



DNA-copper hybrid nanoflowers as efficient laccase mimics for colorimetric detection of phenolic compounds in paper microfluidic devices

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ABSTRACT

Laccases are important multicopper oxidases that are involved in many biotechnological processes; however, they suffer from poor stability as well as high cost for production/purification. Herein, we found that DNA-copper hybrid nanoflowers, prepared via simple self-assembly of DNA and copper ions, exhibit an intrinsic laccase-mimicking activity, which is significantly higher than that of control materials formed in the absence of DNA. Upon testing all four nucleobases, we found that hybrid nanoflowers composed of guanine-rich ssDNA and copper phosphate (GNFs) showed the highest catalytic activity, presumably due to the affirmative coordination between guanine and copper ions. At the same mass concentration, GNFs had similar K_m but 3.5-fold higher V_{max} compared with those of free laccase, and furthermore, they exhibited significantly-enhanced stability in ranges of pH, temperature, ionic strength, and incubation period of time. Based on these advantageous features, GNFs were applied to paper microfluidic devices for colorimetric detection of diverse phenolic compounds such as dopamine, catechol, and hydroquinone. In the presence of phenolic compounds, GNFs catalyzed their oxidation to react with 4-aminoantipyrine for producing a colored adduct, which was conveniently quantified from an image acquired using a conventional smartphone with ImageJ software. Besides, GNFs successfully catalyzed the decolorization of neutral red dye much faster than free laccase. This work will facilitate the development of nanoflower-type nanozymes for a wide range of applications in biosensors and bioremediation.

1. Introduction

Laccases are copper-containing oxidases that catalyze the oxidation of various biologically and environmentally important aromatic compounds, such as di-, poly-, and amino-phenols, aryl diamines, and polyamines (Giardina et al., 2010; Mate and Alcalde, 2015). In these reactions, dioxygen molecules are directly reduced to water without producing hydrogen peroxide (H_2O_2), and thus, laccases have been considered as green catalysts in many environmental applications, including pollutant detection (Lou et al., 2020; Mei et al., 2015), dye degradation (Gahlout et al., 2017; Ladole et al., 2020), and cathode preparation for biofuel cells (Rincón et al., 2011; Vo et al., 2020; Zhang et al., 2019). Nevertheless, laccases are naturally unstable due to the intrinsic structural weakness of protein molecules and their current fermentation-based production and purification involve a long and tedious process that results in low yield; these factors critically hinder the practical applications of laccases (Jones and Solomon, 2015). To overcome these limitations, many studies have been conducted to

develop laccase-mimicking materials. For example, chemical compounds containing copper ions conjugated with ligands such as imidazole (Verónica Rivas et al., 2011) or porphyrin (Zhou et al., 2007) have been developed; however, they yield lower catalytic activity than natural laccases and require harsh synthetic conditions.

Nanomaterial-based enzyme mimics, so called nanozymes, are an important class of enzyme mimics, because they are highly stable and robust even under harsh conditions, can be cost-effectively manufactured by facile mass-production, and have tunable and high catalytic activity (Wu et al., 2019). Their catalytic activity originates from the atoms present on the surface or inside core of the nanozymes, which act as active sites of natural enzymes. Since the coppers in laccases have a central role in their catalytic activity, the laccase-mimicking nanozymes developed so far commonly contain copper elements to mimic the catalytic function, such as copper-containing carbon dots (Ren et al., 2015), guanosin-monophosphate-coordinated Cu^{2+} -containing metal organic frameworks (Liang et al., 2017), and cysteine-histidine- Cu^{2+}/Cu^+ hybrid nanoparticles (Wang et al., 2019). Further efforts are required to

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find or develop superior laccase-mimicking nanozymes, particularly those that allow for easy scale-up production, with high activity and stability, hold significant incentives in laccase-mediated biotechnological fields.

Micrometer-sized organic-inorganic hybrid particles having nano-scale architectures with flower-like morphology, also known as nanoflowers, have attracted considerable attention because of their extraordinary capability to retain high activity with excellent stability of entrapped organic components, by allowing for efficient confinement within the hierarchically-ordered flower matrices (Ge et al., 2012; Tran and Kim, 2018). They can be easily synthesized under mild conditions, by making use of the coordination between amine or amide moiety of biomolecules and inorganic ions, including Cu, Zn, Mn, Co, and Fe (Cui and Jia, 2017), and intensively applied for the development of biosensors (Ye et al., 2016) and biocatalytic systems for protein digestion (Yin et al., 2015), cancer therapy (Guo et al., 2017), dye removal (Li et al., 2017), and antibacterial treatment (Lee et al., 2020; Mohammad et al., 2020). However until now, organic-inorganic hybrid-based approaches have rarely been extended to the development of nanozymes, except several peroxidase-mimicking cases (Batule et al., 2019; Thawari and Rao, 2016).

In the present study, we hypothesized that hybrid nanoflowers containing copper elements coordinated with appropriate biomolecules could serve as new laccase-mimicking nanozymes, since such coordination is known to be essential for producing laccase-like activity (Wang et al., 2019). Upon employing guanine-rich single-stranded (ss)-DNA with copper ions to bloom the respective DNA-copper hybrid nanoflowers, the nanoflowers were found to have efficient laccase-like catalytic activity, yielding up to 3.5-fold higher $V_{\max}$ with similar K_m as those of natural laccase, with extremely high operational stability. The developed nanoflowers were further immobilized onto paper to construct paper microfluidic devices, which could be conveniently used for the colorimetric determination of diverse phenolic compounds. Since phenolic compounds are one of the major industrial contaminants having high toxicity to human and ecosystems when exposed above certain levels, their sensitive and reliable determination method is of significant importance (Barríos-Estrada et al., 2018). Among many detection methods for phenolic compounds, colorimetric strategy is considered as the most convenient platform, which is suitable for instrumentation-free point-of-care testing (POCT) environments. However, the conventional colorimetric assays often involve natural laccase, and thus, the poor stability of laccase severely hampers its practical applications (Lin et al., 2014). On the other hand, the developed nanoflowers were much more stable and robust, and thus, they efficiently retained their laccase-like catalytic activity after the immobilization on paper devices. Using the nanoflowers-embedded paper devices, small amounts of phenols were rapidly, selectively, and sensitively determined by simply dropping samples on the devices.

2. Experimental section

2.1. Materials

Laccase from *Trametes versicolor*, horseradish peroxidase (HRP), (–)-epinephrine, copper (II) sulfate pentahydrate, neutral red, 2-(N-morpholino) ethanesulfonic acid (MES), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), H_2O_2 (30 wt %), 4-aminoantipyrine (4-AP), phenol, 2,4-dichlorophenol (2,4-DP), hydroquinone, dopamine hydrochloride, pyrocatechol, 4-aminophenol, paraoxon-methyl, dichlorvos, and malathion were obtained from Sigma-Aldrich (St Louis, MO, USA). Phosphate-buffered saline (PBS, pH 7.4) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). All solutions were prepared by dissolving the reagents with UltraPure™ DNase/RNase-free distilled water (Thermo Fisher Scientific). DNA oligonucleotides were synthesized from Integrated DNA Technologies (Coralville, IA, USA), and their sequence information is summarized in

Table S1. All other chemicals were of analytical grade and used without further purification.

2.2. Preparation of DNA-copper hybrid nanoflowers

DNA-copper hybrid nanoflowers were synthesized according to our previously reported protocol (Park et al., 2017). Briefly, 20 μ L of aqueous $CuSO_4$ solution (120 mM) was added to 3 mL of PBS (pH 7.4) containing diverse DNAs at different concentrations, followed by 3 days of incubation at room temperature (RT). The nanoflowers were then collected using centrifugation at 10,000 rpm for 5 min and washed three times with UltraPure™ DNase/RNase-free distilled water. Based on the types of ssDNA molecules (guanine-rich, thymine-rich, cytosine-rich, and adenine-rich), we designated their respective hybrid nanoflowers with copper phosphate (GNFs, TNFs, CNFs, and ANFs, respectively). The control sample formed in the absence of DNA was designated as copper phosphate $[Cu_3(PO_4)_2]$ precipitate.

The nanoflowers were visually analyzed using scanning electron microscopy (SEM) images obtained using a Magellan™ 400 Field Emission Scanning Electron Microscope. Their elemental composition was analyzed using an energy-dispersive spectrometer (EDS) (Bruker, Billerica, MA, USA). For the preparation of samples, the suspension of DNA-copper hybrid nanoflowers was dropped and dried on a silicon wafer. For X-ray diffraction (XRD) analysis (D/MAX-2500, Rigaku Corporation, Tokyo, Japan), the collected DNA-nanoflowers were washed with deionized water and dried at 80 °C for 1 day. X-ray photoelectron spectroscopy (XPS) (Sigma Probe, Thermo Scientific, WI, USA) was performed to examine the electronic state of the copper within the DNA-copper hybrid nanoflowers. Specific surface area and pore diameter distribution were obtained from nitrogen physisorption isotherms with a physisorption analyzer (3Flex, Micromeritics, GA, USA) using Brunauer-Emmett-Teller (BET) and Barrett-Joyner-Halenda (BJH) methods. The encapsulation yield of DNA within the nanoflowers was calculated by measuring the amount of DNA in the supernatant with a NanoDrop 1000 system (Thermo Fisher Scientific). The weight percentage of DNA in the nanoflowers was calculated based on the encapsulation yield and weight of the powder.

2.3. Determination of laccase-like activity of the DNA-copper hybrid nanoflowers

Laccase activity was measured using the chromogenic reaction of phenolic compounds with 4-AP (Liang et al., 2017). Typically, catechol (1 mg/mL, 100 μ L) was mixed with 4-AP (1 mg/mL, 100 μ L) in MES buffer (30 mM, pH 6.8, 700 μ L), followed by addition of free laccase or nanoflowers (1 mg/mL, 100 μ L). After reaction for 1 h at RT, the mixture was centrifuged at 10,000 rpm for 2 min, and the obtained supernatant was subjected to recording of the absorption intensity in scanning mode or at 510 nm using a microplate reader (Synergy H1, BioTek, VT, USA). Other phenolic substrates (phenol, dopamine, 2,4-DP, and hydroquinone) were also assayed using the same procedures described above, but they were used as target phenolic compounds instead of catechol.

Steady-state kinetic assays were performed at RT in a 1.5 mL tube with GNF or free laccase (both at concentrations of 0.1 mg/mL) in MES buffer (30 mM, pH 6.8). Catechol at various concentrations (10, 20, 40, 60, 80, and 100 μ g/mL) was added in 1 mL reaction buffer with an excess amount of 4-AP (0.15 mg/mL). After the substrates were mixed, the color changes were monitored in the kinetic mode at 510 nm. The kinetic parameters were calculated based on the Michaelis–Menten equation $v = V_{\max} \times [S]/(K_m + [S])$, where v is the initial velocity, $V_{\max}$ is the maximal velocity, $[S]$ is the concentration of substrate, and K_m is the Michaelis constant. Catalytic constant k_{cat} was derived from $k_{cat} = V_{\max}/[E]$, in which $[E]$ is the molar concentrations of GNF or free laccase, calculated as reported previously (Jiang et al., 2018).

To measure the thermal stabilities of GNF and free laccase, these were incubated in aqueous buffer (MES, 30 mM, pH 6.8) over a range of

temperatures (22, 37, 50, 60, and 70 °C) for 3 h, followed by measurement of the residual activities for catechol sensing using standard procedures, as described above. pH stabilities of GNF and free laccase were measured by incubating them in aqueous buffer (MES, 30 mM) at diverse pH values (3.0–9.0) for 7 h, followed by determination of the residual activities. The effects of ionic strength were measured by incubating GNF and free laccase in aqueous MES buffer containing diverse amounts of NaCl (0, 150, 300, and 500 mM) for 12 h, followed by evaluation of the residual activities. The long-term operational stabilities of GNF and free laccase were measured by assessing their activity at each time point during their incubation at RT under mild shaking conditions. The relative activity (%) was calculated as the ratio of the residual activity to the initial activity of each sample.

To evaluate the oxidation of epinephrine by the laccase-like activity of GNF and free laccase, epinephrine at diverse concentrations was mixed with GNF or free laccase (0.1 mg/mL) in MES buffer (30 mM, pH 6.8) for 1 h at RT. After the reaction, the mixture was centrifuged at 10,000 rpm for 2 min, and the supernatant was used to record the absorption intensity at 485 nm, which corresponds to oxidized epinephrine, using a microplate reader.

2.4. Decolorization of neutral red by GNFs or free laccase

Dye decolorization by laccase-mimicking GNFs or free laccase was assessed using neutral red as a model dye (Li et al., 2017). In a typical reaction, GNF or free laccase (1 mg/mL, 1 mL) was added to the reaction solution containing neutral red (9 mL, 5 mg/mL). The mixture was then incubated under dark conditions at RT, with slight stirring. Dye degradation efficiency was analyzed by measuring the absorption intensity at 523 nm, which corresponds to the original neutral red, using a microplate reader.

2.5. GNFs-incorporated paper microfluidic devices for colorimetric determination of phenolic compounds

Paper microfluidic devices consisting of GNFs were prepared using the wax printing method (Carrilho et al., 2009). The pattern was designed using AutoCAD 2018, followed by printing the wax on Whatman chromatography paper (grade 1) using a wax printer (ColorQube 8570DN, Xerox, Japan). The patterned papers were placed on a hot plate at 150 °C for 2 min to melt the wax, which forms hydrophobic barriers according to the printed pattern. To prevent sample leakage through the barriers, the wax printing thickness was controlled to ~0.7 mm. The microfluidic pattern consisted of four circular detection zones (6 mm in diameter) in which GNFs and 4-AP were immobilized, one sample zone (15 mm in diameter) for sample dropping, microfluidic channels (3 mm in width and 6 mm in length) that connect the detection zones and sample zone, one circular control zone (6 mm in diameter) where both GNFs and 4-AP were immobilized, and one square control zone (6 mm in edge) where only 4-AP was immobilized without GNFs.

To construct GNFs-incorporated paper microfluidic devices, GNFs (2 μ L, 0.2 mg/mL) and 4-AP (2 μ L, 0.2 mg/mL) were consecutively dropped onto detection zones and circular control zone. At the same time, 4-AP (2 μ L, 0.2 mg/mL) in MES buffer without GNFs was dropped onto a square control zone. The paper devices were then dried overnight at RT. To detect phenolic compounds, 50 μ L of the sample solution containing phenolic compounds at diverse concentrations (5–500 μ g/mL), including dopamine, hydroquinone, or catechol, was dropped onto the sample and square control zones. After 20 min, the reacted paper devices were directly used to obtain images with a smartphone (GALAXY S8 NOTE, Samsung, Korea), followed by conversion to grayscale, which was subjected to quantitative image processing using ImageJ software (NIH). Selectivity of the GNF-based paper devices was evaluated following the same procedures described above with phenolic compounds [dopamine, catechol, and hydroquinone] at 100 μ M as well as many interfering molecules [metal and non-metal (Zn, Hg, Fe, Pb, Ca, F,

Cl, and S) ions, protein (BSA), small molecules (glucose, cysteine, and ascorbic acid), and organophosphorus compounds (paraoxon-methyl, dichlorvos, and malathion)] at 1 mM. Long-term stability of the GNF-based paper devices was assessed by measuring the residual color intensities toward dopamine (100 μ M) during storage at RT. The relative color intensity (%) was calculated using the ratio of the residual color intensity to the initial one.

The conventional high-performance liquid chromatography (HPLC) method for the determination of phenolic compounds was performed at KIST (Seoul, Korea) according to the manufacturer's instructions and protocols. Dopamine, catechol, and hydroquinone were prepared in acetonitrile solution and used for the analysis. The correlation between GNF-based paper device and HPLC (Agilent Technologies, CA) was measured using solutions of dopamine, catechol, and hydroquinone (0–250 μ M).

3. Results and discussion

The overall strategy for the preparation of laccase-mimicking DNA-copper hybrid nanoflowers and their application to paper microfluidic devices for detecting phenolic compounds is illustrated in Fig. 1. Since the Cu coordinated with guanine were essential to mimic laccase activity (Liang et al., 2017), we believe that guanine-rich DNA molecules would be the best synthetic template to bloom laccase-mimicking DNA-copper hybrid nanoflowers. There are four coppers in the active site of natural laccase, which catalyze electron transfers among them where molecular oxygen is converted to water (Giardina et al., 2010). Likewise, multi-coppers present throughout the hybrid nanoflowers would facilitate electron transfer, where the target substrates are oxidized while producing water from dioxygen. The hybrid nanoflowers were then immobilized on the detection zones of wax-printed paper, followed by co-immobilization of 4-AP substrate. We envisioned that the paper microfluidic device incorporating the laccase-mimicking nanoflowers would serve as an efficient biosensor that is capable of being used for the colorimetric detection of diverse phenolic compounds.

We first investigated the effects of nucleobases on blooming the nanoflowers by reacting 51-bp ssDNAs [a) guanine-rich ssDNA, b) thymine-rich ssDNA, c) cytosine-rich ssDNA, and d) adenine-rich ssDNA] with copper ions. Owing to the spontaneous self-assembly process during the 3 days of incubation in PBS buffer at RT, flower-like morphologies were observed in all four cases, with an average size of ~10 μ m (Fig. 2a–d). The encapsulation yield and weight percentage of ssDNA (over 95% and 9–14 wt%, respectively) remained at a higher level than that obtained with the conventional nucleic acid encapsulation method (Raval et al., 2019) (Table S2). The maximized encapsulation yield as well as weight percentage of DNA in the nanoflowers were achieved when 51 bp ssDNA at 0.5 μ M was employed regardless of the kinds of base. The control sample formed in the absence of DNA did not yield any flower-like morphology, confirming that the DNA component is essential to bloom the nanoflowers (Fig. S1). These observations confirmed the successful formation of DNA-copper hybrid nanoflowers (Park et al., 2017).

XPS characterizations were then performed to investigate the oxidation state of Cu within the DNA-copper hybrid nanoflowers. The full scan spectrum shows Cu, O, N, C, and P, which correspond to the DNA and copper phosphate crystals within the nanoflowers (Fig. S2). In the high-resolution XPS spectra, the peaks at 934.9 and 954.8 eV represented the Cu 2p_{3/2} and Cu 2p_{1/2} electrons of Cu²⁺, respectively (Fig. 2e–h) (Ma et al., 2018). The lower binding peaks at 933.4 and 953.2 eV suggested the presence of Cu⁺ (Ghodselahi et al., 2008). Therefore, Cu⁺ as well as Cu²⁺ were present in the DNA-copper hybrid nanoflowers. Importantly, it was observed that GNFs have a much higher Cu⁺/Cu²⁺ ratio (~400%), as compared to other DNA-copper hybrid nanoflowers (<130%), presumably due to the affirmative interaction between the amine and amide moieties of guanine with copper ions, to reduce Cu²⁺ into Cu⁺. The higher amount of reduced copper

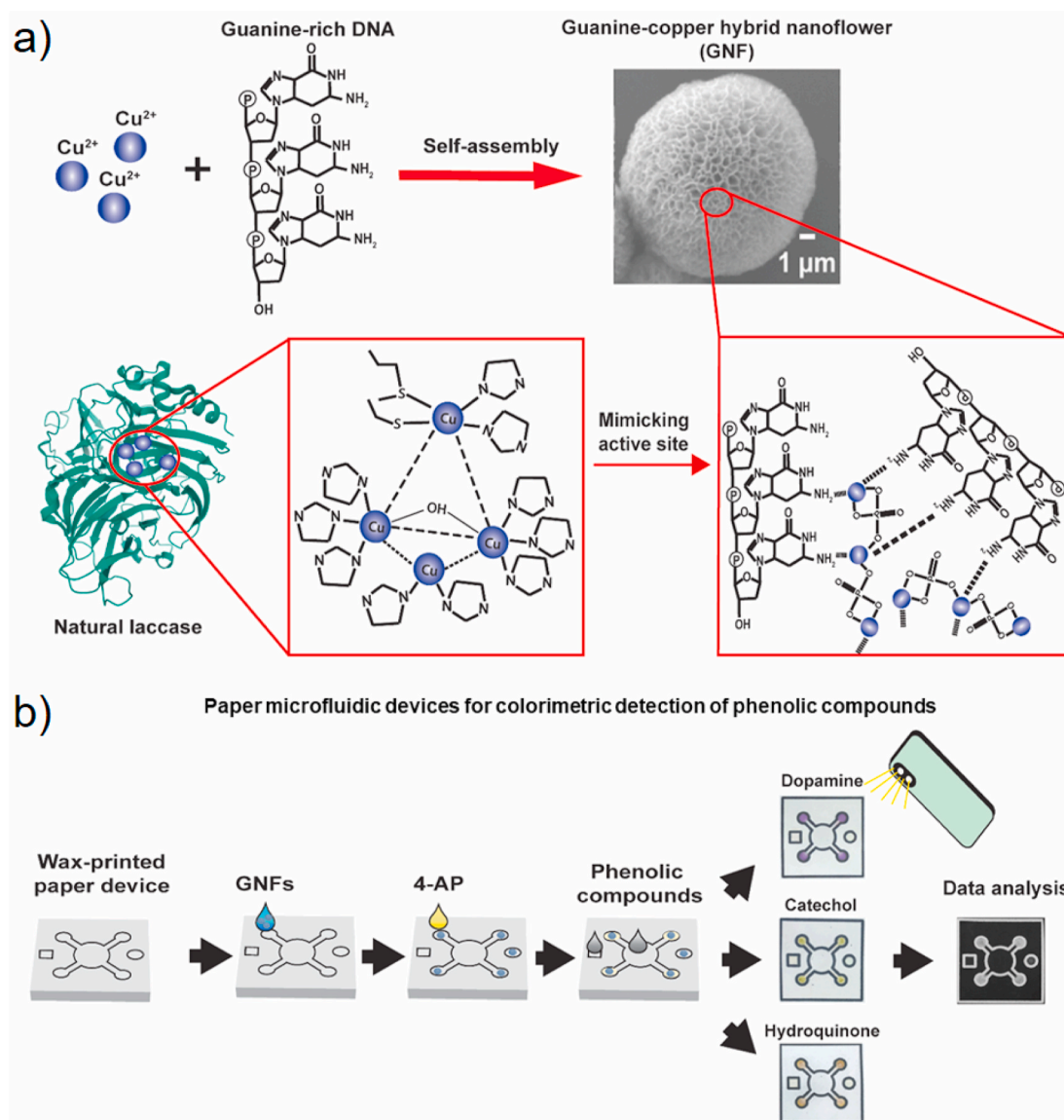


Fig. 1. Schematic illustration of a) the preparation of laccase-mimicking guanine-copper hybrid nanoflowers (GNFs) and b) their application to a paper microfluidic device for colorimetric detection of phenolic compounds.

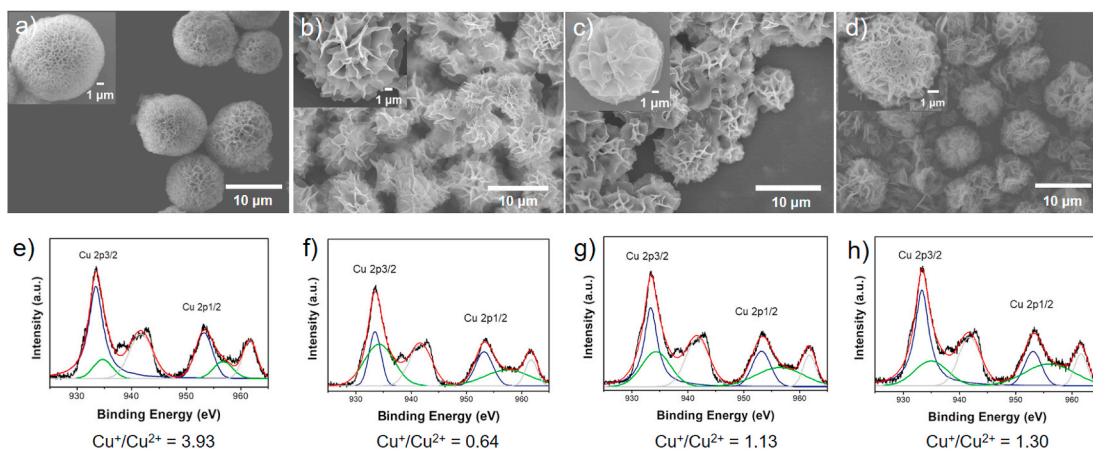


Fig. 2. SEM image and Cu 2p XPS spectrum of (a & e) GNFs, (b & f) TNFs, (c & g) CNFs, and (d & h) ANFs. In XPS spectra, green and blue lines correspond to Cu^{2+} and Cu^+ , respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

species in the form of Cu^+ of GNFs might be a theoretical basis for their higher laccase-like activity (Wang et al., 2019).

The GNFs were further characterized using XRD, EDS, and BET analyses (Fig. S3). The XRD patterns fit well with those of $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ [Joint Committee on Powder Diffraction Standards (JCPDS) 00-022-0548], confirming the presence of copper phosphate crystals within the nanoflowers (Cheon et al., 2019). EDS maps provide a direct proof for the formation of DNA-copper nanoflowers where representative elements corresponding to copper phosphate (Cu, P, and O) and nucleic acid (N) were clearly observed throughout the nanoflowers. The surface area, pore volume, and average pore diameter were determined using nitrogen physisorption and their corresponding values were $16.93 \text{ m}^2 \text{ g}^{-1}$, $0.03 \text{ cm}^3 \text{ g}^{-1}$, and 2.8 nm , respectively, all of which indicated a porous structure and high surface-to-volume ratio of the GNFs. All these characterizations confirmed that GNFs have typical hierarchical nanostructures, similar to conventional DNA-copper hybrid nanoflowers.

Laccase activities of GNFs were examined by performing the representative chromogenic reaction of catechol with 4-AP, and the responses were monitored using absorption spectroscopy. In the presence of catechol, free laccase or laccase mimics catalyzed its oxidation, to further react with 4-AP, to produce an orange-colored product with absorption at 510 nm . Compared to free laccase, all four kinds of DNA-copper hybrid nanoflowers, including GNFs, TNFs, CNFs, and ANFs, showed much higher activity and among them, GNFs displayed the highest activity (Fig. 3a). GNFs also exhibited higher laccase activity compared to the hybrid nanoflowers composed of adenine-thymine dsDNA (AT-NFs) or guanine-cytosine dsDNA (GC-NFs) with copper phosphate (Fig. S4a). These results showed that GNFs are the most efficient laccase-mimicking DNA-copper hybrid nanoflowers, presumably due to the affirmative coordination between the amine and amide moieties of guanine with copper, to efficiently generate reduced copper species, as confirmed in the XPS analyses (Fig. 2e). We also demonstrated that negligible activity was detected using the supernatant solution after removing GNFs by centrifugation (data not shown), indicating that the laccase-like activity originated from the GNFs themselves and not from the leached components in the supernatant. Another control experiment was also performed to prove that GNFs have the laccase activity to directly convert oxygen into water without producing H_2O_2 , while other oxidases, such as glucose oxidase, produce H_2O_2 (Fig. 3b) (Liang et al., 2017). For this, GNFs were incubated with only catechol for 1 h , followed by collection of the supernatant by separating the GNFs using centrifugation. Following that, the peroxidase substrate ABTS and HRP were added into the supernatant, but no color change was observed. Upon addition of H_2O_2 , the color of the supernatant obviously turned green, with high absorption intensity at 417 nm , which corresponds to the oxidized ABTS. These results clearly indicated that GNFs are laccase mimics, and not other oxidases that

produce H_2O_2 . The GNFs displayed highly enhanced laccase activity, and consequently, the highest colorimetric responses among the other control samples such as BSA-copper hybrid nanoflowers (BSA NFs), $\text{Cu}_3(\text{PO}_4)_2$ precipitates, guanine-rich ssDNA, and free laccases (Fig. 3c). Observations made in the study on the effects of the employed concentration/size of guanine-rich ssDNA and reaction buffer pH on the catalytic activity of GNFs revealed that $0.5 \mu\text{M}$, 51 bp , and $\text{pH } 6.8$ (MES, 30 mM) were ideal conditions for this reaction, which were then adopted for further studies (Figs. S4b–d).

To elucidate the laccase activity of GNFs, their steady-state kinetic parameters using catechol as a substrate were determined and compared with those of free laccase (Table 1 and Fig. S5). Importantly, the apparent K_m value of the GNFs was similar to that of free laccase, indicating that the mass transfer limitation of GNFs is not high and nearly the same as that of free laccase. Moreover, the V_{max} value of GNFs was found to be higher, up to 3.5-fold, than that of free laccase. Furthermore, the k_{cat} value of GNFs was significantly higher, up to over ten-order, than that of free laccase, based on the theoretical calculation of the molar concentration of GNFs, as previously reported (Jiang et al., 2018). With 2,4-DP as a substrate, the GNFs yielded similar K_m values with significantly-enhanced k_{cat} values compared with those of free laccase as well as recently-reported laccase mimics (Table S3). These V_{max} and k_{cat} values proved that the GNFs are more active laccase-like catalysts than free laccase. GNFs were also proven to be stable over a broad ranges of temperature (from 22 to 70°C), pH (from 3 to 9), ionic strength (up to 500 mM NaCl), and also during the 20 days of operating conditions (Fig. S6). On the other hand, free laccase was not stable and lost most of its activity under extreme conditions of pH, temperature, and ionic strength, as well as extended incubation time. It is also noteworthy that GNFs exhibited increased activity as the ionic concentration increased, while free laccase showed decreased activity. We believe that a salting-out effect might occur at higher ionic concentrations, resulting in lowered solubility of the phenolic substrate, and thus decreased

Table 1

Kinetic parameters of GNF and free laccase, with various concentrations of catechol as substrate.

Catalyst	Used concentration (mg/mL)	[E] (M) ^a	K_m (mM)	V_{max} ($\mu\text{M}/\text{min}$)	k_{cat} (min^{-1})
GNF	0.1	7.65×10^{-17}	1.84 ± 0.37	5.40 ± 0.39	7.06×10^{10}
Free laccase	0.1	1×10^{-6}	1.88 ± 0.09	1.57 ± 0.04	1.57

^a Molar concentrations of GNF and free laccase were calculated as reported previously (Jiang et al., 2018).

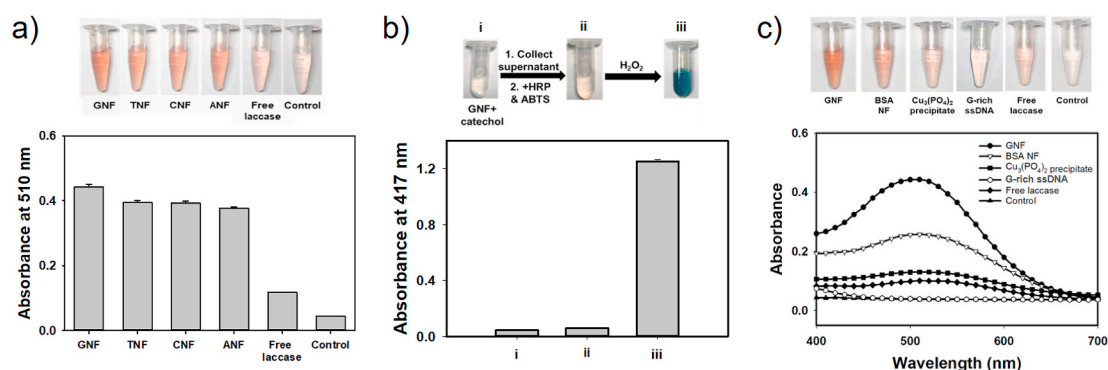


Fig. 3. Photographs and their corresponding absorption intensities or spectra for a) comparing the laccase-like activity of GNF with those of other DNA-copper hybrid nanoflowers (TNF, CNF, and ANF) and free laccase, b) confirming the laccase activity of GNFs rather than other oxidase activity [Sample specifications: i) GNFs reacted with catechol, ii) supernatant with ABTS and HRP, added after centrifugation of i), and iii) after adding H_2O_2 into ii)], and c) comparing the laccase-like activity of GNF with those of control samples such as BSA NF, $\text{Cu}_3(\text{PO}_4)_2$ precipitate, guanine (G)-rich ssDNA, and free laccase.

catalytic activity of free laccase. Additionally, chloride ions can inhibit the catalytic activity of free laccase by binding to the nuclear copper sites (Wang et al., 2019). In contrast, GNFs showed even higher activity at higher ionic strengths, presumably due to more efficient binding of the substrate to the GNFs. Overall, these studies clearly show that GNFs have higher activity and stability, as compared to free laccase, that are quite advantageous for their practical application.

Since laccase exhibits substrate diversity to catalyze the oxidation of a range of phenolic substrates, we next explored the catalytic capability of GNFs. The results showed that GNFs catalyzed five different phenols, such as phenol, dopamine, 2,4-DP, hydroquinone, and 4-aminophenol. Moreover, their catalytic activity was higher than that of free laccase, up to 4-fold in cases of phenol and dopamine (Fig. S7). GNFs was also used for the detection of epinephrine, which is a hormone in the adrenal medulla that is frequently used as a marker to diagnose and treat anaphylactic shock, bronchial asthma, and organic hear disease (Lu et al., 2011). The results of this investigation showed that GNFs successfully catalyzed the oxidation of epinephrine at a ~ 4 -fold faster rate than free laccase (Fig. 4a). As the concentration of epinephrine was increased, the color intensity corresponding to the oxidation of epinephrine increased, and as expected, color intensities with GNFs were much denser than those with free laccase (Fig. 4b). Epinephrine could be determined quantitatively with free laccase and GNFs, yielding a limit of detection (LOD) of 2.5 and 0.3 $\mu\text{g/mL}$, respectively, with $R^2 > 0.99$ in both cases, using the respective calibration plots (Fig. 4c). The much lower LOD of GNFs was attributed to the higher catalytic activity and concomitant slope value of their calibration plot for detecting epinephrine. These studies demonstrated that the GNFs have the ability to accept diverse phenols as their substrates, with higher detection sensitivity than free laccase.

Another important merit of laccase-mimicking GNFs is their possible utilization to degrade phenolic pollutants. To assess this feature, degradation efficiencies of the GNFs for a model phenolic substance, neutral red, were determined, along with those of free laccase. After 1 day of incubation, over 90% of the dye was decolorized and degraded by simple incubation with GNFs at RT, whereas free laccase displayed about 70% degradation (Fig. 4d). The capability of GNFs to rapidly degrade phenolic substances is highly beneficial to make them effective

and versatile for further environmental applications.

Finally, we applied the new laccase-mimicking GNFs to develop a paper microfluidic device for the colorimetric identification of phenolic compounds. Colorimetric paper microfluidic devices are recognized as the most convenient sensing platform, particularly in a resource-limited POCT environment; however, the limited catalytic activity and stability of signalling enzymes often cause low sensitivity and reproducibility, which critically hinder their practical applications (Nguyen and Kim, 2020; Zhu et al., 2017). In the present study, we designed and constructed a simple wax-printed paper microfluidic device, which was composed of one sample zone, four detection zones enabling quadruplicate detection, and two control zones confirming that the color changes originate from the reaction among the target phenolic compounds, GNFs, and 4-AP substrate (Fig. 5a). By just dropping 50 μL of sample solution onto the sample zone, the sample rapidly moves to the detection zones due to capillary force. In the presence of phenolic compounds in the sample solution, the nanoflowers immobilized in the detection zones catalyze their oxidation to further react with 4-AP, to produce a colored adduct, displayed within 20 min at RT on all four detection zones. Importantly, the color signals within the detection zones were quite conveniently quantified by taking real images using a smartphone, followed by image processing with ImageJ software.

We first compared the color signals of the GNF-based paper microfluidic device to detect the model compound dopamine with those from BSA NFs, $\text{Cu}_3(\text{PO}_4)_2$ precipitates, and free laccase (Fig. S8a). The results clearly revealed that the GNF-based paper microfluidic device yielded the highest color intensity, at least 2-fold higher, as we similarly observed from the solution-based sensing results. The concentrations of 4-AP and GNFs immobilized on the detection zones were then optimized to 20 and 25 mg/mL, respectively, based on the resulting color intensity values toward dopamine (Figs. S8b–c). Under the optimized conditions, target phenolic compounds, such as dopamine, catechol, and hydroquinone, were successfully detected from the color changes (Fig. 5b). On the other hand, negligible color intensity was detected in the negative control samples, where metal and non-metal (Zn, Hg, Fe, Pb, Ca, F, Cl, and S) ions, protein (BSA), small molecules (glucose, cysteine, and ascorbic acid), and organophosphorus compounds (paraoxon-methyl, dichlorvos, and malathion) were used, even at ten-fold higher

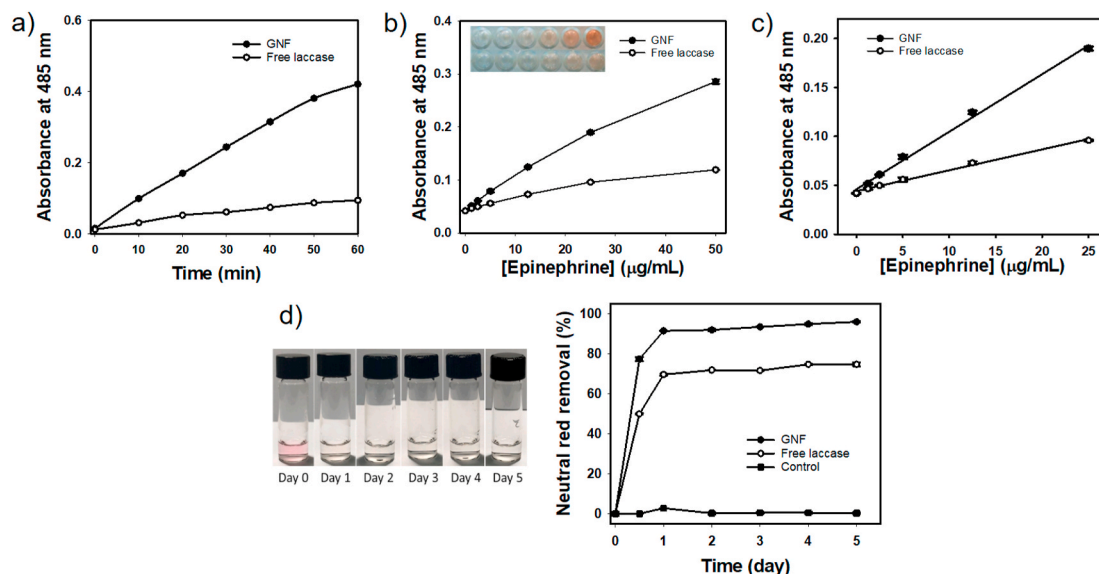


Fig. 4. Comparison of the performances of GNF and free laccase in epinephrine sensing and neutral red decolorization. a) Kinetics for oxidation of epinephrine (100 $\mu\text{g/mL}$) in the presence of GNF or free laccase. b) Dose-response curves for detecting epinephrine using GNF or free laccase. The inset shows the corresponding images. c) Linear relationships between the absorption intensity at 485 nm and the concentration of epinephrine in the presence of GNF or free laccase. d) Photographs for neutral red removal by GNF and the corresponding decolorization efficiencies by GNF or free laccase. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

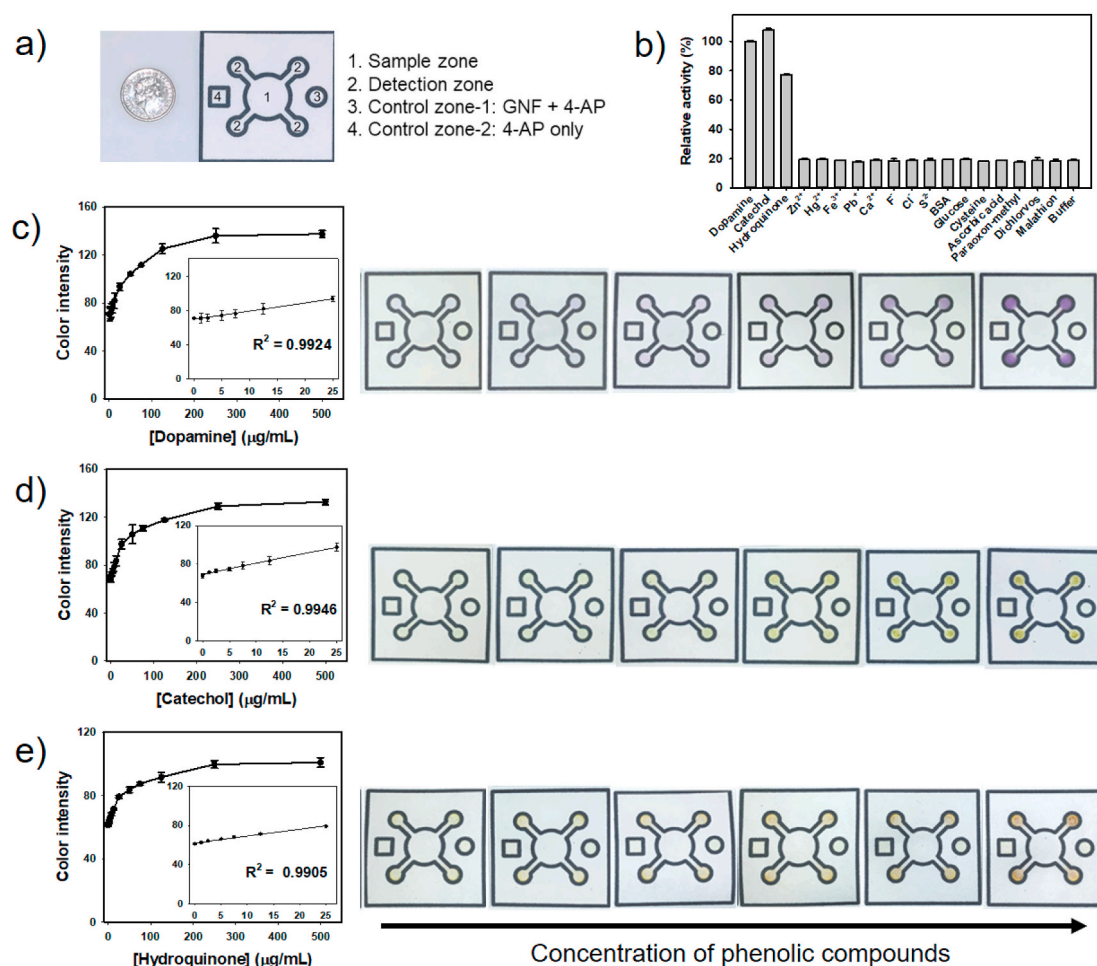


Fig. 5. a) Real images showing the shape and organization of the paper microfluidic device. b) Specificity of GNF-based paper microfluidic device for detecting phenolic compounds. Phenolic compounds were used at a concentration of 100 μM, whereas all control samples were used at a concentration of 1 mM. Sensitivity of GNF-based paper microfluidic device for detecting c) dopamine, d) catechol, and e) hydroquinone. Dose-response curves, as well as the corresponding linear calibration plots, were presented. Photographs show the real images of the device for detecting each target phenolic compound at six representative concentrations (5, 12.5, 25, 50, 125, and 500 μg/mL).

concentrations. These results clearly demonstrated that the GNF-based paper microfluidic device promoted a specific color-generating reaction of only the target phenolic compounds. With increasing concentrations of dopamine, catechol, and hydroquinone, their respective color intensities, shown on all four detection zones without any significant variation, gradually increased (Fig. 5c–e). Analysis of dose-response curves determined the LODs for dopamine, catechol, and hydroquinone as 4.5, 3.0, and 4.5 μg/mL, respectively, with dynamic linear ranges up to 25 μg/mL, with $R^2 > 0.99$ in all cases. The LOD values of our GNF-based paper microfluidic device were among the best describing colorimetric detection of phenolic compounds (Abdullah et al., 2007; Jafarinejad et al., 2017). Additionally, our paper device was compared with a conventional HPLC method for quantitatively determining the three phenolic compounds (dopamine, catechol, and hydroquinone). The observed correlation between the two methods was $R^2 > 0.99$ (Fig. S9). This high concordance between the two methods ensured the validity of the GNFs-based paper microfluidic device for reliable quantitative determination of phenolic compounds.

We evaluated the long-term stability of the developed GNF-based paper microfluidic device. The stabilities were examined by measuring the residual color intensities toward dopamine, during storage at RT. The investigation clearly indicated that the paper microfluidic device showed excellent storage stability during 2 months (Fig. S10). We also demonstrated the practical utility of the paper device by determining three different concentrations (5, 10, and 20 μM) of dopamine, catechol,

and hydroquinone, in spiked tap water solutions. The results were in good agreement with the added amount of each phenolic compound, yielding coefficient of variations (CVs) in the range of 2.0–7.8% and recoveries of 98.3–102.6% (Table S4). These results demonstrate that the developed GNF-based paper microfluidic device can be employed as a promising analytical tool for convenient identification of diverse phenolic compounds in POCT environments.

4. Conclusions

In conclusion, we developed an innovative strategy to prepare DNA-copper hybrid nanoflowers possessing intrinsic laccase-like activity. We demonstrated that GNFs had the highest catalytic activity, even when compared to free laccase, because of the affirmative coordination between guanine and multi-coppers throughout the nanoflower matrices, which helped to generate reduced copper species and mimic the active site of laccase. Furthermore, GNFs exhibited excellent stability over the ranges of temperature, pH, ionic strength, and incubation period tested in this study, with substrate diversity to oxidize diverse phenolic compounds. By applying GNFs in paper microfluidic devices, target phenolic compounds were conveniently quantified by simple processing of real images acquired using a smartphone, with excellent selectivity, sensitivity, stability, and reliability. This study should serve as the foundation for continuing efforts aimed at the development of hybrid nanoflower-type nanozymes incorporating diverse organic and inorganic

substances, as well as, nanoflower-based paper microfluidic devices with significant potential for diverse applications in biosensors and bioremediation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bios.2021.113187>.

Author statement

T.D. Tran and M.I. Kim designed the experimental scheme and analyzed the experimental data. T.D. Tran, P.T. Nguyen, and T.N. Le performed experiments. T.D. Tran, P.T. Nguyen, and M.I. Kim wrote and revised the manuscript.

References

- Abdullah, J., Ahmad, M., Heng, L.Y., Karuppiyah, N., Sidek, H., 2007. *Sensors* 7 (10), 2238–2250.
- Barríos-Estrada, C., de Jesus Rostro-Alanis, M., Munoz-Gutierrez, B.D., Iqbal, H.M.N., Kannan, S., Parra-Saldivar, R., 2018. *Sci. Total Environ.* 612, 1516–1531.
- Batule, B.S., Park, K.S., Gautam, S., Cheon, H.J., Kim, M.I., Park, H.G., 2019. *Sensor. Actuator. B Chem.* 283, 749–754.
- Carrilho, E., Martinez, A.W., Whitesides, G.M., 2009. *Anal. Chem.* 81 (16), 7091–7095.
- Cheon, H.J., Adhikari, M.D., Chung, M., Tran, T.D., Kim, J., Kim, M.I., 2019. *Adv. Healthcare Mater.* 8 (9), 1801507.
- Cui, J.D., Jia, S.R., 2017. *Coord. Chem. Rev.* 352, 249–263.
- Gahlout, M., Rudakiya, D.M., Gupta, S., Gupta, A., 2017. *Int. Nano Lett.* 7 (3), 195–208.
- Ge, J., Lei, J., Zare, R.N., 2012. *Nat. Nanotechnol.* 7 (7), 428–432.
- Ghodselahi, T., Vesaghi, M., Shafiekhani, A., Baghizadeh, A., Lameii, M., 2008. *Appl. Surf. Sci.* 255 (5), 2730–2734.
- Giardina, P., Faraco, V., Pezzella, C., Piscitelli, A., Vanhulle, S., Sannia, G., 2010. *Cell. Mol. Life Sci.* 67 (3), 369–385.
- Guo, Y., Li, S., Wang, Y., Zhang, S., 2017. *Anal. Chem.* 89 (4), 2267–2274.
- Jafarinejad, S., Ghazi-Khansari, M., Ghasemi, F., Sasanpour, P., Hormozi-Nezhad, M.R., 2017. *Sci. Rep.* 7 (1), 1–8.
- Jiang, B., Duan, D., Gao, L., Zhou, M., Fan, K., Tang, Y., Xi, J., Bi, Y., Tong, Z., Gao, G.F., Xie, N., 2018. *Nat. Protoc.* 13 (7), 1506–1520.
- Jones, S.M., Solomon, E.I., 2015. *Cell. Mol. Life Sci.* 72 (5), 869–883.
- Ladole, M.R., Pokale, P.B., Patil, S.S., Belokar, P.G., Pandit, A.B., 2020. *Bioresour. Technol.* 317, 124035.
- Lee, I., Cheon, H.J., Adhikari, M.D., Tran, T.D., Yeon, K.-M., Kim, M.I., Kim, J., 2020. *Int. J. Biol. Macromol.* 155, 1520–1531.
- Li, H., Hou, J., Duan, L., Ji, C., Zhang, Y., Chen, V., 2017. *J. Hazard Mater.* 338, 93–101.
- Liang, H., Lin, F., Zhang, Z., Liu, B., Jiang, S., Yuan, Q., Liu, J., 2017. *ACS Appl. Mater. Interfaces* 9 (2), 1352–1360.
- Lin, Z., Xiao, Y., Yin, Y., Hu, W., Liu, W., Yang, H., 2014. *ACS Appl. Mater. Interfaces* 6 (13), 10775–10782.
- Lou, C., Jing, T., Zhou, J., Tian, J., Zheng, Y., Wang, C., Zhao, Z., Lin, J., Liu, H., Zhao, C., 2020. *Int. J. Biol. Macromol.* 149, 1130–1138.
- Lu, X., Li, Y., Du, J., Zhou, X., Xue, Z., Liu, X., Wang, Z., 2011. *Electrochim. Acta* 56 (21), 7261–7266.
- Ma, B., Wang, S., Liu, F., Zhang, S., Duan, J., Li, Z., Kong, Y., Sang, Y., Liu, H., Bu, W., 2018. *J. Am. Chem. Soc.* 141 (2), 849–857.
- Mate, D.M., Alcalde, M., 2015. *Biotechnol. Adv.* 33 (1), 25–40.
- Mei, L.-P., Feng, J.-J., Wu, L., Zhou, J.-Y., Chen, J.-R., Wang, A.-J., 2015. *Biosens. Bioelectron.* 74, 347–352.
- Mohammad, M., Ahmadpoor, F., Shojaosadati, S.A., 2020. *ACS Omega* 5 (30), 18766–18777.
- Nguyen, Q.H., Kim, M.I., 2020. *Trends Anal. Chem.* 116038.
- Park, K.S., Batule, B.S., Chung, M., Kang, K.S., Park, T.J., Kim, M.I., Park, H.G., 2017. *J. Mater. Chem. B* 5 (12), 2231–2234.
- Raval, N., Jogi, H., Gondaliya, P., Kalia, K., Tekade, R.K., 2019. *Sci. Rep.* 9.
- Ren, X., Liu, J., Ren, J., Tang, F., Meng, X., 2015. *Nanoscale* 7 (46), 19641–19646.
- Rincón, R.A., Lau, C., Luckarift, H.R., Garcia, K.E., Adkins, E., Johnson, G.R., Atanassov, P., 2011. *Biosens. Bioelectron.* 27 (1), 132–136.
- Thawari, A.G., Rao, C.P., 2016. *ACS Appl. Mater. Interfaces* 8 (16), 10392–10402.
- Tran, T.D., Kim, M.I., 2018. *Biochip J.* 12 (4), 268–279.
- Verónica Rivas, M., Méndez De Leo, L.P., Hamer, M., Carballo, R., Williams, F.J., 2011. *Langmuir* 27 (17), 10714–10721.
- Vo, T.N., Tran, T.D., Nguyen, H.K., Kim, M.I., Kim, I.T., 2020. *Mater. Lett.* 281, 128662.
- Wang, J., Huang, R., Qi, W., Su, R., Binks, B.P., He, Z., 2019. *Appl. Catal. B Environ.* 254, 452–462.
- Wu, J., Wang, X., Wang, Q., Lou, Z., Li, S., Zhu, Y., Qin, L., Wei, H., 2019. *Chem. Soc. Rev.* 48 (4), 1004–1076.
- Ye, R., Zhu, C., Song, Y., Lu, Q., Ge, X., Yang, X., Zhu, M.J., Du, D., Li, H., Lin, Y., 2016. *Small* 12 (23), 3094–3100.
- Yin, Y., Xiao, Y., Lin, G., Xiao, Q., Lin, Z., Cai, Z., 2015. *J. Mater. Chem. B* 3 (11), 2295–2300.
- Zhang, L., Cui, H., Zou, Z., Garakani, T.M., Novoa-Henriquez, C., Jooyeh, B., Schwaneberg, U., 2019. *Angew. Chem. Int* 58 (14), 4562–4565.
- Zhou, L., Powell, D., Nicholas, K.M., 2007. *Inorg. Chem.* 46 (19), 7789–7799.
- Zhu, X., Huang, J., Liu, J., Zhang, H., Jiang, J., Yu, R., 2017. *Nanoscale* 9 (17), 5658–5663.