Reagent Filming for Universal Point-of-Care Diagnostics

Gyeo-Re Han, Hyungjun Jang, Hangil Ki, Hyeon Lee, and Min-Gon Kim*

Simplifying assays while maintaining the robustness of reagents is a challenge in diagnostics. This problem is exacerbated when translating quality diagnostic assays to developing countries that lack resources and infrastructure such as trained health workers, high-end equipment, and cold-chain systems. To solve this problem, in this study, a simple solution that films assay reagents to simplify the operation of diagnostic assays and preserve the stability of diagnostic reagents without using cold chains is presented. A polyvinyl-alcohol-based water-soluble film is used to encapsulate premeasured and premixed reagents. The reagent film, produced through a simple and scalable cast-drying process, provides a glassy inner matrix with abundant hydroxyl groups that can stabilize various reagents (ranging from chemicals to biological materials) by restricting molecular mobility and generating hydrogen bonds. The reagent film is applied to an enzymatic glucose assay, a high-sensitivity immunoassay for cardiac troponin, and a molecular assay for viral RNA detection, to test its practicability and universal applicability. The film-based assays result in excellent analytical/diagnostic performance and stable long-term reagent storage at elevated temperatures (at 25 or 37 °C, for six months), demonstrating clinical readiness. This technology advances the development and distribution of affordable high-quality diagnostics to resource-limited regions.

1. Introduction

Point-of-care testing (POCT) simplifies medical diagnostics through decentralized and patient-centered diagnostic testing.[1] For example, at emergency departments in hospitals, POCT can shorten the turnaround time and lessen the workload stipulated by conventional laboratory tests (which typically require precise weighing, pipetting, and complex steps operated by experts) by enabling bedside testing.[2] More importantly, at community health centers or outdoor clinics, compact and simple POCT platforms, such as lateral flow assay (LFA), make diagnostic tests highly accessible, affordable, and easily operable by nonexperts without complex instrumentation, allowing for disease diagnosis in resource-limited settings.[3] As such, because POCT can be used in a wide range of environments, the components used for POCT, especially bio/chemical reagents involved in assays, must be maintained robustly to ensure reproducible and reliable test results regardless of the resource setting.[4] However, diagnostic tests for POCT are engineered based on assays and sensing principles employed in solution-based conventional laboratory tests; thus, they share labile biochemical reagents (i.e., antibodies (Ab), enzymes, nucleic acids, and organic/inorganic compounds) that benefit from a cold chain (4 or −20 °C) for stable storage. Nevertheless, most communities relying on POCT have uncontrolled environments, such as much higher temperatures than in laboratory settings and restricted cold-chain infrastructure, limiting the facile translation of well-validated diagnostic assays toward accessible POCT in the field.[5] This situation introduces a critical challenge in POCT, namely, realizing cold-chain-free storage of assay reagents that require a cold chain during transportation to and storage at the end-user location.

Maintaining the stability of assay reagents is essential to ensure the quality and reliability of diagnostic tests. Healthcare issues, such as the COVID-19 pandemic and the shifting of cardiovascular diseases (CVD) toward developing countries, continue to occur globally.[6] Therefore, there is an increasing need for preventive and impactful disease control via POCT-based high-performance diagnostic measures (e.g., a high-sensitivity immunoassay or point-of-care nucleic acid test [POC-NAT]),[7] which utilize multiple reagents that are more sensitive than those in conventional POCT and therefore require strict maintenance of reagent stability. To resolve this problem, many commercial assays, such as POC-NAT, have adopted lyophilized reagents stored in disposable cartridges for cold-chain-free storage.[8] However, this requires centralized manufacturing facilities entailing costly maintenance/repair,[9] thereby increasing the assay cost and potentially limiting local distribution amidst a pandemic.[10] An approach under research, wherein the assay reagents are encapsulated in tablet shapes (using compression or casting methods), has been demonstrated as effective for cold-chain-free reagent storage.[11] However, the rigid
conformations of such tablets limit their flexible implementation in various POCT platforms. Further, a short stability-test period (1–2 months) and incomplete applications to practical assays impede their immediate use. Moreover, to date, most diagnostic assays that meet clinical standards rely on high-end instruments (such as automated analyzers and thermocyclers) primarily based on a cold chain, worsening the global diagnostic inequity. Therefore, an alternative and universal reagent-storage platform is urgently required to realize the application of high-performance assays in POC environments.

Reagent filming holds great potential for simplifying and strengthening biochemical assays and can provide a highly durable, affordable, scalable, and flexible approach to advance cold-chain-free diagnostics. Thus far, the application of filmed reagents has focused on the pharmaceutical field, for example, drug delivery in the oral cavity via orodispersible films, because of its benefits in terms of dosing flexibility, fast absorption, and continuous manufacturing.[12] Nevertheless, its application for medical diagnostics has been neglected. In this study, we present a film-based reagent-storage platform for universal application to POCT. By using polyvinyl alcohol (PVA)—a water-soluble, transparent, and inert polymer—as a matrix for reagent stabilization, we designed an all-in-one film that incorporates premeasured, premixed, and prepackaged reagents. This film can not only deliver assay reagents without cold storage but also simplify quality diagnostics at POC (Figure 1A). We prepared the reagent film through a simple and cost-effective process of casting, vacuum drying, and peeling of the polymer/reagent mixture (Figure 1B), which allows for the multiscale production of uniform films covering a single test dose to mass production levels (>5000 tests/film) (Figure 1C). The casting-based fabrication, coupled with the superior

Figure 1. Schematic overview of the PVA-based reagent film. A) Comparison of the workflow between solution-based diagnostics (conventional) and film-based diagnostics (this study). B) Production process of the reagent film. C) High scalability of production of the reagent film, ranging from a single test/film to >5000 tests/film. D) Photograph of reagent films with various processible forms. E) Interior scheme of the reagent film. F) Stabilizing mechanism 1; restriction of molecular mobility by vitrification of the surrounding matrix. G) Stabilizing mechanism 2; stabilization of the protein structure by substituting H-bonds with PVA and trehalose after eliminating water.
mechanical properties of PVA, facilitates the post-processing of reagent films with various appearances (i.e., coat, roll, and pellet) (Figure 1D), which enhances its applicability to various assay systems. The inherent hydrophilicity (−OH rich) and excellent film-forming property of PVA enable a rapid dissolution of the film with a subsequent release of reagents upon mixing with biological samples and the kinetic/thermodynamic stabilization of the reagents in the solid-phase film matrix (Figure 1E–G). Furthermore, we demonstrated the practical utility of our system for cold-chain-free diagnostics by evaluating the PVA-based reagent film through thermal/optical/spectral analysis, and by applying it to multiple clinical assays and conducting long-term stability tests.

2. Results and Discussion

2.1. Stabilization Mechanisms of Filmed Reagents

Biochemical reagents are labile to physical and chemical stresses (i.e., heat, moisture, aggregation, and oxidation) that degrade their original functionality. The storage of reagents in a solution accelerates the degradation process by increasing molecular motion or the risk of conformational change in proteins, which makes cold-chain storage essential to lower the reagent mobility for most existing clinical assays. Otherwise, the storage of the reagents in the PVA film can eliminate the potential risks of reagent instability concomitantly by restricting molecular mobility in the vitrified matrix (Figure 1F, kinetic mechanism) and substituting hydrogen bonds (H-bonds) between water and the protein strands by stabilizing excipients (i.e., PVA and trehalose) (Figure 1G, thermodynamic mechanism). These are in line with the slaving model of protein dynamics that highlights the conformational stability of protein affected by the surrounding matrix and hydration shell.

We hypothesized that a solid-state PVA film provides a flexible yet dehydrated glassy matrix that can stabilize the reagent molecules kinetically by restricting the mobility and diffusion to a highly confined space, such as an ancient bee trapped in amber (Figure 1F). We measured the glass transition temperature ($T_g$), at which the vitrified polymer matrix starts to exhibit a rubbery state that enhances the molecular mobility, to evaluate the potential of our film matrices for reagent protection. The $T_g$ values of the PVA film matrices designed for an enzymatic/immunoassay (Film 1, 43.1 °C) and a molecular assay (Film 2, 34.0 °C) are higher than 25 °C, which allows for minimized mobility of filmed reagents at the RT storage range.

![Figure 2](image_url)

**Figure 2.** Experimental validation for the stabilization mechanism of filmed reagents. A) DSC thermogram for analyzing $T_g$ of reagent films. Film 1 and 2 indicate PVA film matrix designed for enzymatic/immunoassay (Film 1) and molecular diagnostics (Film 2). B) Optical microscope images for monitoring mobility of MMPs in the PVA solution and the film. A magnetic field was used to test the particle rearrangement. Inset shows the retained magnetic property of filmed MMPs. C) FTIR spectra of the PVA film and the other PVA films with the native HRP (Day 1: line; Day 30: dotted) or the heat-denatured HRP. D) Correlation analysis of the FTIR spectra in specific wavenumber ranges displayed in (C). The left column indicates a comparison in the FTIR spectrum of native HRP stored at 25 °C for 30 d. The right column shows a comparison of the FTIR spectrum between native HRP and the heat-denatured HRP.
(10–30 °C) (Figure 2A). The smaller $T_p$ values of the reagent films compared with that of the original PVA film (i.e., 52.5 °C) are attributed to the plasticizing effect of the assay reagents and buffer contents doped in the films.[16] To demonstrate the molecular mobility in the PVA film matrix on the invisible scale to the naked eye, we cast a PVA film doped with magnetic microparticles (MMPs) and imaged the molecular mobility using optical microscopy. Contrary to the rearranging motion of MMPs in the solution, the filmed MMPs showed restricted mobility regardless of the presence of the magnetic field, which corroborates our hypothesis (Figure 2B).

The stability of protein reagents is affected directly by the protein structure underpinned by internal and external H-bonds.[37] Our film matrix, composed of PVA with trehalose, provides abundant hydroxyl groups that can replace the water molecules around proteins and the associated H-bonds (hydration shell) during the dehydration process. This can preserve the hierarchical structures of the proteins and their functionalities during long-term storage (Figure 1G). The PVA and trehalose have high molecular flexibility, ensuring close interaction with protein strands by circumventing steric hindrance, which enables a facile substitution of the hydration shells.[15b] Further, trehalose has a high hydration capacity for water, which facilitates the molecular isolation of the remaining water content from assay reagents in the film matrix.[18]

To evaluate the effectiveness of our film matrix in preserving the protein structure during storage, we adopted horseradish peroxidase (HRP) as a protein model and prepared PVA films that, respectively, contain the native HRP and heat-denatured HRP. We analyzed the change in the local 3D structure (secondary structure, i.e., $\alpha$-helix and $\beta$-sheet) of HRP (Figure 2C) by comparing the peak intensity and correlation between the Fourier-transform infrared (FTIR) spectra. Compared with the spectrum of the PVA film including denatured HRP, the PVA film with the native HRP revealed relatively intense spectrum peaks of amide I (1650 cm$^{-1}$; C=O stretching vibration), amide II (1540 cm$^{-1}$; N–H bending vibration), and the disulfide bond (550 cm$^{-1}$; S=S stretching vibration; contributes to protein folding),[19] which reflect the secondary structure and possibly the primary component (i.e., disulfide bridges) for the tertiary structure of proteins. A 2D spectral comparison revealed low correlations between the films with the native HRP and denatured HRP ($r = 0.895$ for 1400–1680 cm$^{-1}$ and $r = 0.916$ for 400–600 cm$^{-1}$), which represents distinct structural differences (Figure 2D). Further, after aging for a month under exposure to ambient air at 25 °C, the PVA film with the native HRP showed an identical spectrum pattern with excellent correlations ($r = 0.999$ for 1400–1680 cm$^{-1}$ and $r = 0.992$ for 400–600 cm$^{-1}$) to the initial analysis (Day 1) (Figure 2D). This result implies that the conformation of HRP was preserved successfully in the PVA film, thus demonstrating the feasibility of our film matrix for stabilizing the protein reagents.

### 2.2. Characterization of Reagent Film

A candidate material for use as a film matrix for reagent storage must not only maintain reagent stability but also possess the following features, including minimum interference with assay reagents and sensing methods, to obtain accurate test results; high solubility in aqueous solutions, for preparing a homogeneous assay mixture with rapid and simple steps; and uniform production of reagent films, for reproducible tests. Given these requirements, we evaluated the properties of our PVA-based reagent film for application to practical assays.

In contrast to the water-insoluble pharmaceutical excipients such as sodium stearyl fumarate and croscarmellose sodium (used as a lubricant and disintegrant in a prior study for tabletting reagents), which might interfere with the assay results or require extra washing steps,[18] PVA provides a water-soluble and optically transparent matrix suitable for straightforward assay processes. The absorption spectrum ($\lambda_{\text{max}}$ at 216 nm) of PVA did not interfere with the spectral range ($\lambda > 300$ nm) commonly used for various sensing modalities (i.e., colorimetric, fluorescence, and luminescent) that can transduce biochemical interactions into measurable signals (Figure 3A). Using the gold nanoparticle (AuNP)-based immunocomposite, we confirmed no shift in the absorption spectral peak ($\lambda_{\text{max}}$ = 528 nm) after rehydrating the filmed conjugate compared to unfilmed aqueous conjugate; this indicates that its original shape and dispersity were maintained in the PVA film matrix (Figure S1, Supporting Information). Next, with regard to multiple biochemical tests, the PVA film-based assays resulted in comparable test signals (coefficient of variation, CV < 5%) with those obtained using conventional assay reagents stored in solution (Figure 3B). Consequently, these results demonstrate the inertness of PVA toward biochemical reagents and the subsequent reactions.

Notably, we found that using the PVA-based reagent film could potentially impact the viscosity of the assay mixture, resulting in a slight deviation of assay intensity from that of the unfilmed assay reagents within the negligible range in terms of repeatability, reproducibility, and assay interference (as shown in Figure 3B,D). For example, in the case of an enzymatic assay using the reagent film, the enhanced viscosity of the assay mixture can decrease the enzymatic reaction rate by delaying the diffusion of the substrate,[20] resulting in lowered signal intensity compared to the use of unfilmed reagents (Figure 3B; the enzymatic assay result). In contrast, in the case of LFA, the enhanced viscosity of the assay mixture might increase the assay intensity and sensitivity because it provides a lowered flow rate of the assay mixture,[21] making antibodies recognize/capture targets much effectively than those in less viscous solution (Figure 3B; the immunoassay result). Meanwhile, in the loop-mediated isothermal amplification (LAMP) assay processed at 60 °C, we attributed that the temperature could offset the viscosity effect,[22] resulting in the LAMP assay likely being less affected by the viscosity of the assay mixture (Figure 3B; the LAMP assay result).

The dissolution test of reagent films with various PVA concentrations revealed a dissolving time of less than 50 s regardless of the mixing method (Figure 3C), this can facilitate the reagents’ release through the fast disintegration of the film matrix. Casting a uniform reagent film that contains evenly distributed compounds with equal amounts per unit volume is a critical factor for precise and reliable testing. The PVA concentration-dependent film-forming test revealed a linear correlation of the PVA concentration with the thickness and weight of the reagent films (Figure S2, Supporting Information), which allows quantitative scaling in film casting. Further,
a film matrix with a PVA concentration greater than 4% (w/w, final) showed a uniform distribution of reagents (Figure S3, Supporting Information) and improved assay reproducibility (Figure S4, Supporting Information). This is attributed to the better reagent encapsulation efficiency of the casting mixture that comprises higher PVA amounts with enhanced matrix viscosity, thereby preventing a local enrichment of reagents during the cast-drying process. Furthermore, the batch reproducibility test using reagent films for enzymatic glucose assay resulted in consistent signal intensity ($CV_{\text{intra-batch}} = 2.9–4.1\%$ and $CV_{\text{inter-batch}} = 3.1\%$) (Figure 3D), which demonstrates highly reproducible and uniform film production for reliable testing.

### 2.3. Application 1: Enzymatic Assay for Urinary Glucose Testing

We selected a glucose enzymatic assay—a standard method for monitoring diabetes mellitus—as the first application subject.\(^{[23]}\) This provided an ideal testbed to evaluate the robustness of the filmed reagent because the assay mixture comprises various reagent types, such as glucose oxidase (GOx), HRP, and chemical chromogens. The reagent film was prepared based on the standard matrix that includes PVA, storage buffer contents, and trehalose (Table S1, Supporting Information). We vacuum-packaged the processed reagent films (4 mm × 4 mm) to prevent contact with moisture and oxygen until use. The filmed reagents enabled one-step glucose testing by rehydrating the reagent film through sample injection (100 µL) (Figure 4A). We designed the assay system to detect glucose by generating a colorimetric signal via 4-aminoantipyrine (4-AAP) and N,N-bis(4-sulfobutyl)-3,5-dimethylaniline disodium salt (MADB) (Figure 4B). This reaction was catalyzed by GOx and HRP to oxidize glucose and generate $\text{H}_2\text{O}_2$. We measured the serially diluted glucose in DW to evaluate the glucose quantification quality of the filmed reagents. This resulted in a linear calibration plot over glucose levels ($R^2 = 0.998$) with a limit of detection (LoD) of 0.29 mg dL$^{-1}$ (Figure 4C).

We validated the practical utility of the filmed glucose assay reagents by testing various concentrations of glucose spiked in pooled human urine because of the need for noninvasive glucose testing, which helps avoid repeated pain and the risk of infection during blood collection.\(^{[24]}\) We observed an excellent correlation ($r = 0.999$) of the spiked glucose level with the measured concentration (i.e., calculated from the calibration plot) in the range of 0–25 mg dL$^{-1}$ (Figure 4D); this includes both diabetic (>14.4 mg dL$^{-1}$) and nondiabetic urinary glucose levels (0–14.4 mg dL$^{-1}$).\(^{[25]}\) This result demonstrates the filmed reagents’ clinical potential for precise glucose testing with minimum interference from the undiluted urine sample matrix. The filmed reagent showed retained activity of 103% (at 4 °C,
control), 94% (at 25 °C), and 76% (at 37 °C) after storage for six months (Figure 4E). However, the activity of the other reagent group dried in a test tube without PVA content decreased sharply to 34% after two months under storage at 25 °C. This result indicates the successful protection of the enzymatic assay reagents against elevated temperature, demonstrating stable reagent storage using the PVA-based film matrix.

2.4. Application 2: Immunoassay for High-Sensitivity Cardiac Troponin I (cTnI) Testing

To evaluate the practical utility of the reagent filming strategy, we applied it to LFA. The LFA is a representative immunoassay platform for POCT to perform high-sensitivity detection of cTnI, which is a gold-standard cardiac biomarker used for CVD diagnosis. As a part of an effort to enhance the analytical performance of LFA, in a previous study, we demonstrated high-performance cTnI testing in chemiluminescence (CL)-based LFA using a new immunoconjugate (AuNP-polyHRP-Ab) that has a highly improved loading capacity for bioreceptors. However, the lyophilized conjugate retained its original activity only in cold storage (at 4 °C), which makes it necessary to extend the stability of the conjugate for practical application in a cold-chain-free environment. To resolve this problem and test the potential of the filmed reagents for extended stability, we cast the PVA-based reagent film comprising AuNP-polyHRP-Ab conjugate for CL-based LFA. Figure 5A illustrates the assay procedure. The assay solution can be prepared by simply rehydrating the conjugate film by adding an assay buffer (45 µL) and an undiluted serum sample (5 µL) in sequence. After incubating the conjugate-sample mixture for the primary recognition of cTnI, the assay proceeds by following the sequential steps of CL-based LFA, immunoreaction at the test line, test-strip washing, and CL reaction using an HRP-luminol-H2O2-based CL system.

The concentration-dependent test using cTnI spiked in standard serum (cTnI-free pooled normal human serum) resulted in test line signals being produced proportionally to the cTnI amounts captured in the immunocomplex (Figure 5B). This demonstrates the highly sensitive (calculated LoD, 0.1 pg mL$^{-1}$) and precise (CV of 1.8–8.5%, from entire testing) measurement of cTnI (Table S2, Supporting Information), satisfying the clinical requirement for high-sensitivity cTnI testing (CV of ≤10%, at and below 99th percentile cTnI levels of health populations; ≈10 pg mL$^{-1}$). The test results of the clinical serum sample were closely fitted with the calibration plot of standard samples (Figure 5C), and they revealed excellent correlation ($r = 0.986$) with the cTnI values measured by the standard clinical analyzer (Atellica from Siemens Healthcare) (Figure 5D). This suggests the practical utility of the filmed conjugate in clinical applications. In contrast to the consistent activity decrease in the lyophilized conjugate stored at 25 °C, we observed a highly conserved activity of the filmed conjugate during the storage for six months, which showed a retained activity of 99% (at 4 °C, control), 93% (at 25 °C), and 74% (at 37 °C). Collectively, these results demonstrate that the reagent film has high compatibility with the immunoassay system, and that it can be used for high-performance POCT while maintaining the original reagent activity without cold-chain storage.
2.5. Application 3: Reverse Transcription (RT)-LAMP Assay for Viral RNA Testing

The RT-LAMP assay is a nucleic acid amplification technique that can exponentially multiply specific viral RNA sequences at a constant temperature. The LAMP assay outperforms the conventional polymerase chain reaction (PCR) in terms of simplicity, affordability, and specificity because of its advantages such as a one-step single-temperature reaction processible at low cost using a portable incubator or water bath, closed-tube assay that can minimize carryover contamination, and higher amplification efficiency and resistance to inhibitors; these make it more suitable for molecular testing at POC situations. However, thus far, all reagents for the RT-LAMP, such as reverse transcriptase, polymerase, primers, dNTPs, and buffers, are recommended to be stored at $-20^\circ C$, which limits their practical use in a cold-chain-free environment. Further, commercial LAMP kits provide assay reagents split into 2–3 packages, which increases the preparation steps and risk of reagent contamination.

The LAMP assay can be simplified and the reagents can be stabilized under ambient conditions by applying our reagent filming strategy. The LAMP reagent film contains premixed contents quantified for a single test, and it can be rehydrated by adding an amplification buffer (21 $\mu$L) and an extracted RNA sample (1 $\mu$L) for the amplification (Figure 6A), which enables a straightforward and one-step molecular assay that can be operated by nonexpert personnel. We used hydroxynaphthol blue (HNB), which functions by binding with Mg$^{2+}$, as an assay indicator because it offers colorimetric and fluorescent sensing features that are suitable for both on-off testing and quantitative analysis (Figure 6B). We adopted the fluorescence modality in this study to compare our system with quantitative RT-PCR (RT-qPCR) by normalizing the fluorescence intensity ($F$). When benchmarked against the test results of classical RT-LAMP using aqueous reagents (LoD: 195 copies $\mu$L$^{-1}$) and conventional RT-qPCR (LoD: 56 copies $\mu$L$^{-1}$) that uses serially diluted Influenza B virus (IBV) control RNA, the reagent film-based RT-LAMP allowed for a comparable quantitative analysis in the ranges of $10^2$–$10^4$ copies $\mu$L$^{-1}$ with a calculated LoD of 222 copies $\mu$L$^{-1}$ (Figure 6C).

We tested the extracted RNA from clinical samples (i.e., obtained via a nasopharyngeal swab) infected with IBV ($n = 15$, not infected with Inf. A) and Inf. A ($n = 10$, not infected with IBV) to verify the filmed RT-LAMP reagents’ clinical applicability. The reagent film-based RT-LAMP assay clearly detected samples with an IBV infection; the details exactly matched with the corresponding test results using classical RT-LAMP and RT-qPCR (Figure 6D); this demonstrates its clinical capability for specific viral RNA detection without cross-reactivity. The clinical samples with IBV infection showed a significantly higher test intensity ($P < 0.0001$) than the control group (Figure 6E). Further, analysis of the clinical sample test results using the receiver operating characteristic (ROC) curve resulted in an area under the curve of 1 (Figure 6F), which indicates its outstanding diagnostic accuracy.

We monitored the stability of the filmed reagents stored at 4, 25, and $37^\circ C$ by testing an IBV RNA mixture (3.3 $\times$ 10$^4$ copies $\mu$L$^{-1}$, quantified by RT-qPCR) extracted from the 15 clinical samples to...
evaluate the effectiveness of the reagent film in stabilizing RT-LAMP assay reagents. Figure 6G shows the stability test result. After storage for 150 d, the filmed reagents stored below 25°C showed a retained activity of >80%, which is sufficiently high to classify a viral infection. The amplification buffer stored at 25°C for 100 d also showed a retained activity of 99% (Figure S6, Supporting Information), which is equivalent to that stored at −20°C. The reagent film aged at 37°C lost its activity (0.8% remained) and was unable to detect IBV RNA from ≈60 storage days. This activity decrease in filmed reagents can be attributed to the Tg of the LAMP reagent film matrix (34°C; Figure 4A) being lower than 37°C. To clarify the reason for the lowered Tg, we compared ΔTg values depending on PVA concentration (5% w/w PVA for Film 1 and 4.4% w/w PVA for Film 2) or addition of glycerol (2.6% w/w) with a fixed PVA level (4.4% w/w), resulting in a relatively larger reduction in Tg value (ΔTg = 73°C) in case of adding the glycerol than that of using different PVA concentration (ΔTg = 1.8°C) (Figure S7, Supporting Information). This result indicates that Tg of PVA film matrix is mainly affected by the addition of glycerol (used as film plasticizer and protein preserving matrix) from enzyme solutions than the slight difference in PVA concentration.

Nevertheless, the stabilizing effect of our system (stable storage for six months at 25°C) is clearly superior to that of previous approaches based on a polymer, lyophilization, or sugar/polyol-based matrix, which typically resulted in short storage periods (≤45 d) of LAMP reagents below 25°C (Table S3, Supporting Information). These results demonstrate the excellent potential of the filmed reagents for cold-chain-free nucleic acid testing, which can significantly improve their accessibility toward quality diagnostics at POC.

2.6. Discussion

As an ideal platform for reagent storage/delivery, the PVA-based reagent film can accelerate the deployment and distribution of cost-effective yet quality diagnostic tests to resource-limited regions. Because the industrial production of PVA is based on chemical processes, the unit price of PVA (290 USD kg⁻¹) is considerably lower than that of other stabilizing excipients such as pullulan (2240 USD kg⁻¹) and trehalose (1800 USD kg⁻¹) (used as primary excipients in previous studies for tableting reagents), which are produced via bioprocesses using enzymes or fungus. In particular, replacing the major excipient of trehalose with PVA followed the use of minimum amounts of trehalose, allowing us to create an extremely low-cost matrix (0.0024 USD/test) for reagent filming. Accordingly, the estimated costs of single-dose reagent films are at affordable levels for each pilot assay: 0.031 USD (enzymatic assay), 0.087 USD (immunoassay), and 1.894 USD (LAMP assay) (Table S4, Supporting Information). Further, PVA-based reagent films can be integrated easily into various conventional assays and biosensing platforms. For example, the reagent mixture can be directly cast on assay consumables (plastic tubes, microplates, and syringes), microfluidic channels (reagent storage parts).
porous materials (membranes, pads, and filters), or any available interfaces/surfaces in sensors; this increases its technical accessibility. The benefits of reagent film-based diagnostics exceed those of the conventional framework stipulated by solution-based assays, demonstrating better compatibility for POCT (Figure 7).

Thus far, most quality diagnostic assays have been produced and supplied by centralized facilities and systems of global manufacturers. However, as the world struggles with the COVID-19 pandemic, notably because of the lack of diagnostic kits and congestion in global logistics networks, such centralized approaches exhibit limitations in the rapid and equitable distribution of diagnostics, thus exacerbating the existing diagnostic inequity in developing countries. Considering the current circumstances and the perspective of diagnostic equity, the filming of reagents provides a unique value in that they consider the local development and distribution of quality diagnostics in resource-limited settings. For example, reagent films can be fabricated in local facilities, including clinical middleware laboratories that usually contain essential equipment such as balances, centrifuges, vacuum/drying ovens, and cutting/packing tools. Then, the filmed reagents can be distributed immediately to sub-local areas under ambient conditions (they could also be assisted by air conditioning). Such distribution can offset the costs for the cold-chain logistics required for transportation from centralized production units, which can account for 80% of bioproduct distribution cost. Thus, reagent filming can enable the delivery of quality diagnostic reagents to end-users in resource-limited settings at affordable prices.

Our future research directions include the design of a hybrid film matrix consisting of PVA and other excipient materials for realizing a better reagent-stabilizing effect; use of glycerol-free stabilents results in quality and reliable diagnostic assays, leading to accurate diagnosis and better patient outcomes. Thus, stabilizing labile reagents is a challenge in diagnostics, especially for implementing diagnostic tests in POC situations lacking cold-chain infrastructures. In this respect, our approach for reagent filming can provide a new framework that helps overcome the risk of reagent instability and deliver high-performance POCT in a cold-chain-free environment. This platform can be readily applied to multiple clinical tests, including enzymatic glucose assay, cTnI immunoassay, and IBV LAMP assay, resulting in excellent analytical/diagnostic performances and stable long-term reagent storage at elevated temperatures (at 25 or 37 °C, for six months). This demonstrates the practical, clinical, and universal applicability of our system for diagnostics.

3. Conclusion

This study has demonstrated that reagent filming using PVA can stabilize biochemical reagents and simplify diagnostic assays, thus making significant strides toward cold-chain-free diagnostics for POCT. Encapsulating premeasured and premixed diagnostic reagents using a PVA film matrix that provides mobility restriction and H-bond formation can stabilize various reagents, ranging from chemical compounds to bioreagents, against thermal or chemical degradation. The use of robust reagents results in quality and reliable diagnostic assays, leading to accurate diagnosis and better patient outcomes. Thus, stabilizing labile reagents is a challenge in diagnostics, especially for implementing diagnostic tests in POC situations lacking cold-chain infrastructures. In this respect, our approach for reagent filming can provide a new framework that helps overcome the risk of reagent instability and deliver high-performance POCT in a cold-chain-free environment. This platform can be readily applied to multiple clinical tests, including enzymatic glucose assay, cTnI immunoassay, and IBV LAMP assay, resulting in excellent analytical/diagnostic performances and stable long-term reagent storage at elevated temperatures (at 25 or 37 °C, for six months). This demonstrates the practical, clinical, and universal applicability of our system for diagnostics.

4. Experimental Section

Materials: GOx, HRP, and a protein saver were purchased from Toyobo (Osaka, Japan); MADB was purchased from Dojindo (Tokyo, Japan); pooled normal human urine was purchased from Innovative Research (Novi, MI); AuNP was purchased from BBI Solutions (Cardiff, UK); and anti-mouse IgG Ab, 4-AAP, n-glucose, glycerol, iron(II) chloride tetrahydrate, luminol, hydrogen peroxide, 4-iodophenol, MMP, polyHRP, PVA (80% hydrolyzed, molecular weight of 9000–10000), Trehalose, Triton X-100, and Tween-20 were purchased from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (BSA) was purchased from Fitzgerald (Acton, MA); anti-cTnI Ab (4T21-19C7, 560), troponin I-T-C complex (BT62), and cTnI-free pooled normal human serum (BTF5) were purchased from Hytest (Turku, Finland); anti-cTnI Ab was purchased from Calbioreagents (Foster City, CA); and cTnI clinical serum samples were obtained from Chonnam National University Hwasun Hospital with IRB approval (CNUHH-2019-016). A plastic backing card was purchased from PJEAGO (Seoul, South Korea); nitrocellulose (NC) membrane (FF80HP) was purchased from Whatman (Maidstone, UK); an absorbent pad (grade 222) was purchased from Whatman (Maidstone, UK); and anti-mouse IgG Ab, 4-AAP, d-glucose, glycerol, iron(II) chloride tetrahydrate, luminol, hydrogen peroxide, 4-iodophenol, MMP, polyHRP, PVA (80% hydrolyzed, molecular weight of 9000–10000), Trehalose, Triton X-100, and Tween-20 were purchased from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (BSA) was purchased from Fitzgerald (Acton, MA); anti-cTnI Ab (4T21-19C7, 560), troponin I-T-C complex (BT62), and cTnI-free pooled normal human serum (BTF5) were purchased from Hytest (Turku, Finland); anti-cTnI Ab was purchased from Calbioreagents (Foster City, CA); and cTnI clinical serum samples were obtained from Chonnam National University Hwasun Hospital with IRB approval (CNUHH-2019-016). A plastic backing card was purchased from PJEAGO (Seoul, South Korea); nitrocellulose (NC) membrane (FF80HP) was purchased from Whatman (Maidstone, UK); an absorbent pad (grade 222) was purchased from Boreda Biotech (Gyeonggi-do, South Korea); and Bst 2.0 WarmStart (WS) DNA polymerase, WS reverse transcriptase,
magnesium sulfate, and isothermal amplification buffer (10x), containing 200 × 10⁻⁶ M Tris-HCl, 100 × 10⁻⁶ M (NH₄)₂SO₄, 500 × 10⁻⁶ M KCl, 20 × 10⁻⁶ M MgSO₄, and 0.1% Tween-20, were purchased from New England Biolabs (Ipswich, MA); a viral RNA purification kit was purchased from Qiagen (Darmstadt, Germany). Oligonucleotides for LAMP primers, previously validated,[34] were synthesized from GenoTech (Daejeon, South Korea); the primer sequences are listed in Table S5 (Supporting Information).

The dNTP mixture was purchased from Thermo Fisher Scientific (Waltham, MA); a viral RNA purification kit was purchased from MyBioSource (San Diego, CA); and patient clinical samples were directly cast on the aluminum pan used for the DSC analysis under the same drying condition described in the preparation of all-in-one reagent films. The samples were heated at a rate of 10 °C/min from 0 to 180 °C. Tg was defined using Proteus thermal analysis software (Netzsch). For the structural analysis of the filmed protein, native HRP and heat-denatured HRP (95 °C, 12 h) were filmed with PVA, respectively; the films were analyzed using a FTIR spectrometer (Vertex 70X, Bruker, Billerica, MA). The mobility of MMPs in 5% (w/w) PVA solution and PVA film was monitored using an optical microscope (Axioskop 40 A Pol, Carl Zeiss, Oberkochen, Germany). For evaluating the batch reproducibility of filmed reagents, the enzymatic assay films were prepared using a petri dish with a 50 mm diameter (corresponding to ~122 tests) in three batches. Among the sliced film batches, three films were randomly selected from each group to test inter-/intra-batch reproducibility. The absorbance spectrum of the PVA solution and AuNP conjugate was measured using a UV–vis spectrometer (UV-2450, Shimadzu, Kyoto, Japan).

Optimization of PVA Concentration for Casting Reagent Film: To determine the optimum concentration of PVA suitable for forming a uniform film matrix, a PVA concentration-dependent film-forming test was performed, the thickness and weight of each film were measured, and assay reproducibility was evaluated. Using the reagents of glucose enzymatic assay as a model and using 10% (w/w) PVA as a stock solution, the PVA concentration-dependent reagent solutions were prepared based on the content described in Table S1 (Supporting Information). For the reagent mixtures less than 4% (w/w) PVA, the insufficient volume occurred from the use of a smaller volume of the PVA stock solution was supplemented by adding a related volume of DW (i.e., for preparing 4% w/w of PVA matrix, 4 mL 10% w/w of PVA stock solution and 1 mL DW was added). The PVA concentration-dependent reagent films were cast in a polystyrene petri dish (Figure S3, Supporting Information). After drying and processing into each film with a square pellet shape (4 mm × 4 mm), the weight and thickness of randomly selected films were measured using microbalance and precision vernier calipers (Figure S2, Supporting Information). Assay reproducibility using randomly selected films from each PVA concentration was measured using 10 mg dL⁻¹ glucose in DW (Figure S4, Supporting Information). Details of the assay step are described in the following section.

Application to Enzymatic Assay: Various concentrations of glucose samples were prepared using DW or human urine. 100 µL glucose solution was added to the reagent film (stored at 25 °C) and subsequently mixed for 1 min to dissolve the film. The mixture was incubated for 10 min at 25 °C. Then, the colorimetric intensity of the solution was measured at 450 nm (absorption λ) using Cytation 5 plate reader and GenS analysis software (BioTek, Winooski, VT).

Application to CL-Based LFA: The LFA test strip for the dip-stick assay was prepared by laminating the NC membrane (25 mm × 300 mm) and absorbent pads (32 mm × 300 mm, double-layered by double-sided tape) to a plastic backing card (40 mm × 300 mm) with 2 mm overlapse between the materials. Test (0.9 µg of capture Abs per single test strip) and control (0.038 µg of IgG Abs per single test strip) lines on the NC membrane were dispersed using a programmable reagent dispenser (DCI-100, Zeta Corporation, Gyeonggi-do, South Korea).[35] The NC membrane was dried for 20 min at 37 °C and then cut into single test strips (55 mm × 3.8 mm) using the automatic cutter. Details of the preparation of the LFA test strip are described as a scheme in Figure S9 (Supporting Information). The cTnI standard samples were prepared by serially diluting troponin I-T-C complex with the cTnI-free serum. To rehydrate the filmed conjugate (AuNP-polyHRP-Ab, stored at 25 °C) in a test tube, an assay buffer (45 µL containing 10 µM H₂O₂ in Tris-HCl buffer (100 mM, pH 7.4)) was added instead of the viral RNA template for the negative control test. The fluorescence signal of the LAMP reaction was measured using the Cytation 5 plate reader and GenS analysis software (BioTek, Winooski, VT).
(exposure time, 2 s; excitation $\lambda$ at 540 nm; and emission $\lambda$ at 610 nm) and Image Lab software. The normalized fluorescence intensity ($F$) calculated by dividing the intensity difference between negative control ($I_0$) and test ($I$) with $I_0$ (i.e., $F = (I - I_0)/I_0$) was used to evaluate the LAMP assay. \(^{[12]}\) The extraction of viral RNA from clinical samples followed Qiagen’s protocol.

1Bv viral RNA control samples and clinical samples were validated using RT-qPCR (CFX96 Real-Time System, Bio-Rad) for comparison with the film-based RT-LAMP system. The original fluorescence intensity of the RT-qPCR result was normalized by dividing with 2000 for comparison with the LAMP assay.

Stability Study: The long-term stability of the filmed reagents was tested for six months. The vacuum-sealed reagent films were stored at 4, 25, and 37 °C, respectively. 10 mg dl$^{-1}$ glucose in DW, 10$^5$ pg ml$^{-1}$ cTnI spiked in serum, and a mixture of IBV viral RNA extracted from 15 clinical samples were used to test the filmed reagents’ stability for the enzymatic assay, immunoassay, and LAMP assay, respectively. The stability of the isothermal amplification buffer (stored at 25 °C in a dark tube) was tested using aqueous LAMP reagents stored at −20 °C. The signal intensity of each assay measured on the day of film preparation was defined as the initial activity (100% at Day 0). The remaining activity (%) of the filmed reagents was calculated by dividing the intensity measured on each storage day by the original assay intensity at Day 0.

Statistical Analysis: All experimental data were presented as the mean of at least three measurements ± standard deviation (SD). Detailed information on experimental replicates is provided in the corresponding figure legends. The CV (%) refers to the SD divided by the mean (%). FTIR spectral patterns were correlated using Pearson’s correlation coefficient. For all assays, the LoD was calculated as follows: LoD = Blank + 3 × SD. The two-sample t-test was used to estimate the difference between IBV negative/positive clinical sample groups. Statistical significance was set at $P < 0.01$.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Data Availability Statement
Research data are not shared.

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