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A Portable Platform for the Quantification of Vitamin D Levels Using Image Recognition and Paper-based Microfluidics

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Vitamin D deficiency has been associated with various diseases and adverse health outcomes, including diabetes, cardiovascular diseases, and cancer. Currently, the majority of vitamin D testing is conducted in large-scale laboratories upon the request of a physician as part of an annual panel of blood tests. In this paper, we present a novel paper-based microfluidic system that enables the rapid quantification of vitamin D levels on a portable platform. The system comprises a smartphone accessory, an app, and a test strip that allows for the colorimetric detection of 25-hydroxyvitamin D3 using a gold-nanoparticle-based immunoassay. We demonstrate that the system can accurately measure the physiological levels of 25-hydroxyvitamin D with an accuracy higher than 25 nM and a precision of 10 nM. We compared our system with the well-established ELISA test kits for serum samples of unknown concentration and demonstrated the equivalency of the obtained results.

1. Introduction

Vitamin D is a vital nutrient that plays a pivotal role in bone metabolism by regulating calcium and phosphate homeostasis. Additionally, emerging evidence suggests its involvement in immune system regulation. The World Health Organization has acknowledged the significance of vitamin D in maintaining optimal health. While the primary source of vitamin D is sunlight exposure, it is also found in certain food products, including oily fish, eggs, and fortified items.[1–3]

Infants, in particular, are at a high risk of vitamin D deficiency owing to their limited endogenous vitamin D stores. During the initial months of life, infants rely on breast milk, sunlight, or supplements as sources of vitamin D. However, breast milk often contains insufficient levels of vitamin D, which is affected by the maternal vitamin D status. Furthermore, infants residing in regions with restricted sun exposure or cultural constraints face additional challenges in obtaining adequate vitamin D. Consequently, infants are highly susceptible to

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vitamin D deficiency, which can result in detrimental consequences such as bone malformation (rickets), seizures, and respiratory difficulties.[4,5]

Furthermore, recent findings have indicated that a substantial percentage of adults in the United States (approximately 77%) exhibit inadequate levels of vitamin D. This insufficiency not only heightens the susceptibility to bone diseases but also increases the risk of cardiovascular diseases and infections.[6] Given the heterogeneous nature of sample types, it is imperative to develop a diagnostic device that can accommodate specific processing requirements for each type. Additionally, careful consideration should be given to the inherent form in which biomarkers are present within the sample.

For instance, it has been reported that the biomarker 25-hydroxyvitamin D [25(OH)D] predominantly exists in a bound state to vitamin D-binding proteins (VDBPs), constituting approximately 95–99% of its presence. This binding significantly hampers the interactions essential for most vitamin D tests based on immunoassays. Consequently, it becomes essential to incorporate an integral sample processing step into the design of the diagnostic device to effectively liberate 25(OH)D from VDBPs, thereby enabling accurate detection and quantification.

Moreover, it is crucial to acknowledge that the deficiency levels of specific micronutrients, including vitamin D, can be affected by the presence of infection or inflammation in the patient. The failure to account for these factors has posed significant challenges in the current methodologies employed for identifying vitamin D deficiency. Consequently, any diagnostic device developed should thoroughly consider and address the potential impact of infection or inflammation on biomarker levels, ensuring the attainment of precise and reliable results.

Current research in the field of detecting vitamin D levels has seen significant advancements, driven by the urgent need to accurately assess and address vitamin D deficiency. Numerous studies have focused on developing innovative methodologies and diagnostic devices to enhance the detection and quantification of vitamin D biomarkers, including biosensing strategies, immunoassay-based techniques, nanomaterial-based approaches, point-of-care devices, electrochemical biosensors, microfluidic platforms, optical sensing technologies, and mass spectrometry-based methods.[7–9] Nevertheless, the studies also suggest that it is important to improve the sensitivity, miniaturization, multiplexing, and data analysis techniques.[7]

Microfluidic paper-based analytical devices (μPADs) are widely used for performing diagnostic assays. Weng *et al.* applied colored wax to enhance and control the time delay valves in 2D/3D assays.[10] Their findings showed that μPADs provide a feasible low-cost alternative to conventional methods for performing diagnostic assays.[10] Anusha *et al.* proposed a paper sensor made using a silver-cobalt (Co-Ag)-doped polyaniline-polypyrrole copolymer and an ionic liquid composite.[11] The paper electrode was designed in a specific dimension in Microsoft PowerPoint software, and a patterned electrode was printed on photocopy paper. This fabrication method demonstrated the successful detection of vitamin D_3 and the potential diagnosis of vitamin D deficiency in remote areas where limited resources are available. However, the signal of chemical oxidative polymerization was still measured in situ.[11]

To reduce the redundancy of measurements in the lab, Yang *et al.* proposed a mobile measurement composed of enzyme-linked immunosorbent assay (ELISA)-on-a-Chip (a

microfluidic device), a microcontroller, and a mobile app.[12] While a CCD camera captured the pattern on the microfluidic device, it could also measure pH. They did not directly detect vitamin D and reported that the pollution outside the lab due to the direct exposure of operators was a significant issue.[12]

Walter *et al.* also combined the mobile measurement with a smartphone to detect 25(O)HD in human serum samples using a AuNP-enhanced aptamer-based assay.[13] However, the image processing technique for the mobile device varies with the brand and may be the drawback of using the application even though the sensor fabrication method used is cost-efficient.[13] Nevertheless, in the development of our portable device for vitamin D quantification, we drew inspiration from the successful utilization of high-density zinc oxide (ZnO) nanostructures with AuNPs for gas sensing applications, as demonstrated by Chu *et al.*[14–17] Huey *et al.* developed a portable device that is integrated with a microcontroller and a spectrophotometer chip, which allows the measurement of Vitamin A concentration.[18] Li *et al.* reviewed the application of sensing using a smartphone, including the optical sensors often used for color, image, and spectrum detection. The main reasons for mobile sensing are that a continuously increasing number of physical quantities can be measured and there are advantages of wired and wireless connections.[19] Moreover, a portable device is a key to bringing the measurement out of the lab, such as through a point-of-care device.

Despite previous research efforts in developing mobile and portable devices for vitamin D detection, there is still a lack of experimental and comparative data, especially regarding concerns about color variation and differences in mobile phone brands. In this study, we developed a cost-effective and portable device using color recognition and paper-based microfluidic chips to address these challenges. Experimental data showed that the device has the potential to significantly reduce measurement costs and accelerate the popularization of vitamin D detection.

2. Materials and Methods

In this study, we developed a paper-based microfluidic device for the detection of vitamin D levels. The platform utilizes a colorimetric assay to quantify vitamin D levels in blood samples. The portable device consists of a paper-based microfluidic chip designed to separate plasma from whole blood and to perform the colorimetric assay. The portable device is used to measure the color intensity of the assay and to calculate the vitamin D concentration.

2.1 Aptamer selection and characterization

The method of preparing paper-based microfluidic chips was introduced in a previous study.[12] In this study, a novel approach was employed, utilizing a nitrocellulose membrane-based aptamer assay for the detection of Vitamin D. Aptamers, which are short nucleic acid sequences known for their high sensitivity and specificity, were utilized as molecular probes instead of traditional antibody-based assays. Aptamers were first described in the early 1990s and have since been recognized as promising bioreceptors for analytical applications owing to

several distinct advantages over antibodies. These advantages include their low cost, high stability, ease of synthesis, and ability to target a wide range of compounds. Furthermore, aptamers can be chemically modified to expand their target range, enhance their binding affinity and conjugation efficiency to targets or nanomaterials, and improve their overall stability.

The aptamers were chemically synthesized *in vitro* at a relatively low cost. The aptamers obtained from the systematic evolution of ligands by exponential enrichment (SELEX) were characterized by AuNP-based analysis. It was found that when a specific aptamer was combined with AuNPs and encountered vitamin D, the AuNPs aggregate and change color. In the absence of vitamin D, no color change occurs, as shown in Fig. 1. The complete sequence of the aptamer is 5'AGCAGCACAGAGGTCATGGGGGGTGTGACTTTGGTGTGCCTATGCGTGCTACGGA A-3'.

2.2 Sample preparation

To assess the interaction between the selected aptamers and AuNPs, a solution mixture containing 253 µL of 2.4 nM AuNPs and 23 µL of 8 µM selected aptamers was prepared. The mixture was allowed to react for 30 min at room temperature. Subsequently, 1.62 µL of 1 M NaCl was added to each incubated sample. Changes in solution color were observed both visually (Fig. 2) and using a UV/Vis spectrophotometer. The experiment sessions involved

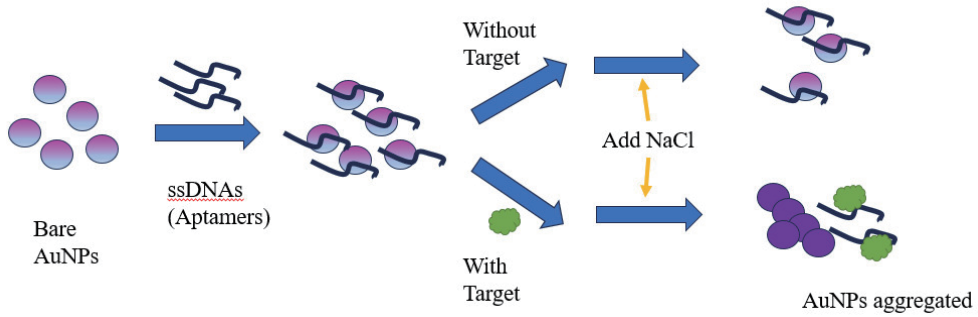
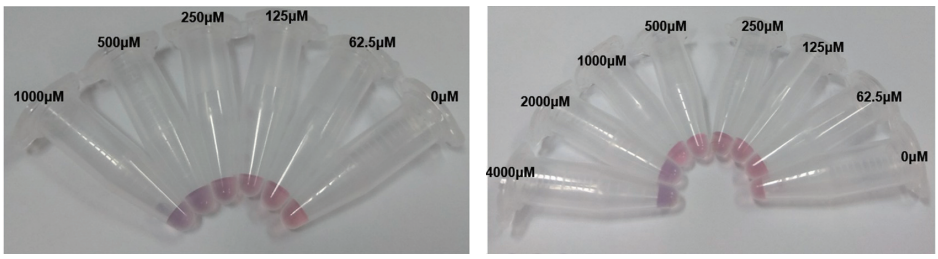


Fig. 1. (Color online) Highly sensitive detection of 25-hydroxyvitamin D3 by target-induced displacement of aptamer.



(a) (b)

Fig. 2. (Color online) Colorimetric results in a tube: (a) digoxin (500 mM) and (b) biotin (2000 µM).

aptamer selection and characterization, AuNPs–aptamer reaction analysis, and the investigation of the dose-dependent response of the selected aptamers to 25-hydroxyvitamin D3.

2.3 Dose-dependent response

To evaluate the dose-dependent response of the selected aptamers to the target (25-hydroxyvitamin D3), the assay was conducted using various concentrations of 25-hydroxyvitamin D3 (ranging from 0 to 50 μM). The aim was to observe the aptamer's response to different concentrations of the target molecule. The experimental matrix is shown in Table 1.

2.4 Portable device and color recognition

The portable device designed in this study is compact and lightweight, as shown in Fig. 3, with a total length of approximately 90 mm, a width of 160.25 mm, and a height of 83 mm. The rectangular container was produced using 3D printing technology and features an opening on one side to allow for the insertion of a paper-based microfluidic chip. The top of the container is equipped with a lid, and the lightweight design allows for handheld operation by the user. The device is also equipped with an internal microcontroller board, allowing for direct connection to a computer program.

Table 1
Experimental matrix for the dose-dependent response of aptamers to 25-hydroxyvitamin D3.

	Configuration										
Formula of aptamers and AuNPs	253 μL of 2.4 nM AuNPs and 23 μL of 8 μM selected aptamers										
Crosslink	Digoxin: 500 mM						Biotin: 2000 μM				
Concentration (μM)	0	0.19	0.39	0.78	1.56	3.12	6.25	12.5	25	37.5	50

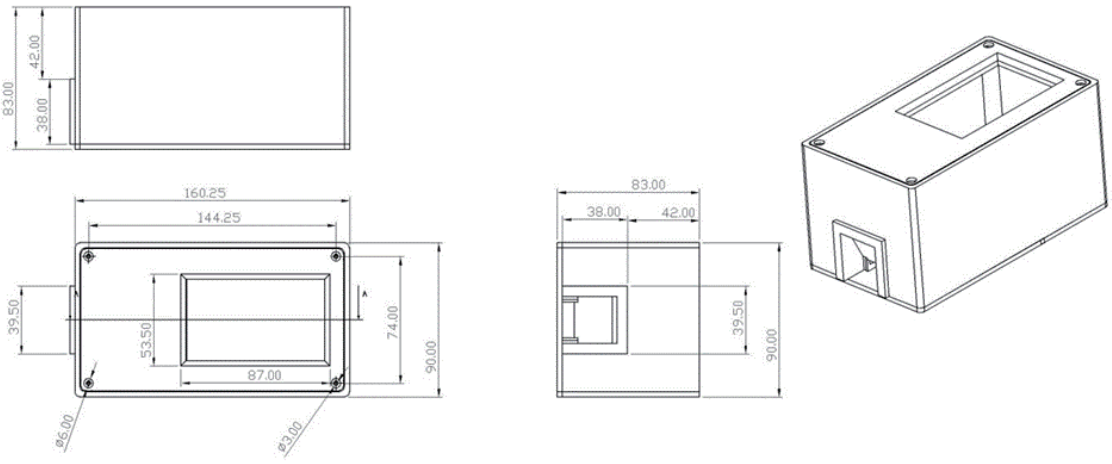
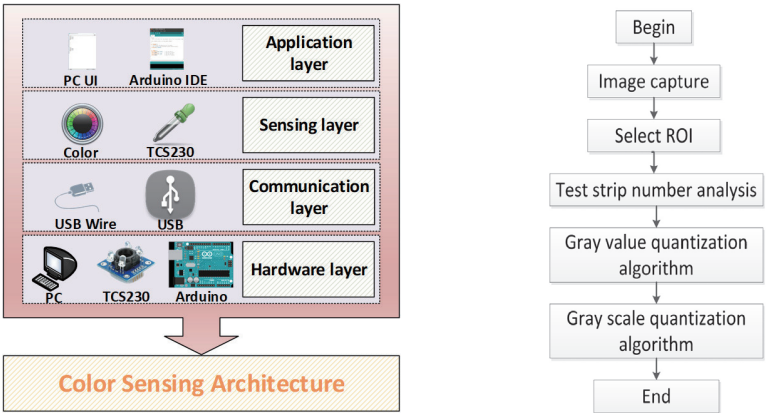


Fig. 3. Computer-aided design of portable device.

The microcontroller board employed in this study is Arduino Uno, which is powered via a USB cable. Specifically, a USB port designed for charging purposes is utilized. Note that a standard USB 2.0 port should be capable of delivering 5 V and 500 mA (equivalent to 0.25 W) to comply with the USB 2.0 standard. Accordingly, the power consumption of Arduino Uno is calculated as 5 V * 500 mA, resulting in a total of 2500 mW. It is connected to a TCS230 sensor for color recognition. The device uses a programming language for logical analysis and decision-making. The design architecture is illustrated in Fig. 4(a). We utilize image recognition technology to measure the color intensity of the test (T) line on the paper-based microfluidic chip after a waiting period. The system captures the color changes in the test line using the TCS230 sensor, which allows for the quantification of vitamin D levels based on the intensity values obtained through image recognition. The computational algorithm was introduced in a previous work[20] and is capable of processing, analyzing, and interpreting the color intensity to simultaneously examine the flow profile. This approach enables the system to provide accurate and precise measurements of 25-hydroxyvitamin D levels, demonstrating the usability and effectiveness of the developed portable device for vitamin D quantification.

The TCS230 sensor is then packaged in an 8-pin SOIC surface-mounted package and integrates 64 photodiodes on a single chip. These photodiodes are divided into four types: 16 with red filters, 16 with green filters, 16 with blue filters, and the remaining 16 without any filters, capturing the full spectrum of light information. The photodiodes are arranged in a cross-grid pattern within the chip to minimize the unevenness of incident light radiation and increase the color recognition accuracy. Additionally, 16 photodiodes of the same color are connected in parallel, evenly distributed within the photodiode array, to eliminate color position errors.

Figure 4(b) shows the program flowchart for color recognition. Upon powering on, if the paper-based microfluidic chip is inserted into the portable device, the image capture process begins. The captured image undergoes preprocessing to retain the regions of interest. Color block calibration is then performed, followed by conversion to grayscale for quantifying color depth. Finally, the quantified grayscale values are converted into vitamin D concentration levels.



(a) (b)

Fig. 4. (Color online) (a) Design architecture of portable device with Arduino Uno microcontroller. (b) Program flowchart for color recognition and quantification.

3. Results and Discussion

In this study, the experiment began by comparing the absorption sensitivity levels of the aptamers for detecting different concentrations of vitamin D and determining the optimal spectral range. The data in Fig. 5 show absorption peaks for different vitamin D concentrations (0, 0.9, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 37.5, and 50 nM) with a fixed digoxin concentration of 500 μM. The results indicate that the maximum absorption wavelength confirmed by UV/Vis spectroscopy is 525 nm, which is characteristic of the binding of AuNPs and the aptamer. Furthermore, it is evident that different vitamin D concentrations can be distinguished into three distinct groups.

Additionally, we found the optimal concentration for crosslinking. Two crosslinking agents, digoxin and biotin, were used at different concentrations to modify the aptamer. We observed that the maximum aggregation of vitamin D occurred when digoxin was at a concentration of 500 μM or biotin was at a concentration of 2000 μM. To minimize costs, subsequent experiments used a digoxin concentration of 500 μM to modify the aptamer for binding to AuNPs.

Figure 6 shows the prototype of a paper-based microfluidic system developed in-house. This innovative analytical tool utilizes cellulose as its functional substrate material and finds extensive application in the analysis of minute sample volumes. The paper channels are typically obtained through manual cutting, the employment of an X-Y cutter/plotter, or the employment of a CO_2 laser cutting apparatus. Subsequently, in the majority of cases, the cut channels are reinforced with adhesive tape to enhance the structural integrity of the device.

After selecting the desired aptamer from the previous experiments, the paper-based microfluidic chip used in the study was improved. The test sample results are shown in Fig. 7(a) as examples. In Fig. 7(b), the principle of AuNP binding in the lateral flow test on the paper-based microfluidic chip is explained. The internal structure of the chip mainly consists of four components: sample pad, conjugated pad, nitrocellulose membrane (containing the test and control lines), and wicking pad.

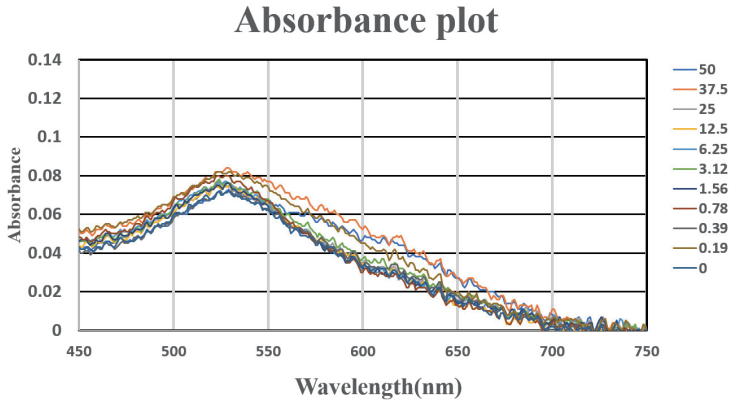


Fig. 5. (Color online) Colorimetric results in tube with 500 μM digoxin to detect various vitamin D concentrations ranging from 0 to 50 nM.

Fig. 6. (Color online) Prototype of in-house-fabricated paper-based microfluidic system.

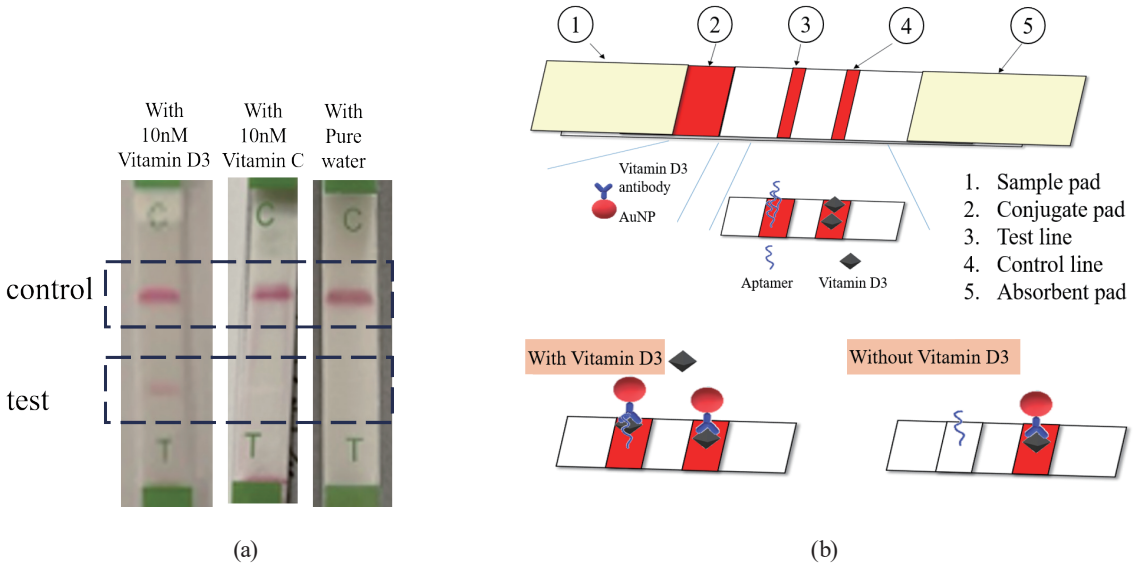
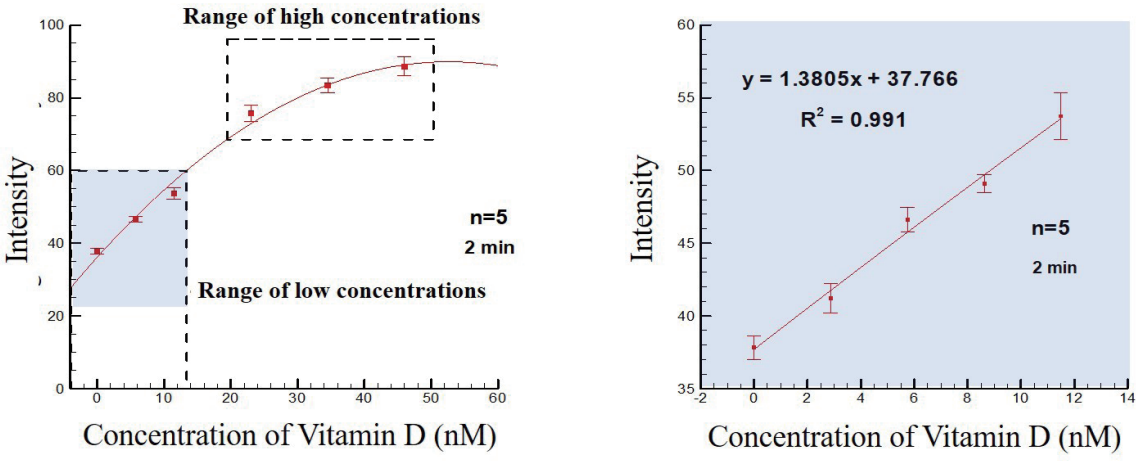


Fig. 7. (Color online) (a) Results of paper-based microfluidic chip for different targets. (b) Principle and explanation of the paper-based microfluidic chip.

When it is added, the sample first comes into contact with the sample pad. Through capillary action, the liquid molecules in the sample slowly move towards the wicking pad through the fibers in each layer. If vitamin D is present in the sample, it will be recognized and bound by the aptamer in the conjugated pad. (In this case, the monoclonal antibody is a special antibody that has been conjugated with nanoscale colloidal Au/fluorescent particles, allowing the test result to show a dark red line.) The vitamin D-aptamer complex will then flow over the nitrocellulose membrane, which has two lines: the test line, containing antibodies that can capture the aptamer, and the control line, containing antibodies that can capture the AuNPs.

Regarding the verification of accuracy, Fig. 8(a) shows the data obtained from the portable device designed in this experiment when measuring six different vitamin D concentrations after a 2-min waiting period. It is evident that there is a significant difference



(a) (b)

Fig. 8. (Color online) (a) Experimental results of color intensity versus different vitamin D concentrations. (b) Linear trend in low concentration range.

in intensity obtained through image recognition. The high concentration region exhibits nonlinear changes, particularly when exceeding 50 nM, where the intensity values decrease and become less suitable for measurement. Conversely, Fig. 8(b) shows a linear trend in the low concentration region, with a linear accuracy of $R^2 = 0.991$. Since the low concentration range is commonly used, and it can be observed from the figure that the reaction reagent can linearly measure the baseline concentration starting from 0, the considerable usability of the developed portable device is verified.

4. Conclusions

The findings outlined above highlight several key points. First, the method was implemented using 25-hydroxyvitamin D3 spiked in human serum, and in future work, various concentrations of 25-hydroxyvitamin D3 will be diluted with human serum to further validate the detection capabilities of the AuNP-based colorimetric assay. We hope that the results will demonstrate the successful detection of 25-hydroxyvitamin D3 spiked in human serum.

Additionally, the conclusion emphasizes the potential for future improvements in terms of the precision and accuracy of the vitamin D quantification method. We described a portable system that quantifies 25(OH)D levels by evaluating the difference in brightness between the detection area and a reference area on the test strip.

Furthermore, the conclusion states that in the future, the ability of the system to quantify vitamin D levels will be demonstrated by evaluating samples with unknown 25(OH)D concentrations and comparing the results with those obtained using a commercial ELISA method. Overall, the conclusion highlights the current findings and suggests potential avenues for further research and refinement of the method.

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