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FrameQR®-code-embedded Paper Sensors: One-click Screening Solution to Analyze Colorimetric Output and On-chip Test and Patient Information

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Microfluidic paper-based analytical devices (microPADs) have significantly strengthened telehealth services by providing on-site disease biomarker screening, with the additional provision of colorimetric output quantification by image analysis applications. The analyte quantification results can be stored or shared in a centralized database accessible to healthcare professionals for disease analysis and e-prescription. However, to automatically share on-device test and patient details to a distant centralized database, it is necessary to upgrade the current microPAD format. To enhance the readability of microPADs, we have proposed laser-printed FrameQR®-code-coupled microPADs (FrameQR®-PADs) to add on-chip information. The canvas area of FrameQR®-PADs was used to design microchannels, while channel barriers were made hydrophobic using wax printing. This modified sensor was used for screening of alkaline phosphatase (ALP) from human serum samples, wherein the colorimetric output was scanned using a smartphone to compute the pixel intensity of images using ImageJ. By designing an integrated smartphone application to scan both on-chip and colorimetric information, FrameQR®-PADs can overcome the limitation of manual test labeling associated with traditionally designed microPADs and support the use of proposed assays in telehealth settings by adding the feature of online storage of patient and test details at a centralized location accessible to healthcare professionals.

1. Introduction

The advancement in technology has substantially shifted our physically active lifestyle to a relatively sedentary one. Human beings have become increasingly dependent on remote control appliances for basic chores of daily life. Not only a lack of physical activity but also scientific and technological advancements have also affected the lifestyle, food, and environment of individuals, which in turn have resulted in several severe health issues and a variety of diseases.

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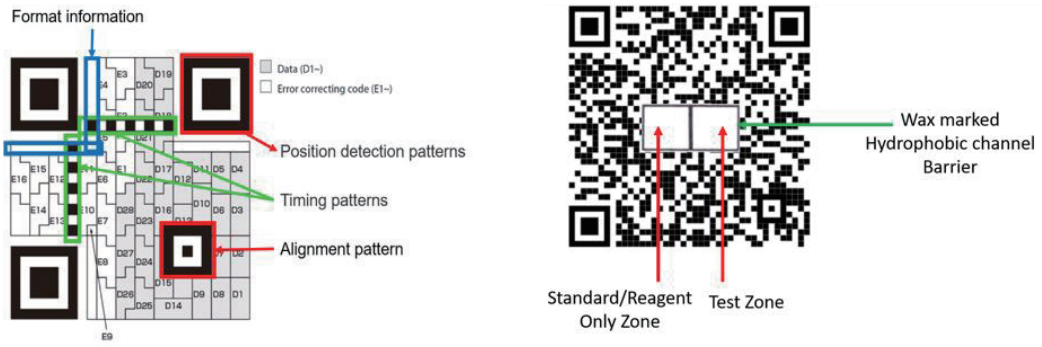
Most of these diseases are linked to changes in the concentration of disease-specific biomarkers. This variability in biomarker concentration is used as an initial screening or diagnostic parameter of disease onset. An initial diagnosis is a prerequisite for early therapeutic intervention or to control disease progression, and currently, a number of techniques are available to perform biochemical and molecular analyses of biofluids to monitor changes in the concentration of disease biomarkers and determine physiological and pathological states of individuals. Although these techniques have very high accuracy, they have some shortcomings such as an unavoidable high cost and the requirement of a specialized laboratory environment along with skilled workers to carefully handle the sensitive equipment.

Along with other fields, the advancement in technology has revolutionized diagnostic approaches. Instead of high-cost laboratory-based equipment and trained professionals, biomedical science aims to provide equipment-free diagnostic solutions to patients at their doorsteps, i.e., on-site or point-of-care (POC) diagnostics,[1–4] including lateral flow assays (LFAs), dipstick assays, and microPADs, e.g., home pregnancy strips and hemoglobin and glucose sensors.[5] Among the different formats of POC assays, microfluidic paper-based analytical devices (microPADs) have gained popularity as a promising alternative to currently available laboratory diagnostic techniques as they provide low-cost and equipment-free diagnostic solutions.[6–11] These devices have been used in diverse medical and clinical applications.[12–19]

Along with their fabrication, different modifications have been proposed to make the output of microPADs more meaningful,[20–22] but a few issues such as readability still remain to be resolved fully. For example, after taking a biofluid sample (e.g., serum, urine, etc.), proper labeling of the sample including patient name/sample ID, sample date, and test information is required. Also, as the shelf life of microPADs is usually short, the manufacturing and expiry dates should be recorded. So far, this has been done manually. It is clearly evident that either improper labeling or swapping of samples among different patients may lead to serious consequences, such as biased or incorrect test results or the patient being prescribed incorrect medication or given incorrect treatment, which may even lead to the patient's death. Thus, to avoid manual labeling, QR-code-embedded/imprinted paper-based assays offer a better solution.

The information embedded in a QR code, such as text, website address, or other data, is encrypted in a square layout consisting of black and white modules, position detection patterns, timing patterns, format information, data area, and an error correction code, usually on a white background [Fig. 1(a)], which can be read by imaging devices such as QR readers, scanners, and smartphones.[23] Among the variants of QR codes, the FrameQR® code has been designed to display customized images (e.g., company logo) or a text in a special zone called the canvas area. The canvas area can be modified without affecting the QR graphic contents and hence does not require error correction. Owing to this unique feature, FrameQR® codes have been customized for multiple commercial applications.[24]

Currently, semi-automated or automated clinical chemistry-based laboratory procedures are used for many clinical analytes including alkaline phosphatase (ALP) analysis. However, these laboratory techniques are often not affordable or inaccessible in some developing countries and resource-limited settings (such as remote or rural areas) and in emergencies. Moreover, these



(a) (b)

Fig. 1. (Color online) Design and fabrication of FrameQR®-PAD. (a) General layout of QR code.[23] (b) Our FrameQR® code design for microPAD.

techniques are not only expensive but also require skilled professionals to operate the equipment, specialized clean laboratory conditions, and 24–72 h procedural time. Thus, to overcome the limitations of currently designed microfluidic paper-based assays and enhance the readability of paper-based assays, we have proposed FrameQR®-code-printed paper-based analytical devices (FrameQR®-PADs) for the quantitative determination of clinically important analytes and demonstrated their use by optimizing them for ALP analysis. We divided the canvas area into two zones, i.e., a standard zone and sample zone, which can be used for the analysis of a single analyte. However, the customized canvas area can be further modified to draw complex channels/patterns to perform multiple analyses on a single microPAD device.

2. Materials and Methods

2.1 Sample preparation

This study was approved by the institutional ethical review committee. Individuals who participated in the study by providing blood samples were briefed about the research and written informed consent was obtained. After collecting 100 blood samples, the standard procedure was followed to isolate serum. Briefly, 5 mL of blood was collected in gel-coated vacutainers and processed within 1 h. Serum was separated by centrifugation at 15000 rpm for 10 min at room temperature and stored in Eppendorf tubes at −20 °C until further use.

2.2 Fabrication of FrameQR®-PAD

To fabricate the FrameQR®-PAD, the fault tolerance area (canvas area) of a FrameQR® code was used to design the hydrophilic channels while the channel barriers were coated by wax printing. This customized FrameQR®-PAD was designed using the ZXing open-source barcode generator library available at https://github.com/zxing/zxing [Fig. 1(b)]. Whatman No. 1 chromatography paper was used as a substrate to fabricate the FrameQR®-PAD. To maintain the

FrameQR® code quality/readability and lower the fabrication cost, the microPAD was printed by a standard office laser printer and the hydrophobic channel barriers were made by wax printing [Fig. 2(a)]. The patterned paper was then heated on a hot plate for 1 min at 120 °C to allow wax to penetrate across the width of the paper [Fig. 2(b)]. The width of the hydrophobic channel barriers was ~0.2 mm.

2.3 Screening of ALP in human serum

The ALP for each serum sample was measured using commercial kits on a semi-automated clinical chemistry analyzer (Microlab-300). To optimize and validate the existing protocol for paper-based assay for serum analytes, the ALP for the serum sample was analyzed through an in-house-developed paper-based assay. For this, chromatography paper (Whatman No. 1) was cut into small circles of 0.5 mm diameter and reagents were spotted serially using a micropipette according to a procedure reported previously.[26] Briefly, 0.7 μL of the reagent solution containing 0.15% nitro blue tetrazolium (NBT) and 0.1% 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 5% dimethyl sulfoxide (DMSO) in water was added twice. After air drying for 10 min at room temperature, 0.7 μL of 500 mM Tris buffer (pH 9.5) was added. The same procedure was followed to spot the reagents on the FrameQR®-PAD.

2.4 Image processing

After 10 min of reagent spotting, 0.4 μL of human serum samples was pipetted in the reaction/test zone of the paper-based device. After 10 min of air drying, the FrameQR®-PAD was initially scanned using CliniPAD, our custom-developed Android application (Fig. 3), to generate test data. Afterwards, the colorimetric output of the paper-based assays was captured through a smartphone camera and analyzed using ImageJ[26] software to obtain mean intensity values. These values were also calculated for different concentrations of serum samples to generate a calibration curve.

(a) (b) (c)

Fig. 2. (Color onlne) In-house-designed FrameQR®-code-based microPAD. (a) After laser and wax printing but prior to hot plate treatment. (b) After hot plate treatment at 120 °C for 1 min. (c) Colorimetric output of FrameQR®-PAD for ALP using reported reagents for this format assay.[25]

Fig. 3. (Color online) CliniPAD: Custom-developed prototype Android-based mobile application for initial scanning of FrameQR®-code-printed paper-based assays for telehealth applications.

2.5 Reaction mechanism

The colorimetric detection of ALP is based on the chemical reaction of BCIP and NBT. BCIP is hydrolyzed by ALP to form a blue intermediate, i.e., 5-bromo-4-chloro-3-indole [Eq. (1)]. In the presence of NBT, this intermediate undergoes dimerization and produces a dehydroindigo dimer, while the hydrogen ions released during dimerization reduce NBT and yield an insoluble NBT-formazan, which is a purple dye [Eq. (2)].

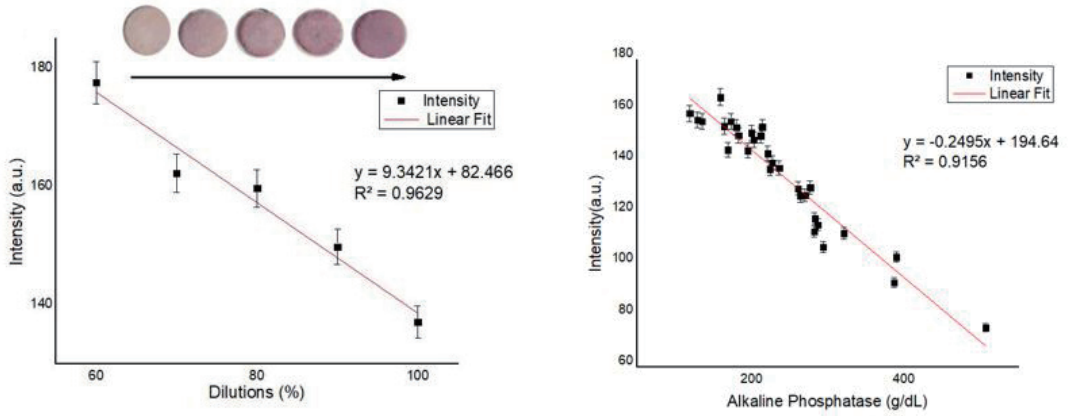
$$\text{BCIP} + \text{Alkaline phosphatase} \rightarrow \underset{}{PO_4^{-3}} + \underset{\text{(Indoxyl intermediate, blue color)}}{\text{5-bromo-4-chloro-3-indole}} \quad (1)$$

$$\text{5-bromo-4-chloro-3-indole} + \text{NBT} \xrightarrow{\text{(Oxidation)}} \underset{\text{(Dehydroindigo dimer)}}{\text{5,5′-dibromo-4,4′-dichloro-indigo-blue}} + \underset{\text{(Purple dye)}}{\text{NBT-formazan}} \quad (2)$$

3. Results

ALP was analyzed through a laboratory-based assay using a semi-automated clinical chemistry analyzer (Microlab-300) and a paper-based assay on both self-customized circular devices and FrameQR®-PADs. The quantitative output of Microlab-300 was recorded for later use. For ALP, paper-based devices showed a visible color change from pale yellow to purple after adding serum samples on reagent spots [Fig. 2(c)]. In particular, a significant decrease in the purple color intensity was noticed in serially diluted serum samples [Fig. 4(a)].

The mean intensity values of ALP, calculated from the colorimetric output of captured images of circular and FrameQR®-PADs, were plotted to generate a calibration curve, which showed a linear relationship (R^2 = 0.96) [Fig. 4(a)]. The entire procedure was performed in less than 40 min. Also, the outputs of the assays obtained by the laboratory-based method using Microlab-300 and through the paper-based device were plotted for 30 random serum samples, which showed a linear (R^2 = 0.9156) relationship [Fig. 4(b)].



(a) (b)

Fig. 4. (Color online) Colorimetric and quantitative outputs of paper-based assay for ALP analysis. (a) Colorimetric and quantitative outputs of paper-based assay for ALP analysis. The color change from pale yellow before adding the serum to purple after adding the serum showed an increase in the purple color intensity for a higher concentration of ALP. The five data points represent the intensities of various serum dilutions (60, 70, 80, 90, and 100%) of one sample. The last data point represents the original (undiluted) serum sample. (b) Comparison of ALP analysis by paper-based assay and conventional laboratory-based assay. The intensity on the *Y*-axis is that for samples obtained from the paper-based assay, whereas the ALP concentration (g/dL) on the *X*-axis represents values obtained from the conventional laboratory-based assay using the clinical chemistry analyzer. Error bars represent one standard deviation from the mean.

A schematic representation of the proposed FrameQR®-PAD fabrication and quantification is shown in Fig. 5. Furthermore, its canvas area can be modified for use with multiple analytes (Fig. 6).

4. Discussion

Paper-based assays are a promising addition to POC testing devices. In developing countries such as Pakistan, the financial crisis, poor food quality, physical inactivity, and polluted environment have contributed significantly to increasing the disease burden. Thus, there is an urgent need for low-cost, user-friendly, and easy-to-access healthcare facilities. Considering all these requirements, as an attempt to explore paper-based assays as an alternative to laboratory procedures, we have tested a routinely used liver biomarker, ALP, and investigated the relationship between the quantitative output of ALP screening by a Microlab-300 laboratory-based semi-automated clinical chemistry analyzer and the color intensity of a paper-based POC device calculated through ImageJ. Interestingly, the good agreement (R^2 = 0.9156) between the outputs of both diagnostic approaches suggests the promising use of paper-based assays for other standard clinical analytes, such as hemoglobin, triglycerides, cholesterol, and urea.

To meet the need for low-cost paper-based assays, different design and fabrication techniques have been proposed. Moreover, nanoparticles have been used with or without reagents to enhance the sensitivity and selectivity of the assay. Although the cost of paper-based assays has decreased and their colorimetric output has undoubtedly improved owing to these modifications,

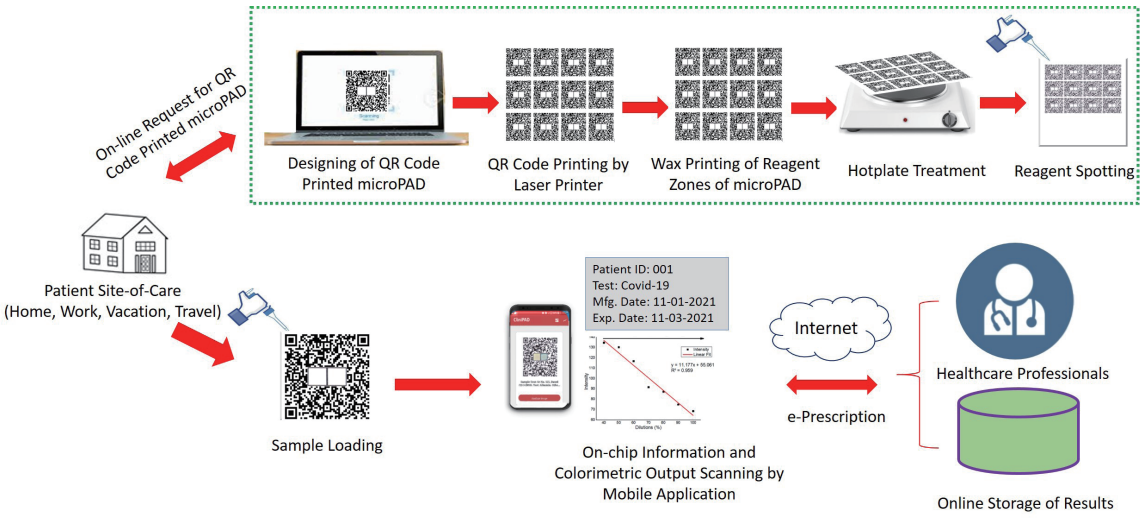


Fig. 5. (Color online) Layout of FrameQR®-PAD fabrication and application.

Fig. 6. (Color online) Various designs proposed for FrameQR®-code-printed microPAD fabrication.

there are still challenges that need to be addressed.[21,22,27,28] In laboratory-based assays, specimens must be labeled correctly with the patient ID, sample date, test type, and so forth. Owing to the escalating disease burden, the number of samples is increasing day by day and sometimes it becomes difficult to label samples manually. Moreover, there is a high likelihood of mislabeling of test/patient data, which can lead to erroneous screening results and hence an incorrect prescription or treatment. Similarly, although the output of microPADs, proposed for ALP screening,[26,29,30] is comparable to that of laboratory-based tests, these devices lack on-chip testing and patient information, which is added manually to keep track of test results, making these devices unsuitable for telehealth.

Considering the versatile applications of QR codes in domestic, industrial, and scientific research fields, we have proposed the use of the information storage capacity of QR codes to record patient IDs, test types, and especially manufacturing and expiry dates, and the use of the canvas area of the FrameQR® code to print hydrophobic channels. Here, the application of the proposed FrameQR®-PAD for ALP was demonstrated. To read on-chip data, CliniPAD was used and the colorimetric output was analyzed using ImageJ. This FrameQR®-code-printed paper-based diagnostic assay will make the results of such assays more reliable and readable than laboratory procedures. Furthermore, its use is not limited to one analyte per assay; the canvas area can be further modified for multiple analytes (Fig. 6), and in one study, an error-correctable QR code-based system was used to perform 30 colorimetric assays in a single device.[31] The multiple zones of the FrameQR®-PAD can be used for diverse biomedical applications. One significant application is genotype-based studies,[32,33] where one simple run can determine the genotype responsible for disease. This knowledge can be used to prescribe personalized medicines. These results can be stored online to develop a worldwide repository, which can help in the preparation of personalized medicines and their genotype-based distribution in specific geographic areas of the world. In another study, a QR code was used as the time component of a device to avoid inaccurate detection time monitoring of the device.[34]

In our laboratory, we have optimized the FrameQR®-PAD for ALP analysis. However, the current Covid-19 pandemic has exposed the limitations of both traditional sophisticated laboratory-based techniques and POC testing techniques (e.g., LFA and microPADs) available for screening of infectious diseases. Within days of a local outbreak of Covid-19, the number of infected individuals often becomes large, which renders diagnostic procedures unaffordable in terms of time, reliability of results, and cost. Also, these methods rely on expensive reagents, skilled laboratory staff, and well-equipped laboratories. Moreover, the physical/clinical examination of patients results in disease transmission from patients to frontline healthcare professionals and, as a result, many doctors and paramedics involved in the treatment or screening of Covid-19 patients become infected, with some losing their lives. Screening of diseases is a prerequisite to initiating evidence-based treatment, so the diagnostic time should be minimized to avoid the pathogenesis of severe diseases. We believe that the current study can be used as a model for designing paper-based devices for diverse infectious diseases including Covid-19, in addition to analyzing clinically relevant analytes.

Our in-house-developed FrameQR®-PAD is advantageous over other conventional procedures. The first major benefit is its low cost, which is one of the main economic barriers for most low-income societies. Instead of using high-cost commercial software (e.g., Adobe Photoshop, AutoCAD), we have designed a FrameQR®-PAD using a freely available online library. Also, to further lower the fabrication cost associated with wax printing, wax was only used to print hydrophobic barriers whereas laser printing was used to outline the hydrophobic channel barrier with the FrameQR® code. Second, as these assays have been targeted for smartphone users, instead of needing professional staff to perform and analyze the test, the output of this paper-based assay can easily be retrieved by scanning it through a smartphone scanner. Third, the FrameQR®-PAD will be highly beneficial for the screening of infectious diseases as it can be customized according to the user identity and the requirement of the

screening test, and can be delivered to the patient after it is ordered online. Fourth, the user can easily use such a device for the screening of infectious diseases where saliva, a nasopharynx/throat sample, urine, tear, or sweat is needed without any of the sample handling, preparation, or processing required for the analysis of blood. Fifth, the user can scan paper-based devices to obtain a colorimetric output and online prescription, and results can be shared with a central repository accessible to healthcare professionals. Moreover, such paper-based devices can be disposed of after use in an environment-friendly manner. In this way, direct physical contact between the patient and healthcare professionals and other members of the public can be minimized or avoided. In the future, such devices could be linked with location-based expert systems to further facilitate disease diagnosis through telehealth settings. These factors make these assays more user-friendly for screening or pre-diagnosis in remote areas and also favor the development of such paper-based devices for the diagnosis of common diseases such as infectious diseases, diabetes, cardiovascular diseases, liver or kidney disorders, and cancer. The deployment of such low-cost POC tests/assays can help reduce the morbidity and mortality by timely diagnosis.

5. Conclusions

Paper-based assays have great potential to revolutionize the field of POC diagnostics. In this study, we have validated the linear relationship between the outputs of a traditional laboratory approach and a paper-based assay for ALP screening, showing the potential use of paper-based assays as an analytical and estimation tool for the initial diagnosis of communicable and non-communicable diseases. To meet the requirements of telehealth and avoid manual recording/labeling of diagnostic test detail and patient data, we have optimized a FrameQR®-PAD and demonstrated its use by scanning it with CliniPAD for ALP analyses. To meet the challenge of an increasing disease burden, a FrameQR®-PAD could be used as a screening tool for prevailing diseases in resource-deprived societies, wherein the results can be made available online to healthcare professionals for prescription by linking smartphone applications to a central database.

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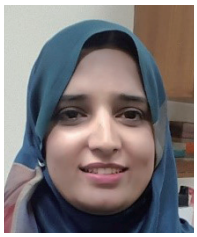

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