LONG-TERM DRY-STORAGE OF ENZYME-BASED REAGENT SYSTEM FOR ELISA IN POINT-OF-CARE DEVICE

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ABSTRACT

Lateral flow devices commonly used for many point-of-care (POC) applications in low resource settings lack the sensitivity needed for many analytes relevant in the diagnosis of diseases. This limitation mainly arises from the lack of signal amplification, which is commonly used in laboratory assays but uses temperature-sensitive reagents and inherently requires multiple assay steps not normally compatible with POC settings. Here, we describe a method for the long-term dry storage of ELISA reagents: horseradish peroxidase (HRP) conjugated antibody label and its substrate diaminobenzidine (DAB). Further, we incorporated these dry reagents into a two-dimensional paper network (2DPN) device and demonstrate an automated ELISA.

KEYWORDS: dry reagent, horseradish peroxidase, signal amplification, diagnostics, ELISA

INTRODUCTION

Point-of-care (POC) devices that are sensitive, reliable, low cost, rapid, easy-to-use and disposable could improve the ability to diagnose disease in low-resource settings. Lateral flow tests are simple wicking-based devices that often use colloidal gold for detection of proteins or antibodies, but they lack the sensitivity needed for the clinically relevant detection of many analytes. Enzyme-based signal amplification such as the one used in ELISA could greatly improve the limit of detection (LOD) if it could be used in POC devices. This poses two challenges: 1) demonstrating the on-board stability of the reagents, namely the enzyme horseradish peroxidase (HRP) conjugated antibody and its substrate, in anhydrous form, and 2) demonstrating the capability of the POC device to perform the sequential multistep processes needed for the enzyme-based signal amplification without user intervention.

There have been only a few reports on the preservation of the enzyme HRP [1-5], and the incorporation of enzyme-based assays in manually-operated lateral flow-like devices [6, 7], but the long-term stability of the enzyme conjugate and its substrate at elevated temperatures has not been reported. In addition, these assays have not been translated to an automated POC format. Herein we describe a method for the long-term elevated-temperature dry storage of reagents for signal amplification – antibodies conjugated to horseradish peroxidase (HRP) and the HRP substrate diaminobenzidine (DAB). Further, we demonstrate that these reagents can be incorporated into a two dimensional paper network (2DPN) device [8] that performs an automated ELISA for POC use.

EXPERIMENTAL

A malarial assay secondary antibody (anti-PfHRP2) conjugated to HRP (HRP-antibody) was used for the study of long-term stability of the enzyme during dry storage. A 10 µl mixture of 0.01 M Fe^{2+}-EDTA, 4 % trehalose and 0.1 % BSA containing 1 µg of HRP-antibody was added to glass fiber pads placed in polystyrene microtiter strips and dried in a vacuum centrifugal evaporator. The pads were vacuum-sealed, placed in a vapor barrier pouch and stored desiccated at 22 °C or 45 °C. The HRP substrate DAB, prepared in water at a concentration of 2 mg/ml containing 4 % trehalose, was also dried in glass fiber pads and stored similarly to the HRP-antibody. The reagents in the pads were periodically rehydrated and tested for HRP-antibody and DAB functionality for over 5 months.

For testing the HRP stability, a colorimetric assay using the tetramethylbenzidine (TMB) substrate was used. Kinetics of the TMB oxidation by HRP to a blue color product was measured at 650 nm every 15 seconds over a period of 5 minutes. The initial rate of the reaction was calculated by taking the first 8 readings and percent activity calculated by comparing to the fresh HRP-antibody.The functionality of the rehydrated secondary HRP-antibody was also tested in a dipstick-style lateral flow immunoassay using nitrocelluose striped with PfHRP2 capture antibody. The assay was performed in sequential steps using a malaria biomarker (recombinant PfHRP2 antigen) at 50 ng/ml spiked into fetal bovine serum, HRP-antibody rehydrated to 10 µg/ml in PBST, and fresh DAB substrate at 0.125 mg/ml in PBST buffer containing sodium percarbonate (0.025 %) as the hydrogen peroxide source. The images were scanned and quantified using ImageJ. The assay signal was calculated as the background-subtracted intensity, and the percent activity retained after dry-storage of HRP-antibody was calculated from fresh HRP-antibody control.

The DAB substrate dry preservation was also tested by the dipstick malarial immunoassay as described above. The substrate samples were rehydrated to a concentration of 0.125 mg/ml in PBST containing hydrogen peroxide and fresh secondary HRP-antibody was used. The assay signal intensities for fresh and dry-preserved DAB were measured as above and percent activity was calculated.

A folding 2DPN device was used to demonstrate automated ELISA using dry reagents. The device is composed of a nitrocellulose three-inlet network cut using a CO_2 laser cutting system and patterned with PfHRP2 antibody at the detection region. The nitrocellulose network along with a cellulose wicking pad was housed on one side of a foldable Mylar laminate card. Glass fiber pads containing dry- preserved HRP labeled secondary antibody, DAB and buffer were located on the other side of the foldable card. Malarial antigen spiked in fetal bovine serum, and PBST buffer containing

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hydrogen peroxide was added to rehydrate the dry HRP-antibody and DAB respectively. The device was folded to simultaneously activate reagent flows through the nitrocellulose to the detection zone. The detection regions of the devices were scanned after 15 minutes.

RESULTS AND DISCUSSION

Figure 1a shows the long-term stability of the enzyme HRP at 45 °C. The HRP-antibody dry-stored in the presence of Fe^{2+}-EDTA and trehalose retained 80% of the activity of the fresh control as determined by colorimetry. In the presence of trehalose alone the enzyme retained only 20% activity. The activity of the HRP-antibody was also evaluated in a malarial assay by a dipstick sandwich immunoassay using DAB as the substrate. The signal is seen as a brown precipitate due to oxidation of DAB by HRP. The HRP-antibody activity was fully functional with signal intensity identical to the fresh HRP-antibody (Figure 1b). The DAB dry preservation was also tested in a dipstick immunoassay and is shown to retain > 90% activity after storage at 45 °C for 3 months (Figure 2).

Figure 3a shows a folding 2DPN device with integrated dry reagent pads that performs an automated ELISA for the malaria biomarker with a single user activation step. The antigen-antibody complex with the HRP label moved through the first inlet and across the detection zone followed by the DAB substrate from the second inlet, and finally a wash buffer from the third inlet. Figure 3b shows an example result from an automated ELISA card. The signal from the DAB precipitate can be easily visualized by eye at the detection zone. This method of on-card enzyme signal amplification can also be quantified using a webcam or a flat-bed scanner. Figure 3c shows images for an antigen dilution series for the automated ELISA cards with on-board dry reagents.

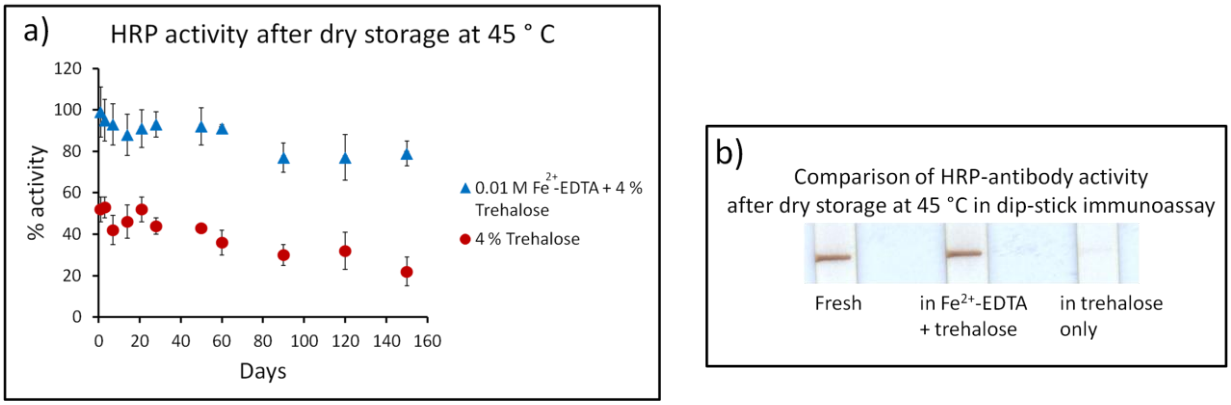

Figure 1: Activity of HRP-labeled antibody after dry storage. a) Chart showing the HRP enzyme activity retained after dry storage at 45 °C at different time points as determined by colorimetry. The HRP enzyme in the presence of 0.01 M Fe^{2+}-EDTA and 4 % trehalose retained ~ 80% activity on dry storage at 45 °C after 5 months. In the presence of trehalose alone the enzyme retained only 20% activity b) Images of the dipstick immunoassay using dry-stored (4 months) HRP -antibody and fresh DAB substrate in a malarial sandwich immunoassay.

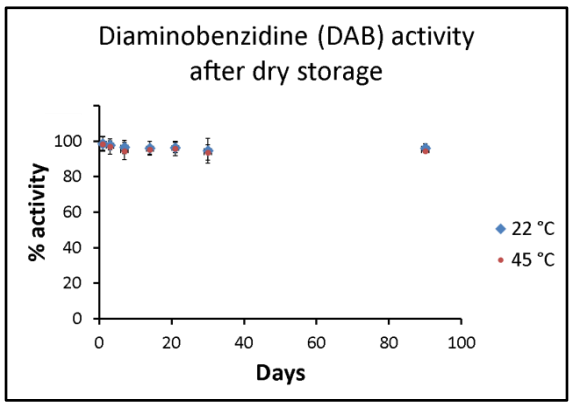

Figure 2: Activity of the HRP substrate DAB after dry storage. DAB substrate stored dry at 22 °C and 45 °C in the presence of 4% trehalose in a glass fiber pad was tested periodically for its functionality in a dip-stick malarial sandwich immunoassay using fresh secondary HRP-labeled antibody. The DAB substrate retained functionality upon rehydration at >90 % after 3 months of dry storage when compared to fresh DAB.

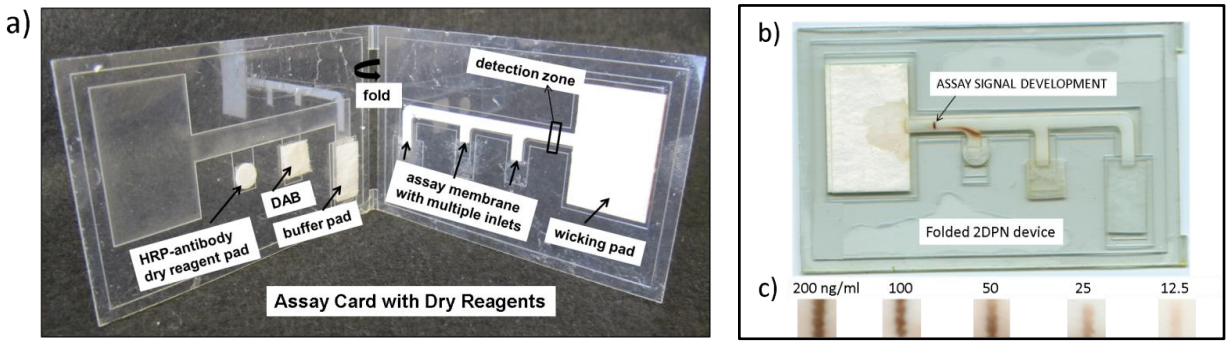

Figure 3: a) A folding 2DPN device with incorporated dry reagents for automated ELISA b) Folded device showing malarial sandwich immunoassay signal development. c) Images of the malarial antigen dose response in the 2DPN assay with dry reagents.

CONCLUSION

We report a method for dry preservation of reagents used in ELISA. We have shown that HRP-antibody and its substrate DAB retained their activity on long-term elevated temperature dry storage and were fully functional in an immunoassay. Our method for drying down the reagents in a glass fiber pad has the benefit of easy incorporation into POC devices, including conventional microfluidic or paper-based devices. Further we have demonstrated an automated ELISA using a 2DPN device with dry reagents integrated into the device. The dry preservation of enzyme-based signal amplification reagents could be used for a variety of applications and have particular advantages for use in POC devices for enhanced sensitivity, portability, and ease-to-use for low resource settings.

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