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Label-free polymerization amplified potentiometric sensing platform for radical reactions using polyion sensitive membrane electrodes as transducers†

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Taking advantage of the cascade amplification abilities of the radical polymerization reaction and the high sensitivity of the ion exchanger doped polymeric membrane electrodes to polycations/polyanions generated from radical reactions, an amplified label-free potentiometric sensing platform for radical reactions was developed.

Introduction

Benefiting from the extremely large number of propagation steps that result from a single initiation event,[1] free radical polymerization reactions offer an attractive signal amplification platform that is sensitive to the presence of trace concentrations of radicals.[2] When a free radical is generated and transferred to carbon–carbon double bonds containing vinylic or aromatic monomers, rapid step-growth polymerization results in the formation of large macromolecule chains.[1,3] Harvesting the cascade processes inherent in a free radical polymerization reaction may amplify any event which results in the generation of radicals.[2b,4] Design and synthesis of a dual-functional macromolecule or an ensemble that is capable of selective target recognition and initiating polymerization reactions may convert a single recognition event into plenty of radical polymerization processes.[1,3a,5] Various kinds of elegant readout strategies have been explored for the generated polymers. While semi-quantitative results can be obtained from the macroscopically observable polymers,[1,3a] quantitative results are available from electrochemical[6] or spectral methods.[2b] Although high sensitivities were obtained by these elegant strategies, they may suffer from the dependence on tedious synthesis, decoration and labelling processes.[2b,6] Moreover, although many kinds of vinylic type monomers have been used for amplified detections, aromatic monomers have received little attention.[7] Limitations in appropriate readout mechanisms and monomers have restricted largely the utility of these "polymerization for amplification" approaches in biosensing. Straightforward and label-free readout methods that can sensitively and directly detect polymers are urgently needed for broadening the application of this promising strategy.

Although polymeric membrane electrodes for small ions have been extensively explored,[8] investigations on polymers/macromolecules sensitive electrodes are far behind. In 1980s, Meyerhoff *et al.* reported that biological polymers such as heparin and protamine could induce large potential responses on ion-exchangers doped polymeric membrane electrodes.[9] Deviating from the Nernst response slope for these two kinds of polyions can be ascribed to steady-state kinetic processes defined by the fluxes of the polyions both to the surface and into the bulk of the polymeric membrane.[9a] Over the past two decades, polyion sensors have been used as transducers for potentiometric sensing of biological polymers and their related reactions.[9b,9c,10] Moreover, based on the electrostatic interactions between protamine and aptamers, a general and label-free potentiometric sensing platform has been developed.[11] To further improve the reversibility of polyion sensors, pulsed polymeric membrane electrodes were developed.[12] However, polymeric membrane electrodes have hardly been used to directly detect chemically synthesised polyelectrolytes, which are widely used in many fields and convenient detection methods are highly desired. Moreover, although sensors for enzymatic hydrolysis reactions have been developed by using the different sensitivities of the membrane electrodes to protamine and to their hydrolysed "monomers",[7a,10b] the differences in sensitivities have not been investigated systematically and utilized efficiently. Harvesting the different sensitivities mentioned above may develop highly sensitive potentiometric sensing platform for free radical reactions. This will broaden the application of membrane electrodes and provide

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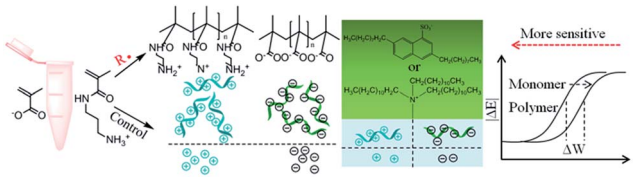

Scheme 1 Response mechanism of the proposed polymerization amplified potentiometric sensing platform.

straightforward detection strategy for polymers and their related reactions.

In this work, after carefully inspecting the response characteristics of the ion-exchanger doped polymeric membrane electrodes to polycation/polyanion and their corresponding monomers, we find that the membrane electrodes are at least two orders of magnitudes more sensitive to the polymers. And thus highly sensitive polyion detection can be achieved even with the presence of large concentrations of background monomers. Taking advantage of the "polymerization for amplification" strategy by using enzymes or other radical initiators to produce polymers *via* free radical polymerization of cationic/anionic monomers (taking *N*-(3-aminopropyl)methacrylamide (APMP) and methylacrylic acid (MAA) as examples), and using the polyion sensitive membrane electrodes as transducers, highly sensitive and label-free potentiometric sensing platform for free radical reactions can be developed (Scheme 1). We demonstrate here the use of this platform to detect low concentrations of horseradish peroxidase (HRP), catalase, G-quadruplex, as well as trace concentrations of Fe^{2+} and Cu^{2+}.

Results and discussion

Because this platform relies on the use of the potential responses of the polymeric membrane electrodes to polymers in the presence of unconverted monomers, we began by comparing the sensitivities of the membrane electrodes to various kinds of polymers with those to corresponding monomers (Fig. 1). Mass concentration was used to show clearly the different sensitivities because analytes at the same mass concentration contain equal numbers of charges or groups. Reversible addition chain transfer (RAFT) polymerization was used to obtain polymers with certain lengths and low molecular weight distributions, and ^{1}H-NMR was used to estimate the conversation rates and molecular weights of the polymers (Table S1, ESI†). It is clear in Fig. 1 that both of these two kinds of polyions could be detected sensitively by the ion-exchangers doped polymeric membrane electrodes. Comparing with APMP, the response curves of PAPMP (polymers of APMP, positively charged at pH = 7.4) were shifted at least two orders of magnitudes to low concentrations (Fig. 1(a)). 0.1 μg mL^{-1} PAPMP could be readily detected while 30 μg mL^{-1} APMP induced little potential response (Fig. S1, ESI†). Moreover, TDMACl-doped membrane electrodes were at least two orders of magnitudes more sensitive to PMAA (polymers of MAA, negatively charged at pH = 7.4) than to MAA (detection limit:

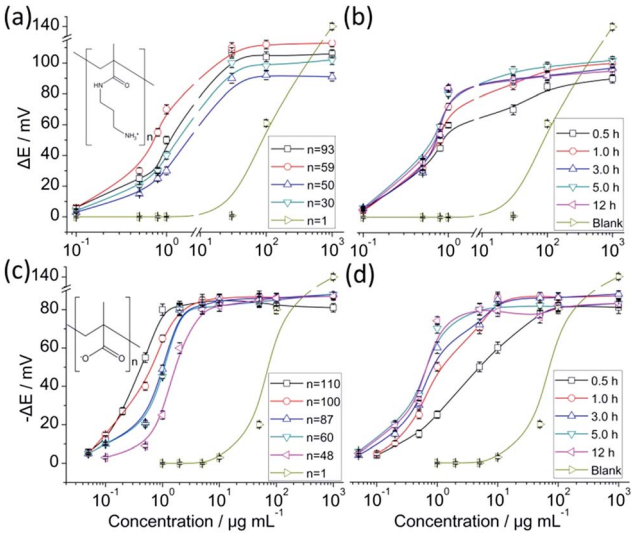

Fig. 1 Potentiometric responses of the polymeric membrane electrodes to varying concentrations of monomers and (a) PAPMP or (c) PMAA of different lengths (*n*) generated from RAFT reactions or (b) PAPMP and (d) PMAA generated by FRP reactions over time. Each error bar represents one standard deviation of 3 replications, the same below.

0.05 μg mL^{-1} *vs.* 10 μg mL^{-1}, Fig. 1(c) and S2, ESI†). The potential responses of PAPMP were independent of the polymer lengths, while those of PMAA increased with polymer's molecular weights (Fig. 1(a) and (c)).

To further examine the correlations between the response behaviours and polymer lengths, the potential responses of the membrane electrodes to another two kinds of polyions with different functional groups and lipophilicity were recorded. While little correlation between the potential responses and the polymer lengths was found for poly-diallyldimethylammonium, different result was obtained for polyacrylic acid (Fig. S3, ESI†), the reasons for these phenomena are not clear now. To further confirm the feasibility of the proposed sensor, uncontrolled free radical polymerization (FRP) was used to polymerize the monomers. Hydrogen abstraction reactions can be promoted by HRP to generate radical species, which can initiate the polymerization reaction subsequently.[13] Direct polymerization of bulky monomers are sometimes difficult due to unfavourable steric interaction or redox potentials. In such cases, enzymatic chain initiation can proceed indirectly *via* a small and oxidizable mediator such as acetyl acetone (acac).[14] HRP was used to polymerize monomers with the assistant of H_2O_2 and acac in HEPES buffer deoxygenated by N_2 bubbling. The reactions were carried out at 70 °C for up to 12 h. At selected time points, the potential responses to the purified products were recorded (Fig. 1(b) and (d), S4 and S5, ESI†). Similar with the products from RAFT, it was observed that polyions generated by FRP could also induce large potential responses on the membrane electrodes. These data complement that seen in Fig. 1(a) and (c), which demonstrate that potential responses can be observed at <0.3% (APMP) or <0.5% (MAA) conversion rate after excluding the signals of monomers by diluting the reaction mixtures. The

above results altogether confirm that polymeric membrane electrodes can be used as transducers for polymerization amplified potentiometric sensing. Further-more, although aromatic monomers have received little attention, phenols can also be used in this "polymerization for amplification" strategy.[3b,15] Based on the oxidation reactions of phenols in aqueous buffer, we have developed a biosensor for DNAzyme sensing.[7a] The amplification ability deteriorated in this previous assay because the formed oligomers precipitated immediately from aqueous buffer, and this process diminished the chain propagation steps.[16] Polymerization of phenol derivatives in organic/aqueous buffer mixture could give rise to polymerized phenols containing C–C and C–O units with large molecular weights.[3b,15] After incubation of HRP, H_2O_2 and *p*-methoxyphenol in 1,4-dioxane (80%)/phosphate buffer (PBS, pH = 7.0, 20%) and PBS for 2 hours, respectively, the potential responses of the TDMACl-doped membrane electrodes to the reaction mixtures were investigated. Fig. S6 (ESI†) shows that the products generated from organic/aqueous buffer mixture can induce more large signals. The high sensitivities of the membrane electrodes to polymerized phenols and the high conversion rates in organic/aqueous buffer mixture may be the main reasons for this phenomenon.

The components of the membrane electrodes were optimized for the two kinds of polyions (Fig. S7, ESI†). For PAPMA, DNNS is the best recognition element and the membrane doped with NPOE shows the best sensitivity. Membranes doped with other anionic additives and plasticizers also work. For PMAA, TDMACl-NPOE is the best combination (see Fig. S8, ESI† for the SEM of the membranes). These results are in good accordance with previous work for protamine and heparin sensitive polymeric membrane electrodes.[9a] Prolonging the reaction time of FRP can improve the sensitivity of this polymerization amplified potentiometric assay. From the perspective of practical reality, 0.5 h was used for the following detection. Phenols were not used in the following detection owing to the inconvenience in dealing with organic solvents, rather than the low sensitivity. It is believed that using co-monomers with large lipophilicity or tightening up the polymer chain with cross-linkers may further improve the performance of the proposed sensing platform.

Applications

HRP detection

As O_2 is a potent radical quencher, the FRP reactions are very sensitive toward small amounts of O_2.[13] To achieve sensitivity at low concentration of radical in an open air assay format, it was necessary to incorporate a mechanism for scrubbing O_2 from solutions instead of the tedious N_2 protection. Through the use of glucose oxidase (GOx), H_2O_2 can be generated *in situ* with the consumption of O_2, and thus polymerizations can be performed in open well plates under normal atmosphere.[2b,17] For HRP detection, after preclude the interference from Fenton reaction, polymerization reactions were performed in 96-well plates containing 330 μL of GOx (200 nM), glucose (10 mM), APMA (0.5 M), acac (0.5 mM) and varying concentrations of HRP (50 mM HEPES buffer, pH 7.4) at room temperature for 0.5 h. Then the reactions were quenched using oxygenated buffer, and the diluted mixtures (with a dilution factor that the concentration of monomer is below the detection limit, the same below) were added to HE-PES buffer (50 mM, pH 7.4) to induce the potential responses. It is clear in Fig. 2(a) and S9 (ESI†) that HRP can be detected in the range of 1×10^{-5} to 5×10^{-7} U mL^{-1} with a detection limit of 2×10^{-7} U mL^{-1}. Moreover, control experiments confirmed that the polymerization reactions were related with HRP rather than other enzymes (Fig. S10, ESI†).

Fe^{2+} and Cu^{2+} detection

Redox-active transition metals such as Fe^{2+} and Cu^{2+} are usually sequestered by protein ligands under physiological conditions, including as static enzyme cofactors, in part because of their potential to trigger oxidative stress and damage *via* Fenton chemistry.[18] Based on the redox degradation of H_2O_2 into hydroxyl radicals by Fe^{2+} and Cu^{2+} *via* the Fenton reaction, trace concentrations of these two kinds of metals can be detected without interferences from other metals, which are redox-inactive in this condition. When 0.5–10 ppb Fe^{2+} and 0.1–5 ppb Cu^{2+} were used to initiate the polymerization reactions, significant potential signals can be observed (Fig. 2(b)). This GOx/monomer system provides a highly sensitive sensing platform for Fe^{2+} and Cu^{2+}.

Catalase detection

Interestingly, it has been reported that radicals can also be generated through the direct oxidation of high concentration of acac by GOx.[2b] After incubation of GOx (200 nM), glucose (10 mM), acac (2 mM) and APMP in HEPES for 0.5 h, the potential responses of the DNNS-doped membrane electrodes to the products were recorded. The significant potential responses confirm the direct oxidation of acac by GOx, which leads to small amounts of polymerization (Fig. 3(a) and (c)). Considering that catalase is a very active enzyme which is able to consume H_2O_2 and produce O_2, we hypothesized that an inverse assay for catalase can be developed based on the inhibited polymerization reactions. To test this hypothesis, we performed a set of polymerizations in the presence of catalase at different concentrations. Fig. 3(c) shows that when catalase was added into the

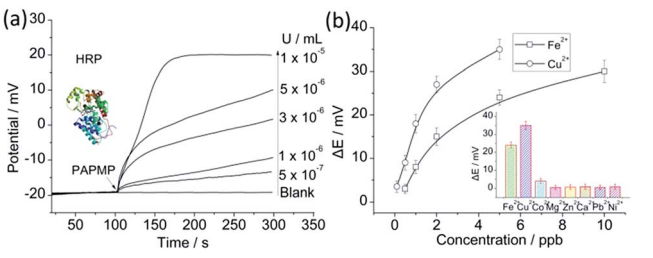

Fig. 2 (a) Potentiometric responses of DNNS-doped membrane electrodes to PAPMP generated from polymerization initiated by different concentrations of HRP. (b) Potentiometric detection of Fe^{2+} and Cu^{2+} using the proposed sensing platform. Inset: potential changes for detection of 5 ppb Fe^{2+}/Cu^{2+} and 100 ppb other metal ions.

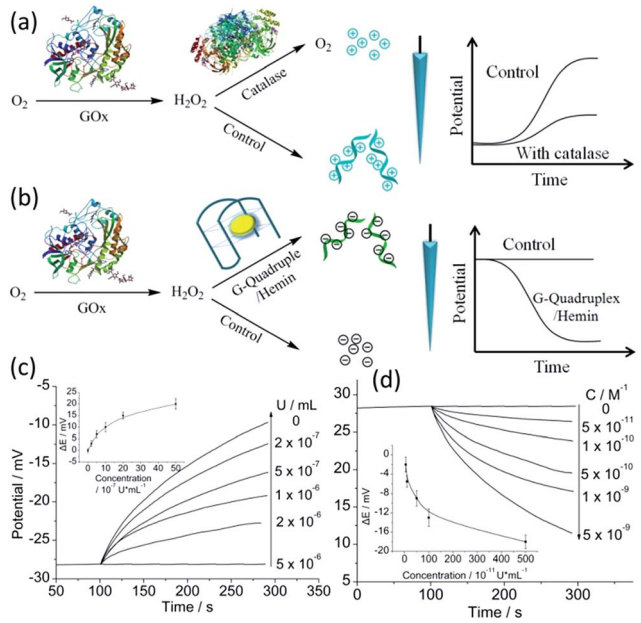

Fig. 3 Schematic representation of (a) catalase and (b) G-quadruplex detection based on the proposed sensing platform. Potentiometric responses of (c) DNNS-doped membrane electrodes to PAPMP generated by polymerization in the presence of catalase at different concentrations (d) TDMACl-doped membrane electrodes to PMAA generated by polymerization initiated by different concentrations of G-quadruplex/hemin. Insets: calibration curves.

system, the potential responses diminished, indicating the decreased amounts of PAPMP owing to inhibited polymerizations. This assay format was extremely sensitive to the presence of catalase, a calibration curve in the range of 5×10^{-6} to 2×10^{-7} U mL^{-1} with a detection limit of 1×10^{-7} U mL^{-1} can be obtained.

G-quadruplex detection

G-quadruplex HRP-mimicking DNAzymes have been extensively used as a catalytic labels for the detection of biological analytes such as DNA, proteins, and even metal ions.[19] Considering that PAPMP may bind with G-quadruplex through electrostatic interaction and thus diminish the activity of the DNAzymes and interference with the potentiometric detection, MAA was used as a monomer for amplified G-quadruplex detection. After incubation of GOx (200 nM), glucose (10 mM), acac (0.5 mM), and MAA (0.5 M) with varying concentrations of G-quadruplex/hemin in HEPES for 0.5 h, the potential responses of the TDMACl-doped polymeric membrane electrodes to the products were recorded. Fig. 3(b) and (d) show that G-quadruplex can be detected sensitively in the range of 5×10^{-9} to 5×10^{-11} M with a detection limit of 2×10^{-11} M. We have confident that DNA/aptamers based detection can also be achieved using this platform with similar sensitivities.

Conclusions

Taking the advantage of the cascade amplification abilities of the free radical polymerization reactions to produce polyions, and using convenient and straightforward polyion sensitive polymeric membrane electrodes as transducers, amplified label-free potentiometric sensing platform for radical reactions was developed. Less than 0.5% monomer-to-polymer conversion rate is required to initiate potentiometric responses without the interference with the background monomers, providing high sensitivity toward the radical generating species. Good sensitivities of this assay toward HRP, catalase, G-quadruplex and ppb concentrations of Fe^{2+} and Cu^{2+} are shown. Given that polymerization amplified detection is currently limited by appropriate readout strategies, it is anticipated that such a label-free and straightforward design will provide a useful platform for sensitive detection of a broad range of biomolecules.

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