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Polymeric Membrane Neutral Phenol-Sensitive Electrodes for Potentiometric G-Quadruplex/Hemin DNAzyme-Based Biosensing

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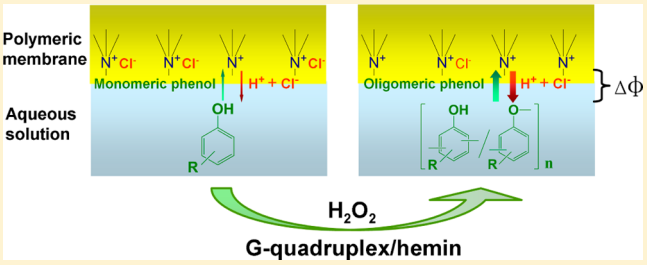
Supporting Information

ABSTRACT: The first potentiometric transducer for G-quadruplex/hemin DNAzyme-based biosensing has been developed by using potential responses of electrically neutral oligomeric phenols on polymeric membrane electrodes. In the presence of G-quadruplex/hemin DNAzyme and H_2O_2, monomeric phenols (e.g., phenol, methylphenols, and methoxyphenols) can be condensed into oligomeric phenols. Because both substrates and products are nonionic under optimal pH conditions, these reactions are traditionally not considered in designing potentiometric biosensing schemes. However, in this paper, the electrically neutral oligomeric phenols have been found to induce highly sensitive potential responses on quaternary ammonium salt-doped polymeric membrane electrodes owing to their high lipophilicities. In contrast, the potential responses to monomeric phenolic substrates are rather low. Thus, the G-quadruplex/hemin DNAzyme-catalyzed oxidative coupling of monomeric phenols can induce large potential signals, and the catalytic activities of DNAzymes can be probed. A comparison of potential responses induced by peroxidations of 13 monomeric phenols indicates that *p*-methoxyphenol is the most efficient substrate for potentiometric detection of G-quadruplex/hemin DNAzymes. Finally, two label-free and separation-free potentiometric DNA assay protocols based on the G-quadruplex/hemin DNAzyme have been developed with sensitivities higher than those of colorimetric and fluorometric methods. Coupled with other features such as reliable instrumentation, low cost, ease of miniaturization, and resistance to color and turbid interferences, the proposed polymeric membrane-based potentiometric sensor promises to be a competitive transducer for peroxidase-mimicking DNAzyme-involved biosensing.



Catalytic DNA molecules (DNAzymes) have found wide use in biosensing, biotechnology, and logic gate applications with advantages of simple preparation, easy modification, low cost, and high thermal stability.[1,2] Among various DNAzymes, the G-quadruplex showing superior peroxidase-like activity in the presence of hemin has attracted considerable attention in recent years. The G-quadruplex/hemin complexes act well as the amplifying readout units of various nucleic acid-based biorecognition events, by which a number of facile biosensing protocols have been developed for nucleic acids, metal ions, small organic targets, proteins, and enzymes.[3,4]

With the wide use of G-quadruplex/hemin DNAzymes, effective transducers of their catalytic activities are also highly desired. The most commonly used transduction modes are spectrophotometry and chemiluminometry, for which 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and luminol are usually employed as the reducing substrates, respectively.[5] Fluorescence detection can also be used to probe peroxidatic activities of G-quadruplex/hemin DNAzymes using substrates such as fluorescein.[6] For electrochemical transduction, amperometry has proven to be competent based on the redox behaviors of the products from the G-quadruplex/hemin DNAzyme-catalyzed oxidations.[7]

Potentiometry is a powerful electrochemical technique for approaching 100 analytes.[8] Its instrument is simple, reliable, and cheap. It is easy to be miniaturized and integrated. Also, compared with spectrometric methods, potentiometry is resistant to color and turbid interferences, which are usually encountered for real-sample bioanalyses. These potential advantages have been manifested by its worldwide applications in blood electrolyte analyses.[9] However, potentiometry has never been used to transduce peroxidase-mimicking G-quadruplex/hemin DNAzyme-involved biorecognition events. Fluoride ion-selective electrodes and iodide ion-selective electrodes were used to measure peroxidatic activities several

Received: December 8, 2012
Accepted: January 5, 2013
Published: January 5, 2013

decades ago. However, their sensitivities are poor and they can only be used under acidic pH conditions to avoid the interference from hydroxyl ion,[10,11] which limit their applications. Although other peroxidatic reactions have also been screened for potentiometric peroxidase detection, no significant advances have been made.[12]

Traditionally, to monitor an analytically important chemical reaction by potentiometry, one will examine ionic species involved in the reaction and find a suitable ion-sensitive membrane electrode to detect the ionic reactant or ionic product.[10−13] In contrast, electrically neutral species would not be considered unless they could be directly converted into ions in the aqueous phase (e.g., CO_2 to HCO_3^-). In the past two decades, several classes of nonionic organic species such as surfactants, phenols, and lipids have been found to induce potential responses on solvent polymeric membrane electrodes via perturbing charge separation on the membrane−sample interfaces.[14−18] However, these nonclassical potential responses have never been utilized in potentiometric biosensing presumably owing to their limited sensitivities.

Here, potential responses to neutral phenols are addressed, because phenolic compounds are involved in the H_2O_2-mediated oxidation reactions catalyzed by G-quadruplex/hemin DNAzyme. It has been found that highly lipophilic neutral phenols such as phenolic dimers show much higher sensitivities on the quaternary ammonium salt-doped polymeric membrane electrode as compared to phenolic monomers under near-neutral pH conditions. Based on the superior potential responses to the neutral oligomeric phenols generated in the G-quadruplex/hemin DNAzyme-catalyzed oxidations of monomeric phenols, the potentiometric probing of catalytic activities of peroxidase-mimicking DNAzymes has been fulfilled. By using the proposed oligomeric phenol-sensitive polymeric membrane electrode, two label-free and separation-free potentiometric DNA hybridization assay protocols based on G-quadruplex/hemin DNAzymes have been developed.

■ EXPERIMENTAL SECTION

Reagents and Materials. Tridodecylmethylammonium chloride (TDMAC), tetradodecylammonium chloride, trihexyltetradecylphosphonium chloride, di-*n*-octyl phthalate (DOP), *o*-nitrophenyl octylether (*o*-NPOE), dibutyl phthalate (DBP), bis(*o*-ethylhexyl) sebacate (DOS), high molecular weight poly(vinyl chloride) (PVC), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Sigma-Aldrich. All phenols are purchased from J&K Scientific Ltd. All DNA products were synthesized by Shanghai Sunny Biotech Co., Ltd. Aqueous solutions were prepared with freshly deionized water (18.2 MΩ cm specific resistance) obtained with a Pall Cascada laboratory water system.

ISE Preparation and EMF Measurements. Polymeric membranes containing PVC and plasticizer in a weight ratio of 1:1 and TDMAC (10 mM/kg) were prepared by solvent-casting with tetrahydrofuran as the casting solvent. After transfer of the cocktail to a glass ring fixed on a glass plate and evaporation of the tetrahydrofuran overnight, a uniform membrane was obtained. Disks of 5-mm diameter were punched from the parent membrane and glued to plasticized PVC tubes (i.d. 3 mm, o.d. 5 mm) to fabricate the polymeric membrane electrodes. All electrodes were conditioned overnight in HEPES−NH_4OAc−NaOAc−KCl buffer (25 mM HEPES−NH_4OH, 20 mM KCl, 50 mM NH_4OAc, 150 mM NaOAc, pH 7.4), and the same solution was used as the inner filling medium.

All electromotive force (EMF) values were measured using a CHI 760D electrochemical workstation (Shanghai Chenhua Apparatus Corporation, China) in the following galvanic cell in a Faraday cage: Ag, AgCl/3 M KCl/1 M LiOAc/sample solution/sensing membrane/HEPES−NH_4OAc−NaOAc−KCl buffer/AgCl, Ag. Potential responses to various concentrations of monohydric and dihydric phenols were recorded when the potential drifts became smaller than 1 mV/min, and the response time was generally less than 5 min.

HPLC Analysis. An HPLC-PDA system (Waters, Milford, MA) equipped with a Sunfire C18 reverse phase column was used to quantify phenols. Elutions were isocratic with mobile phases (1.0 mL/min) consisting of water and methanol (v/v) (50:50 for *m*- and *p*-hydroxyphenol, 30:70 for methylphenols, and 40:60 for phenol, *o*-hydroxyphenol, and methoxyphenols).

DNA Hybridization Assays. In the first approach, the hairpin DNA probe (DNA1: 5′-CCCTACCCAACAGGCGG-CCTTAACTGTAGTACTGGTGAAATTGCTGCCTGG-GTAGGGCGGGTTGGG-3′) at 100 nM and different concentrations of target DNA (DNA2: 5′-GGCAGCAAT-TTCACCAGTACTACAGTTAAGGCCGCCTGT-3′) or non-complementary DNA (DNA3: 5′-CCGTCGTTAAAG-TGGTCATGATGTCAATTCCGGCGGACA-3′) in the HEPES−NH_4OAc−NaOAc−KCl buffer at pH 7.4 containing 1% DMSO were heated at 95 °C for 5 min and gradually cooled to room temperature. Then 50 nM hemin was added to the samples. After a 1 h incubation, the electrodes were inserted into the samples, and *p*-methoxyphenol (*p*-MOP) and H_2O_2 were added to induce the potential responses.

In the second approach, two DNA probes (DNA4: 5′-ACAGGCGGCCTTAACTGTAGTTGGGTAGGGCGGG-3′ and DNA5: 5′-TGGGTCTGGTGAAATTGCTGCC-3′) both at 100 nM and different concentrations of target DNA (DNA2) or noncomplementary DNA (DNA 3) in the HEPES−NH_4OAc−NaOAc−KCl buffer containing 1% DMSO were heated at 95 °C for 5 min and gradually cooled to room temperature. After a 1 h incubation with 50 nM hemin, *p*-MOP oxidation-induced potential signals on the polymeric membrane electrodes were recorded.

■ RESULTS AND DISCUSSION

Principle of Responses. The unexpected potential responses of neutral phenols were first reported by the group of Umezawa.[15] Under near-neutral pH conditions (e.g., pH 6.0), neutral phenols and their derivatives induce anionic potential responses on quaternary ammonium salt-doped polymeric membranes via complexations with quaternary ammonium salts and subsequent ejections of dissociated acids from the membrane into the aqueous phase. Unfortunately, most of these phenols can only induce significant potential responses at concentrations of higher than 10^{-3} M even using the elegantly optimized membrane and the chloride ion-free background solution in a low ionic strength. Such poor sensitivities preclude the potential bioanalytical applications of these nonclassical responses although the response principles were elucidated 15 years ago.

From the potential responses of chloro-, methyl-, and hydroxy-substituted phenols, it has been found that phenols with higher lipophilicities show higher response sensitivities.[15] Enlightened by this rule, we pay attention to a list of phenols bearing more than one benzene ring rather than the previously

investigated phenols with only one benzene ring. The introduction of more benzene rings could significantly increase the lipophilicities of phenols and, therefore, is expected to yield improved sensitivities. In Figure 1, we compare the responses

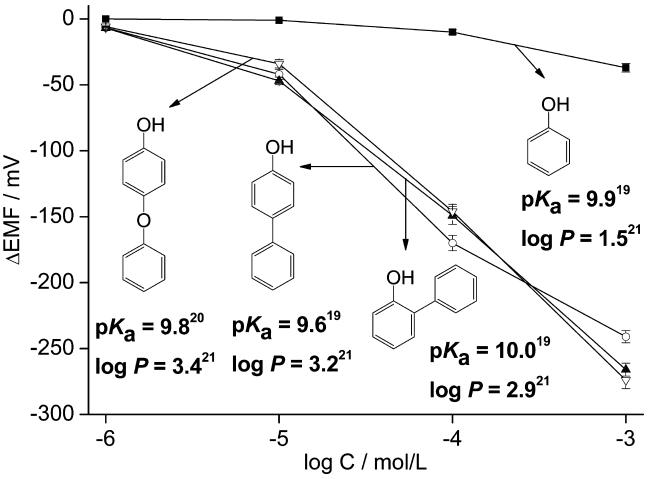


Figure 1. Potential responses to phenol, *p*-phenoxyphenol, *p*-phenylphenol, and *o*-phenlylphenol in the HEPES−NH_4OAc−NaOAc−KCl buffer at pH 7.4 using the TDMAC−DOP−PVC electrode. log *P* denotes the logarithm of the partition coefficient in the *n*-octanol/water system. Each error bar represents the standard deviation of three measurements.

of several monohydric phenol analogues bearing two benzene rings on the TDMAC-doped polymeric membrane electrode with that of phenol. Indeed, neutral phenylphenol and phenoxyphenol show sensitivities much higher than that of phenol. Similarly, for dihydric phenols, the introduction of more benzene ring also largely enhances the potential responses (Figure S1, Supporting Information).

The products of the H_2O_2-mediated oxidations of monomeric phenols are various condensed phenols formed by the coupling of phenoxy radicals.[22] Interestingly, these condensed products possess more benzene rings and higher lipophilicities as compared to the monomeric phenolic substrates. For example, the peroxidation products of the unsubstituted phenol are condensed phenols including *p*-phenoxyphenol shown in Figure 1 and 4,4-dihydroxybiphenyl shown in Figure S1.[23] Because these condensed products could induce potential responses significantly larger than those of the monomeric phenolic substrates, potentiometric sensing of these oxidation reactions may be fulfilled by using suitable polymeric membrane electrodes.

To explore this possibility, the G-quadruplex/hemin DNAzyme-catalyzed oxidation of *p*-methylphenol (*p*-MP) was employed because *p*-MP was found to be an excellent DNAzyme substrate with high reaction efficiency (see below), and its oxidation products are relatively well identified.[24,25] As shown in Figure 2, although the potential response of *p*-MP on the TDMAC-doped polymeric membrane electrode is rather low, a large potential response can be obtained upon its H_2O_2-mediated oxidation catalyzed by the G-quadruplex/hemin complex. The condensed phenolic products of *p*-MP mainly include its C−C, C−O coupling dimers and trimers (Scheme 1). In analogy to phenols with two benzene rings in Figures 1 and S1, these condensed phenolic products are also dominatingly nonionic and possess quite high lipophilicities.

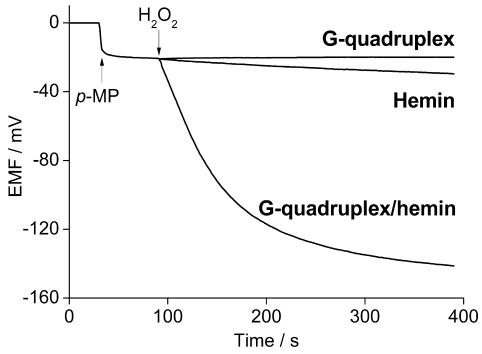
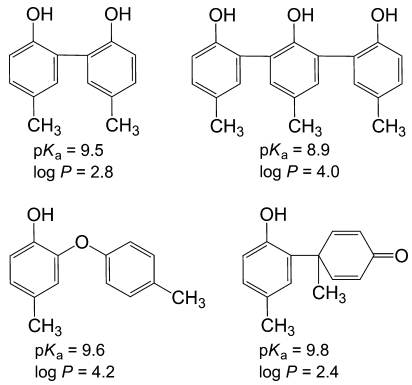


Figure 2. Potential responses to the H_2O_2-mediated oxidation of *p*-MP catalyzed by the G-quadruplex/hemin DNAzyme, the G-quadruplex, and hemin, respectively, in HEPES−NH_4OAc−NaOAc−KCl buffer at pH 7.4: *p*-MP, 0.1 mM; H_2O_2, 5 mM; G-quadruplex (5′-GGGTAGGGCGGGTTGGGT-3′),[26] 0.1 μM; hemin, 0.1 μM. The G-quadruplex and hemin were incubated for 1 h to form the DNAzyme.

Scheme 1. Products of the H_2O_2-Mediated Oxidation of *p*-Methylphenol[24,25] [a]



[a]The shown pK_a values are all the first pK_a values calculated by ACD/PhysChem Suite,[21] below which phenols are non-ionic. log *P* is also calculated by ACD/PhysChem Suite.

Therefore, they should be responsible for the potential responses of the *p*-MP oxidation although the direct observation of responses of these products was not conducted owing to their commercial unavailability.

Optimizations of Phenolic Substrate and Sensing Membrane. To choose the phenolic substrate most suitable for potentiometric biosensing based on neutral condensed phenols, potential responses induced by the G-quadruplex/hemin DNAzyme-catalyzed peroxidations of 10 commonly used phenols were investigated. As shown in Figure 3, *p*-methoxyphenol (*p*-MOP) and *p*-MP induce much larger potential responses upon peroxidation than other phenols on the TDMAC-doped polymeric membrane electrode. By using HPLC, the degradation degrees of *p*-MOP and *p*-MP for oxidation of 5 min were determined to be 13% and 10%, respectively, while those of *o*-, *m*-methylphenol, *o*-, *m*-methoxyphenol, *m*-hydroxyphenol, and phenol are lower than 1%. Therefore, the superior responses induced by oxidations of *p*-MOP and *p*-MP may be attributed to their larger reaction rates.

However, in contrast to the oxidations of the above-mentioned eight phenolic substrates, those of *p*-hydroxyphenol and *o*-hydroxyphenol show largely different relationships

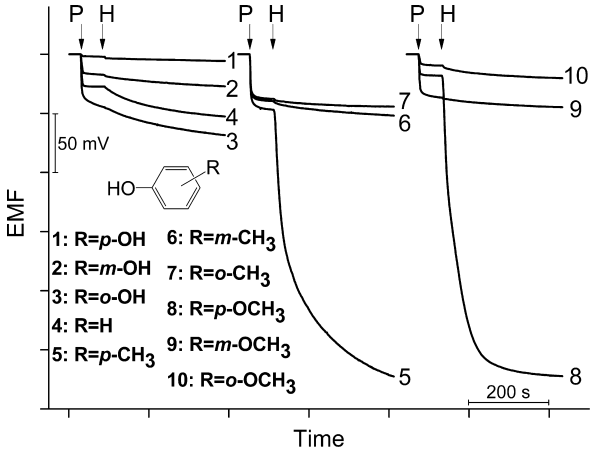


Figure 3. Potential responses to the G-quadruplex/hemin DNAzyme-catalyzed peroxidations of 10 phenols on the TDMAC−DOP polymeric membrane electrodes in HEPES−NH_4OAc−NaOAc−KCl buffer at pH 7.4: phenolic substrate, 0.5 mM; H_2O_2, 25 mM; G-quadruplex, 0.2 μM; hemin, 0.2 μM. G-quadruplex and hemin were incubated for 1 h to form the DNAzyme. "P" and "H" indicate the phenolic substrates and H_2O_2, respectively.

between the reaction rates and potential responses. The degradation degrees of *p*-hydroxyphenol and *o*-hydroxyphenol are as high as 58% and ca. 40%, respectively, whereas no large potential responses were observed. This is probably due to the different response sensitivities of different products depending on their acidities and lipophilicities.[15] The clear knowledge of the exact structure, pK_a and log *P* of each product, and its proportion in total products would enable the elucidation of the different response behaviors. However, because many kinds of products could be formed via various phenolic coupling modes and some of them are unstable, the identification and characterization of the products of every phenolic oxidation reaction are quite difficult. Here, only the oxidation of *p*-hydroxyphenol is noted. Unlike other examined phenols which undergo intermolecular coupling, the H_2O_2-mediated oxidation product of *p*-hydroxyphenol is *p*-benzoquinone (this product was confirmed by its retention time of 4.1 min and characteristic absorption peak at 245 nm in HPLC-PDA), which could not be condensed.[27] Because the employed electrode does not respond to *p*-benzoquinone, this reaction is supposed to induce no potential response according to the condensed phenol-based response principle. Indeed, in addition to an immediate potential decrease of ca. 1.5 mV caused by H_2O_2 itself, no appreciable potential response was observed for *p*-hydroxyphenol upon its DNAzyme-catalyzed oxidation. Moreover, the increase of G-quadruplex/hemin DNAzyme concentration does not cause any improvement in this potential response although it accelerates the oxidation reaction (data not shown), which further demonstrates that the employed electrode is not responsive to the oxidation of *p*-hydroxyphenol.

Considering the small substrate response and superior reaction response of *p*-MOP, it seems to be the best phenolic substrate for potentiometric measuring of DNAzyme activities. We further investigated the potential responses induced by the oxidations of its homologues including *p*-ethoxylphenol, *p*-propoxylphenol, and *p*-butyoxylphenol (Figure S2, Supporting Information). *p*-MOP also shows the largest potential responses upon oxidation owing to its largest reaction rate. Therefore, in the subsequent applications, *p*-MOP was used as the substrate of the G-quadruplex/hemin DNAzyme.

For the sensing membrane, TDMAC was used as the recognition element because it is the most commonly used ionic additive for anion-selective polymeric membrane electrodes and has better commercial availability. Besides TDMAC, other quaternary ammonium salts (e.g., tetradodecylammonium chloride) and phosphonium salts (e.g., trihexyltetradecylphosphonium chloride) could also work (data not shown). The sensing membrane plasticized with DOP shows the best sensitivity as compared to DOS, DBP, and *o*-NPOE (Figure S3, Supporting Information). This is probably because of the solvation effect of DOP on the neutral oligomeric phenols, which could favor the extraction of phenols into the polymeric membrane and enhance the charge-separation process across the membrane−sample interface. Therefore, the TDMAC−DOP membrane was used throughout the study. After each measurement on this membrane, the baseline potential could readily return to the initial value upon a 2 × 3 min washing step using a mixture of HEPES−NH_4OAc−NaOAc−KCl buffer and ethanol (4/1, v/v). The standard deviation of potential responses toward *p*-MOP oxidation (0.5 mM *p*-MOP, 25 mM H_2O_2, 5 nM G-quadruplex/5 nM hemin, and 5 min oxidation) for five consecutive measurements with the same electrode is 1.6 mV (the mean potential response is 33 mV). However, because the ion exchanger-based polymeric membrane is quite inexpensive and easily prepared, the electrodes were singly used in this study to avoid the regeneration steps.

G-Quadruplex/Hemin DNAzyme-Based DNA Assays Using the Neutral Phenol-Sensitive Electrode. To examine the applicability of the neutral condensed phenol-sensitive polymeric membrane electrode in biosensing, two label-free and separation-free potentiometric DNA assay protocols were developed based on the peroxidatic activity of the G-quadruplex/hemin DNAzyme. In one approach, a hairpin structure with a G-rich sequence partly hybridized in the stem region and a recognition domain in the loop was employed as the probe DNA. The hybridization of the target DNA with the recognition sequence could open the hairpin structure and release the G-rich sequence to assemble into the peroxidase-mimicking G-quadruplex DNAzyme in the presence of hemin (Figure 4A).[28] As shown in Figure 4B, by using the electrode responsive to the *p*-MOP oxidation, potential responses proportional to the concentration of the target DNA (1 to 100 nM) were obtained and the target DNA at a concentration as low as 0.2 nM could be detected. For the noncomplementary DNA (DNA3), however, negligible changes in the potential responses as compared to that of the blank solution were observed even at a high level of 100 nM (data not shown), which confirms that the potential signals were induced by the specific hybridization of the target DNA with the probe DNA. Notably, the potential response for the blank solution is larger than that induced by the hemin-catalyzed *p*-MOP oxidation. This is probably due to the fact that a small amount of the G-quadruplex might exist even in the absence of the target DNA, which could form the peroxidase-mimicking DNAzyme with hemin and thus induce a catalytic activity larger than that of hemin itself.

The second approach utilizes two single-stranded DNA probes both with the recognition sequence and the split G-quadruplex-forming sequence. The target DNA could hybridize with the recognition sequences of two probes and induce the assembly of the G-quadruplex from two overhanging split G-

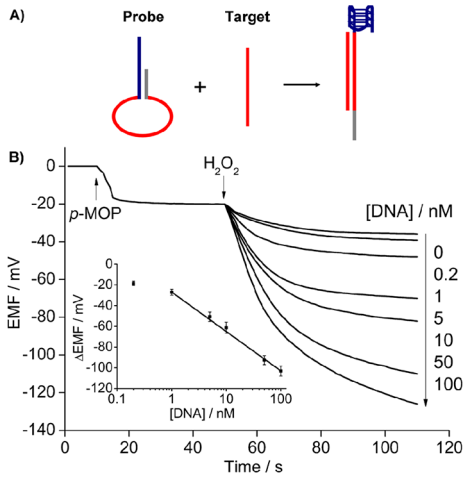


Figure 4. A: Schematic representation of the DNA hybridization assay based on the hairpin DNA probe. B: Potential traces for H_2O_2-mediated *p*-MOP oxidation (*p*-MOP, 0.5 mM; H_2O_2, 25 mM) in the presence of 100 nM hairpin probe DNA (DNA1) and different concentrations of target DNA (DNA2). The inset shows the calibration curve for DNA detection, which is plotted by using potential decreases at 60 s after additions of H_2O_2.

rich sequences (Figure 5A).[29,30] In the presence of hemin, the assembled G-quadruplex could show the peroxidase-like activity

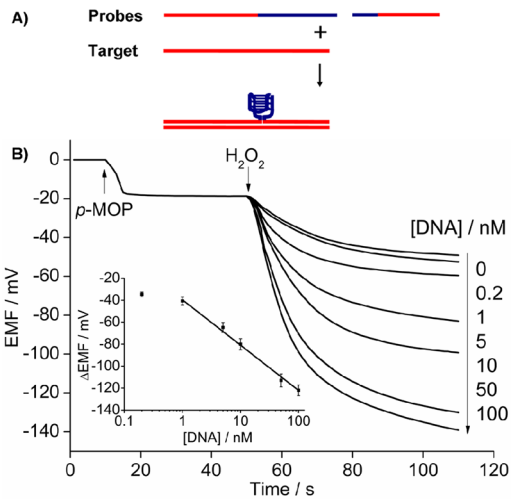


Figure 5. A: Schematic representation of the DNA hybridization assay based on the two probes possessing split G-quadruplex. B: Potential traces for H_2O_2-mediated *p*-MOP oxidation (*p*-MOP, 0.5 mM; H_2O_2, 25 mM) in the presence of probe DNA (DNA4 and DNA5 both at 100 nM) and different concentrations of target DNA (DNA2). The inset shows the calibration curve for DNA detection, which is plotted by using potential decreases at 60 s after additions of H_2O_2.

and catalyze the oxidation of *p*-MOP, which would be detected by the TDMAC-doped polymeric membrane electrode. By using this protocol, the characteristics of DNA assays similar to those of the approach based on the hairpin probe have been obtained (Figure 5B).

The subnanomolar detection limit achieved by the potentiometric polymeric membrane electrode is lower than those in spectrophotometric and fluorometric DNA assay protocols based on similar DNAzyme-based principles (Table 1). Combined with other amplification strategies such as DNAzyme nanowire assembly[5] and circle-rolling amplification,[31] the sensitivity is expected to be further improved. Moreover, based on the target-induced release of blocked G-rich sequences (similar to Figure 4A) and assembly of two split G-quadruplex-forming sequences (similar to Figure 5A), biosensing protocols for other analytes such as Hg^{2+}, ATP, AMP, lysozyme, and cocaine could be developed by using the proposed potentiometric DNAzyme sensor.[32−35]

Table 1. Comparison of the Detection Limits of Different DNA Hybridization Assays Using Catalytic Beacons or Split G-Quadruplex DNAzymes

detection method	substrate	detection limit, nM	reference
potentiometric detection using catalytic beacon or split G-quadruplex	*p*-MOP	0.2	present study
colorimetric detection using catalytic beacon	ABTS	200	28
colorimetric detection using split G-quadruplex	ABTS	1	29
fluorescence detection using split G-quadruplex	H_2DCFDA^a	7	30

[a] 2′,7′-Dichlorodihydrofluorescein diacetate.

CONCLUSIONS

A potentiometric biosensing methodology based on the potential responses of neutral phenols has been demonstrated. The highly lipophilic condensed phenols generated by peroxidation reactions show much larger potential responses as compared to the monomeric phenols on the polymeric membrane electrode doped with a quaternary ammonium slat, by which sensitive potentiometric detection of the G-quadruplex/hemin DNAzyme-catalyzed phenolic oxidations has been fulfilled. The potentiometric DNAzyme sensing platform has been successfully applied in homogeneous DNA hybridization detection and promises to be an effective transducer for G-quadruplex/hemin DNAzyme-based bioanalyses toward other analytes such as proteins, metal ions, and drugs. The proposed strategy may also function as a simple and facile technique for monitoring of phenolic compound degradation catalyzed by peroxidase or its nanoparticle mimetics.[36−38] Moreover, since neutral species such as phenols and lipids that could induce potential responses are widely involved in various enzymatic reactions, the attention to these neutral species may promote the development of more potentiometric biosensing systems.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was financially supported by the Instrument Developing Project of the Chinese Academy of Sciences (YZ201161), the National Natural Science Foundation of

China (41176081), and the Taishan Scholar Program of Shandong Province (TS20081159).

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