Encapsulating chromogenic reaction substrates with porous hydrogel scaffolds onto arrayed capillary tubes toward a visual and high-throughput colorimetric strategy for rapid occult blood tests

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A porous hydrogel scaffold was fabricated for the first time to encapsulate chromogenic reaction substrates onto arrayed capillary tubes, resulting in a visual and high-throughput colorimetric method for rapid occult blood tests (OBTs) based on the hemoglobin (Hgb)-catalyzed chromogenic reactions. Gelatin (Gel), a biodegradable and biocompatible polymer, was introduced to couple with p-hydroxyphenyl-propionic acid (HPA) yielding the Gel–HPA hydrogel scaffold. Chromogenic reaction substrates of 3,3,5,5-tetramethylbenzidine and H2O2 were then encapsulated into the Gel–HPA matrix and further attached onto the amine-derivatized capillary tubes by forming porous chromogenic composites through the HPA-mediated bridging of Gel by the oxidation of H2O2. The developed Hgb catalysis-based OBT platform can facilitate the detection of Hgb with the level down to 0.125 μg mL−1 in human excreta (i.e., saliva, urine, and feces) through capillarity-enabled automatic sampling. This simple, sensitive, selective, and high-throughput colorimetric method may be promising for the bedside OBT for point-of-care monitoring and rapid diagnostics of clinical bleeding diseases.

1. Introduction

Occult blood (OB) is the potential bleeding in human excreta like saliva, urine, and feces, in which red blood cells generally get damaged and cannot be seen by naked eye or under the microscope.1 OB can be associated with many diseases like inflammation, physiological stones, and tumors. For example, the OB in feces may be observed in colorectal cancer, a most common malignancy worldwide.2 Hemoglobin (Hgb) from OB shows the average amounts of 5.0 μg mL−1 and 75.7 μg mL−1 for healthy people and patients, respectively.3 Therefore, the development of an accurate, high-throughput, and bedside applicable analysis method for OB tests (OBTs) is very important for rapid diagnosis of clinical bleeding diseases.4

In recent years, many OBT analyses and screening methods have been developed mainly including the radio-analytical, microscopical, fluorimetric,5 immunochemical, and chemical analysis methods.6,7 The radioactivity-based determination for OBTs is highly specific and quantitative, but too complicated for the routine usage.7 The physical OBT methods, such as the microscopic examination of red blood cell count,8 were rapid and practical but remain academic curiosities. The immunochemical test methods9,10 are highly selective and reproducible; however, they can involve time-consuming processes and specialization-entailed operation. In recent years, increasing attention has alternatively been drawn to the sensitive and selective chemical OBT methods by the use of the hematin portion of the Hgb molecules released from the OB. Hgb with pseudo-peroxidase activity can catalyze the chromogenic reaction of 3,3,5,5-tetramethylbenzidine (TMB) and H2O2 to develop a colorimetric analysis system.11 Also, it can be modified to fabricate enzyme mimics for improved catalysis applications.12 Nevertheless, the current OBT based on Hgb catalysis may still encounter some disadvantages such as being time-consuming, cost-ineffectiveness, and complex instrumental operation, which are unsuitable for on-site applications for the bedside monitoring and rapid diagnosis of clinical bleeding diseases.13 Moreover, porous hydrogels have been widely synthesized but mostly applied in the tissue engineering field. In particular, the hydrogels with high oxygen permeability and a high water-content matrix were employed as biomimetic substrates for cell growth.14,15 In addition, to date, many rapid and point-of-care analysis methods have been developed for blood glucose assay,16 anemia testing,17 and drug monitoring.18 To the best of our knowledge, however, the analysis strategy using porous hydrogels has hardly been reported for the OBT in the clinical laboratory.
In the present work, a capillary array-based colorimetric analysis method has been initially proposed for the visual and high-throughput OBT based on the Hgb-catalyzed chromogenic reactions. A hydrogel scaffold was fabricated by covalently coupling gelatin (Gel) with p-hydroxyphenyl-propanoic acid (HPA), and further employed to in-site encapsulate chromogenic reaction substrates of TMB and H$_2$O$_2$ onto the amine-derivatized capillary tubes by the porous Gel–HPA–TMB composites formed. A colorimetric OBT platform with capillary arrays was thereby constructed for the visual and high-throughput detection of Hgb targets. Here, the capillary tubes could offer a space-confined micro-environment for the Hgb-catalytic chromogenic reactions to realize rapid and sensitive analysis. In particular, the capillarity-enabled automatic sampling could also be expected. Moreover, the hydrogel composites with a porous structure could allow for the TMB–H$_2$O$_2$ substrates to be largely and firmly anchored onto the capillary tubes. Also, the introduction of specific Hgb-catalyzed chromogenic reactions would help to achieve the highly selective OBT analysis. The practical application feasibility of the developed capillary array-based analysis method was demonstrated for rapid and high-throughput OBT evaluations by probing the Hgb levels in human excreta (i.e., saliva, urine, and feces).

2. Experimental section

2.1 Materials and instruments

Hemoglobin (Hgb), gelatin (Gel), p-hydroxyphenyl-propanoic acid (HPA), 3,3,5,5-tetramethylbenzidine (TMB), hydrogen peroxide, N-hydroxysulfosuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), glucose (Glu), urea, uric acid (UA), vitamin C (Vc), tyrosine (Tyr), histidine (His), serine (Ser), and lysine (Lyr), 2-mercaptoethanol, (3-aminopropyl)triethoxysilane (APTES), and 2-(N-morpholino)ethanesulfonic acid (MES) were purchased from Sigma-Aldrich (Beijing, China). Medical capillary tubes (inner diameter: 0.9–1.1 mm, length: 100 mm) were obtained from Huaxi Medical University. The ultra-filtration membrane (MWCO 8–10 KD, W. 77 mm) and phosphate buffered saline (PBS) tablets were purchased from Sangon Biotech. The specimens of saliva, urine, and feces were kindly provided by the local hospital, and informed consent was obtained from the providers whose saliva, urine, and feces were taken. All colorimetric experiments for the analysis of Hgb spiked separately in these human specimens were conducted in accordance with the hospital’s guidelines and approved by the university’s ethics committee. All chemicals used were of analytical grade. The glass containers were cleaned using aqua regia and ultrapure water before usage. Deionized water (>18 MΩ) was obtained from an Ultra-pure water system (Pall, USA).

A UV-3600 spectrophotometer (Shimadzu, Japan) with the home-made holder for capillary tubes and an Infinite M 200 PRO (TECAN, Switzerland) were applied separately for the colorimetric measurements. Characterization of the as-prepared composites was performed by using scanning electron microscopy (SEM, Hitachi E-1010, Japan).

2.2 Fabrication of the colorimetric analysis platform

The colorimetric analysis platform was fabricated using the arrayed capillary tubes that were covalently coated or encapsulated with the H$_2$O$_2$-loading Gel–HPA–TMB composites, as schematically illustrated in Scheme 1. First, a 20.0 mL mixture containing HPA (100.00 mg), EDC (73.50 mg), and NHS (22.10 mg) in MES (50.0 mM) was prepared under stirring for 30 min, followed by the addition of 2-mercaptoethanol (20 mM) for quenching the excessive EDC. Furthermore, Gel powder was suspended at 4.0% (w/v) in the activated HPA and heated up to 50 °C under stirring until the dissolution of the Gel. Then, the mixture was cooled to room temperature and stirred overnight. The resultant Gel–HPA hydrogel was further dialyzed against de-ionized water for 6 h by using an ultrafiltration membrane, producing the Gel–HPA hydrogel. Second, capillary tubes were cleaned with the piranha solution consisting of H$_2$SO$_4$ and H$_2$O$_2$ overnight. After being rinsed separately with water and alcohol three times each, the capillary tubes were dried and then immersed into the APTES (6.0 wt%) solution for 6 h, followed by rinsing with alcohol, yielding the APTES-derivatized capillary tubes. Third, the above Gel–HPA hydrogel was activated again by the EDC–NHS chemistry and then mixed with an aliquot of TMB and H$_2$O$_2$ at the optimized
concentrations. Following that, the APTES-derivatized capillary tubes were immersed into the mixture of H$_2$O$_2$-loaded Gel–HPA–TMB composites to be coated for 24 h. The resulting capillary tubes were rinsed twice with water, and subsequently stored at 4.0 °C for future usage.

2.3 Colorimetric measurements using the developed colorimetric platform

The optimization of synthesis conditions of the capillary array-based colorimetric platform was first conducted separately using different concentrations of Gel (0.50%, 1.0%, 2.0%, 4.0%, 6.0% (w/v)), HPA (0.50, 2.5, 5.0, 10, 20 mg mL$^{-1}$), TMB (1.0, 5.0, 10, 20, 40 mg mL$^{-1}$), and H$_2$O$_2$ (1.0, 2.0, 4.0, 6.0, 8.0, 10, 12, 14, 16 mM). Colorimetric measurements of peroxidase-like catalysis activities of Hgb were conducted for the cross-linked H$_2$O$_2$-loaded Gel–HPA–TMB composites on capillary tubes. Aliquots of Hgb samples were introduced into the capillary tubes coated with H$_2$O$_2$-loaded Gel–HPA–TMB composites. After the reactions proceeded for 15 min, colorimetric measurements were performed for the reaction products of the composites on capillary tubes with the UV-vis spectra or absorbance values being recorded.

By following the same procedure above, the optimization of the detection conditions of the developed capillary array-based colorimetric platform with the H$_2$O$_2$-loading Gel–HPA–TMB composites was performed by using reaction mixtures consisting of different ionic strengths (0.0, 2.5, 5.0, 10, 15, 20 mM NaCl), temperatures (4.0, 10, 15, 20, 25, 37 °C, 45 °C, 60 °C), pH values (2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10, 11, 12), and reaction times (0.0, 2.0, 4.0, 6.0, 8.0, 10, 12, 14, 16, 18, 20, 22, and 24 min). The comparable tests were also carried out for the commercially-available TMB–H$_2$O$_2$ substrates.

Moreover, the selective colorimetric responses of the developed capillary array-based colorimetric platform to Hgb (120 µg mL$^{-1}$) were explored by comparing it with other ions (100 mM) or substances (240 µg mL$^{-1}$) such as Fe$^{2+}$, Fe$^{3+}$, Cl$^-$, Na$^+$, K$^+$, Ca$^{2+}$ ions, ammonia (Am), Glu, protein (Pr), urea, UA, Vc, Tyr, His, Ser, and Lyr, including their mixtures separately co-existing in Hgb samples. Also, the storage stability of the H$_2$O$_2$-loading Gel–HPA–TMB composites and the detection reproducibility of the developed capillary array-based colorimetric platform were examined by storage at 4 °C over different time periods (2.0, 4.0, 6.0, 8.0, 10, and 12 months) for the repeated colorimetric detection of Hgb (120 µg mL$^{-1}$).

2.4 Colorimetric analysis for practical samples

The colorimetric analysis for Hgb levels separately spiked in the practical samples of saliva, urine, and feces was carried out using the capillary array-based colorimetric platform, with the Hgb concentrations ranging from 0.50 to 120 µg mL$^{-1}$ in saliva, 0.25 to 110 µg mL$^{-1}$ in urine, and 1.00 to 110 µg mL$^{-1}$ in feces. Herein, the samples of saliva and urine were used directly for Hgb spiking. As for the faecal samples, an aliquot of 1.0 g of feces was added to the centrifugal tube, followed by the addition of 10 mL of PBS. Hgb was then spiked separately with the resulting mixtures to yield different Hgb concentrations which are stored at 4.0 °C for future usage.

3. Results and discussion

3.1 The fabrication and analysis protocol of the capillary array-based colorimetric platform

Gelatin (Gel) as a biodegradable and biocompatible polymer was commonly applied for the fabrication of injectable 3D hydrogels for studying the cell functional responses.14,19 On the other hand, the capillary tubes, which are routinely used in the clinical laboratories for the capillarity-aided instantaneous suction of blood,20 were used for the fluorimetric analysis of pyruvic acid but entailing an external power to suck the sample solutions.21 In the present work, Gel was alternatively coupled with HPA to yield a hydrogel scaffold for covalently encapsulating the chromogenic reaction substrates onto the arrayed capillary tubes. A visual and high-throughput colorimetric platform was thereby fabricated with capillary arrays for the OBT based on specific Hgb-catalyzed chromogenic reactions, with the main reaction mechanism and analysis procedure being schematically illustrated in Scheme 1. As can be seen from Scheme 1A, HPA with carboxyl groups was first activated by an EDC/NHS agent and then bound to Gel with amine groups. The obtained Gel–HPA was further activated to couple with TMB to be attached onto the APTES-derivatized capillary tubes, where H$_2$O$_2$ was simultaneously encapsulated into the Gel–HPA matrix. Herein, H$_2$O$_2$, on the one hand, could conduct the oxidation of HPA toward Gel bridging so as to form porous Gel–HPA–TMB composites.22 On the other hand, it would work with TMB to form chromogenic substrates for Hgb-catalytic reactions. As described in Scheme 1B, the so prepared capillary tubes were arrayed and employed to probe the Hgb levels in different human excreta of saliva, urine, and feces, of which the sample solutions were automatically fetched by the capillary. A capillary array-based colorimetric OBT analysis was thus performed by probing the Hgb levels based on the Hgb-catalyzed chromogenic reactions on the capillary tubes, showing a change in the visible blue gelled products.

3.2 Studies on the Hgb-catalyzed chromogenic reactions in Gel–HPA–TMB composites

The formation of H$_2$O$_2$-loaded Gel–HPA–TMB composites was investigated using a UV-vis spectrophotometer by comparing them with TMB, Gel, and Gel–HPA (Fig. 1). As shown in Fig. 1A, the UV-vis spectra of the prepared chromogenic composites could include the characteristic absorbance peaks of TMB, Gel, and HPA. An obvious shift, however, was comparably observed. Also, a color change was witnessed for the component materials after being covalently bound to form the composite, i.e., the yellowish Gel was changed to a more intense color, as evidenced from their photographs (Fig. 1B). These results indicate that the Gel–HPA–TMB composites should be yielded by way of H$_2$O$_2$-oxidized covalent attachment. Furthermore, scanning electron microscopy (SEM) was applied for characterizing the H$_2$O$_2$-loaded Gel–HPA–TMB composites, revealing the porous structure of the hydrogel-based composites decorated with numerous conglomerations (Fig. 1C). Notably, the morphology of Gel–HPA–TMB composites is different from that of the Gel–HPA...
hydrogel reported elsewhere\(^1\) presumably due to the additional presence of amine-derivatized TMB. Importantly, the developed hydrogel matrix of the porous chromogenic composites would confine the encapsulated TMB–H\(_2\)O\(_2\) substrate within a nano-scaled space\(^2\) so as to minimize the diffusion barrier and especially reducing the decomposition of active intermediates. More rapid and effective chromogenic reactions catalyzed by Hgb could thus be expected towards Hgb detection with high sensitivity. Additionally, the storage stability of the TMB–H\(_2\)O\(_2\) substrate would be significantly improved as demonstrated afterwards.

To investigate the sensing performances of the H\(_2\)O\(_2\)-loaded Gel–HPA–TMB composites on capillary tubes in the Hgb-catalyzed chromogenic reactions, a comparison of reaction results was carried out among the reaction substrates of different compositions (Fig. 2). One can note that Hgb could catalyze the chromogenic reactions of a common TMB–H\(_2\)O\(_2\) substrate only in citrate buffer solution (Fig. 2b), where no reaction was surprisingly observed for the one in water (Fig. 2a). However, the H\(_2\)O\(_2\)-loaded Gel–HPA–TMB composites could be catalyzed by Hgb to yield the blue product (Fig. 2c), the UV absorbance of which was consistent with that of H\(_2\)O\(_2\)-loaded Gel–HPA–TMB composites alternatively in citrate buffer solution (Fig. 2d). The data imply that the Hgb-catalyzed chromogenic reactions could proceed well in the H\(_2\)O\(_2\)-loaded Gel–HPA–TMB composites anchored on capillary tubes, the practical potential or adaptability of which is comparable to the common reaction substrate in buffer solution. Remarkably, the so prepared porous chromogenic composites may provide an ideal reaction microenvironment for the Hgb-catalyzed chromogenic reactions in producing the visual products toward the sensitive OBT.

### 3.3 Optimization of main fabrication and OBT conditions for the capillary array-based colorimetric platform

The synthetic components of H\(_2\)O\(_2\)-loaded Gel–HPA–TMB composites on the capillary tubes were optimized for the Hgb-catalyzed chromogenic reactions with the colorimetric variations for OBTs (Fig. 3). It was observed that the dynamic Hgb responses of the chromogenic composites could separately depend on the amounts of Gel (A), HPA (B), and TMB (C), showing the optimal concentrations at 4.0% (w/v), 5.0 mg mL\(^{-1}\), and 20 mg mL\(^{-1}\), respectively. Notably, too high concentrations of Gel and HPA might lead to gradually decreased Hgb responses, presumably due to the large cross-linking degree of the hydrogel, and that the resulting porosity might be too dense to be accessed by Hgb for catalyzing the chromogenic reactions. Meanwhile, too high TMB dosages would result in their worse dispersion in the hydrogel matrix showing decreased Hgb responses. Furthermore, H\(_2\)O\(_2\) could play a dual role in HPA oxidization for the formation of porous hydrogel composites and in TMB oxidization for the chromogenic reactions. Accordingly, 10 mM H\(_2\)O\(_2\) was selected suitably for modifying the capillary tubes with the H\(_2\)O\(_2\)-loaded Gel–HPA–TMB composites (Fig. 3).

The main Hgb-sensing conditions, including ionic strengths (NaCl), temperature, pH values, and reaction time, of the H\(_2\)O\(_2\)-loaded Gel–HPA–TMB composites on capillary tubes were investigated in comparison to the commercially available TMB–H\(_2\)O\(_2\) buffer solution. The data imply that the Hgb-catalyzed chromogenic reactions could proceed well in the H\(_2\)O\(_2\)-loaded Gel–HPA–TMB composites anchored on capillary tubes, the practical potential or adaptability of which is comparable to the common reaction substrate in buffer solution. Remarkably, the so prepared porous chromogenic composites may provide an ideal reaction microenvironment for the Hgb-catalyzed chromogenic reactions in producing the visual products toward the sensitive OBT.
photograph (inset). It is worth noting that Fe$^{2+}$ and Fe$^{3+}$ ions might have a highly selective response to Hgb, as also visually observed in the gel–HPA–TMB composite solution. Gel–HPA–TMB composites on capillary tubes could allow for the Hgb-catalyzed chromogenic reactions under mild conditions. That is, the H$_2$O$_2$-loaded Gel–HPA–TMB composites could serve as a robust chromogenic reaction substrate to be practically applied to probe Hgb levels for the OBT. Yet, they could exhibit additional advantages such as the capillarity-aided automatic sampling and the porous composite-enabled micro-environment for more effective chromogenic reactions, and especially the higher storage stability as demonstrated afterwards.

### 3.4 Investigation on the OBT performances of the capillary array-based colorimetric platform

It is well established that peroxidase can present high specificity in catalyzing the TMB–H$_2$O$_2$ chromogenic reactions. Herein, the detection selectivity of the OBT with peroxidase-like Hgb was explored by using 17 kinds of interfering substances possibly co-existing in the Hgb samples (Fig. 5).

The colorimetric measurements manifest that the H$_2$O$_2$-loaded gel–HPA–TMB composites on capillary tubes could present a highly selective response to Hgb, as also visually observed in the photograph (inset). It is worth noting that Fe$^{2+}$ and Fe$^{3+}$ ions might in a way display a little catalysis for the chromogenic reactions, the colorimetric responses of both of which were, however, significantly low as compared to that of Hgb. The above results of chromogenic reactions indicate that the H$_2$O$_2$-loaded Gel–HPA–TMB composites on capillary tubes could achieve the high selectivity of OBTS.

It is well recognized that common TMB–H$_2$O$_2$ substrates may suffer from the major shortcoming of low storage stability over time. Here, the H$_2$O$_2$-loaded Gel–HPA–TMB composites on capillary tubes were stored at 4 °C over different time intervals for colorimetric Hgb detection, with the results being shown in Fig. 6. It is riveting to witness that the capillary tube-carried composites could be stored up to one year without any significant change in the Hgb-sensing performance promising high detection reproducibility. Also, it could be stabilized for about three months if stored at room temperature (data not shown). Again, such a desirable high storage stability of the composites are thought to benefit from the porous Gel–HPA hydrogel on capillary tubes that could provide the porous scaffolds for the covalent loading of TMB and the in-site encapsulation of dispersed H$_2$O$_2$ to perform the aforementioned Hgb-catalyzed chromogenic reactions.

### 3.5 Practical sample analysis with the capillary array-based colorimetric platform

Under the optimized conditions, the developed colorimetric platform with a capillary array was applied for the high-throughput OBT by probing Hgb of different levels spiked separately in human excreta samples of saliva, urine, and feces (Fig. 7). Fig. 7A shows the UV-vis spectra for the colorimetric responses to Hgb at different concentrations in saliva samples. One can note that the absorbance values could typically increase with Hgb concentrations, as also illustrated in the corresponding photographic results (inset).
detection range of Hgb concentrations was obtained from 0.50 to 120 μg mL⁻¹, with the limit of detection (LOD) of 0.125 μg mL⁻¹, estimated using the 3σ rule (Fig. 7B). Moreover, the colorimetric detection of Hgb spiked in urine samples was conducted, with the Hgb concentrations linearly ranging from 0.25 to 110 μg mL⁻¹, with the LOD of 0.050 μg mL⁻¹ (Fig. 7C). Also, the capillary array-based analysis was conducted for different levels of Hgb spiked in feces samples, showing the linear detection range of 1.00 to 110 μg mL⁻¹, with the LOD of 0.270 μg mL⁻¹ (Fig. 7D). A comparison of LODs was conducted among the developed OBTs and the documented ones (water) 5 and colorimetric assay. Therefore, the developed capillary array-based colorimetric strategy could be practically applied for the OBT with the advantage of rapid and sensitive analysis, which is thought to benefit from the porous hydrogel composites with a porous structure could allow for the high immobilization stability, and especially the space-confined micro-environment for the Hgb-catalytic chromogenic reactions toward rapid and highly sensitive detection. Third, highly selective OBT analysis can be achieved by the evaluation of Hgb levels based on the highly specific Hgb-catalyzed reactions. Finally, the developed capillary array-based colorimetric method with the capillarity-aided automatic sampling can feature the simple, substantially cost-effective, and portable device for the on-site analysis of the analyzers using the micro-dosage human excreta samples such as saliva, urine, and feces, thus promising the extensive applications of bedside OBTs for the point-of-care monitoring and rapid diagnosis of clinical bleeding diseases.

4. Conclusions

To summarize, a porous hydrogel composite was fabricated successfully for the first time to covalently bind amine-derivatized TMB and in-site encapsulate H₂O₂ onto the arrayed capillary tubes toward a visual and high-throughput colorimetric platform for OBTs by probing the Hgb levels in human excreta. The developed OBT strategy with the Hgb-catalyzed chromogenic reactions can possess some advantages over the current OBT ones (i.e., the microscopical analysis). First, the use of the disposable and transparent capillary tubes arrayed as the sensing platforms could facilitate the visual and high-throughput detection of targets. In particular, the power-free automatic sampling enabled by the capillarity could also be expected. Second, the application of hydrogel composites with a porous structure could allow for the minimized diffusion barrier of reactant substrates to be firmly anchored on capillary tubes with high immobilization stability, and especially the space-confined micro-environment for the Hgb-catalytic chromogenic reactions toward rapid and highly sensitive detection. Third, highly selective OBT analysis can be achieved by the evaluation of Hgb levels based on the highly specific Hgb-catalytic reactions. Finally, the developed capillary array-based colorimetric method with the capillarity-aided automatic sampling can feature the simple, substantially cost-effective, and portable device for the on-site analysis of the analyzers using the micro-dosage human excreta samples such as saliva, urine, and feces, thus promising the extensive applications of bedside OBTs for the point-of-care monitoring and rapid diagnosis of clinical bleeding diseases.

Table 1 Comparison of the LODs among different methods for probing Hgb separately in water and urine

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References