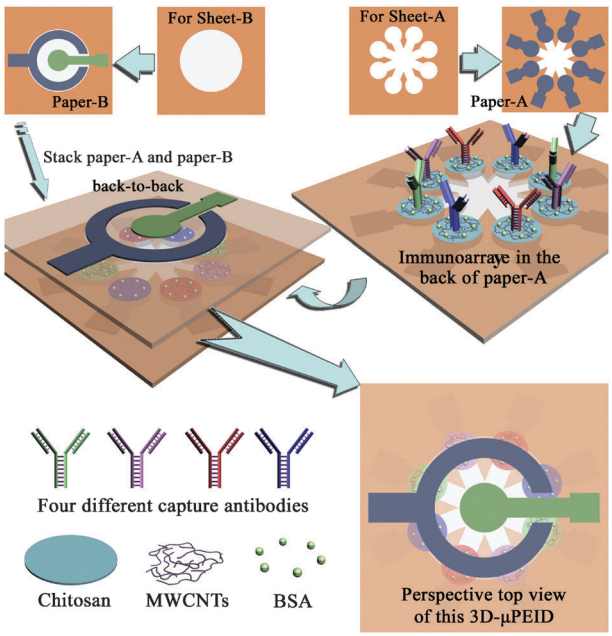
A high-throughput, simple, fast, low-cost and sensitive paper-based electrochemical immunodevice has been demonstrated based on a functionalized 3D paper-based device for point-of-care diagnosis.

Whitesides and co-workers[1] firstly demonstrated lab-on-a-paper systems using patterned paper as a substrate, named microfluidic paper-based analytical devices (μPADs), to combine the simplicity, portability, disposability and low-cost of paper strip tests and the multiplex analysis and complex function of the conventional lab-on-a-chip devices. They aimed at developing a simple, inexpensive, portable, disposable, easy-to-use and multiplex point-of-care testing (POCT) platform for developing countries, resource-limited and remote regions. This is a promising technology for POCT, public health and environmental monitoring applications for which highly sensitive methods and complex function must be combined with low-cost, rapid, and simple fabrication and operation. Much effort has been directed toward the development of fabrication,[2] functionalization[3] and quantitative methods[4] for μPADs.

Multiplexed immunoassay of a panel of markers has recently attracted considerable interest due to its advantages in early screening of diseases, evaluating the extent of diseases, and monitoring the response of diseases to therapy in POCT. Electrochemical methods have gained considerable interest as bioanalytical methods in recent years because of the advantages of simple instrumentation, easy signal quantification, low cost of the entire assay, and convenient miniaturization and integration.[5] These advantages of electrochemical methods mentioned above make electrochemical immunoassays (EI)[6] an innately exciting strategy for building a new generation of simple, low-cost, disposable, portable, sensitive and specific POCT devices. To the best of our knowledge, no reports on establishing EI on μPADs have been published.

To combine the EI with μPADs for high-performance, high-throughput, low-cost, and simple POCT, we demonstrated a 3D microfluidic paper-based electrochemical immunodevice (3D-μPEID) based on functionalized 3D μPADs. 3D μPADs[3b] are particularly useful because they permit fluid movement in the *x*-, *y*-, and *z*-directions, and therefore, they can accommodate more assays on a smaller footprint than the typical 2D, lateral-flow devices. A 3D μPAD can distribute a sample from a single entry point to hundreds of test regions.



Scheme 1 Schematic diagram of the fabrication of the 3D-μPEID.

As shown in Scheme 1, this 3D-μPEID is comprised of two stacked layers of patterned square papers with the same size (30.0 mm × 30.0 mm, named Paper-A and Paper-B, respectively, below), which were produced in bulk (named Sheet-A and Sheet-B, respectively, below, details in ESI†).

Wax-screen-printing, as a rapid, inexpensive, and simple method, is used to pattern Sheet-A and Sheet-B according to our previous work.[2d] The shape for patterning these patterns and screen-printing paper-electrodes on Sheet-A and Sheet-B was designed using Adobe illustrator CS4. As shown in Scheme 1, the wax-patterns on Paper-A contain eight paper working zones (4 mm in diameter) with crossed paper channels to connect them. The unprinted area constituted a reservoir of electrochemical cells. The wax-patterns on Paper-B contain only an auxiliary zone (15 mm in diameter). Owing to the 3D porous structure of paper, the melted wax can penetrate into the paper to decrease the hydrophilicity of paper remarkably.[2d] In addition, the unprinted area maintains good hydrophilicity, flexibility and 3D porous structure and will not affect the further

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screen-printing of electrodes and modification of paper working zones.[2d]

The wax-patterned Sheet-A and Sheet-B were then ready for printing electrodes on their hydrophilic zones after cooling to room temperature (within 1 min) (details in ESI†). In addition, due to the small size of this device, the silver wires and contact pads in traditional screen-printed electrodes were unnecessary and can be directly replaced by carbon ink and Ag/AgCl ink, respectively, to decrease the cost of this device. Furthermore, the screen-printing paper-electrodes will be more important for further development of μPED in low-cost and disposable application[4b] than the commercialized ones.[4c] The eight working electrodes will share the same reference and counter electrodes with the aid of the wax-patterned electrochemical cells after stacking back-to-back (Scheme 1).

Fast immunoassay is highly desired for diagnosis. Many technologies have been explored to accelerate the immunoreaction in a microarray format.[7] All these incubation processes need additional equipment and operation, leading to the increased cost and inconvenience. Whitesides and co-workers proposed a paper-based ELISA[8] to develop a fast and simple colorimetric immunoassay method based on the high surface-to-volume ratio porous paper fibers for accelerating immunoreaction. Based on this acceleration of paper substrates, our group has demonstrated a fast and sensitive chemiluminescence immunoassay on μPADs.[2d]

Therefore, to accelerate the immunoreaction in this 3D-μPEID, as shown in Scheme 1, the immunoarrays were assembled into porous paper working zones on the back of working electrodes through multi-walled carbon nanotubes (MWCNTs) modification, chitosan coating and glutaraldehyde cross-linking (details in ESI†). MWCNTs, due to their high electronic conductivity, were used to modify the paper fibers in paper working zones to enhance/increase the electronic conductivity/effective surface area of the working zones/electrodes with the aid of the porous structure of paper for the first time. Chitosan was used to coat the MWCNTs modified paper fibers not only for avoiding the leakage of MWCNTs, but also for providing active amino-groups to immobilize antibodies through glutaraldehyde cross-linking. Finally, the 3D-μPEID was rinsed (detailed procedures in ESI†) and stored in a dry environment prior to use.

The morphologies of modified paper working zones were characterized by scanning electron microscopy (SEM). Fig. 1B exhibits that MWCNTs were assembled on paper fibers through physical adsorption in the form of small bundles or single tubes. In comparison with the pure paper fibers (Fig. 1A), the morphology of chitosan coated MWCNTs modified paper fibers (Fig. 1C) displayed a more stable assembly surface which was advantageous to improve the reproducibility of immunodevice preparation. After antibodies immobilization and BSA blocking, the modified paper working zones still showed a non-compact porous micro-structure (Fig. 1D), which can increase the effective surface area of the screen-printed working electrodes, enhance the loading of biomolecules, facilitate the approach of immunoreagents to the immobilized capture antibodies, accelerating the rate of electron transfer and resulting in fast immunoreaction and sensitive electrochemical response.

To further confirm the successful immobilization of immuno-arrays, the electrochemical impedance spectroscopy (EIS) of the resulting paper working zones is shown in Fig. 2. The electron

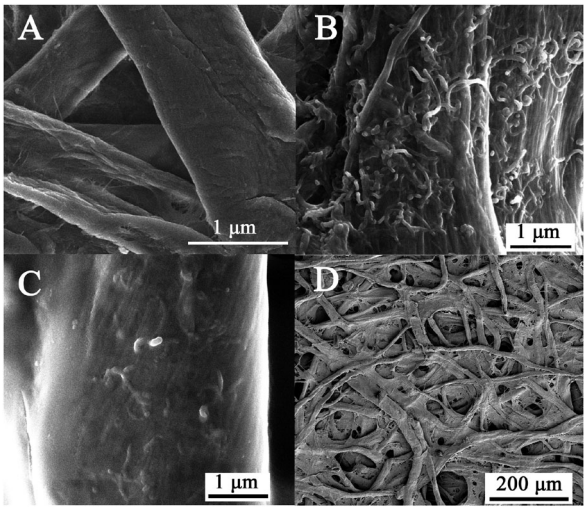

Fig. 1 SEM images of (A) pure paper fibers; (B) MWCNTs assembled paper fibers; (C) chitosan/MWCNTs modified paper fibers and (D) BSA/antibodies/chitosan/MWCNTs modified porous paper of paper working zones.

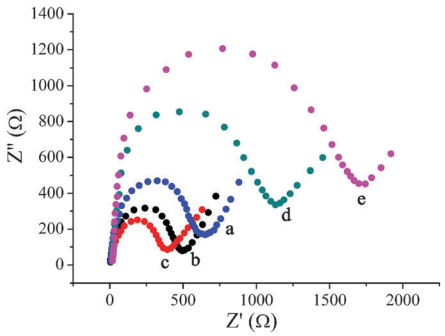

Fig. 2 EIS of paper working zones upon the stepwise assembly processes in 0.1 M KCl containing 5.0 mM $[Fe(CN)_6]^{3-}$ and 5 mM $[Fe(CN)_6]^{4-}$.

transfer of $[Fe(CN)_6]^{3-/4-}$ can be blocked by the formation of BSA/antibodies/chitosan/MWCNTs composites in the paper working zone, which results in an increase in the electron transfer resistance. The bare paper working zone in Paper-A revealed a relatively small semicircular domain (curve a), which implied a low-electron-transfer resistance of the redox couple. After MWCNTs were assembled into paper working zones, the resistance was decreased (curve b), as the existence of the MWCNTs leads to the increase in the electron transfer kinetics of $[Fe(CN)_6]^{3-/4-}$ and the effective conductivity to the electrode surface. It could be found that chitosan coated paper working zones showed a much lower resistance for the redox probe (curve c), the reason of which may be the abundant amino groups can adsorb much more negatively charged $[Fe(CN)_6]^{3-/4-}$ and it can be easily reached at the fiber surface to accelerate electron transfer. However, for the antibodies modified paper working zone through glutaraldehyde linking, the diameter increased markedly (curve d), which can be accounted for the association of glutaraldehyde with chitosan obturated many amino groups of chitosan and formation of protein barriers for electron transfer. Similarly, BSA could also resist the electron-transfer kinetics of the redox probe at

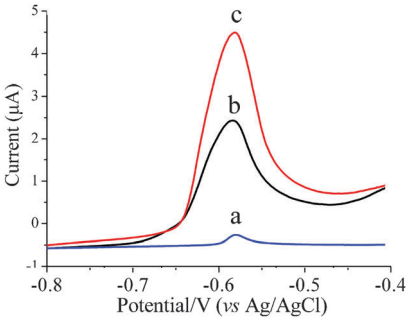

Fig. 3 DPV curves of CEA as a model: (a) background without CEA; (b) after a sandwich immunoreaction with 0.25 ng mL^{-1} CEA and (c) 1.5 ng mL^{-1} CEA.

the paper–electrode interface, resulting in the increasing impedance of the paper working zone (curves e), which testified the immobilization of BSA.

The electrochemical assay procedures of this 3D-μPEID are shown in ESI† using cancer markers, r-fetoprotein (AFP), carcinoma antigen 125 (CA125), carcinoma antigen 199 (CA199) and carcinoembryonic antigen (CEA) as models (every two working electrodes for one cancer marker as shown in Scheme 1). A typical horseradish peroxidase (HRP)-*O*-phenylenediamine-H_2O_2 electrochemistry system was adopted as the detection strategy in this 3D-μPEID as a model. Briefly, after the sandwich incubations between capture antibodies, antigens and HRP-labeled signal antibodies, this 3D-μPEID was connected to an electrochemical detector with a simple home-made device-holder (details in ESI†). With the aid of a section-switch, eight working electrodes were sequentially placed into the circuit to trigger the electrochemical reaction. The differential pulse voltammetric (DPV) signals were measured using a portable electrochemical workstation (PalmSens Electrochemical Portable Apparatus).

The DPV responses of the chitosan/MWCNTs modified paper working zone in this 3D-μPEID immobilized with sandwich cancer markers (CEA as a model), immunocomplexes labeled with HRP, are shown in Fig. 3. One DPV peak appeared at about −0.57 V *vs.* Ag/AgCl in the cathodic process, generated from 2,2′-diaminoazobenzene that is formed by the redox reaction of *O*-phenylenediamine.[9] Clearly, the sandwich immunocomplexes (curve b) giving a much higher DPV response than the nonspecific adsorption of signal antibodies without antigens (curve a) were observed in the absence of antigens, indicating very low levels of nonspecific adsorption of labeled signal antibodies. Furthermore, the DPV response increased with the increasing concentration of antigens (curve c). Therefore, it could be applied to the sensitive determination of cancer markers in this 3D-μPEID.

The optimum conditions such as pipetting volume of solutions are investigated in ESI.† Especially, the successful development of the multiplex immunoassay required that the common incubation time must be suitable for all analytes, and a total incubation time of 4 min was adopted in the further study. The analytical performance, the resistance to cross-talk and the reproducibility are shown in ESI.†

In conclusion, we have demonstrated a 3D-μPEID for high-performance, high-throughput, simple, fast, low-cost, and sensitive POCT. The advantages of this configuration includes: (1) the separated paper-electrode system will be very beneficial for the high integration and various distributional patterns of working electrodes on paper without considering the position and pattern of reference and counter electrodes; (2) bulk operations of the immunoreactions on working electrodes can be realized without considering the influence and contamination to reference and counter electrodes, and (3) this could further decrease the analysis cost due to the increased service life of the electrodes, especially the expensive Ag/AgCl reference electrode; (4) it will be easily understandable to obtain the electrochemical response just through stacking two papers in a simple home-made device-holder. In addition, this 3D-μPEID can be easily integrated and combined with the recently emerging paper electronics to further develop a simple, sensitive, low-cost, disposable and portable point-of-care testing device without a device-holder in our future work.

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