Lab on a Chip

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Cite this: *Lab Chip*, 2013, **13**, 4103

Paper-based electroanalytical devices with an integrated, stable reference electrode†

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Received 27th June 2013,
Accepted 7th August 2013

DOI: 10.1039/c3lc50771h

www.rsc.org/loc

This paper describes the development of a *r*eferenced *E*lectrochemical *P*aper-based *A*nalytical *D*evice (rEPAD) comprising a sample zone, a reference zone, and a connecting microfluidic channel that includes a central contact zone. We demonstrated that the rEPADs provide a simple system for direct and accurate voltammetric measurements that are referenced by an electrode with a constant, well-defined potential. The performance of the rEPADs is comparable to commercial electrochemical cells, and the layout can be easily integrated into systems that permit multiplexed analysis and pipette-free sampling. The cost of this portable device is sufficiently low that it could be for single-use, disposable applications, and its method of fabrication is compatible with that used for other paper-based systems.

Introduction

With the rapid development of portable lab-on-a-chip devices, miniaturized electrochemical systems have attracted increasing attention, because they are sensitive, insensitive to light levels, independent of human interpretation of color, easily interfaced with the web, and require only a small volume (several μL) of sample.[1] We and others are exploring Electrochemical Paper-based Analytical Devices (EPADs)[2–7] that combine the advantages of paper-based analytical devices (light weight, portability, pump-free sample transport) with those of electrochemical systems. EPADs often employ a printed Ag/AgCl "pseudo-reference electrode" whose potential ($AgCl(s) + e^- \leftrightarrows Ag(s) + Cl^-(aq)$) depends on the concentration of chloride ions in the sample solution.[8] These devices cannot maintain a stable potential unless a high and stable concentration of soluble chloride salts is added to the sample prior to measurement. This requirement limits their general applicability.

Several commercial hand-held micro-electrochemical diagnostic systems (for example, the i-STAT® clinical analyzer[9]) determine analytes in human blood through amperometry or potentiometry; these systems use miniaturized reference electrodes that resemble scaled-down versions of conventional glass-bodied Ag/AgCl reference electrodes. These three-dimensional miniaturized reference electrodes require sealing of an inner reference solution with a protective layer, and the implementation of a micro-junction between the reference solution and the sample.[10] They may require complicated lithography, or chip-manufacturing techniques.

Paper has been used as an integral component in electrochemical cells (for example, historically as a salt bridge to connect half-cells in conventional galvanic cells or to join two ion-selective electrodes in a multilayer slide for potentiometric measurements,[11] or more recently as a matrix to hold the internal reference solution in a separate reference electrode[12]). An integrated paper-based device that includes an accurate reference electrode would enable the full range of electrochemical measurements (*e.g.*, voltammetry, or measurements of pH and concentrations of ions) in a low-cost and disposable format.

Here we report the design of a well referenced Electrochemical Paper-based Analytical Device (hereinafter referred to as a "rEPAD", Fig. 1) comprising a sample zone, a reference zone, and a connecting microfluidic channel that includes a mixing zone. This arrangement allows ionic contact between the sample and the reference solutions, but – due to the slow (diffusive), convection-free transport of ions in the liquid-filled paper channels – prevents interchange of ions between the sample and reference zones. These rEPADs provide a stable and well-defined reference potential for direct and accurate measurements of analytes; their performance is comparable to conventional electrochemical cells that contain a commercial glass-bodied Ag/AgCl reference electrode, and they offer several advantages: (i) they are made of paper, which is inexpensive, lightweight, portable, less fragile than glass, and easily disposable (for single use), (ii) they eliminate the

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c3lc50771h

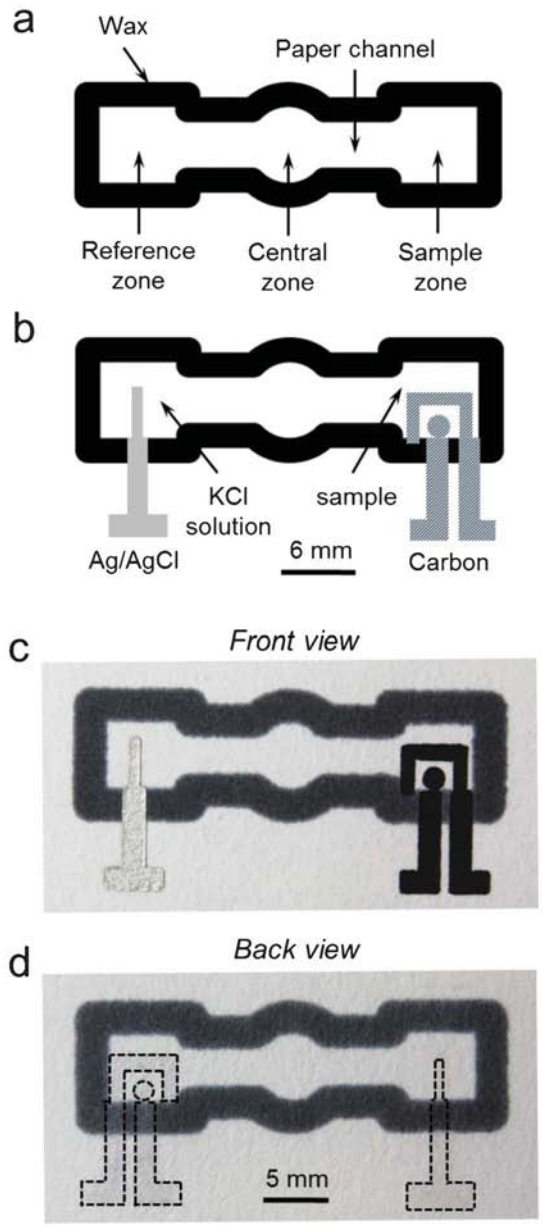

Fig. 1 Schematic illustrations (a and b) and photographs (c and d) of a referenced Electrochemical Paper-based Analytical Device (rEPAD). The paper was patterned by wax printing to define the sample zone, central contact zone, reference zone, and microfluidic channels. The sample and reference zones include stencil-printed carbon and Ag/AgCl electrodes, respectively. The dashed lines in (d) indicate the approximate boundaries of the carbon and Ag/AgCl ink printed on the back.

need for storage of the reference electrode in a solution of KCl, (iii) they are fabricated in a planar structure, and thus appropriate for mass fabrication with roll-to-roll printing, and (iv) they can be easily integrated into systems that permit multiplexed analysis or pipette-free sampling.

Experimental design

Device design and fabrication

Fig. 1 shows the geometry of the rEPAD. We defined the microfluidic channels (Fig. 1a) by wax printing,[13] and added electrodes to the device by stencil-printing carbon and Ag/AgCl inks in the sample and reference zones,[3] respectively. The resulting devices can be used without further modification (for experiments lasting <6 min), or can be sealed with tape to minimize evaporation (for longer experiments). The Supplementary Information (ESI†) provides additional details.

When two different solutions are added to the sample and reference zones, either by pipetting (~10 μL, for devices of the type shown in Fig. 1) or dipping (for the devices shown in Fig. 2), capillarity pulls the liquids (aqueous solutions of electrolytes such as potassium chloride) along the channels to the central zone. Once the liquids meet and completely saturate the hydrophilic network of cellulose fibers, capillary-driven flow and bulk convective transport of liquid stop. Instead, diffusional transport, due to concentration gradients across the interface between the two liquids, dominates the mass transport of ions within the device (subtle differences in the hydrodynamic pressure between the zones might, in principle, result in mass transport by convection, but we have found this contribution to be negligible – see ESI†).

Because the accuracy of the rEPAD depends on the concentration of the solutions in the reference and sample zones, and not in the zone of contact, we undertook experiments and calculations to determine if the sample and reference have constant concentrations within their respective zones. We sealed the tops and bottoms of the devices with transparent tape to minimize evaporation, and placed a solution of cobalt chloride ($CoCl_2$, pink) in contact with the left inlet of the device, and copper sulfate ($CuSO_4$, blue) with the right inlet. The pink Co^{2+} ions arrived (by wicking) at the zone of contact in less than 15 min (Fig. 2a). Following this initial rapid transport of liquid, we observed no pink Co^{2+} diffusing into the right zone for 2 h, and, correspondingly, no blue Cu^{2+} diffusing into the left zone. After 2 h, the device began to dry by evaporation, as indicated by the color change. The times required for both wicking and evaporation were shorter (<1 min and ~20 min, respectively) in devices that were not sealed with tape (Fig. S1 and ESI†).

We estimated the time required for an ionic species to diffuse from the interface of contact to the sample or reference zone using the Einstein relation (eqn (1)).[14]

$$\sigma^2 = 2Dt, \quad (1)$$

Assuming one-dimensional diffusion (a reasonable assumption since the thickness of the paper is about 200 μm, and the length of the mixing zone is ~5 mm), an ion would diffuse, σ = 5 mm, during the time period, $t = 10^3$–10^5 s (~20 min - 1 day), for a value of diffusion coefficient (D) expected for an ion (typically 10^{-8} to 10^{-10} m^2 s^{-1}).[15] As these times are much longer than the duration of a typical electrochemical measurement (<3 min), we conclude that diffusion is unlikely to appreciably change the concentration of ions in the sample

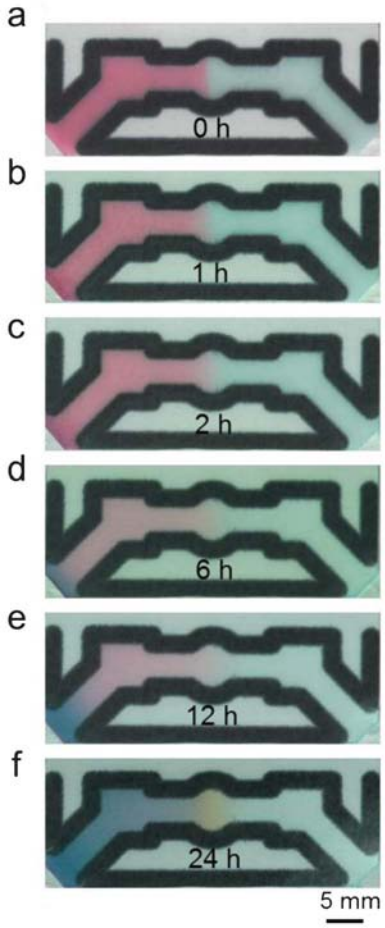

Fig. 2 Photographs of a sealed paper-based microfluidic device taken after the addition of a pink aqueous solution of $CoCl_2$ and a blue solution of $CuSO_4$ to the left and right zones, respectively. The two solutions mixed in the central zone but did not diffuse into the right or left paper zones. The color of the $CoCl_2$ solution became paler after 1 h and changed to blue after 12 h as water in the device gradually evaporated.

and reference zones of the rEPADs. Theoretically, by lengthening the channel by a factor of ten (plausibly using a serpentine pattern to keep the footprint of the rEPAD small), the times required would become very long (~1 day to ~4 months).

The hydrated rEPAD therefore allows physical contact and ionic conductivity between the reference and working electrodes, while preventing large-scale convection that would alter the concentrations of analyte and chloride ions in their respective zones (and thus shift the potential of the reference electrode). The central zone operates in a manner similar to the porous plug or frit used in glass-bodied reference electrodes (Fig. S2, arrow 6, ESI†), in which only a small junction potential is present. Other parts in the paper device (labeled by the arrows) perform functions similar to those of their glass-bodied counterparts.

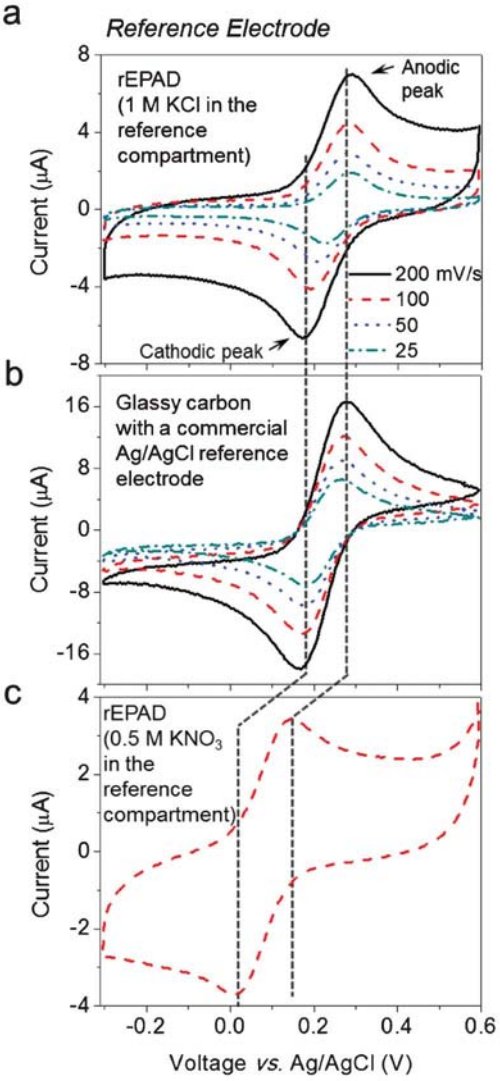

Fig. 3 Cyclic voltammograms of 1 mM $K_3[Fe(CN)_6]$ solution at various scan rates obtained from (a) a rEPAD containing a paper-based reference electrode and (b) a commercial electrochemical cell that includes a 3-mm diameter glassy carbon-disk working electrode and a conventional Ag/AgCl electrode. 1 M aqueous solutions of KCl and 0.5 M solutions of KNO_3 were used as the internal filling solution of the reference electrode and supporting electrolyte in the sample solution, respectively. (c) Cyclic voltammogram of 1 mM $K_3[Fe(CN)_6]$ solution obtained from a rEPAD using a reference solution of 0.5 M KNO_3 instead of 1M KCl.

Results and discussion

rEPADs for voltammetry

Having determined that the ionic constituents in our reference and sample zones were sufficiently isolated for application in a rEPAD, we used cyclic voltammetry, which provides both qualitative and quantitative information (oxidation/reduction potential, half-cell potential, reaction rates, and concentrations),[8] to compare the performance of this device (Fig. 1b–d, no sealing tape) with that of a conventional, glass-bodied, three-electrode system. Fig. 3 shows the effect of the reference solution on the peak potentials for the redox reaction of

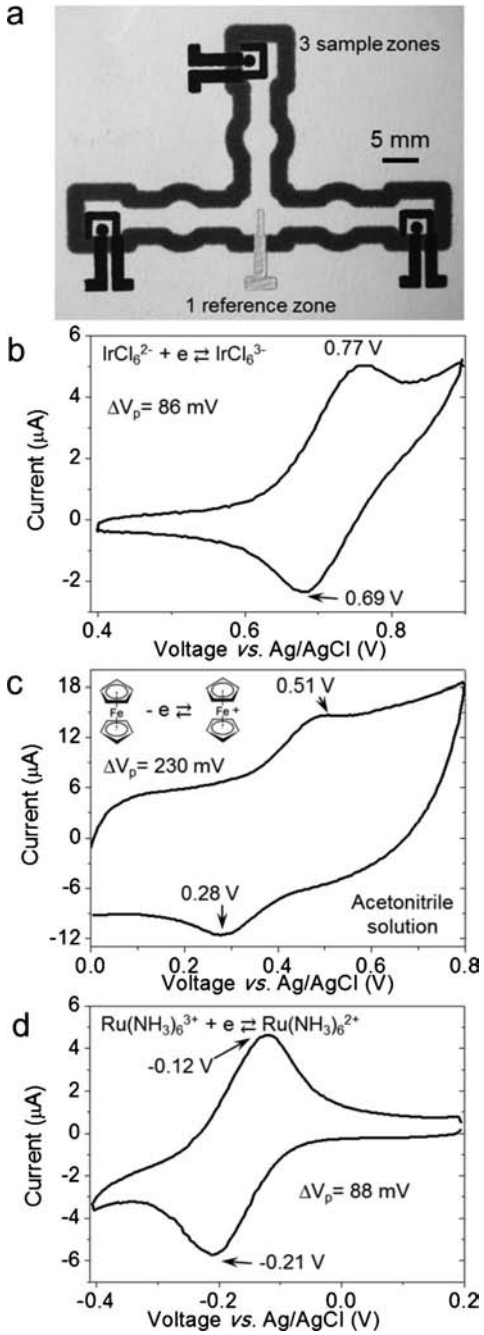

Fig. 4 (a) Photograph of a multiplexed rEPAD. Three pairs of carbon working and counter electrodes (located at the top, bottom left, and bottom right of the Figure) share the same Ag/AgCl electrode (bottom middle); this design allows electrochemical analysis of three (or by extension of the design, more) different samples simultaneously. (b), (c), and (d) Cyclic voltammograms obtained from a single rEPAD shown in (a) for analysis of three different analytes: 1 mM potassium hexachloroiridate(IV) (K_2IrCl_6) in 0.1 M aqueous KNO_3, 0.5 mM ferrocene ($Fe(C_5H_5)_2$) in acetonitrile solution with 0.1 M $TBAPF_6$, and 1 mM hexaammineruthenium(III) chloride ($Ru(NH_3)_6Cl_3$) in 0.1 M aqueous KNO_3. 10 μL each of the sample and reference solutions were added to the respective zones of a device with no sealing tape. The measurements were performed at a scan rate of 50 mV s^{-1}.

potassium ferri/ferrocyanide ($Fe(CN)_6^{3-}$(aq) + e^- ⇆ $Fe(CN)_6^{4-}$(aq)), as measured by cyclic voltammetry. By using rEPADs and a reference solution of 1 M KCl, we estimate anodic and cathodic peak potentials (Fig. 3a) for the ferri/ferrocyanide couple of 0.28 ± 0.01 and 0.19 ± 0.02 V, respectively. These results are in good agreement with the peak potentials (0.28 ± 0.02 and 0.17 ± 0.02 V) (Fig. 3b) obtained using the same sample solution and commercial electrodes (a 3-mm glassy carbon-disk working electrode, a platinum-mesh counter electrode, and a conventional Ag/AgCl reference electrode).

In contrast, the same rEPADs operating without chloride ions in the reference solution – an arrangement similar to that of a Ag/AgCl pseudo-reference electrode in direct contact with the sample solution – showed a large shift (~0.15 V, Fig. 3c) in the peak potentials. The peak shift is due to the ill-defined potential of this quasi-reference electrode, and corresponds to a decrease of approximately three orders of magnitude in the concentration of chloride ions in the solution bathing the Ag/AgCl electrode, based on calculations from the Nernst equation (0.059 V per decade). Under these experimental conditions, the paper-based electrochemical cell is no longer properly referenced, and the voltage information obtained from the voltammetric curves is not accurate. The rEPADs exhibit larger capacitive currents than the glassy carbon electrode, plausibly because these two systems use different carbon electrodes. The ESI provides additional details on the quantitative performance of the rEPADs.

rEPADs for multiplexed voltammetry

Having established that the rEPADs exhibit a voltammetric performance similar to that of commercial reference cells, we evolved the design of the paper-based devices into a multiplexed system that permits multiple samples to be analyzed simultaneously. Fig. 4a shows three separate electrochemical systems in a single device; the sample zones share the same reference electrode without cross contamination. This arrangement would be difficult to achieve with commercial electrochemical cells; an equivalent system would require three separate sample vials, each containing a working and counter electrode, connected *via* salt bridges to a fourth vial containing the reference electrode.

We tested the multiplexed rEPADs using three different redox couples, $IrCl_6^{2-/3-}$ (1 mM aqueous solution), ferrocene/ferrocenium (0.5 mM acetonitrile solution), and $Ru(NH_3)_6^{3+/2+}$ (1 mM aqueous solution). We chose these samples because they have very different peak potentials, and because they are widely used as standard redox couples for evaluating the performance of electrochemical devices, and as calibrants for unknown species. Fig. 4b–d show that the three samples exhibit the expected peak potentials (~0.7,[16] 0.42,[17] and −0.12 [8] V respectively, *vs.* Ag/AgCl).

The peak currents in Fig. 4 are close to each other, a consequence of the similar diffusivities of the redox couples. We emphasize that other electrochemical devices based on a Ag/AgCl pseudo-reference electrode cannot be used to analyze samples prepared in a solvent, such as acetonitrile, that does not dissolve KCl. The ESI discusses the compatibility between

acetonitrile and the wax barrier and the peak splitting in organic solutions.†

rEPAD for pipette-free sample introduction and extended lifetime

We modified the design of the rEPAD to enable application of the sample and reference solutions without the need for a pipette or injection device; this type of design makes the device suitable for use in the field. Fig. 5a shows a device that includes inlets at the corners to allow spontaneous wicking of solutions into the relevant zones. We investigated the working lifetime of the rEPADs by performing repeated cyclic voltammetry measurements at a scan rate of 25 mV s^{-1}. The device, which we sealed with tape in order to minimize the rate of solvent evaporation, was functional for approximately 1.5 h (at $T \sim 23$ °C, RH $\sim$ 15%) after the application of sample (it takes about 15 min for the solutions to wick into the device and mix). Fig. 5c shows that the peak potentials remain constant over the interval; this stability indicates a consistent concentration of chloride ions at the reference electrode. The reason for the decrease of the peak current with increasing time (>30 min) is not clear at this stage, but may be due to the diffusion of the sample away from the working electrode, or adsorption of analyte onto the electrode surface.

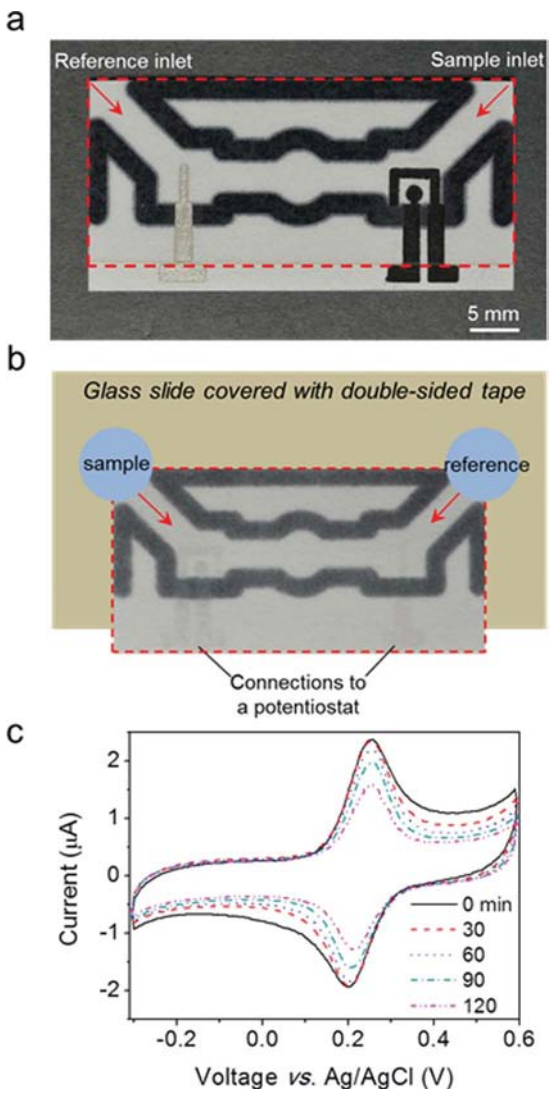

Fig. 5 (a) Photograph of a sealed rEPAD with the capability of performing both sample application and on-site sample analysis. Microfluidic channels are defined at the corners of the paper device. The dashed lines indicate the boundaries of the tape sealing the top of the device. (b) Schematic illustration of an electrochemical cell containing a sealed rEPAD. The rEPAD is attached to a glass slide by double-sided tape, with the electrode side facing the slide, in order to minimize gravity-driven fluid flow, which might cause the contamination of sample or reference zones. Drops of the sample and reference solutions were added to the top of the corresponding inlets to allow controlled and continuous wicking into the device. (c) Time-dependent voltammetric curves obtained from a sealed rEPAD shown in (a) with 1 mM $K_3[Fe(CN)_6]$ as the sample and 1M KCl as the reference solution.

Conclusions

Paper-supported fluids are attractive for electrochemical analyses, and reference electrodes are essential for the development of paper-based electrochemistry. By controlling mass transfer within paper channels (convective during filling; diffusive in use), we have thus designed an electrochemical paper-based analytical device capable of providing accurate voltammetric measurements that are referenced by an electrode with a constant, well-known potential.

The rEPADs have advantages over other miniaturized electrochemical devices: (i) the reference electrode is separate and thus provides a well-defined potential, (ii) the layout can be easily modified, depending on the intended analytical purpose, (iii) the fabrication process does not require complicated thin-film microfabrication processes, and (iv) the device is compatible with samples prepared in some non-aqueous solvents (*e.g.*, CH_3CN). These rEPADs have a simpler structure and a more fully integrated electrochemical reference potential than other paper-based reference electrodes.[12]

These devices are particularly suitable for single-use applications that require a separate reference electrode or an accurate potential, or in cases where chloride ions may interfere with the electrochemical experiment. Combining rEPADs with other, previously demonstrated functions of paper-based devices, including valving, sample pre-concentration, and storage or immobilization of reagents for (bio)chemical assays,[18] should enable more advanced forms of electrochemical analysis in rEPAD-based systems. The concept may also be extended to the construction of electrochemical devices based on other low-cost materials such as nitrocellulose, cloth, Nylon, cotton string, or silk.

Acknowledgements

This work was supported by the Bill and Melinda Gates Foundation (51308). E.J.M. thanks the Natural Sciences and Engineering Research Council of Canada for individual support (NSERC PDF). C.P. acknowledges Generalitat de Catalunya for the fellowship 2011 BE1 00520.

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