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Convenient Colorimetric Detection of Cholesterol Using Multi-Enzyme Co-Incorporated Organic–Inorganic Hybrid Nanoflowers

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We have developed a microscale well-plate colorimetric assay for the multiplexed detection of cholesterol in clinical human blood samples. This system utilizes a novel multi-enzyme incorporated organic–inorganic hybrid nanoflower which entrap both cholesterol oxidase (ChOx) and horseradish peroxidase (HRP) as the organic components with copper phosphate as the inorganic component to detect cholesterol levels in blood samples. The hybrid nanoflowers, synthesized via an extremely simple but rapid sonication-mediated method within 5 min at room temperature, enable an efficient one-pot two-enzyme cascade reaction. The ChOx in the nanoflowers catalyze the generation of H_2O_2 only in the presence of cholesterol in the sample. This subsequently activates the HRP co-entrapped in the nanoflowers, thereby leading to the conversion of the employed chromogenic substrate, 3,3′,5,5′-tetramethylbenzidine (TMB), into a blue-colored product. This strategy can be used to detect target cholesterol concentrations as low as 8 μM, with a linear range from 10 to 70 μM, which is suitable to diagnose high levels of cholesterol (hypercholesterolemia) with excellent stability over three weeks at room temperature. The biosensor also exhibited an excellent selectivity to detect target cholesterol even in the presence of common interfering biomolecules in human blood and showed a high degree of precision when employing human blood serum samples. Therefore, this hybrid nanoflower-based assay can be used in clinical practice for the multiplexed and reliable quantification of cholesterol, and readily extended to other enzymes to prepare multi-step cascade enzymatic reactions for various biotechnological applications.

Keywords: Organic–Inorganic Hybrid Nanoflowers, Cholesterol Oxidase, Horseradish Peroxidase, Stability, Colorimetric Biosensors.

1. INTRODUCTION

Cholesterol, an essential part of the animal cell membrane, is considered a key precursor for the synthesis of several important biomolecules, such as steroid hormones and vitamin D.[1,2] Determination of cholesterol levels is frequently performed during medical checkups, since a high level of serum cholesterol is closely related to serious diseases, including arteriosclerosis.[3] Over the past few decades, diverse analytical methods have been developed for the sensitive detection of cholesterol, such as electrochemical methods,[4,5] fluorescent assay,[6,7] and molecular imprinting technology;[8] however, there is a need to develop a simple, convenient, and cost-effective protocol that is suitable for facility-limited environments.

Colorimetric strategy fulfills most of the aforementioned requirements, as it enables convenient visual detection of target molecules without the need for detection instrumentation.[9,10] To date, colorimetric detection of cholesterol has been performed in two separate steps comprised of ChOx- and HRP-mediated reactions. In the first step, target cholesterol present in a sample is incubated with ChOx, which oxidizes the cholesterol to produce H_2O_2. In the second step, the solution containing H_2O_2 produced from the first reaction is reacted with HRP that catalyzes the oxidation of the employed colorimetric substrate with reduction of H_2O_2 to H_2O. This two-enzyme cascade reaction is highly specific; however, a significant amount of H_2O_2 can be decomposed during the two-step reaction owing to the instability of enzymes, consequently

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resulting in a decrease in accuracy and sensitivity of the assay experiment.

Engineering natural enzymes to enhance the enzyme's performance, including its stability, has been an ongoing major research topic in enzyme-related technology. Recently, a unique strategy to construct micrometer-sized hybrid particles having nanoscale flower-like architectures, so called nanoflowers, using protein and copper phosphate was reported and has garnered significant attention owing to its ability to overcome such limitations of enzymes.[11, 12] The proteins that contain nitrogen atoms in their amide or amine groups of amino acids were suggested to form the complexes with copper ions via coordination interaction, which drives the nucleation of primary nanoparticles and concomitant anisotropic growth to form highly branched flower-like structures.[11] These organic–inorganic hybrid nanoflowers were proven to be highly active, stable, and reusable, probably due to the high surface area and effective confinement of enzyme molecules in the nanoflowers. Several groups have utilized the hybrid nanoflowers for different applications, such as detection of hydrogen peroxide,[13] phenol,[14] and glucose,[15] or protein digestion.[16] We recently reported an innovative strategy to significantly reduce the synthesis time from the conventional 3 days to only 5 min via simple sonication at room temperature, which enables rapid synthesis of the nanoflowers and thus expands their utilization in biotechnology, like our recent application of enzyme nanoflowers in the development of biofuel cells.[17, 18] Although these examples demonstrate the potential of hybrid nanoflowers in diverse enzyme-mediated applications, there have been few studies on the clinical utility of these nanoflowers for the detection of target molecules in human blood.

Thus, in order to accelerate the use of hybrid nanoflowers on the detection of clinically important target molecules, we have developed a microscale well-plate colorimetric assay for the multiplexed detection of cholesterol in clinical human blood serum samples. Our technique is based on the hybrid nanoflowers that entrap ChOx and HRP with copper phosphate (ChOx@HRP hybrid nanoflowers), to detect cholesterol. Various analytical features of the constructed system (e.g., sensitivity, selectivity, precision, and stability) were determined and its clinical utility in detecting diverse levels of cholesterol in human blood samples was also assessed.

2. EXPERIMENTAL DETAILS

2.1. Materials

Cholesterol oxidase from *Streptomyces sp.* (ChOx), copper(II) sulfate pentahydrate, phosphate-buffered saline (PBS), horseradish peroxidase (HRP), 3,3′,5,5′-tetramethylbenzidine (TMB), cholesterol, D-glucose, maltose, sucrose, arabinose, fructose, and lactose were purchased from Sigma-Aldrich (Milwaukee, WI, USA). All solutions were prepared with deionized water from the Milli-Q Purification System (Millipore, USA).

2.2. Sonochemical Synthesis of ChOx, HRP, and ChOx@HRP Hybrid Nanoflowers

For the preparation of sonicated nanoflowers, 20 μL of aqueous $CuSO_4$ solution (120 mM) was added to 3 mL of PBS (pH 7.4) at 25 °C containing either ChOx, HRP, or ChOx with HRP at different concentrations, followed by sonication for 5 min. After this, the samples were washed thrice with PBS using centrifugation at 4,000 rpm for 5 min, and stored at 4 °C in PBS until use. For sonication, we utilized a Branson bath sonicator (length 15.2 cm, breadth 14 cm, height 10.2 cm, operating capacity 1.9 L) at a frequency of 40 kHz and power of 160 W.

2.3. Characterization of Nanoflowers

The resulting suspension of protein-inorganic hybrid nanoflowers was filtered and dried on a silicon wafer, and analyzed by scanning electron microscopy (SEM) images by using a Field Emission Scanning Electron Microscope (Magellan 400). The elemental composition was analyzed by using energy-dispersive spectrometer (EDS) (Bruker, Billerica, MA, USA). Fourier transform infrared (FT-IR) spectra of the nanoflowers were obtained using a FT-IR spectrophotometer (FT/IR-4600, JASCO, Easton, MD). The encapsulation yield of the enzymes in the nanoflowers was calculated by measuring the differences between the initial enzyme amounts and the leached amounts in the supernatant, using a Pierce™ bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific) with BSA as a standard.

2.4. Cholesterol Detection with ChOx@HRP Hybrid Nanoflowers

Cholesterol detection with ChOx@HRP hybrid nanoflowers, which were sonochemically prepared using the PBS solution (3 mL) containing 20 μg mL^{-1} ChOx and 0.2 μg mL^{-1} HRP with $CuSO_4$ solution (20 μL, 120 mM), was performed in a 96-well plate as follows: The ChOx@HRP hybrid nanoflower suspension (40 μL), TMB substrate (20 μL, 5 mM), and sample solutions containing standard or clinical blood samples (40 μL) in PBS (100 μL, pH 7.4) were added into each well and incubated for 10 min. For constructing a cholesterol standard curve, cholesterol (10%, w/v) was first dissolved in Triton X-100, and subsequently diluted with distilled water to the pre-determined concentrations. The reacted solution in a 96-well plate was used to capture color images. Spectrophotometric measurements of the colored reaction products were conducted in scanning mode or at 652 nm using a microplate reader (Synergy H1, BioTek, VT) with a transparent 96-well plate. The cholesterol concentration in our detection system varied from 0.008 mM to 1 mM.

2.5. Comparison of Activity and Stability of ChOx@HRP Hybrid Nanoflowers and Their Corresponding Free Enzymes

Catalytic activities of the cascade reaction of ChOx@HRP hybrid nanoflowers and their corresponding free enzymes, ChOx and HRP, were determined by measuring the absorption intensity of the colorimetric reaction that involved ChOx@HRP nanoflowers or free ChOx and HRP (20 and 0.2 μg mL^{-1}, respectively) with TMB substrate (0.5 mM) and cholesterol (1 mM) in PBS (pH 7.4) for 10 min at room temperature, and other detection procedures were the same as described above. Stability of ChOx@HRP hybrid nanoflowers and their corresponding free enzymes were checked in an aqueous buffer (PBS, pH 7.4) under static conditions at room temperature. First, the initial activities were determined by measuring the absorbance intensity as described above. The relative activity (%) of each sample was then calculated at pre-determined time-points as the ratio of the residual activity to the initial activity.

2.6. Cholesterol Detection in Human Serum

Initial cholesterol concentration of human serum was first determined by cholesterol assay kit (Sigma-Aldrich) and then a predetermined amount of cholesterol was further added into human serum to make spiked samples, which represent normal, boundary, and high level of blood cholesterol. Finally, cholesterol concentration of each spiked sample (40 μL, 10-fold dilution) was determined by the same procedure as described above. The recovery rate [recovery (%) = measured value/actual value × 100] and the coefficient of variation [CV (%) = SD/average × 100] were determined to evaluate the precision and reproducibility of the assay.

3. RESULTS AND DISCUSSION

3.1. Sonochemical Synthesis of ChOx@HRP Hybrid Nanoflowers

The overall scheme for the sonochemical synthesis of multi-enzyme incorporated organic–inorganic hybrid nanoflowers and the detection mechanism for target cholesterol are illustrated in Figure 1. To synthesize the sonicated nanoflowers entrapping both ChOx and HRP with copper phosphate, aqueous PBS solution containing ChOx, HRP, and copper(II) sulfate was sonicated for 5 min, resulting in the formation of flower-like morphology similar to that obtained from the conventional 3 days of incubation.[17] We hypothesized that these rapidly prepared ChOx@HRP hybrid nanoflowers would yield a high catalytic activity and stability for cholesterol detection owing to the effective confinement of both enzymes and reduction in decomposition of H_2O_2 by the close proximity of ChOx and HRP in the nanoflowers.[15] The highly active and stable multi-enzyme incorporated hybrid nanoflowers can be applied to detect cholesterol through a successful cascade reaction. Using the ChOx@HRP hybrid nanoflowers, ChOx generates H_2O_2 by catalyzing the oxidation of cholesterol present in the blood serum sample, which subsequently leads to the activation of HRP that converts the selected substrate, TMB, into a colored end product (Fig. 1).

We first characterized the sonicated nanoflowers prepared with ChOx (40, 20, and 10 μg mL^{-1}), HRP (20, 2, and 0.2 μg mL^{-1}), or ChOx@HRP (20 μg mL^{-1} ChOx with 20, 2, and 0.2 μg mL^{-1} HRP) in the presence of copper(II) sulfate by SEM analyses. As expected, hierarchically-structured flower-like morphologies with an average size of ~20 μm having a large surface-to-volume

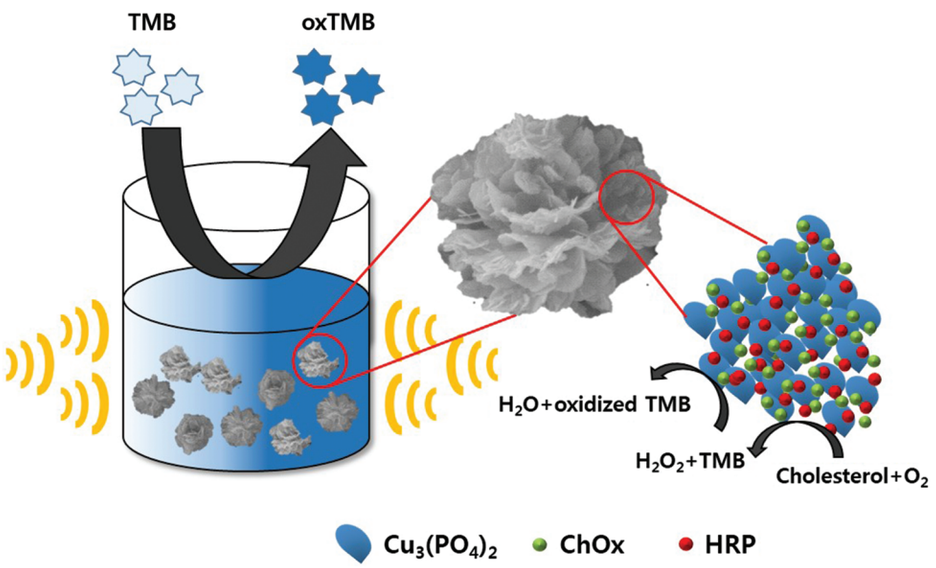


Figure 1. Schematic illustration showing the sonochemical synthesis of multi-enzyme incorporated organic–inorganic hybrid nanoflowers and the mechanism for target cholesterol detection.

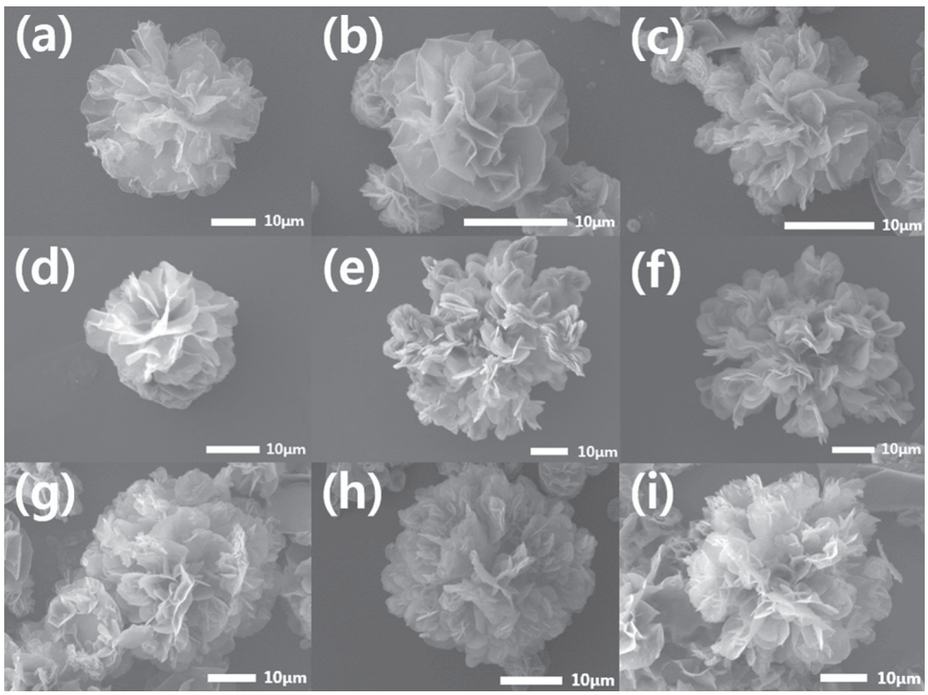


Figure 2. SEM images showing sonicated nanoflowers prepared with different concentrations of ChOx [(a) 40 μg mL^{-1}, (b) 20 μg mL^{-1}, and (c) 10 μg mL^{-1}], HRP [(d) 20 μg mL^{-1}, (e) 2 μg mL^{-1}, and (f) 0.2 μg mL^{-1}], and ChOx@HRP [20 μg mL^{-1} ChOx with (g) 20 μg mL^{-1}, (h) 2 μg mL^{-1}, or (i) 0.2 μg mL^{-1} HRP] in the presence of copper(II) sulfate.

ratio were observed in all cases, confirming that 5-min sonication is enough to bloom the nanoflowers (Fig. 2). Furthermore, as the employed concentrations of ChOx or HRP decreased, the obtained nanoflowers became larger (Figs. 2(a–f)). This result indicates that the decrease in number of enzyme molecules would lead to a corresponding decrease in the number of nucleation sites, resulting in larger nanoflowers.[11] When ChOx@HRP hybrid nanoflowers were created using a constant ChOx concentration (20 μg mL^{-1}), but with decreasing concentrations of HRP (20, 2, and 0.2 μg mL^{-1}), nanoflowers with similar sizes and morphologies were obtained (Figs. 2(g–h)), as observed previously.[15] For the ChOx@HRP hybrid nanoflowers, utilization of 20 μg mL^{-1} ChOx with 0.2 or 2 μg mL^{-1} HRP resulted in a high encapsulation yield of over 50%, calculated as a ratio of the amount of incorporated enzyme to the total amount of enzyme used (Table I). EDS maps provide a direct proof for the formation of enzyme-copper nanoflowers where the representative elements corresponding to copper phosphate (Cu and P) and both the enzymes (N) were clearly observed throughout the nanoflowers (Fig. 3(a)). In addition, the chemical structures of ChOx@HRP hybrid nanoflowers were compared with those of copper phosphate and the free enzymes by FT-IR (Fig. 3(b)). The peaks observed at 985 and 1054 cm^{-1} in the spectrum for copper phosphate and ChOx@HRP hybrid nanoflowers, respectively, were attributed to P–O vibrations, clearly indicating the presence of phosphate groups in the hybrid nanoflowers.[19] Typical peaks at 1400–1600 cm^{-1} for the peptide bond and at 2800–3000 cm^{-1} for –CH_2 and –CH_3 were observed in the spectrum for the hybrid nanoflowers, thereby revealing that the enzymes were encapsulated in nanoflowers.[20] All these characterizations confirm that the simple sonication treatment for 5 min at room temperature could successfully bloom the nanoflowers incorporating ChOx and HRP with copper phosphate.

3.2. Cholesterol Detection Using ChOx@HRP Hybrid Nanoflowers

Catalytic activities for the cascade reaction to detect cholesterol with ChOx@HRP hybrid nanoflowers were

Table I. Encapsulation yield of the employed enzymes in the nanoflowers.

Nanoflowers	Initial enzyme concentration (μg mL^{-1})	Encapsulation yield (%)
ChOx	10	48.7
	20	69.9
	40	20.6
HRP	0.2	91.6
	2	50.2
	20	5.0
ChOx@HRP	20@0.2	56.3
	20@2	50.2
	20@20	32.4

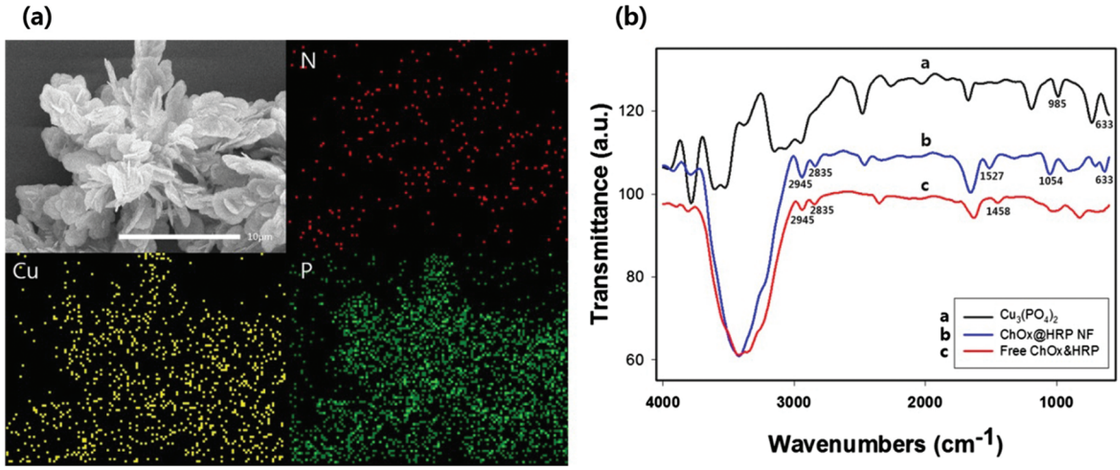


Figure 3. (a) Elemental mapping of ChOx@HRP hybrid nanoflowers via EDS. ChOx@HRP nanoflowers exhibit the element sensitive maps of nitrogen, copper, and phosphorus. (b) FT-IR spectra of $Cu_3(PO_4)_2$, ChOx@HRP hybrid nanoflowers, and free enzyme mixtures of ChOx and HRP.

determined and compared to those with the corresponding free enzymes (Fig. 4(a)). In a typical activity assay using 1 mM cholesterol, the hybrid nanoflowers resulted in ~60% activity compared to that of the corresponding free enzymes. Considering an encapsulation yield of ~56% i.e., about half of the enzyme molecules were employed in the activity assay of ChOx@HRP hybrid nanoflowers compared to that with the corresponding free enzymes; the nanoflowers were able to effectively retain their initial activity probably due to the efficient entrapment of both enzyme molecules with close proximity in the flower-like matrices.[15] We then tested and compared the cholesterol detection activities of the ChOx@HRP hybrid nanoflowers prepared from different enzyme concentrations (Fig. 4(b)). The nanoflowers from 20 μg mL^{-1} ChOx and 2 μ g mL^{-1} HRP showed the highest activity for cholesterol detection and were thus used for further studies.

We then applied ChOx@HRP hybrid nanoflowers for the cholesterol detection in a microscale well-plate format, enabling multiplex analyzing capability. As a result, multiple samples containing cholesterol at gradient concentrations were simultaneously analyzed based on the visible image along with the corresponding absorption spectra (Fig. 5(a)). The well-plate format also yielded near-perfect linearity and was able to detect cholesterol levels as low as 8 μM, with a linear range of 10–70 μM (Fig. 5(b)). The LOD value is among the best results of those recently reported describing colorimetric measurements of cholesterol.[10,21] Considering that the cutoff value of cholesterol in the blood of

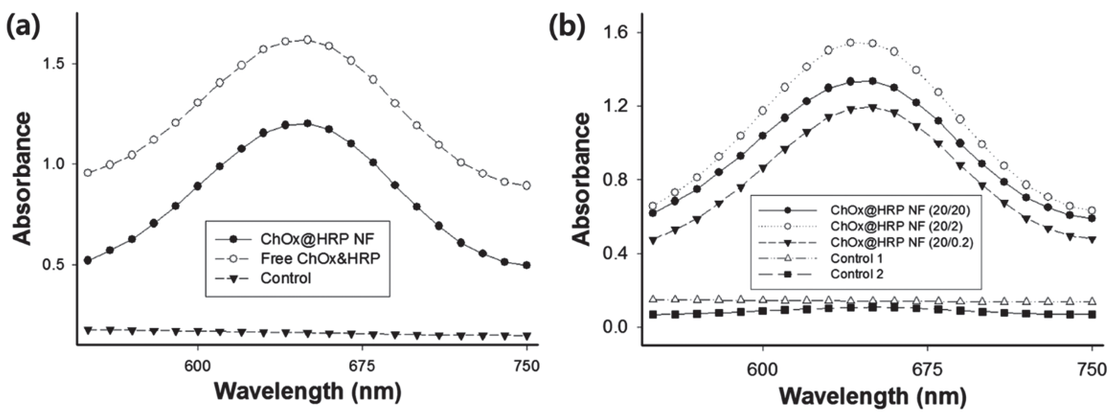


Figure 4. (a) Catalytic activities for cholesterol detection using ChOx@HRP hybrid nanoflowers prepared from 20 μg mL^{-1} ChOx with 0.2 μg mL^{-1} HRP and the corresponding free enzymes (20 μg mL^{-1} ChOx and 0.2 μg mL^{-1} HRP). (b) Catalytic activities of ChOx@HRP hybrid nanoflowers prepared from different enzyme concentrations. Control 1 indicates the catalytic reaction with HRP nanoflowers without ChOx, and control 2 indicates the catalytic reaction with ChOx nanoflowers without HRP.

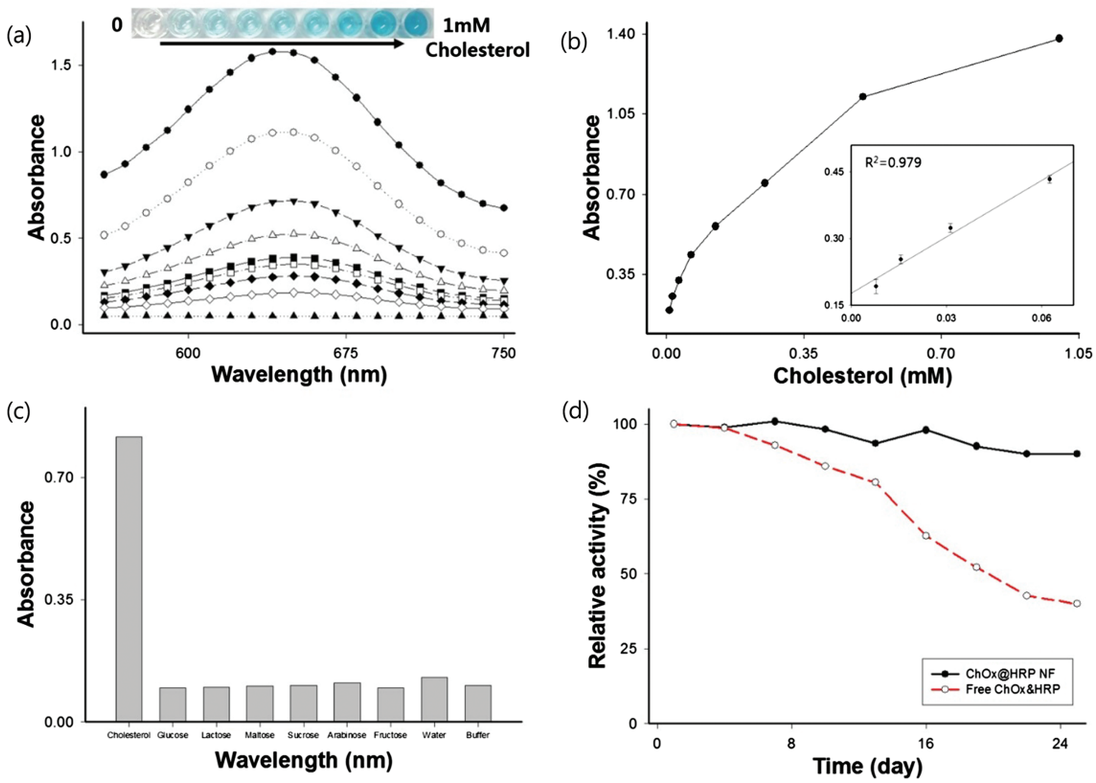


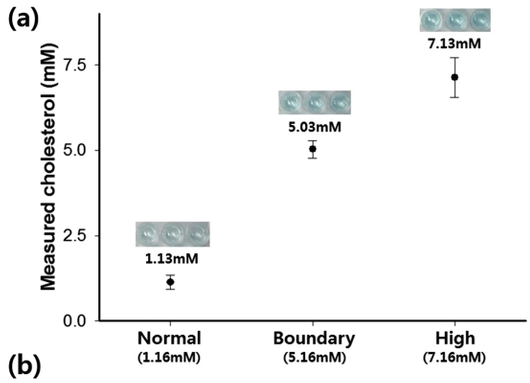
Figure 5. Cholesterol detection using ChOx@HRP hybrid nanoflowers in a well-plate format. (a) Real images and the corresponding absorption spectra for the colorimetric detection of cholesterol using TMB as a substrate. From the bottom of the spectra, 0, 0.0078, 0.0156, 0.0312, 0.0625, 0.125, 0.25, 0.5, and 1 mM cholesterol concentrations were used. (b) Linear calibration plot corresponding to the different cholesterol concentrations. (c) Selectivity to detect cholesterol. In the experiment, 1 mM cholesterol was used while 10 mM of other carbohydrates was used. (d) Long-term stability of ChOx@HRP hybrid nanoflowers.

patients with hypercholesterolemia is about 6 mM,[22] the nanoflower-based biosensor system is sufficiently sensitive to distinguish between diseased and normal patients. The proposed nanoflower-based biosensor system also shows excellent selectivity to the corresponding target cholesterol, while no significant absorbance signal was generated from the negative control samples like glucose, lactose, maltose, sucrose, arabinose, and fructose, which are commonly present in human blood, even when used at 10-fold higher concentrations (Fig. 5(c)). These results demonstrate that a specific, sensitive, and multiplexed colorimetric reaction utilizing only the target cholesterol is promoted by the ChOx@HRP hybrid nanoflowers.

The distinct advantage of the enzyme-inorganic nanoflowers is their enhanced operational stability. To assess this feature, the residual activities of the ChOx@HRP nanoflowers were determined along with those of the corresponding free enzymes (Fig. 5(d)). After about 24 days of incubation, the nanoflowers efficiently retained over 90% of their initial activity whereas free enzyme mixtures showed about 60% loss in their respective activities. This remarkably enhanced stability of the multi-enzyme incorporated nanoflowers could make them effective and versatile for further applications.

3.3. Diagnosis of Representative Stages of Hypercholesterolemia

Finally, we evaluated the diagnostic capability of the ChOx@HRP hybrid nanoflower-based system using real human serum samples that contained representative levels of cholesterol corresponding to normal, boundary, and high stages of hypercholesterolemia (normal: $\leq$5 mM, boundary: 5$\sim$6 mM, and high: >6 mM).[22] The original amount of cholesterol in the serum samples was first determined with a cholesterol assay kit (Sigma-Aldrich, USA), and a predetermined amount of cholesterol was then added to establish the representative levels. Clear differences were observed in the colorimetric signals from the three representative blood samples (Fig. 6(a)). In all cases, the serum cholesterol levels were quantified with excellent precision, yielding CVs in the range of 4.9–8.3% and recovery rates of 97.4–99.6% (Fig. 6(b)), thus verifying the excellent reproducibility and reliability of this method. These observations show that the multi-enzyme incorporated nanoflower-based assay system can serve as a



(b)

	Original (mM)	Added (mM)	Expected (mM)	Measured (mM)	SD	CV (%)	Recovery (%)
Normal	1.16	0	1.16	1.13	0.093	8.23	97.41
Boundary		4	5.16	5.03	0.249	4.95	97.48
High		6	7.16	7.13	0.574	8.05	99.58

Figure 6. Colorimetric quantification of cholesterol for diagnosis of hypercholesterolemia in human blood serum samples. (a) Measurements of different levels of cholesterol in blood samples are plotted with their corresponding real images. (b) Accuracy and precision of the cholesterol measurements.

promising analytical tool for the clinical detection of high levels of cholesterol.

4. CONCLUSION

We herein developed a robust method for the sonochemical synthesis of multi-enzyme incorporated organic–inorganic hybrid nanoflowers comprising ChOx and HRP with copper phosphate, which can be employed for the multiplexed colorimetric detection of the target cholesterol. The nanoflowers were rapidly synthesized via sonication for 5 min but exhibited hierarchically structured flower-like morphologies as well as high activity and stability to catalyze an enzymatic cascade reaction for the determination of cholesterol. Our system in a 96-well plate format displays excellent selectivity, linearity, multiplexing capability, and diagnostic utility, as evident from the precise quantification of cholesterol in multiple human blood serum samples. Based on our encouraging clinical application results, it is anticipated that multi-enzyme incorporated nanoflowers can be utilized for the rapid visual detection of target molecules in a simple, convenient, and cost-effective manner, thereby enabling efficient point-of-care detection in clinical diagnostics.

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