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ORIGINAL ARTICLE

Dopamine and norepinephrine assistant-synthesized nanoflowers immobilized membrane with peroxidase mimic activity for efficient detection of model substrates

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Abstract
Herein, we prepared dopamine assistant-synthesized nanoflowers (dNFs) and norepinephrine assistant-synthesized nanoflowers (neNFs) as novel nano-biocatalysts with intrinsic and effective peroxidase mimic activities. The NFs dispersed in solution or immobilized on the filter membrane served as the Fenton reagent in the presence of hydrogen peroxide (H_2O_2) allowing spectrophotometric and colorimetric detection of *m*-cresol and dopamine. Due to its high polar surface property and porosity, the neNFs exhibited remarkably high catalytic activity than dNFs in both solution or in immobilized states. The activity performance of neNFs as a function of varied concentrations of neNFs or *m*-cresol or dopamine or H_2O_2; and reaction time was also studied. The oxidative reaction of 0.5 mM *m*-cresol (in the presence of 4-AAP and H_2O_2) or 0.25 mM dopamine (in the presence of H_2O_2) catalyzed by the neNFs (1 mg/mL) were visually detected within 10 min in solution. Whereas, after immobilization on filter membrane, the colorimetric detection of the same concentrations of *m*-cresol or dopamine was achieved in 30 min using neNFs (2 mg/mL). Furthermore, the effect of repeated use of the neNFs in solution or immobilized states on the catalytic activity was also investigated. We expect that, the NFs formed from non-enzyme molecules possess peroxidase mimic activities that could imply to a study for bioanalytical and biomedical application in the near future.

Keywords Dopamine nanoflowers · Norepinephrine nanoflowers · Fenton reagent · Peroxidase mimic activities

Introduction

The synthesis of enzyme-inorganic nanoflowers (NFs) set-off an encouraging development among enzyme immobilization methods owing to its superb catalytic activities and stabilities (Ge et al. 2012). The mechanism of the formation of NFs in phosphate-buffered saline (PBS) relies on the coordination reaction between amide groups in the protein backbone and copper (II) ions (Cu^{2+}). Initially, copper phosphate nanocrystals ($Cu_3(PO_4)_2$) are formed as seeds and the enzymes bind to these nanocrystals through self-assembly to produce flower-shaped hybrid structures. Since the discovery of the NFs, various enzymes and metal ions under different experimental conditions have been utilized to fabricate these NFs for various purposes (Wang et al. 2013; Yin et al. 2015; Somturk et al. 2015, 2016; Ocsoy et al. 2015; Altinkaynak et al. 2016a, 2016b, 2017; Zhu et al. 2017; Duan et al. 2018; Celik et al. 2018; Tang et al. 2019). In an earlier study, Ocsoy et al. (2015) achieved superior enzymatic activities towards model substrates using horseradish peroxidase (HRP) enzyme as an organic component for the production of HRP–NFs with the incorporation of Cu^{2+} ion. In addition to that, Wang et al. (2013) also introduced rational design for the fabrication of $CaHPO_4$-α-amylase hybrid nano-biocatalytic system relied upon allosteric effect of calcium ions.

Recently, due to the high cost of enzymes, researchers are trying to focus on the synthesis of the NFs with peroxidase-like activities via using non-enzyme organic molecules (Wu et al. 2016; Ildiz et al. 2017; Baldemir et al. 2017; Kilic et al. 2020; Koca et al. 2020; Celik et al. 2020). In a separate study, Ildiz et al. (2017) and Baldemir et al. (2017) worked

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with *Viburnum opulus* (VO) and green tea extract for the synthesis of plant extract-$Cu_3(PO_4)_2$ NFs with intrinsic peroxidase-like activity along with antimicrobial activities. Wu et al. (2016) produced the NFs with Cu^{2+} ions using 20 natural amino acids which served organic parts where the R groups of amino acids in the NFs contributed towards peroxidase-like activity following Fenton-like reaction. In our previous study, we developed catecholamines (dopamine, epinephrine, and norepinephrine) incorporated NFs and examined their peroxidase mimicking and antimicrobial action. This study revealed that organic components in NF formation does not have to be enzymes, proteins, or amino acids; rather, it can be any organic molecule containing free amine groups for coordination reaction with Cu^{2+}, may lead to the formation of NFs (Celik et al. 2020).

Phenolic compounds are used in various industrial processes mainly in production of dyes, plastics and pesticide. However, they are polluting chemicals accumulated in the environment, easily adsorbed by skin exhibiting detrimental effects on human health (Alkasir et al. 2012). Hence, the determination of such compounds are extremely important.

Although many analytical methods have been developed for determination of these compounds including electrochemical techniques (Liu et al 2013) and chromatography (Kalili et al. 2011), colorimetry is widely used due to its simple, low cost and rapid response (Xiong et al. 2015).

Dopamine, as a family of catecholamines, is an important neurotransmitter due to their involvement to physiological role in central and peripheral nervous system. The dysfunction of dopamine system in human body leads to various nervous disorder such as Schizophrenia, Alzheimer and Parkinson's disease (Zhang et al. 2007). Thus, the detection of dopamine level possess great significant in the early diagnosis of such diseases. Recently, colorimetric methods have been developed for the determination of dopamine level in human body (Zhu et al. 2015).

Oriero et al. (2011) developed composite fibers of silicate-polymer matrix immobilized tyrosinase enzyme for colorimetric detection of phenol. Ge et al. (2012) reported phenol and dopamine oxidation using laccase hybrid nanoflowers. To use enzyme-based NFs, pre-synthesized enzyme NFs have been attached to the surface of the porous materials via post-modification (Zhu et al. 2013). Inspired from this work, we rationally prepared dopamine assistant-synthesized (dNFs) and norepinephrine assistant-synthesized nanoflowers (neNFs) in solution except neNFs which is allowed to immobilized on filter membrane, and used them as novel nano-biocatalysts for spectrophotometric and colorimetric detection of *m*-cresol and dopamine. The dNFs and neNFs exhibited peroxidase-like activities in the presence of H_2O_2 due to the Fenton-like reaction. Although enzyme-based hybrid materials are widely used in detection of phenolic compounds and dopamine, the activity of enzymes gets affected by changes in temperature and pH leading to low operational stability (Xiong et al. 2015). To overcome this bottleneck, we succesfully synthesized non-enzyme organic hybrid nanoflowers (neNFs) having peroxidase mimic activity for detection of *m*-cresol and dopamine.

Moreover, the optimization of peroxidase mimicking activity of the neNFs dispersed in solution or immobilized on filter membrane was also checked using varied concentrations of neNFs, *m*-cresol, dopamine in the presence of H_2O_2 and reaction time.

Experimental

Chemicals and materials

Catecholamines (dopamine and norepinephrine), copper (II) sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$), *m*-cresol, 4-aminoantipyrine (4-AAP) and hydrogen peroxide (H_2O_2, 25% w/v), the salt precursor of PBS (NaCl, KCl, Na_2HPO_4, KH_2PO_4) were purchased from Sigma Aldrich. Cellulose acetate syringe filters (0.45 µm) were obtained from Isolab. All solutions were prepared with ultrapure water. All the chemicals were of analytical grade and were used without any further purification.

Instrumentation

The synthesized NFs were characterized with Scanning Electron Microscopy (SEM, ZEISS EVO LS10). UV–Vis Spectrophotometry (Shimadzu UV1800) was used for detection of *m*-cresol.

Formation of dopamine and norepinephrine assistant-synthesized nanoflowers

Dopamine and norepinephrine assistant-synthesized nanoflowers (dNFs and neNFs) were synthesized according to the literature with slight modifications (Celik et al. 2020). 0.02 mg/mL dopamine and norepinephrine was dissolved in distilled water. $CuSO_4$ solution (120 mM, 660 µL) was separately mixed with prepared dopamine and norepinephrine solutions which was then added to 100 mL phosphate buffer saline (PBS) solution (10 mM, pH 7.4). Each mixture was vigorously stirred and incubated at 25 ºC for 3, 6, 12 and 72 h. The precipitates were collected through centrifugation (5000 rpm, 10 min) and washed several times with pure water. The synthesized NFs were dried at 50 ºC and stored for characterization and catalytic activity studies.

Detection of m-cresol and dopamine in solution

The nanoflowers (1 mg/mL) were added to PBS solution (0.1 M, pH 7.4) containing varied concentrations of *m*-cresol ranging from 0.05 to 0.5 mM, 1 mM H_2O_2 and 4 mM 4-AAP followed by incubation at 25 ℃ for 15 min. Subsequently, the mixture was centrifugated at 8000 rpm for 5 min to remove the neNFs. Finally, the absorbance of the supernatant was recorded using the UV–Vis spectrophotometer. The stock solution of 10 mM dopamine was prepared in 1 mM HCl from which dopamine with different concentrations (0.25 and 0.5 mM) were incubated at 25 ℃ for 20 min with the reaction mixture of 0.25 mg/mL neNFs and 0.5 mM H_2O_2 in PBS (0.1 M, pH 7.4). Thereafter, the suspension was centrifuged at 8000 rpm for 5 min to remove the neNFs and the absorbance of supernatant was measured using UV–Vis spectrophotometer.

Detection of m-cresol and dopamine on filter membrane

The suspension of neNFs in PBS (0.1 M, pH 7.4) was allowed to pass through the cellulose acetate membrane of pore size 0.45 µm. The filter surface was covered with neNFs since the size of the neNFs is greater than the pore size of the filter. Later, the mixture containing different concentrations of *m*-cresol, 4 mM 4-AAP in H_2O_2 was injected to the nanoflower-coated filter and kept for 30 min. After allowing the mixture solution pass through the filter, the filtrate was collected and the absorbance was recorded by UV–Vis spectrophotometer. Similarly, for the detection of dopamine on filter membrane, the above-mentioned procedure was opted except the use of 4-AAP as a substrate.

Results and discussion

The earlier report showed that pre-synthesized laccase NF (0.06 mg/mL) deposited on cellulose acetate filter membrane caused oxidation of phenol (with a series of concentrations) within 5 min (Zhu et al. 2013). However, such study used enzyme and failed to establish the effect of reaction parameters on catalytic activities. In the present investigation, we carried out a systematic work using non-enzyme incorporated NFs (dNFs and neNFs) in solution as well as on filter membrane for simple, cost-effective, rapid and repeated detection of *m*-cresol and dopamine molecules through the Fenton reaction. The oxidation of *m*-cresol with 4-AAP and dopamine in solution and on filter membrane were recorded and compared via both UV–Vis spectrophotometer and naked eyes. Furthermore, the procedure was systematically optimized under some experimental parameters. The oxidation of *m*-cresol and dopamine catalyzed by dNFs or neNFs in solution and on filter membrane is illustrated in Fig. 1. The peroxidase-like activity mechanism of NFs depend on Fenton reaction. The Cu^{2+} ions in NFs can react with H_2O_2 to form Cu^{+}. Highly reactive and short-lived hydroxyl radicals species generated from the reaction between Cu^{+} ions and H_2O_2. These radicals eject electron from hydroxyl groups of *m*-cresol, forming quinone radicals, which react with 4-AAP to produce colored quinone imine (Wu et al. 2020). Moreover, NFs can oxidize dopamine in the presence

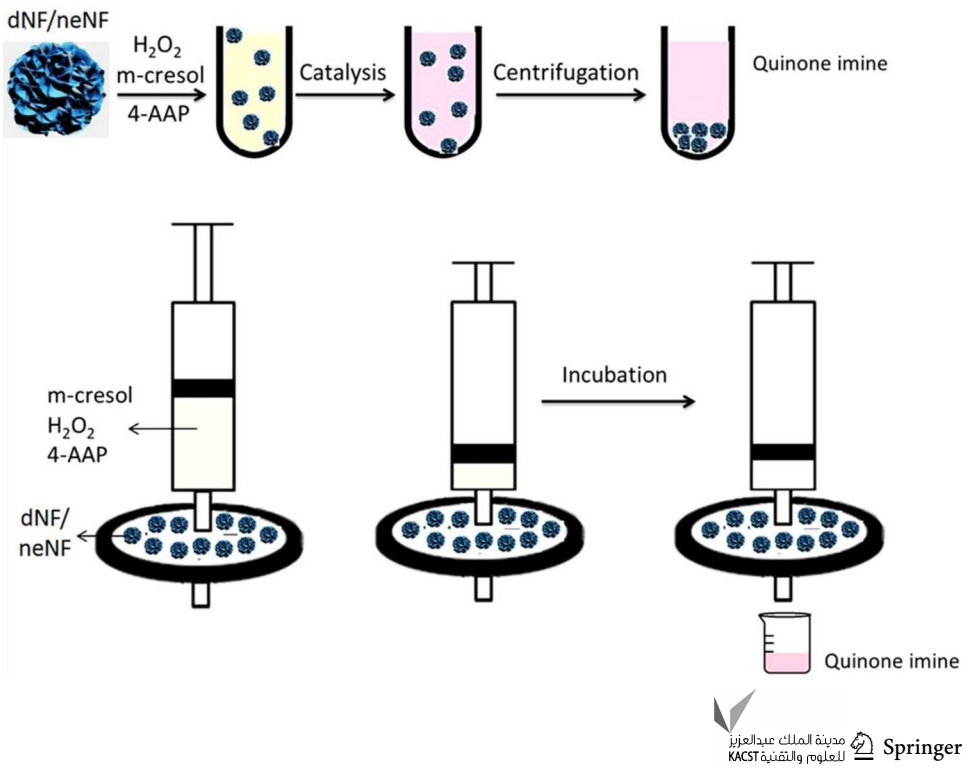

Fig. 1 Illustration of oxidation of *m*-cresol and dopamine by the dNFs and neNFs to colored products in solution and on filter membrane

of H_2O_2 to produce colored quinone type product (Ge et al. 2012). The dopamine and norepinephrine can also serve as green reductant for the formation of NFs eradicate the necessity for toxic reducing agents.

Initially, the activity was carried out in solution under various parameters. To determine the ideal NF with high catalytic activity, the NFs formed with varied incubation time were used for the detection of *m*-cresol. The 1 mg/mL dNF-3 and neNF-3 (formed in 3 h), dNF-12 and neNF-12 (formed in 12 h) and dNF-72 and neNF-72 (formed in 72 h) were separately added into reaction vials containing 4 mM 4-AAP, 1 mM H_2O_2 and 0.5 mM *m*-cresol and was incubated separately for 60 min. Results showed that neNF-3 exhibited much higher peroxidase mimic activity in oxidation of *m*-cresol than dNF-3 (Fig. 2a) due to high surface area with much polar surfaces. BET surface area was measured as 4.0867 and 13.1667 m^2/g for dNF and neNF, respectively, as presented in Electronic Supporting Material (Figure ESM1). The monodispersed and compact morphology of dNF-3 and neNF-3 were visualized under scanning electron microscope (Fig. 2b, c). The EDX analysis and elemental mapping of dNF-3 and neNF-3 were also compeleted and presented in Figure ESM2 and ESM3, respectively.

The effects of experimental parameters on peroxidase mimic activities of the neNF-3 were examined. We demonstrated that the neNF-3 was incubated with *m*-cresol (0.5 mM) and 4 mM 4-AAP in phosphate buffer (0.1 M, pH 7.4) for 10, 30, 60, and 90 min. The oxidized product was detected via spectrophotometer by measuring absorption at 495 nm and via naked eyes for the appearance of pink color (Fig. 3a). After catalysis of series of concentrations of *m*-cresol (from 0.5 to 0.05 mM) by the neNF-3, both faint color changing and absorption [at the lowest *m*-cresol concentration (0.05 mM)] were monitored both visually as well as using spectrophotometer (Fig. 3b). It was noted that the color intensity and absorption of the solution was found to be proportional to the concentration of oxidation product of *m*-cresol. As expected, no color change or no absorption peak was observed in *m*-cresol solution lacking neNFs suggesting intrinsic peroxidase mimic activity of neNFs (Fig. 3c). Interestingly, the neNF-3 itself (without H_2O_2) catalyzed oxidation of the *m*-cresol to antipyrine dye which can be evidenced by absorption spectra as well as the appearance of the distinct pink color. The plausible reason behind it is reduction of Cu^{2+} to Cu^{+}. First, chelation was occurred between Cu^{2+} and *m*-cresol. The redox reaction was observed following oxidation of *m*-cresol and reduction of Cu^{2+} to Cu^{+}. While, the neNF-3 (without H_2O_2) catalyzed oxidation of *m*-cresol in 30 min, the presence of the H_2O_2 may shorten the oxidative coupling reaction time from 30 to 10 min.

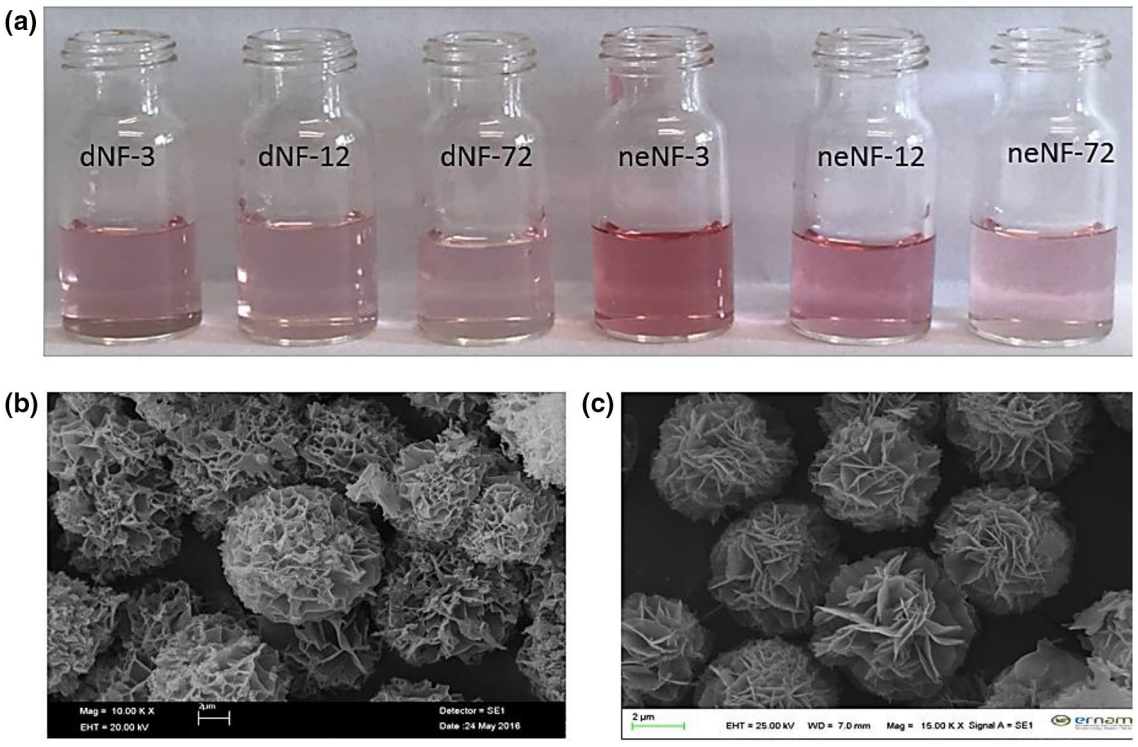

Fig. 2 **a** Peroxidase mimic activities of the dNFs and neNFs formed in various incubation times towards *m*-cresol. SEM images of **b** dNF-3 and **c** neNF-3

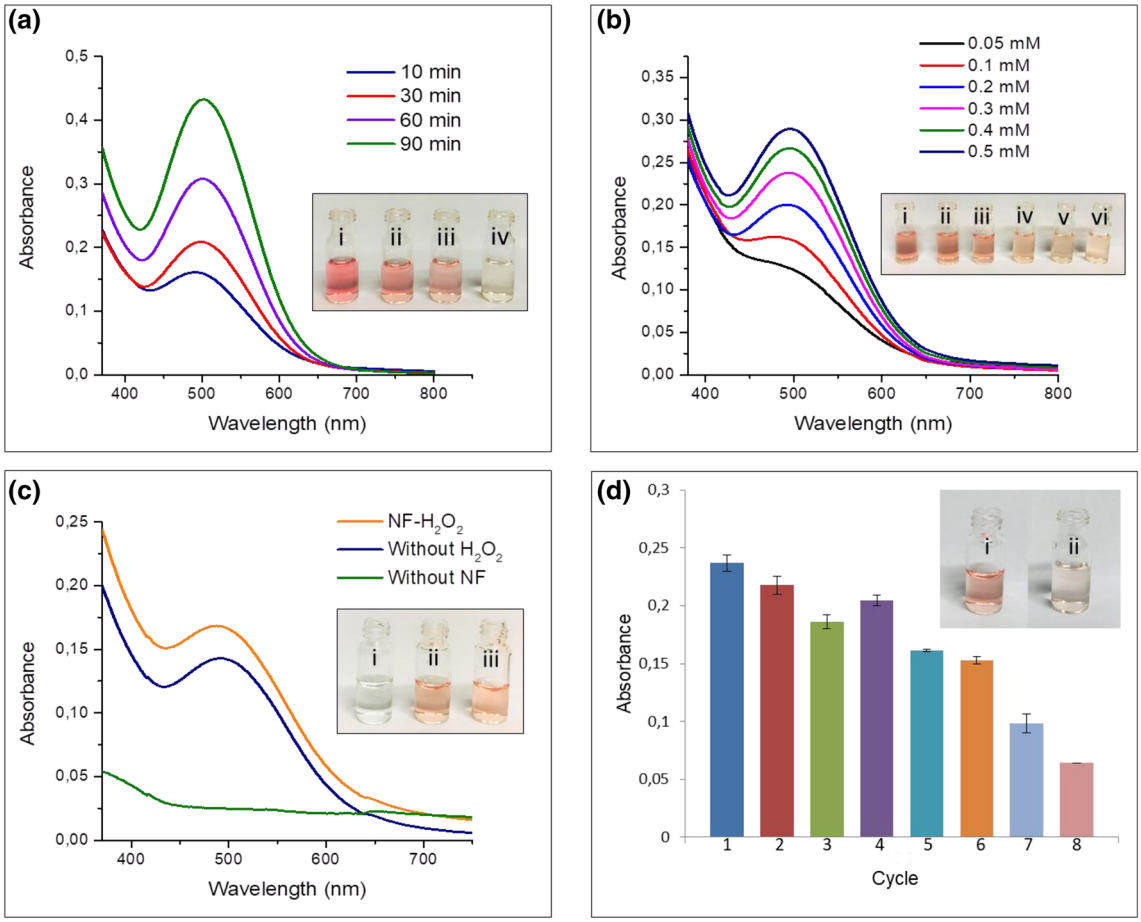

Fig. 3 Peroxidase mimic activities of the neNF-3 dispersed in solution as a function of **a** reaction time, **b** m-cresol concentration, **c** H_2O_2 presence or absence and **d** repeated use

To demonstrate the repeated use capability of the neNF-3, the procedure in Fig. 2 was followed. Basically, neNF-3 (1 mg/mL) was dispersed in phosphate buffer (pH 7.4) containing 4-AAP and H_2O_2. The resulting mixture was incubated at 25 °C for 10 min. After incubation, the mixture was centrifuged at 5000 rpm for 3 min and absorption of the supernatant was measured at 495 nm. The new phosphate buffer containing 4-AAP and H_2O_2 was deposited on the precipitate of the neNF-3. Thereafter, the final mixture was sonicated, incubated and centrifuged followed by measuring the absorbance of the supernatant. The precipitate of neNF-3 was repeatedly used (eight times) for testing the performance of the catalytic cycle (Fig. 3d). This procedure was repeated three times. Error bars indicate standard deviations obtained from three independent measurements. Initial absorption value and color of the reaction solution are represented with the blue column and the inset photograph (i), respectively. After the 8th cycle, neNF-3 lost 70% of its initial activity, which is quite consistent with color of reaction solution as shown in inset photograph (ii).

The peroxidase mimic activity of the neNF-3 immobilized filter membrane was investigated based upon the concentration of neNF-3 and H_2O_2 under various reaction times. The determined amounts of NFs were deposited on filter membrane due to average size of NFs is much larger than pore size of filter membrane. Figure 4a shows that 0.5, 2 and 4 mg/mL of the neNF-3 were separately adsorbed on filter membrane, then 4 mM 4-AAP, 1 mM H_2O_2 and 0.5 mM *m*-cresol were immobilized on filter membrane and incubated for 12 h. When the amount of neNF-3 was increased, peroxidase mimic activity of filter membrane was enhanced and more amount of oxidized *m*-cresol was observed. When 4 mg/mL neNF-3 was used, the intensity of the absorption spectrum of the product solution was increased (red line) and the more intense color of the product solution was observed as shown in the photograph (iii) in Fig. 4a, both of which are proportional to the amount of the neNF-3 immobilized on the filter membrane. In Fig. 4b, the same procedure as of Fig. 4a was applied but only reaction time was fixed to 4 h. The results exhibited that peroxidase mimic activities of neNF-3 (0.5, 1, 2, and 4 mg/mL) adsorbed filter membranes

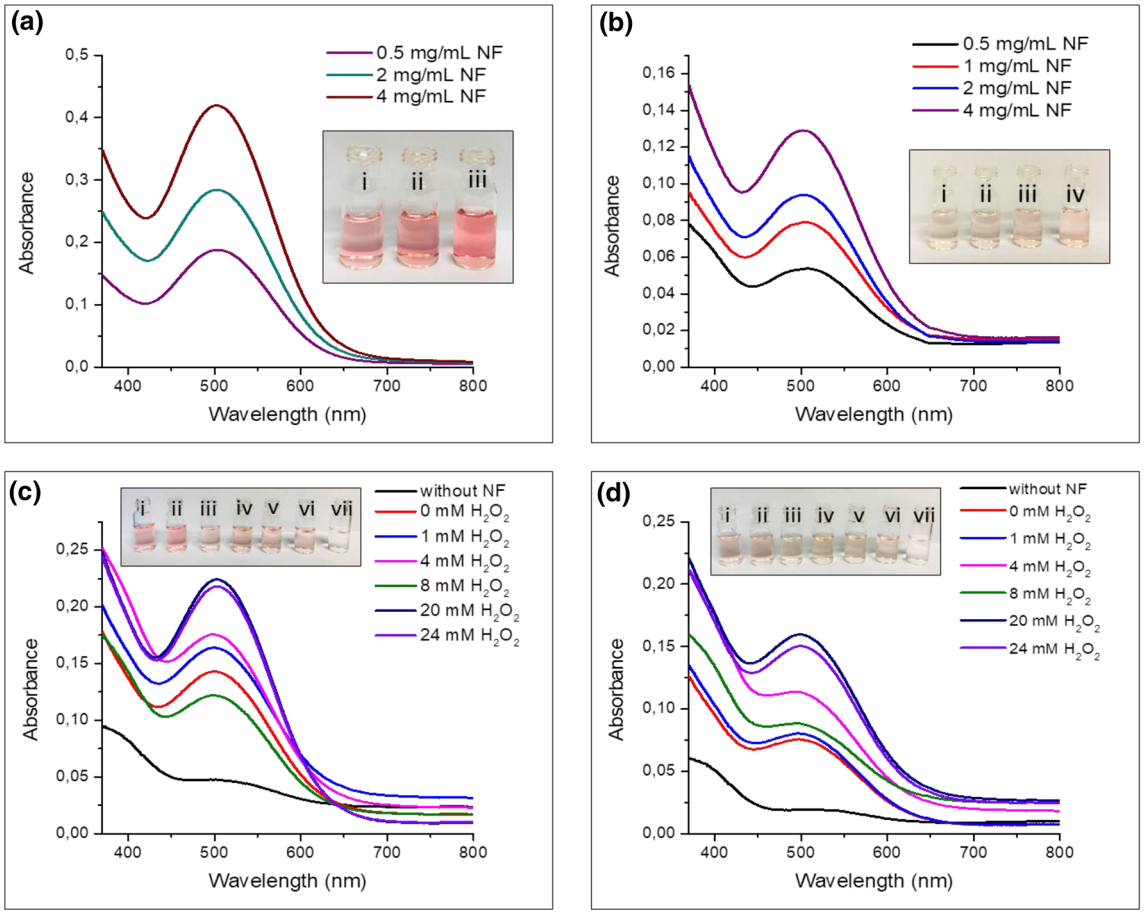

Fig.4 Peroxidase mimic activities of the neNF-3 adsorbed on filter membrane under some experimental parameters. The effect of the neNF-3 concentration in reaction time of **a** 12 h and **b** 4 h. The effect of the H_2O_2 concentration in reaction time, **c** 1 h and **d** 30 min

were drastically decreased as reflecting in the absorption spectrum and color intensity of the product solutions.

To shorten reaction time and produce effective peroxidase mimic activity, we manipulated concentrations of H_2O_2 (1, 4, 8, 20, and 24 mM) in 1 h and also 30 min reaction times as shown in Fig. 4c and d, respectively. We conclude that the optimal peroxidase mimic activity was observed with the concentrations of H_2O_2 arranged between 20 and 24 mM. The absorption spectrum of the oxidized *m*-cresol and its solution colors in photographs (i and ii) also proved that the most efficient peroxidase mimic activity of the neNF-3 was witnessed at corresponding H_2O_2 concentrations with great consistency.

Moreover, peroxidase mimic activity of 2 mg/mL neNF-3 adsorbed filter membrane was evaluated in the presence of 20 mM H_2O_2 against *m*-cresol concentrations, reaction time, and repeated use. Figure 5a shows that oxidation of *m*-cresol with a series of concentrations was successfully catalyzed by the filter membrane in 60 min incubation. As expected, an increase in peroxidase mimic activity of the filter membrane was proportional to *m*-cresol concentration. It was also noticed that increases in absorption value and color intensity (photograph in Fig. 1a) of the oxidized *m*-cresol are consistent with each other. We examined the performance of the filter membrane as a function of reaction time against 0.5 mM *m*-cresol. Although most efficient catalytic activity was observed in 60 min, quite acceptable activity was recorded in 30 min and even in 15 min as shown in Fig. 5b. Results showed that even after cyclic use of the filter membrane the initial activity remained upto 76%. Hence, we interpret that to maintain most of the initial activity, it can be achieved through the limited mobility of the neNF-3 on the filter membrane.

As a final peroxidase mimic study, the same experimental procedure for the filter membrane as used for *m*-cresol was applied against dopamine (DA) as a new and alternative substrate. The 0.25 mM (red line) and 0.5 mM (pink line) concentrations of oxidation of DA molecules catalyzed by the filter membrane were monitored by UV–Vis spectrometer at 485 nm. However, no remarkable absorption values of oxidized DA (quinone imine) (black line and blue line) were observed without neNFs (Fig. 6).

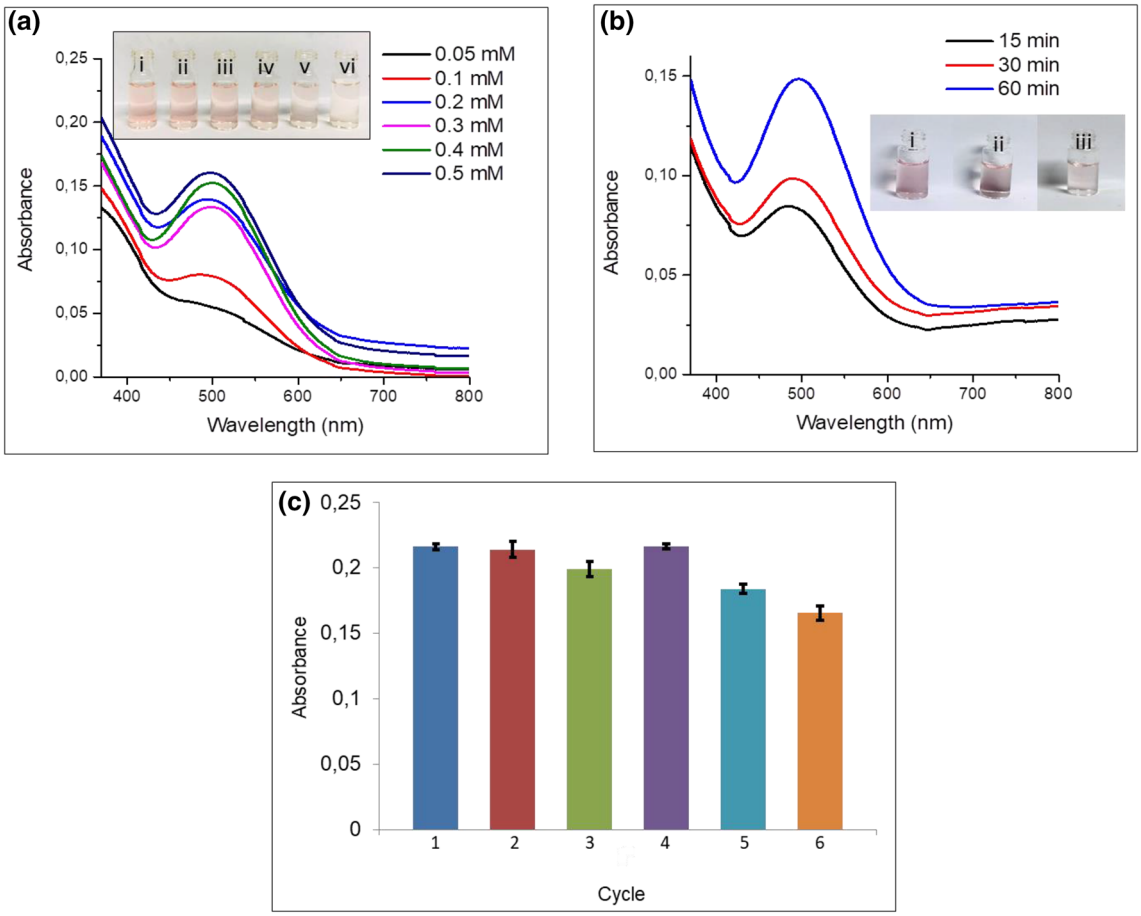

Fig. 5 Peroxidase mimic activities of the neNF-3 adsorbed filter membrane as a function of **a** *m*-cresol concentration, **b** reaction time and **c** repeated use

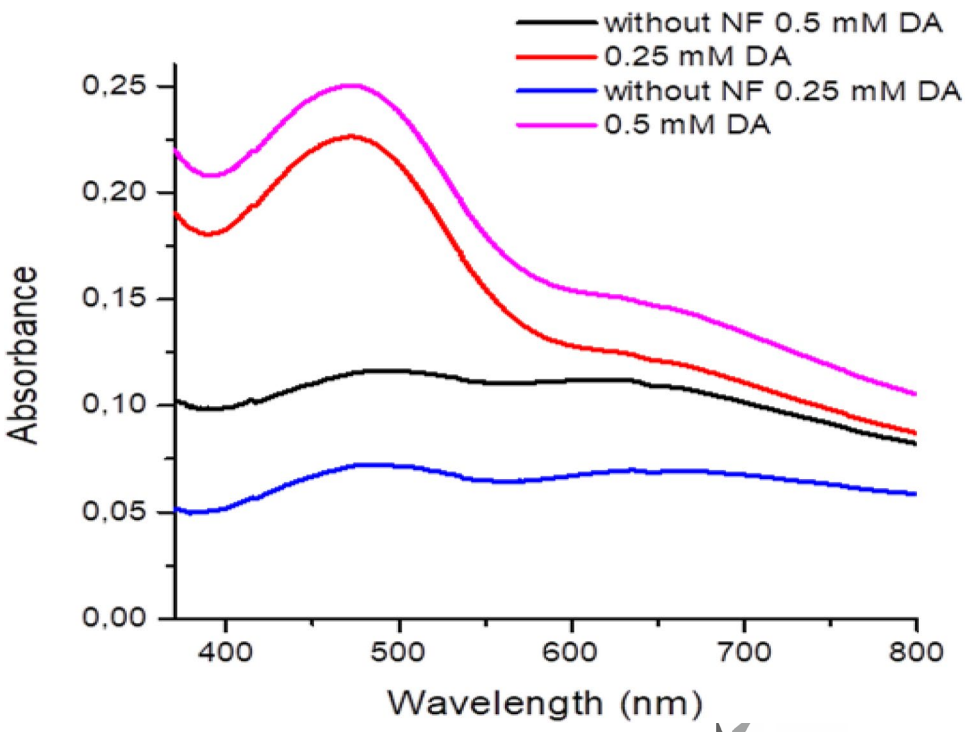

Fig. 6 Peroxidase mimic activities of the neNF-3 adsorbed filter membrane against various concentration of DA

Conclusion

We have developed a novel and simple strategy to use dNFs and neNFs in solution and on filter membrane as nano-biocatalysts for spectrophotometric and colorimetric detection of *m*-cresol and dopamine. The driving force for peroxidase mimic activities of the dNFs and neNFs is based on the principle of the Fenton reaction in the presence of H_2O_2. In terms of the experimental results, the neNF-3 showed much higher peroxidase mimic activity compared to dNFs and neNFs produced in different forms owing to its high polar surface property and porous structure. The peroxidase mimic performance of the neNF-3 adsorbed filter membrane demonstrated that it can be considered as a stable and portable biosensor for simple, rapid, and on-site detection of substrates and also, it can be used in various scientific and industrial applications. We also claim that effective catalytic activities can be achieved without the incorporation of enzymes.

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Author contributions SD performed all experiments as a first author. CC contributed to experiments IO and AKM conceived the original idea and designed the project. SD, CC, AKM and IO mainly wrote the manuscript.

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Compliance with ethical standards

Conflict of interest The author(s) declare that they have no competing interests.

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