Multicolorimetric ELISA biosensors on a paper/polymer hybrid analytical device for visual point-of-care detection of infection diseases

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Abstract
Enzyme-linked immunosorbent assay (ELISA) is widely used for the detection of disease biomarkers. However, it utilizes time-consuming procedures and expensive instruments, making it infeasible for point-of-care (POC) analysis especially in resource-limited settings. In this work, a multicolorimetric ELISA biosensor integrated on a paper/polymer hybrid microfluidic device was developed for rapid visual detection of disease biomarkers at point of care, without using costly equipment. This multicolorimetric ELISA platform was built on multiple distinct color variants resulted from the catalytic oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB) and the etching of gold nanorods (AuNRs). The vivid color changes could be easily distinguished by the naked eye, and their red mean values allowed quantitative biomarker detection, without using any sophisticated instruments. When this multicolorimetric ELISA was integrated on a paper/polymer hybrid analytical device, it not only provided integrated processing and high portability but also enabled fast assays in about 50 min due to the unique advantages of paper/polymer hybrid devices. The limit of detection of 9.1 ng/μL of the hepatitis C virus core antigen, a biomarker for hepatitis C, was achieved using this multicolorimetric ELISA platform. This multicolor ELISA analytical device provides a new versatile, user-friendly, affordable, and portable immunosensing platform with high potential for on-site detections of various viruses, proteins, and biomarkers for low-resource settings such as at home, public venues, rural areas, and developing nations.

Keywords
Multicolorimetric ELISA · Paper/polymer hybrid microfluidic device · Point-of-care detection · Quantitative biomarker detection · Infectious disease

Introduction

Hepatitis C is a liver disease caused by the hepatitis C virus (HCV), a hepatotropic flavivirus. The virus can cause both acute and chronic hepatitis, ranging in severity from a mild illness lasting a few weeks to a serious, lifelong illness. Chronic HCV infections often advance into cirrhosis or liver cancer. Due to its widespread, especially in underdeveloped and developing countries, each year about 399,000 HCV-infected people succumb to cirrhosis, hepatocellular carcinoma, and liver failure [1]. Therefore, screening of HCV infection is mandatory in many epidemiologic settings to begin appropriate treatment. There is an urgent need for rapid HCV detection so that the drugs can get administered to patients quickly, thus decreasing the chance of the patient getting other syndromes [2, 3]. Although advanced and sophisticated diagnostic technologies, including polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA), are extensively used in developed countries, they are not widely available in developing countries because of costly equipment, limited funds, and lack of skilled technicians. These techniques, along with other methods such as cell culture and western blotting, are normally laborious and time-consuming [4–6]. Hence, rapid and sensitive point-of-care
(POC) detection of HCV in low-resource settings (e.g., high-poverty regions) is still a challenge [7, 8].

ELISA is a widely used conventional technique that allows people to detect and quantify substances such as cells, viruses, and molecular antigens due to their specific binding to immobilized antibodies or antigens [4, 9–11]. However, conventional ELISA takes hours to complete because of the hour-long incubation (e.g., overnight) and blocking time. The consumption of large volumes of precious samples and reagents and the dependence on costly laboratory settings make ELISA not suitable for POC detection [6, 12]. Furthermore, the normally colorless property of ELISA reagents and monochromatic solutions with different intensities makes it hard to do the quantitative or semi-quantitative analysis without combining other detection technologies such as absorbance spectra, chromatography, radiolabeling, and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) [10, 11, 13, 14]. This poses another problem of using expensive and bulky instruments, such as spectrophotometers and microplate readers [14–18], which significantly limits their application in POC detection in developing countries. Therefore, a portable, versatile, cost-effective, and instrumental-free ELISA method is highly desirable.

The multicolorimetric ELISA method can be a promising solution that can eliminate the use of these expensive and bulky instruments and allow it to be portable and cost-effective. Particularly, multicolorimetric sensing based on noble metal nanomaterial such as AuNRs [19] has attracted great interest owing to its advantages of visual color readout, simplicity, and robust enzymatic properties of nanomaterials, and those methods have been applied to detect various biomolecules and pathogens, such as pesticides, glucose, and Listeria monocytogenes [20–23]. For instance, a multicolorimetric sensor was developed for the detection of a toxic organophosphorus pesticide, omethoate, in a centrifuge tube based on the inhibition of the enzyme-induced metallization of AuNRs [20]. However, a microplate reader was required to achieve the quantitative analysis based on the wavelength shift. In recent studies, Lin et al. [21] presented a multicolor glucose sensor on a microplate, which allowed people to detect their glucose levels in serum based on different colors corresponding to different concentrations of glucose in sample solutions at the end of the assay. But an ultraviolet–visible spectrometer (UV-Vis) was still needed, which compromised its capability for point-of-care analysis. Liu et al. [22] reported another multicolorimetric aptasensor to determine the amount of Listeria monocytogenes in a centrifuge tube. However, the procedures to prepare samples and aptamer-conjugated magnetic nanoparticles were complex, and the assay required centrifugation and magnetic separation, which significantly prolonged the assay time and had stringent equipment requirements. However, these multicolorimetric assays were performed in a microplate or a centrifuge tube, which consumes more reagents and lacks integration and portability. Furthermore, these multicolorimetric assays are not ELISA; multicolorimetric ELISA is rarely reported. Although Li and co-workers reported a multicolor ELISA based on alkaline phosphatase-triggered growth of AuNRs, it still required overnight incubation and a microplate reader for quantitative analysis [24].

The recent microfluidic lab-on-a-chip technique provides a versatile platform for various bioanalyses particularly for POC analysis with the advantages of low reagent consumption, fast analysis, and high portability [25–33]. Paper-based microfluidic devices are low cost [2, 34, 35]; however, they have low performance in reagent control and handling, especially for ELISA that usually involves repeated washing steps. Because different chip substrates have different advantages and limitations, different paper/polymer hybrid microfluidic devices were developed to draw more benefits from two different substrates [36–41]. For instance, the novel introduction of paper in a paper/polymer hybrid microfluidic device significantly decreased the ELISA duration from approximately 30 to 1 h [13, 14]. In a paper/polymer hybrid microfluidic device, the paper provides a 3D porous substrate for rapid antibody immobilizations, ELISA conjugations, and many other reactions. Leveraging these advantages of paper/polymer hybrid microfluidic devices, the combination of a paper/polymer hybrid microfluidic chip with multicolor ELISA provides new opportunities for low-cost and rapid POC detection of HCV and other infectious diseases.

In this study, we developed a multicolorimetric ELISA biosensor on a paper/polymer hybrid analytical device for visual POC detection of HCV, based on multiple color variants through etching of AuNRs and the oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB). As shown in Fig. 1, the catalytic oxidation of TMB via horseradish peroxidase (HRP) or other enzymes produced oxidized TMB2+, being accompanied by a distinct color change of the system [5]. Meanwhile, oxidized TMB2+ could etch AuNRs quickly, resulting in another color change. During the multicolorimetric ELISA in each microwell of the paper/hybrid chip, the presence of HCV was specifically recognized and captured by a monoclonal antibody and then immunocomplexed with the HRP conjugated secondary antibody. Different amounts of HCV produced different amounts of TMB2+, which modulated different degrees of AuNRs etching processes. Consequently, the combination of the AuNRs etching and the TMB redox reaction resulted in various color changes, which could be easily discerned by the naked eye. The red mean values analyzed using ImageJ were introduced for the first time in the multicolorimetric ELISA for quantitative detection of HCV, eliminating specialized analytical detectors such as spectrometers commonly used in other quantitative multicolorimetric assays. The limit of detection of 9.1 ng/mL of the HCV core antigen (HCVcAg) was successfully achieved without using...
bulky and costly equipment. This on-chip multicolorimetric ELISA biosensor is rapid (<50 min) and portable, and allows rapid quantitative ELISA of infectious diseases at the point of care. This integrated multicolorimetric biosensor could be broadly applied to other target analytes with POC prospect, holding great potential for a wide range of applications.

**Experimental**

**Materials and reagents**

Silver nitrate (AgNO₃), ascorbic acid, 3,3′,5,5′-tetramethylbenzidine (TMB), phosphate-buffered saline (PBS), poly(L)lysine, sodium hydroxide (NaOH), dimethyl sulfoxide (DMSO), and human serum were purchased from Sigma-Aldrich (Burlington, MA, USA). Sodium borohydride (NaBH₄), hydrochloric acid (HCl), horseradish peroxidase (HRP), and glutaraldehyde were purchased from VWR International (Aurora, CO, USA). The HCV core antigen (HCVcAg) and HCV monoclonal antibody were purchased from Abcam (Cambridge, MA, USA). Hydrogen tetrachloroaurate (III) hydrate, cetyl trimethyl ammonium bromide (CTAB), and IgG-HRP secondary antibody (rabbit) were purchased from Fisher Scientific International (Waltham, MA, USA). All aqueous solutions were prepared using ultrapure water purified by a Milli-Q system (Millipore, Bedford, MA, USA).

**Synthesis of the AuNRs**

The AuNRs were synthesized following the previously published seed-mediated method with a slight modification [42, 43]. Briefly, AuNRs were produced with the use of 25 mL of HAuCl₄ (50 mM) mixed in 4.7 mL of CTAB (0.1 M) in a 15-mL centrifuge tube. After the addition of AgNO₃ (10 mM) 100 mL, 30 mL of the Au seed solution was added into the AuNRs growth solution in the centrifuge tube, and they were placed in a water bath at 30 °C for a minimum of 2 h before use.

**Chip design and fabrication**

The three-layer paper/PMMA hybrid chip shown in Fig. 2b was prepared following our previously published study [13]. Briefly, the top two PMMA plates were designed and modulated with Adobe Illustrator CS5 and laser ablated using a laser cutter machine (Epilog Laser, Golden, CO, USA). 6 × 6 microwells (6 mm in diameter) were laser ablated for the multicolorimetric ELISA for the higher throughput analysis. The top two layers were then assembled and sealed with a bottom plate through thermo-adhesion at 130 °C by using a gravity convection oven (VWR International, Aurora, CO, USA). Meanwhile, the chromatography paper was cut into separate paper disks (Φ 6 mm) by a laser cutter and placed into each microwell (3.97 mm in depth), forming a paper/polymer hybrid analytical device, as shown in the cross-section view in Fig. 2a. An assembled hybrid chip filled with oxidized TMB (blue color) is displayed in Fig. 2c.

**Multicolorimetric ELISA procedures**

The TMB-H₂O₂ solution was firstly prepared, consisting of 0.5 mg of TMB, 1 mL of DMSO, 9 mL of PBS (pH 7.4), and 2 mL of hydrogen peroxide (H₂O₂) (30%). The HRP solutions at various concentrations from 0.0125 to 0.25 U/mL were prepared through multiple steps of dilutions with PBS. In each well of the chip, before the ELISA, the TMB-H₂O₂ solution and the HRP solution were mixed with a ratio of 15:1. After 5 min incubation, 0.05 M HCl was added to quench the reaction between TMB and H₂O₂. Ultimately, the AuNRs (2.67 nM) solution was added and became etched, resulting in different color changes from different concentrations of the oxidized TMB²⁺. The multicolorimetric ELISA was firstly tested on a microplate, and the colorimetric
variations were characterized using UV-Vis spectra via a micro-
plate reader (Molecular Devices, Sunnyvale, CA, USA) prior to
on-chip detection. The final multicolorimetric detection results
from on-chip ELISA were captured by Digital Single Lens
Reflex Nikon D3500 (Nikon Corporation, Tokyo, Japan) or a
smartphone camera [44], and analyzed by ImageJ (n = 5). See
Supplementary Information (ESM) for more details in ImageJ
analysis.

Results and discussions
Condition optimization of the TMB-H₂O₂ colorimetric
system
To achieve optimal color changes from this multicolorimetric
system, multiple conditions of the TMB-H₂O₂ were opti-
mized, including TMB reaction time, H₂O₂ concentrations,
and HCl concentrations.

The optimization of the TMB reaction time is a crucial as-
pect of the assay and was processed firstly, because the reaction
time between TMB and HRP can significantly affect color
changes from the TMB-H₂O₂ solution. We first tested the color
changes of the TMB-H₂O₂ system at different reaction times in
the absence of AuNRs. As shown from Fig. 3b, the 5-min
reaction time allowed us to obtain more varieties of colors in-
cluding from yellow, green to blue, while the 20-min reaction
time only resulted in color in blue or colorless. The HRP-
catalyzed TMB-H₂O₂ system is a commonly used ELISA, in
which absorbance at 650 nm is usually used for the target quan-
tification and characterization of the reaction system [5]. Hence,
the UV-Vis absorbance of these samples with different concen-
trations of HRP after the 5-min reaction time was measured
and plotted in Fig. 3a, which also shows the absorbance of the TMB
solution at 650 nm was located in the middle having greater
dynamic change potential among these tested samples.

The concentration of H₂O₂ was optimized as well, because
hydrogen peroxide played the major role of oxidizing TMB in
the presence of HRP. We tested the color changes and UV-Vis
absorbance of the TMB-H₂O₂ system in the presence of vary-
ing concentrations of H₂O₂. Similarly, we can find from Fig.
3d that the 2.22% of H₂O₂ provided us more color variations,
compared with other concentrations of H₂O₂. The absorbance
in 450 nm wavelength that implied the status of the TMB sec-
ondary oxidation (Fig. 3c) increased with the concentration
increase of H₂O₂, and the absorbance from 2.22% H₂O₂ was
located in the middle, allowing a wide range of dynamics
accordingly. Considering the color changes and the spectra,
we chose 2.22% H₂O₂ for the multicolorimetric assay.

Optimization of the concentrations of AuNRs
After the condition optimization of the TMB-H₂O₂ colorimet-
ric reaction, the concentrations of AuNRs, the other major
color influencer in the multicolor TMB/AuNRs system, were
optimized as well. As mentioned earlier and illustrated in Fig.
1, the produced TMB$^{2+}$ will react with AuNRs to produce TMB; meanwhile, AuNRs (reddish/purple) will be oxidized into Au (I) in yellow. Different levels of etching of AuNRs will result in various colors of the solution, i.e., the original concentration of AuNRs will affect the color of the solution collectively. Hence, we tested the color changes of the TMB/H$_2$O$_2$ solution at different concentrations of H$_2$O$_2$ in the presence of different concentrations of HRP. Samples with 0.0625 U/mL HRP from the dashed quadrilateral area in a microplate were scanned by UV-Vis absorbance and are shown in a. c Absorbance spectra and d scanned images of the TMB-H$_2$O$_2$ solution at different concentrations of H$_2$O$_2$ in the presence of different concentrations of HRP. Samples with 0.0625 U/mL HRP from the dashed quadrilateral area were scanned by UV-Vis absorbance and are shown in c.
AuNRs multicolorimetric system with varying concentrations of AuNRs in the presence of different concentrations of the secondary detection antibody conjugated with HRP (i.e., IgG-HRP). It can be seen from Fig. 5a that a light blue color started to be perceived for all three concentrations of AuNRs from the lower concentrations of 0.00098 U/mL IgG-HRP, while higher concentrations of IgG-HRP caused darker blue, green, and light yellow. The clear color in the leftmost two microwells in each row indicates that those two HRP concentrations are not distinguishable by the naked eye (or below the limit of detection) under our TMB/AuNRs colorimetric conditions. Since the low concentration of 0.191 μg/mL AuNRs was capable of indicating similar color changes as other higher concentrations of AuNRs did in our TMB/AuNRs multicolorimetric system, we used 0.191 μg/mL AuNRs for the subsequent experiments. These different color variants in Fig. 5a also demonstrated the feasibility of our TMB/AuNRs multicolorimetric ELISA for semi-quantitative bioanalysis.

To achieve the quantitative analysis of the multicolorimetric ELISA without using specialized equipment, we studied the RGB values of these three different colors, light blue, green, and yellow. As listed in ESM Table S1, red mean values of light blue, dark green, and yellow are 80, 0, and 255, respectively. In contrast, the green values of these three colors are 208, 192, and 224, respectively. Among RGB values, red mean values give the best indicators to distinguish these three colors. Therefore, red mean values were chosen in this work to explore the quantitative multicolorimetric ELISA without using any costly and bulky equipment in the subsequent assays. The red mean values of TMB/AuNRs ELISA solutions in the top row of Fig. 5a were analyzed by using ImageJ and are shown in Fig. 5b. At the AuNRs concentration of 0.191 μg/mL, the red mean values showed a linear relationship along with different concentrations of IgG-HRP (from $4.90 \times 10^{-3}$ to $4.90 \times 10^{-2}$ U/mL), with the $R^2$ values of 0.99 ($P \leq 0.05$), indicating the feasibility of using red mean values for quantitative analysis in this multicolorimetric ELISA. Hence, quantitative multicolorimetric ELISA can be achieved without using sophisticated equipment, a bottleneck problem in current quantitative multicolorimetric assays.

**Multicolorimetric ELISA biosensor on a paper/polymer hybrid device**

After condition optimization of TMB and AuNRs multicolorimetric system, we performed complete multicolorimetric ELISAs of different concentrations of the
target HCV on the paper/polymer hybrid analytical device, following a protocol depicted in Fig. 1. Briefly, the HCV core antigen (0–100 ng/mL in 10 μL PBS pH 7.4 solution) was added to the microwells of the paper polymer hybrid chip. Then, 10 min of incubation was processed for the antigen immobilization on the chromatography paper in the microwells. This 3D porous paper substrate could fasten the antigen immobilization process, decreasing the incubation time from over-night to 10 min, which is a unique benefit of our paper/polymer hybrid microfluidic device [13]. Then, the paper surface was blocked by a blocking buffer (4% BSA w/v in PBS) for 7 min. After washing with PBS (10 mM, pH 7.4), HCV monoclonal antibody and anti-rabbit IgG-HRP (20 μg/mL) were introduced, incubated (10 min), and washed, sequentially. Finally, the TMB-H₂O₂ solution (75 μL) was added and incubated for 5 min, followed with the addition of 0.05 M HCl (2 μL) and 0.191 μg/mL AuNRs (20 μL). After 30 s at room temperature in the paper/polymer hybrid chip, the color variations from different concentrations of the target were captured (Fig. 6a) and their red mean values were analyzed by using ImageJ (Fig. 6b). As shown in Fig. 6a, as the concentrations of the HCV core antigen increased, the color showed vivid color changes from dark green to light yellow, allowing for semi-quantitative HCV detection by the on-chip multicolorimetric ELISA biosensor with the naked eye.

To achieve quantitative multicolorimetric ELISA, as discussed earlier, the captured images were analyzed by ImageJ and their different red mean values were plotted as the calibration curve versus the concentration of HCVcAg. As displayed in Fig. 6b, the red mean values increased with the increase of the HCVcAg concentrations. A linear relationship was obtained between the red mean values and the HCVcAg concentration in the range from 10.0 to 100.0 ng/mL, with the \( R^2 \)-squared value of 0.974. The limit of detection (LOD) of this on-chip multicolorimetric ELISA was calculated to be as low as 9.1 ng/mL based on 3-fold standard deviations above the blank signal. Although this LOD is slightly higher than that of a conventional ELISA using a microplate reader (LOD, 1.0 ng/mL) [45], this multicolorimetric ELISA not only provides semi-quantitative results in the form of different vivid colors to the naked eye but also allows quantitative disease detection without using bulky and costly instruments. All these features make the on-chip multicolorimetric ELISA biosensor particularly suitable for POC detection and other bioanalyses in resource-limited settings such as rural areas and developing nations.

To validate our method, three different concentrations (10, 50, and 100 ng/mL) of HCVcAg spiked in whole human serum were tested using our on-chip multicolorimetric ELISA biosensor and the results are listed in Table 1. The human serum samples without spiked HCVcAg spiked were not detectable by our method. Other spiked samples at concentrations of 10, 50, and 100 ng/mL of HCVcAg showed recovery percentages of 104.53%, 101.55%, and 98.02%, respectively. These results are within the acceptable range for the validation of analytical methods, which not only has validated the accuracy of our method but also indicates the robustness of our method in testing complex human samples.

## Conclusions

A TMB/AuNRs-based multicolorimetric ELISA biosensor on a paper/polymer hybrid microfluidic device has been developed for rapid semi-quantitative and quantitative POC...
detection of HCV in low-resource settings, without using any bulky and costly instruments. The vivid multiple color variants resulted from the oxidation of TMB and the etching of AuNRs enabled visual semi-quantitative detection by the naked eye, while analyzed RGB values were successfully applied for quantitative detection of disease biomarkers, without the aid of specialized analytical detectors. Additionally, the ingenious introduction of paper in this hybrid multicolorimetric ELISA device greatly shortened the assay time from hours to about 50 min. After condition optimization, the LOD of 9.1 ng/μL of HCVAg, an HCV biomarker, was achieved using this multicolorimetric ELISA platform.

This multicolorimetric ELISA biosensor on a paper/polymer hybrid analytical device is portable and low cost, and allows fast semi-quantitative and quantitative analysis without using costly specialized equipment. All these significant features make it highly desirable for POC detection and other bioanalyses, particularly for rural areas and developing nations. Furthermore, this multicolorimetric ELISA platform has great potential for POC detection of a variety of other infectious diseases from hepatitis B and tuberculosis (TB) to recent COVID-19 [13, 46, 47].

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Declarations

Competing interests The authors declare no competing interests.

References

1. Jefferies M, Rauff B, Rashid H, Lam T, Rafaq S. Update on global epidemiology of viral hepatitis and preventive strategies. World journal of clinical cases. 2018;6(13):589.
2. Prasad KS, Abgalyon Y, Li C, Xu F, Li X. A new method to amplify colorimetric signals of paper-based nanobiosensors for simple and sensitive pancreatic cancer biomarker detection. Analyst. 2020;145(15):5113–7.
3. Dou M, Sanchez J, Tavakoli H, Gonzalez JE, Sun J, Dian Bard J, et al. A low-cost microfluidic platform for rapid and instrument-free detection of whooping cough. Anal Chim Acta. 2019;1065:71–8.
4. Sanjay ST, Li M, Zhou W, Li X, Li X. A reusable PMMA/paper hybrid plug-and-play microfluidic device for an ultrasensitive immunoassay with a wide dynamic range. Microsystems & Nanoengineering. 2020;6(1):1–11.
5. Fu G, Sanjay ST, Zhou W, Brekken RA, Kirken RA, Li X. Exploration of nanoparticle-mediated photothermal effect of TMB-H2O2 colorimetric system and its application in a visual quantitative photothermal immunoassay. Anal Chem. 2018;90(9):5930–7.
6. Fu G, Sanjay ST, Li X. Cost-effective and sensitive colorimetric immunosensing using an iron oxide-to-Prussian blue nanoparticle conversion strategy. Analyst. 2016;141(12):3883–9.
7. Albertoni G, Girao M, Schor N. Mini review: current molecular methods for the detection and quantification of hepatitis B virus, hepatitis C virus, and human immunodeficiency virus type 1. Int J Infect Dis. 2014;25:145–9.
8. Fu G, Sanjay ST, Dou M, Li X. Nanoparticle-mediated photothermal effect enables a new method for quantitative biochemical analysis using a thermometer. Nanoscale. 2016;8(10):5422–7.
9. Fu G, Li X, Wang W, Hou R. Multiplexed tri-mode visual outputs of immunoassay signals on a clip-magazine-assembled photothermal biosensing disk. Biosens Bioelectron. 2020;170:112646.
10. Ma L, Tang B, Yang W, Liu Y, Zhao Y, Li M. Integration of a biochip technique with technetium-99m labeling provides zptomolar sensitivity in liver cancer biomarker detection. Anal Methods. 2015;7(4):1622–6.
11. Tang B, Wang J, Hutchison JA, Ma L, Zhang N, Guo H, et al. Ultrasensitive, multiplex Raman frequency shift immunoassay of liver cancer biomarkers in physiological media. ACS Nano. 2016;10(1):871–9.
12. Kai J, Puntambekar A, Santiago N, Lee SH, Sehy DW, Moore V, et al. A novel microfluidic microplate as the next generation assay platform for enzyme linked immunoassays (ELISA). Lab Chip. 2012;12(21):4257–62.
13. Sanjay ST, Dou M, Sun J, Li X. A paper/polymer hybrid microfluidic microplate for rapid quantitative detection of multiple disease biomarkers. Sci Rep. 2016;6(1):30474.
14. Sanjay ST, Li M, Zhou W, Li X, Li X. A reusable PMMA/paper hybrid plug-and-play microfluidic device for an ultrasensitive immunosensor with a wide dynamic range. Microsystems & Nanoengineering. 2020;6(1):28.
15. Patel J, Sharma P. Design of a novel rapid immunoassay for simultaneous detection of hepatitis C virus core antigen and antibodies. Arch Virol. 2020;165(3):627–41.
16. Hassain TM, Abdelrahman EM, Abdellahmeed S, Abdelrazik M, Fouad YM. Detection of hepatitis C virus core antigen as an alternative method for diagnosis of hepatitis C virus infection in blood donors negative for hepatitis C virus antibody. European Journal of Gastroenterology & Hepatology. 9000;Publish Ahead of Print.
17. Kim DD, Hutton DW, Raouf AA, Salama M, Hablas A, Seifeldin IA, et al. Cost-effectiveness model for hepatitis C screening and treatment: implications for Egypt and other countries with high prevalence. Glob Public Health. 2015;10(3):296–317.
18. Datta S, Goel N, Wastal C. Utility of routine real time quantitative PCR monitoring of HCV infection in haemodialysis patients. Indian J Med Microbiol. 2015;33(Suppl):106–11.
19. Mohamad A, Teo H, Kesaberry NA, Ahmed MU. Recent developments in colorimetric immunoassays using nanomaterials and plasmonic nanoparticles. Crit Rev Biotechnol. 2019;39(1):50–66.
20. Zhang Q, Yu Y, Yun X, Luo B, Jiang H, Chen C, et al. Multicolor colorimetric sensor for detection of omeprazole based on the inhibition of the enzyme-induced metallization of gold nanorods. ACS Applied Nano Materials. 2020;3(6):5212–9.
21. Lin Y, Zhao M, Guo Y, Ma X, Luo F, Guo L, et al. Multicolor colorimetric biosensor for the detection of glucose based on the etching of gold nanorods. Sci Rep. 2016;6(1):37879.

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22. Liu Y, Wang J, Zhao C, Guo X, Song X, Zhao W, et al. A multicolorimetric assay for rapid detection of Listeria monocytogenes based on the etching of gold nanorods. Anal Chim Acta. 2019;1048:154–60.

23. Si Z, Li Y, Han S, Liu Y, Hu P, Lu S, et al. Gold nanorods–based multicolor immunosensor for visual detection of enterovirus 71 infection. Microchim Acta. 2020;187(10):1–8.

24. Li Y, Ma X, Xu Z, Liu M, Lin Z, Qiu B, et al. Multicolor ELISA based on alkaline phosphatase-triggered growth of Au nanorods. Analyst. 2016;141(10):2970–6.

25. Sanjay ST, Fu G, Dou M, Xu F, Liu R, Qi H, et al. Biomarker detection for disease diagnosis using cost-effective microfluidic platforms. Analyst. 2015;140(21):7062–81.

26. Tavakoli H, Zhou W, Ma L, Perez S, Ibarra A, Xu F, et al. Recent advances in microfluidic platforms for single-cell analysis in cancer biology, diagnosis and therapy. TrAC Trends Anal Chem. 2019;117:13–26.

27. Tavakoli H, Zhou W, Dou M, Tavakoli H, Ma L, Xu F, et al. Recent advances of controlled drug delivery using microfluidic platforms. Advanced Drug Delivery Reviews. 2018;128:3–28 (*Five-Year impact factor 17.3).

28. Lv M, Zhou W, Tavakoli H, Bautista C, Xia J, Wang Z, et al. Aptamer-functionalized metal-organic frameworks (MOFs) for biosensing. Biosens Bioelectron. 2021;176:112947.

29. Li XJ, Zhou Y. Microfluidic devices for biomedical applications: Woodhead Publishing; 2013.

30. Li XJ, Valadez AV, Zuo P, Nie Z. Microfluidic 3D cell culture: potential application for tissue-based bioassays. Bioanalysis. 2012;4(12):1509–25.

31. Zhang J, Wei X, Zeng R, Xu F, Li X. Stem cell culture and differentiation in microfluidic devices toward organ-on-a-chip. Future Science OA. 2017;3(2):FSO187.

32. Dou M, Macias N, Shen F, Dien Bard J, Dominguez DC, Li X. Rapid and accurate diagnosis of the respiratory disease pertussis on a point-of-care biochip. EClinicalMedicine. 2019;8:72–7.

33. Wei X, Zhou W, Sanjay ST, Zhang J, Jin Q, Xu F, et al. Multiplexed instrument-free bar-chart SpinChip integrated with nanoparticle-mediated magnetic aptasensors for visual quantitative detection of multiple pathogens. Anal Chem. 2018;90(16):9888–96.

34. Prasad KS, Cao X, Gao N, Jin Q, Sanjay ST, Henao-Pabon G, et al. A low-cost nanomaterial-based electrochemical immunosensor on paper for high-sensitivity early detection of pancreatic cancer. Sensors and Actuators B: Chemical. 2020;305:127516.

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