Lab on a Chip

A New Paper-Based Biosensor for Therapeutic Drug Monitoring

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COMMUNICATION

A New Paper-Based Biosensor for Therapeutic Drug Monitoring

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Abstract Tacrolimus is one of the most effective and prevalent drugs used to combat vascularized composite allotransplantation rejection. We have fabricated a rapid and easy-to-use six-layer paper based microfluidic device using the principles of competitive immunoassays and vertical flow microfluidics for colorimetric detection of tacrolimus in a small volume of blood.

1. Introduction

Tacrolimus is one of the most effective drugs in combating vascularized composite allotransplantation (VCA) rejection [1, 2]. Tacrolimus is an immunosuppressant that inhibits cytokine production and blocks cell division, in addition to inhibiting both interleukin (IL-2) production and the expression of the IL-2 receptors [3, 4]. It is the most common calcineurin inhibitor (CNI), which help shut down T-cell activation in the immune system [5]. These T-cell and IL-2 inhibitions make tacrolimus an invaluable drug for preventing rejection of tissues or organs such as the heart [6]. Tacrolimus has recently been shown as highly effective in applications for VCA research for transplanting tissues such as bone, muscle, nerve, and skin to patients with substantial injury from deceased donors [7]. While immunosuppressive medication is used to combat the initial attack against the foreign tissue of these grafts, a long-term defense against this attack is required [8]. Tacrolimus, in combination with mycophenolate mofetil (MMF), mycophenolate sodium, azathioprine (AZA), sirolimus, and steroids, provides these desired effects [8].

While VCA transplants include life enhancing surgeries, there is a severe risk of graft rejection. Tacrolimus reduces this risk because with a sufficiently high concentration (5-10 ng/mL) [7], the patient's immune system can be rendered ineffective in its plight to attack the foreign tissue. However, if the tacrolimus concentration is not elevated enough (<5 ng/mL), the transplanted tissue is attacked and possibly destroyed by the patient's immune system [7]. Tacrolimus has a narrow therapeutic range, and a slightly superfluous amount of it (> 20 ng/mL) can result in numerous side effects, such as renal blood flow and creatinine clearance, microangiopathic hemolytic anemia, hypertension, central-nervous-system demyelination, decrease in pancreatic insulin production, and nephrotoxicity [9, 10]. Common techniques for determining the concentrations of tacrolimus in blood include LC-MS/MS, enzyme-multiplied immunoassay (EMIT), antibody-conjugated magnetic immunoassay (ACMIA), and electrochemiluminescence immunoassay (ELICA) [11]. While LC-MS/MS has been identified as the gold standard, it has the potential for cross-reactivity between parent drug and metabolites [12]. This falsely elevated concentration value, in addition to the cost and labor required to complete the procedure, renders it undesirable. LC-MS also requires highly-trained specialists to use and evaluate the results [12]. The EMIT suffers from nonspecific cross-reactivity, leading to poor repeatability between analytical runs, as well as a wide dispersion of results in proficiency testing [11]. Besides these drawbacks, EMIT uses expensive reagents [13]. The ACMIA has several weaknesses too, including insufficient functional sensitivity, inaccuracy at low analyte concentrations, and shift of assay results over time [14]. Electrochemiluminescence immunoassay (ECLIA) has higher cross-reactivity than ACMIA, and there is up to an 11% bias between ELCIA and LC-MS/MS. Another disadvantage of electrochemiluminescence is the need for specialized instrumentation that can induce generation of electrochemically-excited states coupled with sensitive light detection [15, 16]. The limitations of the currently available approaches require a new technique to be formulated—a low-cost and simple platform for detection and quantification of tacrolimus from human blood.

A paper-based microfluidic enzyme-linked immunosorbent assay (ELISA) is an ideal solution because it is a versatile, flexible, porous, and eco-friendly platform [17]. In addition to these advantages, it provides affordable, simple, accurate, and rapid detection (within 5-10 minutes). It is also portable and can be especially useful in resource-limited settings, such as developing countries and austere environments, where highly trained medical staff and fully-equipped facilities are limited or absent [17-19]. Furthermore, it is repeatable within its error range, providing consistent results.

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In this work, we developed a paper-based point-of-care (POC) device to detect and quantify the concentrations of tacrolimus in human blood. To our knowledge, this is the first paper-based competitive ELISA assay. Key analytical capabilities evaluated in this device for reliable tacrolimus TDM include detection of tacrolimus in whole human blood at the drug's standard therapeutic range of 5-20 ng/mL, quantification of results, and demonstration of these analytical targets being achieved with time frames and sample volumes relevant to minimally invasive POC diagnostics (e.g. <50 µL and <10 minutes). In addition, accelerated shelf-life testing was implemented to ensure proper shelf life parameters, and the device performance was evaluated in the presence of potential interferents. Our results demonstrated the capability of our device to provide rapid detection of tacrolimus in under 10 minutes using only 20 µL of human blood. Colorimetric results were differentiated by the naked eye and accelerated shelf-life testing demonstrated the potential stability of the device to be approximately six months. The device also proved to maintain its detection performance when challenged in the presence of endogenous substances and drugs co-administered with tacrolimus.

2. Materials and Methods

2.1 Materials

The Whatman chromatography paper, nitrocellulose membrane, and blotting paper were purchased from GE Healthcare Life Sciences (Pittsburgh, PA). The Anti-FK-506 Mab was purchased from Creative Diagnostics (Shirley, NY). The FK-506 tacrolimus drug and rabbit anti-IgM Ab (control Ab) were purchased from USBiological Life Sciences (Salem, MA). Tacrolimus BSA conjugate was purchased from Molecular Depot (San Diego CA, USA). Mycophenolate mofetil, rapamycin, and Tween 20 were purchased from Sigma Aldrich (St. Louis, MO). Bilirubin, cholesterol, human albumin, and human gamma globulin were obtained from MP Biomedicals, LLC (Solon, OH). Sucrose, uric acid, and Dulbecco's phosphate buffer saline (DPBS) were obtained from Thermo Fisher Scientific, Inc. (Waltham, MA). Double-sided adhesive was supplied by FLEXcon (Spencer, MA). De-identified whole human blood from healthy donors was obtained from Research Blood Components, LLC (Watertown, MA).

2.2 Sample Preparation

The tested samples were prepared using unmodified fresh human blood spiked with tacrolimus (FK-506) alone, tacrolimus in combination with drugs that interfere with tacrolimus detection (sirolimus and mycophenolate mofetil), and lastly, tacrolimus in combination with endogenous substances (bilirubin, cholesterol, uric acid, albumin, and gamma globulin).

2.3 Synthesis of Colloidal Gold-Anti-Tacrolimus (FK-506)

Our POC diagnostic device was fabricated to specifically detect tacrolimus (FK-506), which is a macrolide antibiotic with a reliable immunosuppressive function proven to be effective in combating VCA rejection. Conjugation of the anti-FK-506 antibody to colloidal gold nanoparticles was accomplished by following the manufacturer's protocol (DCN Diagnostics, Carlsbad, CA). Briefly, prior to conjugation, the anti-FK-506 protein was dialyzed in a 10 mM sodium phosphate buffer solution using 10K MWCO dialysis devices. To facilitate the conjugation reaction, 5 mL of colloidal gold solution (AuNP) titrated at the recommended pH values (7.0-9.0) was mixed with the 0.004-0.012 mg/mL anti-FK-506 protein for 15 minutes at room temperature. Then, the solution was blocked with the kit's blocking solution for 30 minutes. The resulting solution was centrifuged at 14,000 x g for 10 minutes, and the conjugated anti-FK-506 mAb was resuspended in the buffer provided by the kit. The AuNP-FK-506 mAb conjugate had an optical density (OD) of 10, and it was stored at 4° C until use.

2.4 Fabrication of the Paper-Based Diagnostic Devices

Paper-based POC devices comprised of six layers arranged in a vertical flow arrangement were fabricated using the principles of competitive immunoassays for detecting small biomolecules. The patterns on each of the six layers was designed using the Adobe Illustrator. Wax printing was used to establish hydrophobic areas that surround the active hydrophilic test zones of the device, and this was done on every layer except the blotting and the separator layers. The sample pad, conjugate, and incubation layers of the device were printed using a Xerox ColorQube 8580 wax printer on Whatman No 1 chromatography sheets or nitrocellulose membranes. The test readout layer was printed on the nitrocellulose membrane. These paper layers were then baked in an oven at 130˚C to facilitate melting of the wax (30 secs), which created hydrophobic boundaries that defined the sample zones. Finally, the printed paper devices were cut using a guillotine-type paper cutter. To assemble the layers together, adhesive films patterned with opened holes and channels created from a laser cutter machine (GuangZhou Amonstar Trade CO., Ltd, KH-3020) were placed on the back-side of each layer. Lastly, the device was constructed by stacking the layers together, starting with the sample pad as the first layer and the blotting paper as the final bottom layer (Figure 1A). The fully assembled device dimensions are 1.75 cm by 1.75 cm.

2.5 Immunoassay Implementation

The sample pad, incubation layer and blotting layer were not treated. The conjugate layer was treated with 1 µL of conjugate treatment solution (0.05% Tween 20 and 1% BSA). The solution was allowed to air dry at room temperature. This was followed by treating the layer with the colloidal gold anti-FK-506 detection antibody, which was also allowed to air dry for 10 minutes. A 1 µL of a BSA-FK-506 solution at 100 µg/mL was added to the sample test zone located in the readout layer. In addition, a rabbit anti-mouse IgM solution (2 mg/mL) was added to the positive control test zone (Figure 1B). This was followed by blocking the layer with 1 µL of blocking solution (0.05% Tween 20 and 1% BSA). The device was then assembled in preparation for testing. Whole human blood samples spiked with tacrolimus concentrations at 100, 25, 21, 10, 4, 1, and 0 ng/mL were tested in the device. The immunoassay was initiated by adding 20 µL of a sample to the device sample pad which was followed by the addition of a 60 µL of washing DPBS buffer. After the antibody-antigen binding, the results were determined by peeling the devices' layers apart to expose the test readout layer. This allows for color interpretation by the naked eye. The red color formation for tacrolimus detected at these concentrations was quantified using the NIH ImageJ software from the images taken by an Android phone. The grey intensity was quantified, and statistical analysis of the results was performed (Figure 1C). The detection time was less than 10 minutes for all the assays performed. Triplicate experiments were performed for each tacrolimus concentration.

2.6 Accelerated Aging and Shelf Life

As previously detailed in section 2.5, devices were prepared and assembled to test the shelf life of the paper-based POC devices when stored at 50 °C for 15 days. The sample testing was then performed on days 0 and 15. Whole human blood spiked with 10 ng/mL tacrolimus was used to test the shelf life of the devices. The results

were quantified, and statistical analysis was performed to determine if there was a significant difference between the results on days 0 and 15. Triplicate experiments were performed for each condition.

2.7 Testing of Potential Interference from Drugs and Endogenous Substances

To characterize the effects of potential interferents on the device performance, devices were prepared and assembled as specified in section 2.5. The potential sources of interference included sirolimus (rapamycin), mycophenolate mofetil, bilirubin, cholesterol, uric acid, albumin, and gamma globulin. First, tacrolimus was tested in the presence of commonly co-administered drugs to determine whether these drugs would have any interference in the quantification of tacrolimus when assayed by our diagnostic device. We spiked whole human blood with 10 ng/mL of tacrolimus, 300 ng/mL sirolimus (rapamycin), and 100,000 ng/mL mycophenolate mofetil. This mixture was then tested in our device. In addition, we tested the interference for tacrolimus detection in the presence of a mixture of endogenous substances. In this test, whole human blood was spiked with 10 ng/mL of tacrolimus, 0.6 mg/mL bilirubin, 5 mg/mL cholesterol, 0.2 mg/mL uric acid, 120 mg/mL albumin, and 120 mg/mL gamma globulin. The results were quantified, and statistical analysis was performed to determine if there was a significant difference between the results of devices tested with tacrolimus-alone and devices tested with tacrolimus co-administered with other interferent substances. Triplicate experiments were performed for each condition.

2.8 Statistical Analysis

The statistical analyses were performed by using GraphPad Prism (La Jolla, CA, U.S.A.). The statistical data was evaluated by paired and unpaired t-test. In this work, the data was represented as an average ± standard deviation (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$).

3. Results and Discussion

In this work, we have developed a low-cost diagnostic device for rapid therapeutic drug monitoring (TDM). Our platform performs immunoassay-based point-of-care (POC) tests for tacrolimus levels in human blood. The key analytical capabilities necessary for a reliable tacrolimus TDM device include: the ability to detect tacrolimus at ranges relevant to the TDM (5-20 ng/mL) to ensure safe yet effective drug levels in the body, quantification of results relevant to the TDM range to indicate necessary adjustments, and demonstration of these analytical targets to be achieved with time frames and sample volumes relevant to minimally invasive POC diagnostics (e.g. <50 µL and <10 minutes). In addition, our device was evaluated by accelerated shelf life testing. Furthermore, the effects of potential interferents on the assay performance in our device were characterized.

3.1 Device Design for Monitoring Tacrolimus in Whole Blood

Sensitive, low-cost, and portable devices are desirable in TDM because they are less invasive, require a small amount of time to take and analyze samples, provide instant warnings at the indication of potential toxicity, and are able to reduce time used by clinical personnel during diagnosis, in addition to improving the clinical performance during TDM. However, the need for pre-treatment of blood samples before detection of the targeted analyte, and the lack of quantification capabilities by the diagnostic tools are the most common obstacles presented by immunoassays implemented for TDM [20].

In this study, by implementing the principles of both competitive immunoassays and vertical flow microfluidics, a rapid paper-based POC test was developed for colorimetric detection and quantification of tacrolimus in human blood (Figure 1). Vertical flow, rather than lateral flow, was used to wick fluid in the proposed six-layer device (Figure 1A). Vertical flow arrangement was decided because it allows for shorter path for sample flow and shorter diagnosis time for the targeted analyte compared to lateral flow configuration. In addition, vertical flow device eliminates potential hook effect problems that could compromise the detection efficiency of the diagnostic test [21-23]. A specific anti-tacrolimus antibody with high affinity was chosen for the competitive ELISA-based immunoassay. Due to the small size of the tacrolimus molecule (MW: 804.031 g/mol) and the presence of few epitopes, using two highly specific antibodies (Abs) for a sandwich immunoassay is highly challenging [24]. Therefore, a competitive immunoassay approach was chosen in this work.

Throughout the study, there was no interference between test regions in the device. The competitive interactions between the targeted analyte FK-506 (tacrolimus) and the AuNP-FK506 Mab (gold nanoparticle-conjugated anti-tacrolimus antibody) against the BSA-FK-506 (BSA-tacrolimus) conjugate at the test readout layer were successful. This was possible because the red blood cells effectively separated from the unmodified human blood sample by the filter system in the second layer of the device. The glass fiber membrane in the filter system blocked the passage of red blood cells (RBCs) to the subsequent layers of the device by acting as a sieve where the RBCs are bigger than the average pore size of membrane. Subsequently allowing the plasma containing the tacrolimus drug to gradually flow down the subsequent layers of the device. The gold nanoparticle (GNP)-conjugated anti-tacrolimus antibody in the conjugation layer was mixed with the plasma sample and bound to the tacrolimus drug. Capillary flow carried the GNP-antibody/tacrolimus complex and unbound GNP-conjugated anti-tacrolimus antibodies through the test readout layer (capture layer), which allowed for competitive binding with the tacrolimus-BSA conjugate to form a detectable red color signal. In addition, the six-layer vertical flow arrangement in this diagnostic platform allowed for separated interactions between analytes and antibodies at each individual layer (conjugate, incubation, and test-readout layer). Therefore, there are no interference or cross reactivity problems at the final test readout line, which are observed in lateral flow devices as a result of placement and interference between test lines [21].

During the device development process, the cost and colorimetric detection efficiency of the diagnostic test were optimized. To elaborate, gold nanoparticles 20 nm in size were selected to reduce the amount of the antibody during conjugation of the anti-FK506 Ab and increase color intensity during detection. A decrease in the size of gold nanoparticles to approximately 20 nm has been showed to require less antibody during conjugation [25], therefore reducing the cost of the test and improving its scalability as more conjugation solution is obtained by using less antibody. In addition, literature examples have shown that gold nanoparticles around 20-40 nm in size are commonly used in the development of immunoassays due to their high color intensity and the sensitivity during detection of this particle size [25, 26]. Gold nanoparticles with diameters <10 nm compromise the colorimetric efficiency of the assay with a lighter color formation during detection while too large nanoparticles with diameters >40 nm are unstable and cause self-coagulation during the conjugation reaction[25, 27, 28].

Overall, the design pattern of our diagnostic device, which included both the sample and wicking pads as part of the six-layer device, allowed for the efficient and rapid detection of tacrolimus under 10

minutes in unmodified human blood. Pre-analytical steps during sample processing were not required in our platform. The paper-based device did not need pre-treatment for separation of red blood cells to measure the tacrolimus concentration in blood compared to the commercialized immunoassays (EMIT in the Siemens Dade VIVA analyzer, MEIA in the IMx analyzer, and CMIA in the ARCHITECT analyzer by Abbott Diagnostics)[29]. Therefore, our new paper-based biosensor is advantageous over existing immunoassays due to the reduced testing time and absence of pre-analytical preparation steps. In addition, only 20 µL of sample was required to perform the test. These qualities demonstrate the ability of this diagnostic tool to provide a rapid diagnosis of tacrolimus at a low cost within time frames and sample volumes relevant to minimally invasive POC diagnostics.

3.2 Device Performance for Tacrolimus Detection

We have assessed the capability of our paper-based microfluidic device to detect tacrolimus at concentrations within the drug's standard therapeutic range of 5-20 ng/mL in unmodified whole human blood. Due to the high variability in the blood concentration of tacrolimus among patients, close monitoring of the drug is critical for its effective use in patients that are high-risk, namely, those who are at risk for liver or heart allograft rejection. [24, 30]. For these high-

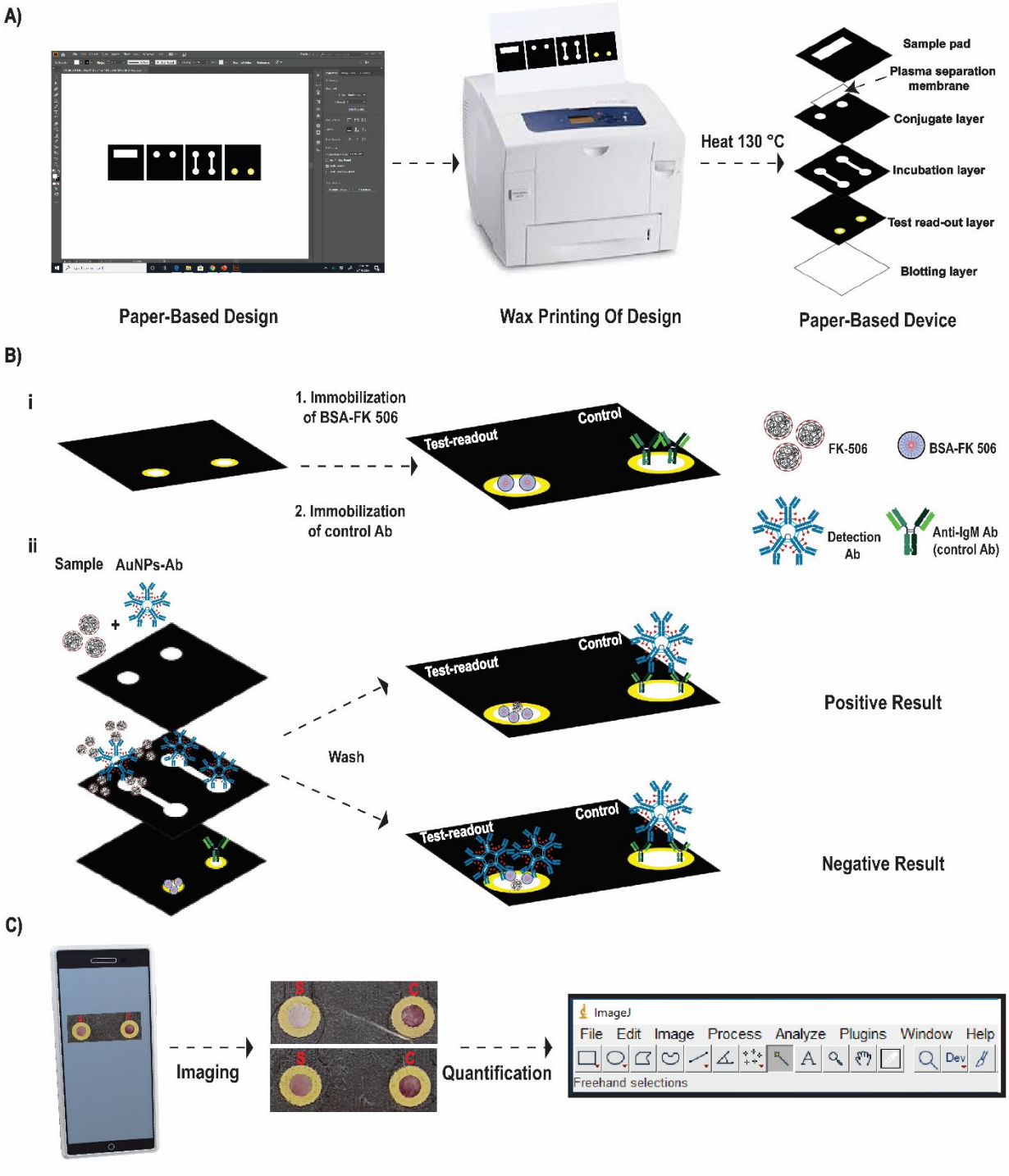

Figure 1. Device fabrication and tacrolimus detection. (A) Schematic representation of the fabrication of a six-layer paper-based POC device using a wax printing method, (B) (i) Tacrolimus-BSA and control Ab immobilization on the detection and control zones of the microfluidic device at the test-readout layer (ii) Competitive assay results (positive and negative), and (C) Color formation, interpretation of the results from the images taken by an android cellphone, and quantification of results using NIH ImageJ software.

risk patients, tacrolimus levels below the recommended therapeutic range may be recommended because low dosages of tacrolimus used with supplemental drugs can reduce the risk of side effects. Because it is critical to monitor the concentration of tacrolimus at levels lower than 3 ng/mL in these high-risk patients, we have optimized our device to detect tacrolimus at concentrations in the higher and lower ends of the recommended drug medical decision range. As a result, concentrations such as 25, 10, and 1 ng/L were tested and detected in our device. In addition, tacrolimus concentrations of 100, 21, 4, and 0 ng/mL were also assayed in the device. It is recommended by the International Association of Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT) that diagnostic devices performing detection of tacrolimus in human blood to show a limit of quantification approximately 1 ng/mL for effective tacrolimus monitoring [30].

During the study, we utilized NIH ImageJ software for quantifying the amount of color formation by images taken on an Android phone, and we performed statistical analysis to determine if the results for each concentration were significantly different from each other. In Figure 2A, the formation of color in the test zone (S) for the 25 ng/mL concentration was lighter than that of the 10 and 1 ng/mL. This result is expected because of the smaller number of free-flowing gold-conjugated antibodies that can travel to the test line when the concentration of tacrolimus in the tested sample is higher. The difference in color intensity was visible to the naked eye in the images for all of these concentrations. In addition, the immense increase in red color formation for the smaller concentration (1 ng/mL) of tacrolimus demonstrated the colorimetric detection efficiency of the device based on the competitive binding assay. Similar detection efficiency has been shown by the ACMIA immunoassay method in the Siemens-Dade Dimension RxL instrument (Siemens Medical Solutions USA, Inc, Malvern, PA) at 1 ng/mL concentration of tacrolimus [31]. The quantified results for these concentrations shown in Figure 2A confirmed the statistically significant differences in the color intensities between each concentration ($P < 0.0001$ and 0.0123, respectively). Additionally, the colorimetric results for the 100, 21, 4, and 0 ng/mL tacrolimus concentrations in Figure 2B can also be differentiated by the naked eye. When comparing the colorimetric results for both figure 2A and 2B, the difference in red color intensity across each concentration can be clearly observed, where the 100 ng/mL concentration shows the lightest color and the concentration with no analyte shows the darkest. The statistical analysis for the quantified results of these concentrations (Figure 2B) also showed significant differences, except between the 10 ng/mL and 4 ng/mL concentrations. Future studies will require a closer determination of additional parameters to obtain differences in the quantification results between these concentrations. For example, varying the optical density (OD) of the gold nanoparticle-conjugated antibody solution for optimal color formation or using another anti-tacrolimus antibody that has higher sensitivity can be used to mitigate this challenge. To further demonstrate the difference among lower concentrations tested in our device, supplemental data for 10 ng/mL and 3 ng/mL concentrations are provided in Figure S1. The red color intensity increased when the tacrolimus concentration decreased from 10 to 3 ng/mL as expected for a competitive immunoassay. The quantified color intensities between the no analyte group and all of the tested concentrations were found to be statistically significant. The reproducibility of the test at each of the detected concentrations (25, 10, and 1 ng/mL, n=3 replicates) were also evaluated and resulted in coefficients of variation (CV) of 2.8, 2.4, and 3.5% for each concentration, respectively. This is below the 20% CV, which is the value recommended by the Food and Drug Administration (FDA), indicating the high performance of the device for repeatable measurements. In the future, these experiments will be performed per the Clinical and Laboratory Standards Institute (CLSI) guidelines, which require testing a higher number of replicates for a period of at least 5-20 days [24, 32, 33].

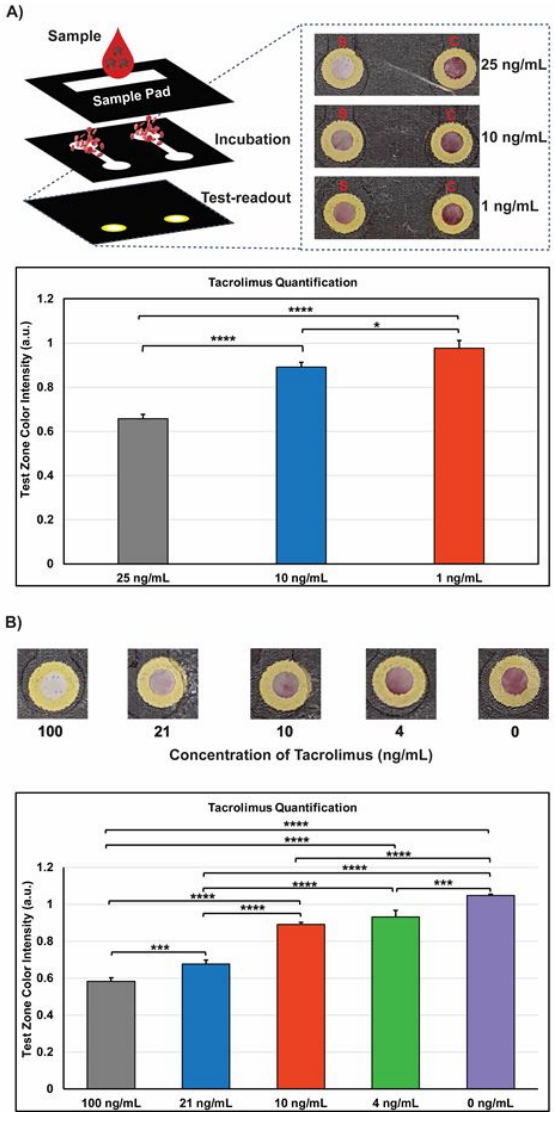

Figure 2. Colorimetric detection and quantification of tacrolimus in human blood. A) Detection and quantification of tacrolimus in unmodified whole human blood at 25, 10 and 1 ng/mL concentrations. The test zone and the control zone were labeled S and C, respectively. Tacrolimus was detected using 20 µL of whole human blood sample in the test zone S. The detection time was <10 minutes. When the concentrations of 25, 10 and 1 ng/mL of tacrolimus were tested, the red color formation increased with decreasing tacrolimus concentration, as expected for the competitive assay. B) Detection and quantification of tacrolimus in whole human blood at 100, 21, 10, 4 and 0 ng/mL concentrations. quantification of red color formation for all concentrations was performed using NIH ImageJ. This was done by measuring the grey intensity in the images taken using an Android cellphone camera. Three replicates for each condition were performed for all concentrations. Statistical analyses were performed using GraphPad Prism (La Jolla, CA, USA). Error bars: ± SD, *p < 0.05 and ****p < 0.0001.

The results of this study demonstrate the ability of our diagnostic tool to efficiently detect tacrolimus in a small volume of blood sample (20 µL) at clinically relevant concentrations. The colorimetric results can be evaluated by the naked eye qualitatively or via image analysis semi-quantitatively. No false positive results were obtained during this work.

3.3 Shelf Life Test

The preservation of the activity of biomolecules on diagnostic devices is a critical factor in determining the shelf life of the product. Parameters such as temperature, humidity, and time are the most critical challenges affecting the shelf life of a diagnostics device intended to be used in resource limited areas where appropriate storing conditions are extremely lacking [34, 35].

In this study, the shelf life stability of our POC diagnostic device was assessed through accelerated aging testing (Figure 3). To provide an initial indication of the shelf life of the POC device, devices were stored at 50 °C for two weeks. The results suggested the detection efficiency of the devices under elevated temperatures maintained similar to the ones that were tested at room temperature when the target analyte (tacrolimus) was detected at a 10 ng/mL concentration (Figure 3A). In addition, no significant difference was found from the statistical analysis of the quantified data between devices tested at day 0 (0.89 ± 0.03) versus 15 days (0.83 ± 0.05) after storage at 50 °C (Figure 3B). The long-term activity of the gold-conjugate anti-tacrolimus antibody on the POC device was preserved in high temperatures (50 °C) during the 15-day storing cycle. The stability of the protein was maintained as a result of using sucrose and casein. The supplementation of these reagents prevented the unfolding of the protein during heating. It is well established that the use of non-reducing sugars such as sucrose or trehalose enhance the stability of proteins in high temperatures. In addition, using a mixture of non-reducing sugars and blocking reagents has been shown to preserve the activity of antibodies at temperatures such as 45 °C in paper-based diagnostic devices used at the POC [35]. The results of this study did not only validate the detection ability of the device to be preserved at 50 °C, which is acceptable under the World Health Organization (WHO) guidelines, but it also indicated its real-time shelf life to be approximately equal to six months at room temperature, as proposed by other literature examples [36-38].

3.4 Assay Performance in the Presence of Potential Interferents

Validation of the immunoassay performance under the presence of endogenous compounds and interferent drugs must be performed to determine the effects of cross reactivity on the efficacy of a diagnostic test. According to the National Committee for Clinical Laboratory Standards "Interference Testing in Clinical Chemistry; Proposed Guideline", tacrolimus should be tested in the presence of endogenous compounds and commonly co-administered drugs at their highest concentration or 10-fold higher than the highest established therapeutic dosage to gain an understanding for the sensitivity of the diagnostic platform intended for tacrolimus detection [39-41].

In this study, to test the influence of interferent drugs and endogenous substances on the detection of tacrolimus, we assessed the potential cross reactivity in the presence of routinely administered medications and physiological compounds. The interferent compounds used in this study were identified based on recommendations by the FDA and the National Committee for Clinical Laboratory Standards (NCCLS) [40, 41]. Figure 4A shows the colorimetric results for tacrolimus tested alone at a 10 ng/mL concentration and tacrolimus tested in the presence of sirolimus (300 ng/mL) and mycophenolate mofetil (100,000 ng/mL) in whole human blood. The quantified results and statistical analysis for this set of data revealed no significant difference between each tested group (0.89 ± 0.02 vs. 0.84 ± 0.02) (Figure 4B). In addition, results for the detection of tacrolimus alone (10 ng/mL, 0.89 ± 0.02) and in the presence of the endogenous substances (0.6 mg/mL bilirubin, 5 mg/mL cholesterol, 0.2 mg/mL uric acid, 120 mg/mL albumin, and 120 mg/mL gamma globulin; 0.88 ± 0.03) recommended by the FDA and the NCCLS showed no significant difference between each tested group (Figure 4C and 4D). As per "Class II Special Controls Guidance Document," 10 ng/mL tacrolimus was the preferred concentration tested in the presence of the interferent compounds assayed in this study because it is in the tacrolimus medical decision range of 5 ng–15 ng/mL [41].

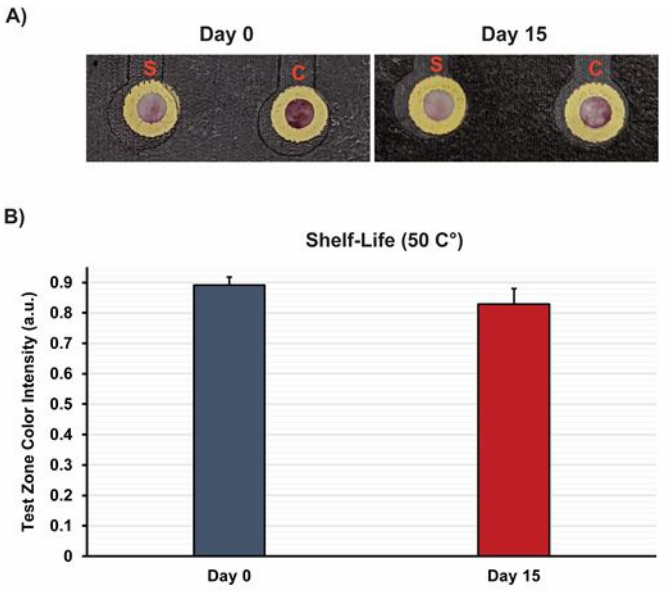

Figure 3. Accelerated aging and shelf life of the paper-based microfluidic devices for tacrolimus detection at 50 °C. A) Colorimetric results for devices containing whole human blood spiked with 10 ng/mL tacrolimus. Devices were stored at 50 °C and tested at day 0

and at day 15. Three devices were tested per time point. B) Quantification of the concentration of tacrolimus on paper-based microfluidic devices in the aging experiment stored at 50 °C. The statistical analysis shows no significant difference between devices after 15 days of aging at 50 °C.

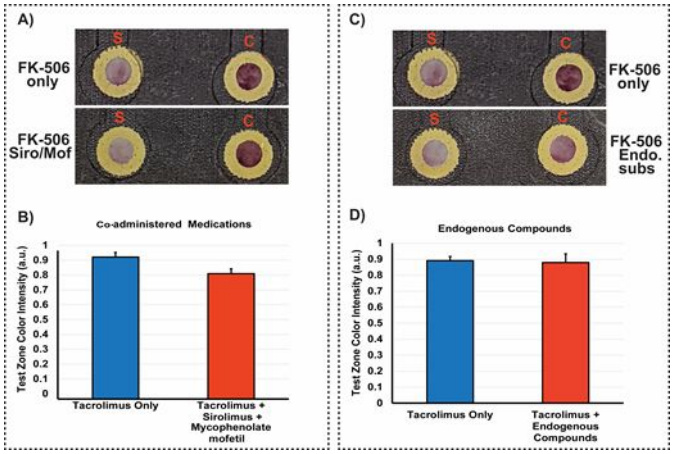

Figure 4. Interference test for detection of tacrolimus in the presence of co-administered medications and endogenous compounds. A) Interference test for the colorimetric detection of tacrolimus in the presence of sirolimus (rapamycin), and mycophenolate mofetil. Three replicates were carried out for each experimental condition. Colorimetric results for tacrolimus tested alone at a 10 ng/mL concentration in whole human blood are shown in the image labeled as "FK-506 only." The image labeled as "FK-506, Siro/Mof" demonstrates the results obtained when a mixture containing 10 ng/mL tacrolimus, 300 ng/mL sirolimus, and 100,000 ng/mL mycophenolate mofetil in whole human blood was tested in the devices. B) Quantification results of tacrolimus tested in the presence of potentially interfering co-administered drugs (sirolimus and mycophenolate mofetil). Quantification of red color was performed using NIH ImageJ for the images that were acquired on an Android cellphone camera. The test mixture contained 10 ng/mL of tacrolimus, 300 ng/mL sirolimus, and 100,000 ng/mL mycophenolate mofetil. The statistical analysis results showed no significant difference between devices tested with tacrolimus-only and devices tested with tacrolimus in the presence of other potentially interfering drugs. C) Interference test in the presence of endogenous substances (bilirubin, cholesterol, uric acid, albumin, and gamma globulin) for the detection of tacrolimus in the paper-based microfluidic devices. Image labeled as "FK-506 Endo.Subs" demonstrates the colorimetric results obtained when a mixture containing 10 ng/mL tacrolimus, 0.6 mg/mL bilirubin, 5 mg/mL cholesterol, 0.2 mg/mL uric acid, 120 mg/mL albumin, and 120 mg/mL gamma globulin in whole human blood was tested in the devices. D) Quantification of tacrolimus in the presence of potentially interfering endogenous substances. The statistical analysis results showed no significant difference between devices tested with tacrolimus-only and devices tested with tacrolimus in the presence of other potentially interfering endogenous compounds.

This amount was also the middle range concentration in our test, which is known to provide reliable cross reactivity data [32, 40]. In addition, our results are in accordance with other immunoassay approaches (ECLIA and CMIA), where cross reactivity against interferent compounds such as bilirubin, hematocrit, or total protein is zero [32, 42]. Overall, the results of the presented study suggest that our diagnostic device is highly effective for the detection of tacrolimus in the presence of interferent substances and co-administered medications.

Conclusions

In this study, we fabricated a low-cost paper-based device for the colorimetric detection of tacrolimus. Our rapid, easy-to-use POC device can be successfully operated using a small volume of blood from a finger prick, and its colorimetric results can be interpreted by the naked eye. The device can be operated by non-trained users, therefore meeting the ASSURED criteria proposed by WHO for devices intended to be used in resource-restrained settings. By using a competitive immunoassay approach in a vertical flow platform, our device was able to efficiently detect tacrolimus at concentrations as low as 1 ng/mL in less than 10 minutes. Cross-reactivity studies demonstrated the ability of the device to maintain its sensitive performance when tacrolimus was co-administered in the presence of other endogenous substances and drugs. In addition, accelerated shelf-life studies confirmed the potential long-term stability in the detection efficiency of the device if used under elevated temperatures. Our technology does not only offer a potential alternative to current immunoassay-based techniques used in tacrolimus monitoring, but also it provides a cost-effective, labor-effective, and less invasive approach beneficial for both medical personnel and their patients. This device, with all it has to offer, can improve the efficiency of tacrolimus monitoring in the clinical setting and bring comfort to patients. The simplicity of our device will not only assure the efficient detection of tacrolimus, but its detection approach can be implemented in the detection of other small molecule drugs.

Author Contributions

G.C.U., B.U., and D.L. conceived of the study; D.L. conducted the experiments; D.L., J.T., B.U., and G.C.-U. wrote, revised, and edited the manuscript. All authors have read and approved the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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