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Article

Polymer Coating for the Long-Term Storage of Immobilized DNA

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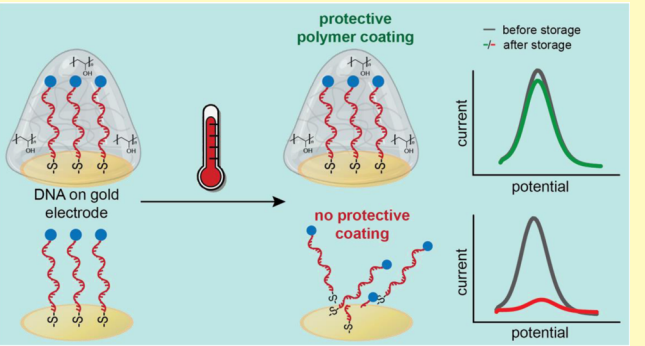
ABSTRACT: As healthcare systems worldwide demand early disease detection and personalized medicine, electrochemical biosensors stand out as a promising technology to meet these demands due to their sensitivity, selectivity, and rapid response. Specifically, DNA-based electrochemical biosensors are versatile and have been used to identify biomarkers of various infectious diseases. However, there is a significant gap between laboratory-scale proof-of-concept systems and commercially viable technologies. Commercialization of such sensors faces many challenges, with one of the most important being the stability and shelf life of the immobilized DNA. Surface-associated DNA faces thermal degradation, structural changes, and oxidation of tethering thiol groups, which causes DNA stripping from the surface. Currently, technology to support the long-term storage of these sensors at ambient temperatures is limited. Here, we report a novel method to preserve DNA in electrochemical biosensors through the application of a protective coating of poly(vinyl alcohol) (PVA). We show that with our PVA coating, the shelf life of dried, DNA-functionalized electrodes at ambient temperature is a minimum of 2 months. We further demonstrate that the protective capabilities of PVA extend to temperatures as high as 65 °C and that the biological relevance of the assay is not impacted by the coating. Our simple approach to DNA protection supports our understanding of how the electrode interfaces with biomolecules and facilitates biosensor scaling and commercialization.



KEYWORDS: *electrochemical sensors, biosensors, nucleic acids, long-term stability, poly(vinyl alcohol)*

Electrochemical biosensors are potentially important technologies for disease diagnosis, offering a combination of sensitivity, selectivity, and rapid response.[1−4] However, their applicability beyond the glucose meter has remained limited due to challenges with platform stability and scalability. Generally, such sensors function by employing biorecognition elements, such as proteins or nucleic acids, to detect disease biomarkers. These approaches do not require extensive sample preparation or sophisticated infrastructure and have the added benefit of low-production costs, portability, and straightforward use.[5,6]

In particular, DNA-based electrochemical biosensors are popular because of their versatility, cost-efficiency, and compatibility with signal amplification of DNA.[7−11] For sensing, DNA is generally modified with a redox-active label, such as methylene blue or ferrocene, at one end and a binding moiety to immobilize the strand on an electrode surface at the other end.[12] Gold electrodes are common substrates because they are readily modified by self-assembly of thiolated DNA,[13] simplifying the preparation of DNA monolayers.[14,15] The combined advantages of DNA-modified electrodes have therefore led to their extensive development for biosensing.[16−21]

Despite the potential of DNA-based platforms to overcome economic barriers associated with electrochemical biosensor commercialization, these technologies remain confined to research laboratories.[22,23] One major challenge in commercializing these sensors is maintaining the stability of the immobilized DNA in suboptimal storage conditions.[24,25] Thiol−gold bonds are susceptible to disruption due to high temperatures or drying, leading to degraded signals.[26,27] Further damage to DNA can occur from reactive oxygen species (ROS) generated by the redox probe or from UV exposure to aqueous storage solutions.[28] Thus, DNA monolayers are generally formed just prior to the use of the device, limiting their use outside of the laboratory.

While the diagnostics industry has had some success commercializing shelf-stable DNA-based sensors, their stability continues to be cited as an ongoing issue in sensor literature.[27] Specifically, prolonging the shelf life of the DNA monolayer is viewed as a critical challenge to overcome for sensor technology commercialization.[27,29] Improving stability in DNA-based sensors has generally involved engineering the

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DNA monolayer, including the use of alkanethiol coadsorbates for surface passivation and overall monolayer stability.[30] However, these molecules still suffer from challenges associated with thiols, including oxidation and degradation.[31] Furthermore, while alkanethiols with longer carbon chains improve the stability of DNA monolayers, they can decrease electron transfer efficiency at the surface, reducing the sensitivity of sensors.[30] Multidentate thiols have also been used to enhance the stability of the DNA monolayer,[32,33] but these necessitate complex syntheses and storage in humid, refrigerated environments. Similarly, implantable DNA sensors have been shown to remain stable for extended timeframes in biological fluids at elevated temperatures,[34] but *in vivo* stability does not extrapolate to long-term, *ex vivo* storage.

In biochemistry, DNA is known to be stabilized by preservation agents such as trehalose, but even these approaches show diminished stability when stored in warm environments.[35,36] Polymer coatings, mainly based on collagen and hydrogels, have also been used to protect DNA-modified surfaces.[37−39] Poly(vinyl alcohol) (PVA), a hydrophilic polymer capable of embedding small particles, has also been used as a protective agent for DNA.[40] The hydrophilicity, high glass transition temperature, and flexible film-forming abilities of PVA are thought to be key contributors to its stabilizing effects.[41] Additionally, PVA is chemically inert and biocompatible, making it favorable for use with biomolecules.[42] Despite these beneficial characteristics, PVA has yet to be evaluated for DNA monolayer protection. Here, we report PVA as a novel preservation method for DNA monolayers in electrochemical systems. Upon application of PVA to DNA-functionalized electrodes, we maintain stability under nonideal storage conditions for a minimum of two months, including at high temperatures. Further, following PVA coating, storage under nonideal conditions, and PVA removal, DNA is found to maintain a biologically relevant conformation. Taken together, these results demonstrate the immense power of a relatively simple approach to enable the storage and transport of DNA-based electrochemical biosensors under nonideal conditions. These capabilities solve a critical challenge in the translation and commercialization of biosensors.

RESULTS AND DISCUSSION

Preparation and Characterization of DNA-Modified Electrodes and PVA Coatings. Though many disposable gold electrode substrates exist, we have previously demonstrated the superior quality of in-house fabricated gold leaf electrodes for DNA-based sensing as compared to popular screen-printed electrodes.[19,20] We therefore employed these electrodes for stability evaluation. Electrodes were fabricated as previously described and functionalized with linear, single-stranded DNA probes modified with a 5′ methylene blue (MB) and a 3′ thiol. Mercaptohexanol was then applied as a passivating agent. Following electrode modification with DNA, the MB electrochemical signal was characterized by square-wave voltammetry (SWV). From these data, proper assembly of the MB-DNA monolayer was confirmed.

Following characterization of the electrochemical behavior of these DNA-modified electrodes, PVA was applied to the DNA monolayer. As PVA is produced with a range of molecular weights, we evaluated multiple polymer molecular weight ranges: 9k−10k, 13k−23k, 30k−70k, and 89k−98k g/mol. Solutions of each PVA molecular weight were prepared as 1% weight by volume (w/v) aqueous solutions. The PVA solution was then drop casted onto the working electrode and allowed to air-dry to form a transparent thin-film coating (Figure 1).

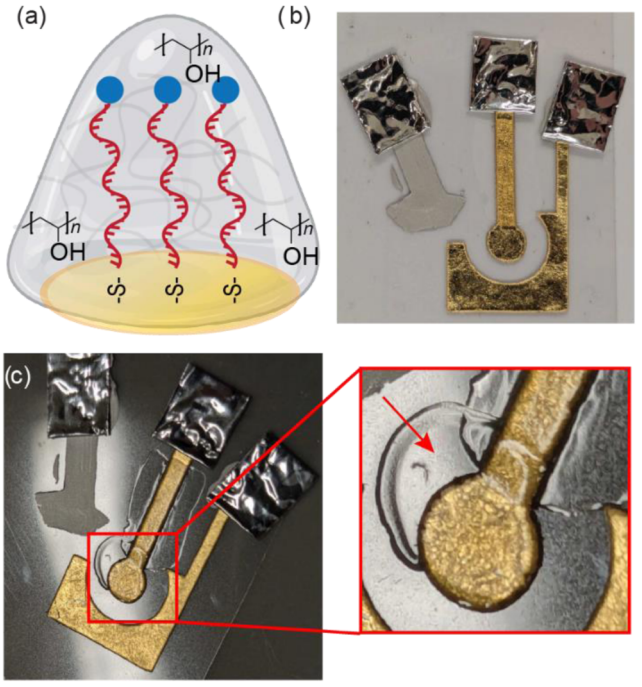

Figure 1. Preparation of the DNA-modified electrodes and PVA coating. (a) Gold electrode was first functionalized with methylene blue (MB)-labeled DNA through thiol−gold interactions. PVA was drop casted onto the surface and allowed to air-dry to form a thin film. (b) Gold electrode before applying the PVA coating. (c) Gold electrode after forming the PVA thin-film coating. The magnified image of the working electrode indicates the dried PVA film.

Extended Shelf Life of DNA-Functionalized Electrodes. Based on existing literature, DNA monolayers on gold can be stored in buffer at 4 °C for up to 1 week while maintaining sufficient signal for subsequent biosensing.[43] Thus, we initially evaluated the protective behavior of PVA under these conditions. Based on previous reports of PVA for biomolecule protection,[44] 13k−23k g/mol PVA was selected for stability studies. Electrodes functionalized with MB-DNA were coated with a 1% w/v PVA solution and allowed to dry. As controls, electrodes were stored in 10 mM Tris buffer (pH 8.5) as well as dried in the absence of PVA. Stability of the DNA monolayer was evaluated by comparing the signal from the MB before PVA coating and storage to that after storage over varying lengths of time (Figures 2a and S1).

Our results confirm previous reports of DNA instability on electrodes. Following dry storage for 3 days, electrodes not treated with PVA showed only 23 ± 8% of the MB signal remaining (Figure 2b). The MB signal continues to decrease over subsequent days, with only 16 ± 6% of the original signal remaining after 7 days. In contrast, for DNA-functionalized electrodes stored in Tris buffer, 67 ± 6% of the signal remained after 3 days and 63 ± 5% after 7 days. However, a drastic signal decrease is observed over the next week of storage, with only 3 ± 3% of the original signal remaining after 14 days. These results agree with literature reports of DNA monolayer stability, with a maximum of 1 week of stability for storage.[43] In contrast, PVA-coated electrodes showed significantly

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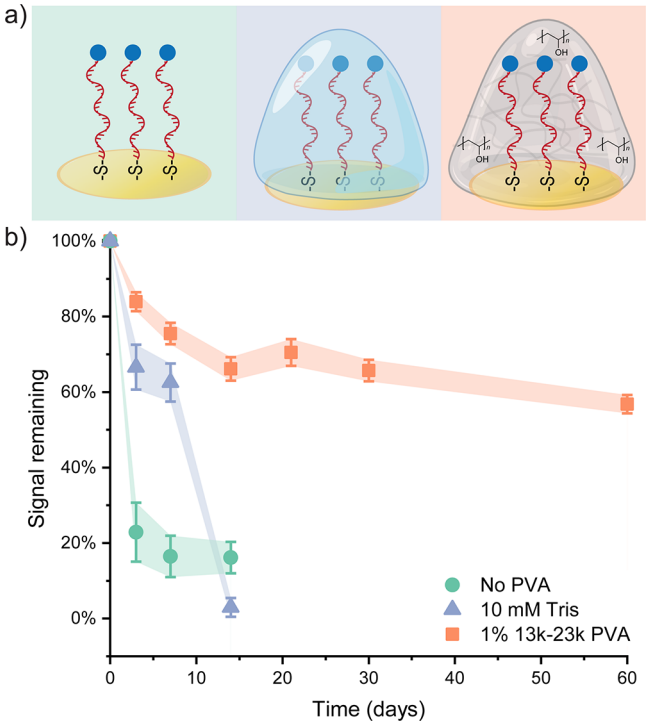

Figure 2. Comparison of the long-term storage of DNA-functionalized electrodes at room temperature. (a) DNA-functionalized electrodes were stored under, from left to right: coated with 1% PVA (13k–23k g/mol), stored in 10 mM Tris buffer (pH 8.5), or left dry. (b) MB signal remains for each of the three storage systems over the course of 60 days. Error bars represent standard error (n = 12, 12, 11, 12, 10, and 15 for PVA; n = 11, 11, and 12 for no PVA; n = 12, 12, and 10 for 10 mM Tris, at each time point).

increased stability; after 14 days, 66 ± 3% of the original MB signal remained. The initial signal drop within the 14 days is likely due to a combination of the rearrangement of the DNA monolayer, initial rapid desorption of the DNA due to polymer-gold interactions, change in DNA orientation, and electrical stress on the monolayer from the baseline scan.[15,34]

Further, after two months of storage, 57 ± 2% of the original signal remained on protected electrodes. This is consistent with previous reports of PVA serving as a barrier to gas diffusion, inhibiting thiol oxidation and DNA desorption, and demonstrates the potential of PVA to serve as a protective agent.[45]

Optimal PVA Conditions for Storage. Following initial demonstrations of the PVA-based protection of DNA-functionalized electrodes, we evaluated the impact of altering the PVA composition on protection. Because the physical properties of PVA change with its average MW, we hypothesized that both the PVA MW and the solution concentration impact the protection ability. Thus, we evaluated a variety of PVA sizes and concentrations to determine the optimal conditions for protection.

We first evaluated multiple polymer molecular weight ranges: 9k–10k, 13k–23k, 30k–70k, and 89k–98k g/mol. DNA-functionalized electrodes were coated with 1% w/v PVA solution, followed by storage at room temperature for 14 days. Two weeks of storage was selected to enable comparison with buffer-stored electrodes that degrade by this point. The SWV MB signal following storage was compared to the prestorage signal to determine a percent signal remaining. Similar protective capabilities were observed for the 9k–10k and 13k–23k g/mol molecular weights of PVA. For both cases, approximately 65% of the signal remained following 14 days of storage (Figure 3a). However, as the molecular weight of the protective polymer increases, we observe a decrease in the remaining MB signal and an increase in the variability. Notably, only 24 ± 6% of the initial signal remained for the 89k–98k g/mol group, a statistically significant difference from

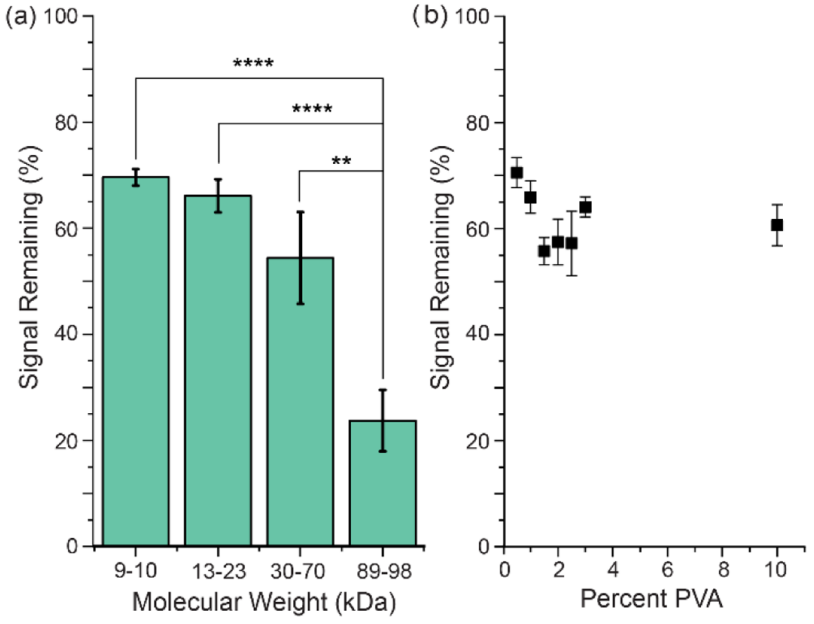

Figure 3. PVA protection depends on the molecular weight of the polymer and the solution weight percentage. (a) MB signal remains after 14 days when electrodes are coated with a 1% PVA solution of various molecular weights. (b) MB signal remains after 14 days when electrodes are coated with PVA (13k–23k g/mol) solution of various concentrations. Error bars represent standard error (n = 12, 11, 12, and 12 for molecular weights from low to high, n = 11, 11, 11, 12, 12, 10, and 11 for percentages from low to high).

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the other molecular weights. We speculate that the significant signal decrease was because the coating does not fully dissolve during the wash phase, and the remaining PVA impeded the electron transfer between the MB and electrode. This hypothesis was subsequently confirmed by SEM imaging, as described below. Thus, we employed 13k−23k g/mol PVA for subsequent stability studies.

Following the determination of the PVA molecular weights that are effective for protection, we then evaluated the impact of the solution concentration. Solutions of 13k−23k g/mol PVA were prepared at concentrations ranging from 0.5−3% w/v, and stability was again evaluated following 14 days of storage. For all PVA concentrations tested, around 60% of the original signal remained, with no statistically significant difference between concentrations (Figure 3b). To determine if equivalent protection is maintained at even higher concentrations, we additionally evaluated a 10% w/v PVA solution. Above 10% w/v, the PVA solution becomes viscous and difficult to drop-cast. Again, following 2 weeks of storage, we observed around 60% signal remaining for the sample treated with 10% w/v PVA. Together, these results demonstrate that the MW of PVA has a greater impact on protection than the percent used. These results are consistent with prior reports that, as the MW of PVA increases, its solubility decreases and both viscosity and tensile strength increase. All of these properties impact our ability to remove the PVA following storage prior to biosensor use. In contrast, the main impact of increasing solution concentration is the viscosity of the solution.[46−48] Thus, we employed 1% w/v solutions of 13k−23k g/mol PVA for additional stability experiments.

Physical Basis for Observed PVA Molecular Weight Differences. Based on the observed differences in PVA protection, we hypothesized that larger polymers do not support signal maintenance from DNA monolayers as efficiently because they are less soluble than their smaller counterparts. We therefore characterized the PVA-coated electrodes with scanning electron microscopy (SEM) both following PVA coating and after dissolution of the films (Figures 4, S2, and S3). As was previously observed with our in-house fabricated gold leaf electrodes, the bare electrode is mostly smooth on the micrometer scale, with some undulating features.[21] Following coating with PVA of all molecular weights evaluated, the surface morphology changed significantly (Figure 4b,c). Further, the morphology of the film differs based on the molecular weight of the PVA. The size of the features and their density in the PVA films were found to depend on the polymer molecular weight. In particular, with 89k−98k g/mol PVA, the features observable in the films from other MWs disappear, and small, bead-like features appear within a mostly smooth surface (Figure 4c). We attribute this unique morphology to a thicker polymer layer formed from drop-casting. The differences in morphology are consistent with the subsequent electrochemical behavior of the electrodes, with PVA-coated electrodes of all MWs other than 89k−98k behaving similarly.

To further confirm that differences in PVA behavior were responsible for observed differences in signal maintenance, we dissolved the coating and reimaged it by SEM. PVA was removed from the electrodes by soaking them in DI water for 5 min, followed by rinsing. PVA films were no longer visible by eye following soaking (Figure S4). We then acquired SEM images of the electrodes after the PVA coating was removed

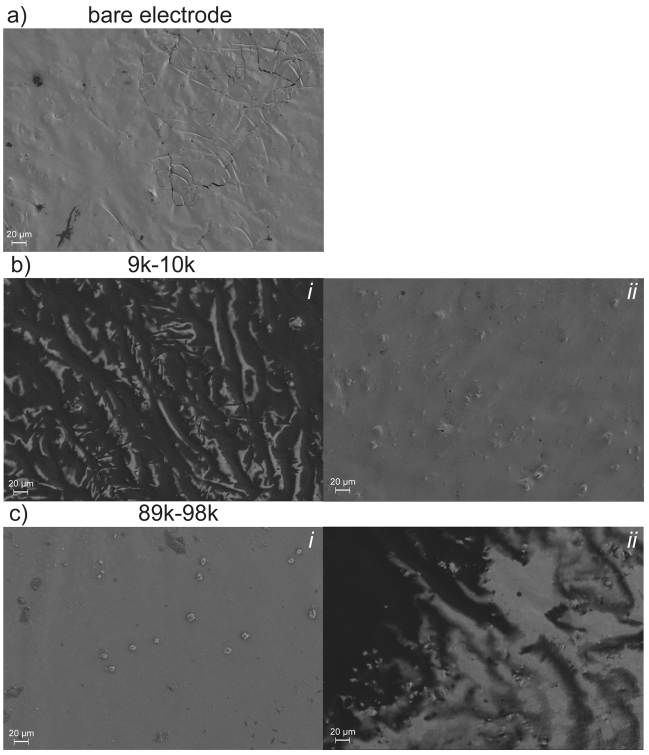

Figure 4. Protective PVA coating on DNA-functionalized gold electrodes. (a) SEM images of a bare gold electrode. (b) SEM images of electrodes following (i) 9k−10k molecular weight PVA coating and (ii) dissolution of the coating. (c) SEM images of electrodes following (i) 89k−98k molecular weight PVA coating and (ii) dissolution of the coating.

(Figure 5). After dissolution, electrodes coated with PVA of MWs lower than 89k−98k g/mol lose their characteristic features and instead show smooth features consistent with the surface of the bare electrode. In contrast, electrodes coated

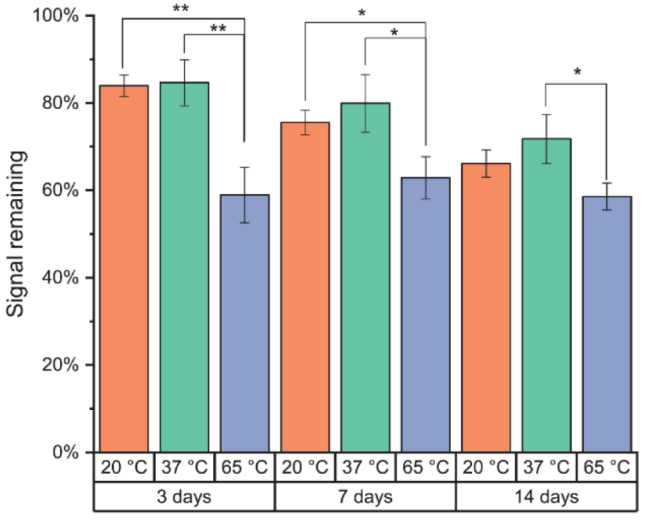

Figure 5. DNA-functionalized electrodes coated with 1% PVA (13k−23k g/mol) can be stored at high temperatures. The remaining MB signal was measured over the course of 14 days for electrodes stored at 20 °C (orange bars), 37 °C (green bars), and 65 °C (blue bars). Error bars represent standard error (n = 12, 12, and 11 for 20 °C, n = 10, 11, and 10 for 37 °C; n = 11, 11, and 12 for 65 °C, from times ranging from 3, 7, and 14 days).

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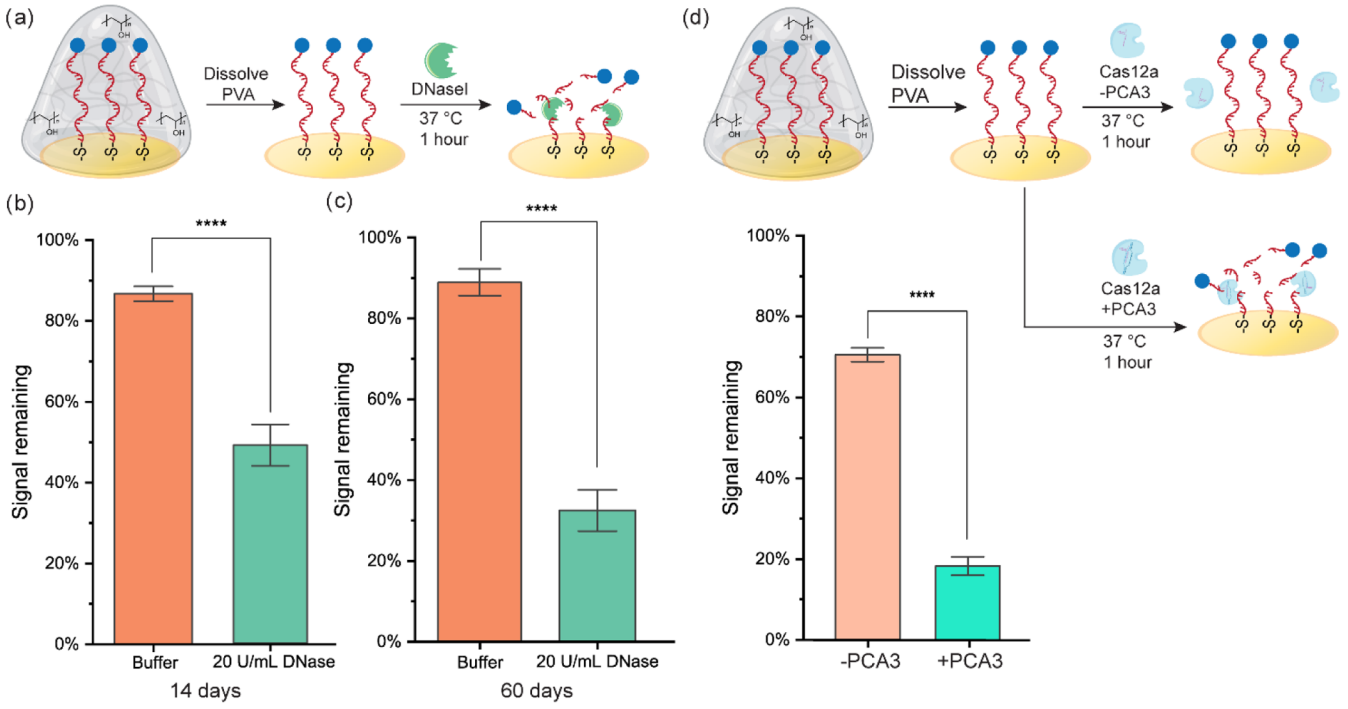

Figure 6. Enzymatic treatment on the electrodes. (a) DNase degradation of PVA-protected electrodes following 14 days or 60 days of storage at room temperature. DNA-functionalized electrodes were rinsed with water to dissolve the 1% PVA (13k−23k g/mol) coating and exposed to DNase I at 37 °C for 1 h. Successful cleavage results in the loss of the MB tag and reduces the MB signal. (b) MB signal following DNase for samples stored for 14 days. (c) MB signal following DNase for samples stored for 60 days. Error bars represent standard error (n = 12 for both groups in 14 days, n = 5 for both groups in 60 days). (d) Cas12a-based *trans*-cleavage of PVA-protected electrodes following 7 days of storage at room temperature. DNA-functionalized electrodes were rinsed with water to dissolve the 1% PVA (13k−23k g/mol) coating and exposed to Cas12a, with or without PCA3, at 37 °C for 1 h. Successful cleavage results in the loss of the MB tag and reduces the MB signal. (d) MB signal following DNase for samples stored for 14 days. Error bars represent standard error (n = 6 for the negative group, n = 5 for the positive group).

with 89k−98k g/mol PVA do not return to the smooth, bare electrode surface. Instead, regions of the surface appear to be bare electrode, while other regions maintain the morphology of a PVA film, indicating that regions of the surface continue to have polymer coatings. These observations are consistent with only partial dissolution of the PVA, as we had hypothesized is the reason for decreased efficacy to protect the electrochemical signal from DNA monolayers. This high MW PVA remains in regions of the surface even following dissolution for 1 h.[49] Thus, our images of the polymer films and the surface following film dissolution confirm our hypothesis that the shorter polymers dissolve completely upon soaking of the electrode, but the longest polymer does not.

Protection under High Temperatures. While DNA exhibits increased thermal stability relative to other biomolecules, such as RNA and proteins, high temperatures can cause thermal degradation of DNA and higher rates of thiol oxidation. Both of these factors contribute to monolayer instability and signal decreases upon DNA-modified electrode storage. Unfortunately, such stressors are often unavoidable during transport under standard conditions. Currently, the main alternative is high-cost, logistically challenging, and energy-intensive end-to-end cold chain shipping. Thus, the ability of DNA-modified electrodes to withstand high temperatures would represent a significant step toward their broad applicability. We challenged our PVA coatings with high temperatures, evaluating both 37 and 65 °C. These temperatures were selected both because they are the temperatures of several biological assays and because they can be reached during nontemperature-controlled shipping. Stability was measured based on the remaining MB signal at three, seven, and 14 days of storage.

At 37 °C, no difference was observed across time points, with protection after 14 days being equally efficient to three-day protection (Figure 5). However, following storage at 65 °C, an immediate decrease in the remaining signal was observed, with 59 ± 6% of the initial signal remaining after 3 days of storage. This decrease is statistically significant (p < 0.001) compared to decreases observed from samples stored at 20 or 37 °C. However, subsequent storage over the course of 2 weeks did not result in additional signal decreases. After 2 weeks of storage, the remaining signal is comparable to signals from samples stored at lower temperatures. Thus, we conclude that our PVA coating confers protection against high-temperature stress and offers improved stability under conditions relevant for technology dissemination.

DNA Viability Studies. DNA-cleavage enzymes, such as restriction enzymes and CRISPR-Cas enzymes, are common components of DNA-based electrochemical diagnostics.[19,20,50−52] Enzymatic degradation of DNA generally removes the redox label from the system. Thus, for our PVA-based storage system to be used in diagnostics, the DNA must retain a biologically relevant conformation following PVA film dissolution. For PVA-coated samples that were stored for 21 or 60 days, after dissolving the coating and measuring the remaining MB signal, we applied either DNase I or enzyme-free buffer to the electrodes and incubated the surfaces for one h, followed by remeasurement of the signal. When DNase I is active, the MB-modified DNA is cleaved, removing the MB

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from the surface and yielding a smaller signal (Figures 6 and S5).

We found that applying DNase I causes a statistically significant decrease in electrochemical signal following storage for both 21 and 60 days. To demonstrate that the signal drop was not due to nonspecific adsorption of the enzyme to the electrode surface, we applied DNase that was heat deactivated and found that the inactive DNase does not induce a loss of signal (Figure S6). These results confirm that the enzyme is active on the immobilized DNA substrates. For the enzyme to be active on DNA, it must recognize the biomolecule. Thus, as our system is effective for cleaving DNA, we can conclude that DNA maintains a biologically relevant conformation. We acknowledge that DNA-cleavage-based signal-off assays represent one model of electrochemical diagnostics, while other formats, such as hybridization or affinity assays, are also commonly employed.[11,54] Further applications of the PVA coating in other DNA-sensing functions will be explored in future work.

Finally, we demonstrated that our PVA-based storage system is compatible with downstream sensing and has real-life applications by performing an electrochemical assay to detect prostate cancer gene 3 (PCA3), a biomarker for prostate cancer diagnostics.[55] Cas12a was engineered to bind to the target DNA sequence PCA3, upon which it exhibits *trans*-cleavage activity that digests MB-DNA and yields a signal drop. Without the PCA3 sequence, Cas12a is inactive and does not cleave the MB-DNA (Figure 6d). This assay was performed on electrodes that were stored for 7 days. We found that applying Cas12a in the presence of PCA3 leads to a statistically significant drop in signal, confirming that our storage system has real-life applications for diagnostic assays.

CONCLUSIONS

A critical limitation of the widespread implementation of electrochemical biosensors is their instability under common storage conditions. Here, we demonstrated that PVA coatings can be used to protect DNA monolayers on electrodes. The preparation process is exceedingly facile, involving only drop-casting a PVA solution onto functionalized electrodes. We further show that this coating extends the shelf life of DNA-functionalized electrodes from the previously reported 2 weeks under buffer to two months at room temperature and also protects against high heat. Finally, the coating can be dissolved with water, and the DNA remaining on the surface is compatible with enzyme-based assays and remains in a biologically relevant conformation. These results represent a significant step toward the scaling and commercialization of electrochemical biosensors using inexpensive and abundant materials.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssensors.5c00937.

Methods and materials, supplemental figures, and supplemental references (PDF)

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Author Contributions

The manuscript was written through the contributions of all authors.

Notes

The authors declare no competing financial interest.

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