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Amplified electrochemical aptasensor taking AuNPs based sandwich sensing platform as a model

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Abstract

Here, we report a sensitive amplified electrochemical impedimetric aptasensor for thrombin, a kind of serine protease that plays important role in thrombosis and haemostasis. For improving detection sensitivity, a sandwich sensing platform is fabricated, in which the thiolated aptamers are firstly immobilized on a gold substrate to capture the thrombin molecules, and then the aptamer functionalized Au nanoparticles (AuNPs) are used to amplify the impedimetric signals. Such designed aptamer/thrombin/AuNPs sensing system could not only improve the detection sensitivity compared to the reported impedimetric aptasensors but also provide a promising signal amplified model for aptamer-based protein detection. In this paper, we realize a sensitive detection limit of 0.02 nM, with a linear range of 0.05–18 nM. Meanwhile, the effect of 6-mercaptohexanol (MCH) and 2-mercaptoethanol (MCE) on the modification of the electrode is investigated.

Keywords: Impedimetric aptasensor; Au nanoparticles; Thrombin; Aptamer

1. Introduction

Since screened through the systematic evolution of ligands by exponential enrichment (SELEX) process from random RNA or DNA libraries (Ellington and Szostak, 1990; Robertson and Joyce, 1990; Tuerk and Gold, 1990), aptamers have been widely used in analytical assays and shown their enormous potential in recognizing and detecting various targets (Hamula et al., 2006; Osborne and Ellington, 1997) due to their generally impressive selectivity, affinity and their multifarious advantages over the traditional recognition elements (Osborne and Ellington, 1997). Until now, a lot of aptamer-based detection systems for proteins have been developed such as optical transduction (McCauley et al., 2003), quartz crystal microbalance (Liss et al., 2002), surface plasma resonance (SPR) (Minunni et al., 2005; Pavlov et al., 2005), fluorescence (Jiang et al., 2004; Li et al., 2007a; Nutiu and Li, 2005; Wang et al., 2005), colorimetry (Huang et al., 2005; Liu and Lu, 2006; Liu et al., 2006; Stojanovic and Landry, 2002), electrophoresis (Li et al., 2007b; Zhang et al., 2006), electrochemistry (Hansen et al., 2006; Polsky et al., 2006; Shen et al., 2007; Xiao et al., 2005; Xu et al., 2005; Zheng et al., 2007; Zuo et al., 2007), etc., among which the analysis based upon electrochemistry has been widely developed and considered to take a very important role in the future research.

In fact, once captured onto the electrode, most proteins are ready to affect the condition of the electrode surface due to their high molecular weight and/or high charge density. Just based on this characteristic, electrochemical impedance spectroscopy (EIS) has been imported to design the aptasensing systems as a sensitive and convenient electrochemical technique (Cai et al., 2006; Radi et al., 2005; Robertson and Joyce, 1990; Xu et al., 2005; Zayats et al., 2006). Up to now, several aptamer-based impedimetric sensors for proteins have been reported (Cai et al., 2006; Radi et al., 2005; Robertson and Joyce, 1990; Xu et al., 2005; Zayats et al., 2006). For example, O'Sullivan's group (Radi et al., 2005) has realized the thrombin assay by detecting the increase of electron-transfer impedance (Ret) after the protein interacting with the aptamer that was immobilized on the gold substrate. Xu's group (Xu et al., 2005) detected IgE with the same principle. By measuring the decrease of the Ret

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for the $Fe(CN)_6^{4-/3-}$ probes, Wang's group (Rodriguez et al., 2005) detected the positively charged protein lysozyme. Though all the sensors above could realize the assays of proteins successfully, the dependence of signal on the protein size or charge could limit the detection sensitivity.

Here, an amplified impedimetric aptasensor for protein is reported, based on the aptamers and rhodamine 6G (R6G) molecules modified Au nanoparticles (AuNPs), where aptamers are used for recognizing protein and R6G are used for blocking the surface. Thrombin, a kind of serine protease that plays important role in thrombosis and haemostasis is taken as a model molecule (Bock et al., 1992; Kankia and Marky, 2001; Padmanabhan et al., 1993; Smirnov and Shafer, 2000). According to the fact that one thrombin molecule has two active sites for its 15-mer aptamer (Polsky et al., 2006; Smirnov and Shafer, 2000), a sandwich manner is easily fabricated with aptamer/thrombin/AuNPs system. During the sensing process, thiolated thrombin binding aptamer (TBA) is firstly immobilized on the gold electrode to capture the target, and once in the presence of the thrombin, the TBA functionalized AuNPs could further bind to thrombin, forming a sandwich sensing system on the electrode as shown in Fig. 1C. The aptamer functionalized AuNPs are negatively charged, which results in an amplified Ret signal of $Fe(CN)_6^{4-/3-}$ probes compared with that of the protein simply without functionalized AuNPs. Through such amplified method, a detection limit of 0.02 nM is realized, which is more sensitive than most impedimetric sensors reported (Cai et al., 2006; Radi et al., 2005). Because the aptamers modified on the AuNPs can also be labeled with other probes to produce signals, and AuNPs can be substituted or fuctionalized with both aptamers and other probes (Pavlov et al., 2004; Zheng et al., 2007), the sensor designed provides a promising signal amplified model for protein detection, which does not limit to the impedimetric detection only. Just like this system, R6G is a well used Raman probe, which provides this sensor a potential application of qualitatively recognizing thrombin using surface enhanced Raman scattering (SERS).

2. Experimental section

2.1. Chemicals and materials

15-mer thiolated TBA (5′-HS-$(CH_2)_6$-GGTTGGTGTGGTTGG-3′) were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. and diluted to 6.5 μM in 34 mM Tris–HCl buffer (B-buffer, pH 7.4, 233 mM NaCl, 8.5 mM KCl, 3.4 mM $MgCl_2$) for use. One millimolar 2-mecaptoethanol (MCE, Fluka), 1 mM 6-mecaptohexanol (MCH, Fluka), 0.1 mM rhodamine 6G (R6G, Fluka), and different concentrations of the thrombin (Sigma) and bovine serum albumin (BSA) were all prepared in the Tris–HCl buffer. All other chemicals were of analytical-grade. The DNA and protein solutions were stored at 4 °C before use.

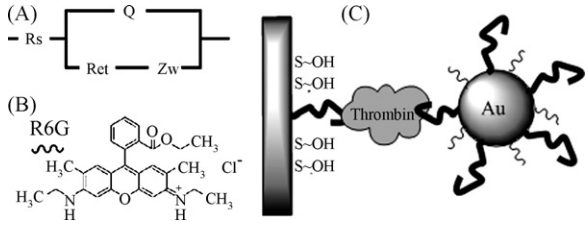

Fig. 1. A circuit for the EIS (A). Molecular structure of R6G (B). Schematic diagram of sandwich amplified impedimetric aptasensor based on functionalized AuNPs (C).

2.2. Fabrication of the sensing interface

The Au electrode (1.2 mm in diameter) was polished with 1.0 and 0.3 μm α-Al_2O_3 and then washed ultrasonically with pure water for three times, subsequently electrochemically cleaned in 0.1 M H_2SO_4 by potential scanning between −0.2 and 1.55 V until a reproducible cyclic voltammetry was obtained. Then, it was sonicated and rinsed with copious amount of pure water, finally blown dry with nitrogen before assembly.

The TBA modified electrode was prepared by placing prepared 18 μL 6.5 μM thiolated TBA solution on the Au electrode held upside-down. Then, the end of the electrode was fitted with a plastic cap to protect the solution from evaporation. The assembly was kept for over 20 h at room temperature and then rinsed with pure water and B-buffer for several times. Both for the MCH and MCE modification, Au/TBA interface was covered with 5 μL corresponding solution (1 mM in B-buffer for each) and kept at room temperature for 1 h, followed by rinsing with pure water and B-buffer.

Then, the resulting monolayer functionalized electrode was incubated for 60 min (28 °C) with various concentrations of thrombin in B-buffer for protein–aptamer interaction (Radi et al., 2005; Zheng et al., 2007), after which the Au/TBA/thrombin electrode was immersed in the prepared AuNPs solution for 120 min (28 °C) to get a Au/TBA/thrombin/AuNPs system (Pavlov et al., 2004; Zheng et al., 2007).

As in control experiment, the TBA modified electrode was treated with 1 μM non-specific protein BSA, followed by immersing the electrode in prepared AuNPs for 120 min.

2.3. Synthesis and modification of AuNPs

AuNPs stabilized with citrate were synthesized according to the procedure of literature (Storhoff et al., 1998). That, 100 mL of 1 mM $HAuCl_4$ was brought to a reflux while stirring and then 10 mL of a 38.8 mM trisodium citrate solution was added quickly, which resulted in a change in solution color from pale yellow to deep red. After the color change, the solution was refluxed for an additional 15 min.

AuNPs modified by TBA and R6G were prepared according to the literatures (Liu and Lu, 2006; Pavlov et al., 2005; Storhoff et al., 1998) with a little modification. That, transfer 3 mL of the already prepared AuNPs to the NaOH-treated glass vials and then add 50 μL 6.5 μM TBA with magnetic stirring to facilitate the reaction for 16 h. The final Tris–HCl concentration is 0.56 mM. Add 18 μL of 10^{-4} M R6G to above vial with stirring for another 8 h and store the vial at 4 °C for another day before use. Centrifuge the modified AuNPs at 14,000 × *g* at room temperature for 25 min twice to remove the free DNA and

R6G. Again, disperse the AuNPs in 2 mL of buffer containing 4.7 mM NaCl, 0.56 mM Tris–HCl, 0.14 mM KCl, pH 7.4.

2.4. Electrochemical measurements and Au nanoparticle (AuNPs) amplified thrombin detection

Electrochemical experiments of CV and EIS were done after each steps of modification and performed on an Autolab PGSTAT30 (Utrecht, The Netherlands, controlled by GPES4 and Fra software) using a conventional three electrode electrochemical cell with Ag–AgCl electrode as reference electrode, Pt coil as counter electrode and gold disk (1.2 mm in diameter) as working electrode. The cell was housed in a homemade Faraday cage to reduce stray electrical noise. All the measurements with the Autolab were carried out at room temperature (~17–18 °C). EIS measurements were performed under an oscillation potential of 5 mV over the frequency range of 10 KHz–0.1 Hz and in the solution of 5 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ in B-buffer.

3. Results and discussion

3.1. Electrochemical behavior of the TBA modified electrode

The self-assembled monolayer of TBA on the Au electrode was investigated using both CV and EIS. At TBA monolayer electrode, its negatively charged interface repels the negatively charged redox probes, $[Fe(CN)_6]^{4-/3-}$ anions, which retards the interfacial electron-transfer kinetics of the probes (Radi et al., 2005). As shown in Fig. S1 (inset, supporting information), compared with curve a (bare gold electrode), the peak current of curve b (TBA modified electrode) decreases and the peak-to-peak separation increases. It is because the negative TBA monolayer blocks the redox probes from effective electron-transfer at the sensing surface. The consistent result was also provided in the impedance spectra (Fig. S1, supporting information). Fig. 1A shows the circuit that includes the commonly existed electrolyte resistance (Rs), constant phase element (Q), Warburg impedance (Zw) and the electron-transfer resistance (Ret/Rct). The bare Au electrode shows a very small semicircle domain which represented the Ret (Fig. S1, curve a), while for the TBA monolayer electrode, the response of the equimolar $[Fe(CN)_6]^{4-/3-}$ anions are retarded, leading to decreased electron-transfer with large semicircle. The results above demonstrate that the TBA monolayer has been self-assembled on the electrode surface successfully.

3.2. Comparison of the MCE and MCH modified Au/TBA electrode

MCH and MCE are two common complexes to block the electrode surface (Pavlov et al., 2005; Radi et al., 2005). For more effective detection, we compared the electrode modified with the two complexes separately. After functionalizing the electrode with thiolated TBA, the electrodes were treated with MCE and MCH, respectively, at the same condition. Compared with the Au/TBA system, it is found that after one hour's interaction, the MCE modified electrode (Fig. 2A) displays a decrease of Ret while that of MCH (Fig. 2B) shows a significant increase. The reason for the difference may be presumed as follow. If without any further blocking, the TBA does not orientate very orderly on the electrode and some of them inclines to the Au surface or enwinds with other ssDNA molecules. Some molecules can even coordinate with Au through nitrogen atoms (Steel et al., 1998). In this case, the negative assembled monolayer almost covers the whole Au surface and obviously repels the $[Fe(CN)_6]^{4-/3-}$ anions. However, for both the conditions with MCE and MCH, the thiolated complexes take up the spare active sites of Au surface and largely lift the TBA molecules from the electrode surface to make sure that each probe is immobilized solely through the thiolate (Steel et al., 1998). Then, for MCE, the molecules are charge-free and relatively smaller/shorter. So, when the electrode is modified with TBA and MCE, the ability to repel $[Fe(CN)_6]^{4-/3-}$ anions would be lower than the only TBA modified one, which results in the decrease of Ret. Differently, the condition for MCH modified electrode is just opposite. MCH

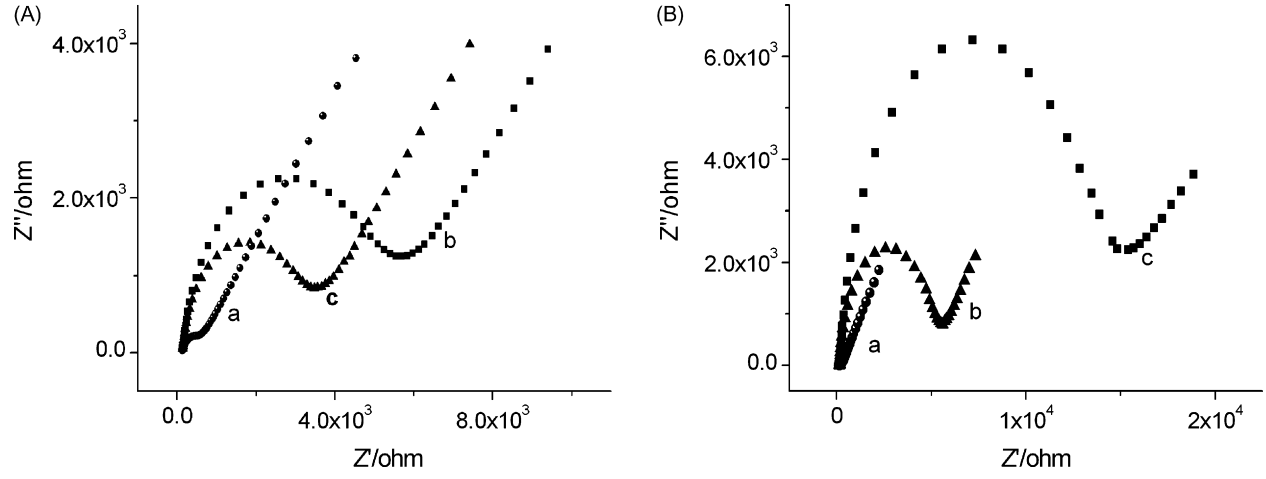

Fig. 2. Comparison between MCE (A) and MCH (B) modified Au/TBA. For two figures: (a), bare Au electrode; (b), Au/TBA system; (c), Au/TBA/MCE system for 2A and Au/TBA/MCH system for 2B.

molecule is longer and its blocking effect is much stronger than MCE. That might be why the TBA/MCH modified electrode could lead to an obvious increase of the Ret compared to the only TBA modified one. As shown, the signal transduced in this sensor depends on the signal-increase after thrombin or AuNPs are added onto the TBA modified electrode. Therefore, it is easy to understand that the lower the baseline (Ret of TBA/blocker modified electrode) it possesses, the better sensing sensitivity it may get. So, we choose MCE to block the sensing interface in all the experiments for detection. For convenience, TBA/MCE modified electrode will be expressed as TBA modified electrode or Au/TBA system.

3.3. EIS detection in the presence of thrombin

Previous works have (Cai et al., 2006; Lohndorf et al., 2005; Radi et al., 2005) demonstrated that after interacting with the solution of thrombin, the Ret would increase compared to the TBA modified electrode, originating from an integrated function of blocking of the electrode surface with the bulky protein and the resistive hydrophobic layer insulating the conductive support (Cai et al., 2006). Fig. 3 provides the consistent result (the CV results are provided as Fig. S2 in supporting information). When the TBA modified electrode was treated with 45 nM thrombin for 1 h, there was an increase of the Ret. Then, the TBA/thrombin modified electrode was immersed in TBA/R6G functionalized AuNPs to realize one more time's recognition and form a sandwich manner of TBA/thrombin/AuNPs. Here, the AuNPs are no longer well conductors (here for $[Fe(CN)_6]^{4-/3-}$ probe) but negatively charged complexes, which interfere with effective electron transfer on the surface. That is largely attributed to the fact that the surface has been covered with ssDNA and other active sites have been taken with R6G molecules. Therefore, after the AuNPs bind to the thrombin on the electrode, the bulk negative charges could further repel the probes of $[Fe(CN)_6]^{4-/3-}$ anions, making the Ret increase more obviously than that of the AuNPs absence one. As shown in Fig. 3, the

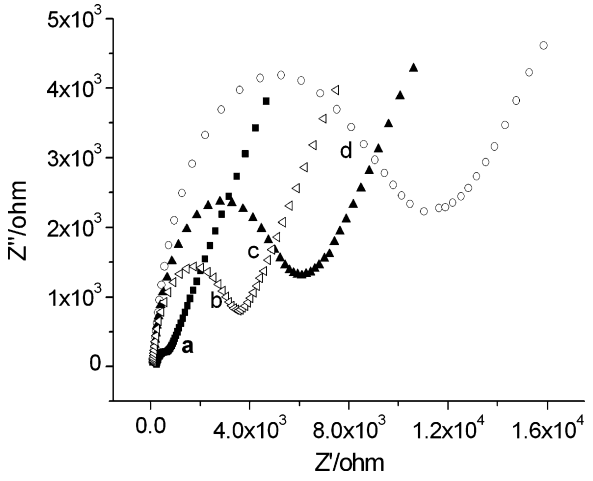

Fig. 3. Niquist plots of the bare Au electrode (a), Au/TBA/MCE system (b), Au/TBA/MCE/thrombin system (c), Au/TBA/MCE/thrombin/AuNPs system (d). The concentration of thrombin was 45 nM.

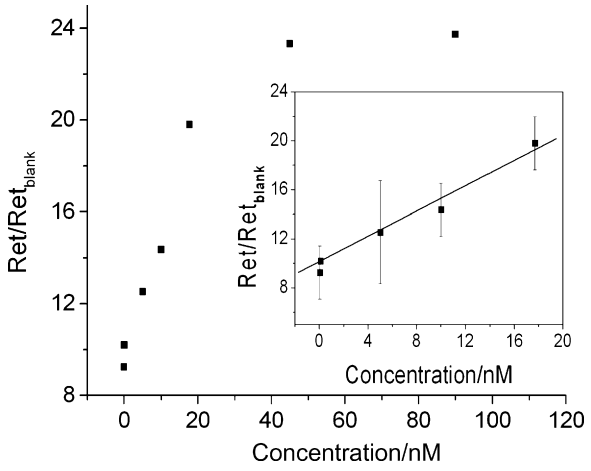

Fig. 4. Response of the sensor to different concentrations of thrombin. Inset: the response to thrombin from 0 to 18 nM. The error bars represent the average standard deviation of two measurements.

AuNPs binding to the thrombin leads to a significant increase of the Ret, which amplifies the signal effectively. Just adopting the signal amplification process of the sandwich manner and ultimately detecting the Ret after interacting with the AuNPs, a linear detection range of 0.05–18 nM (Fig. 4) is obtained, and the detection limit is as low as 0.02 nM. It is a sensitivity at least 10-fold higher than the one without AuNPs (0.1 nM we get) and most other existed impedimetric aptasensors, the detection limit of which are down to ~nM, for example 2 nM (Radi et al., 2005) or 0.1 nM (Cai et al., 2006).

3.4. Function of the R6G

In this work, the AuNPs are functionalized with not only TBA but also R6G. The TBA is chosen just for recognizing thrombin and providing long chains of negative charges for the AuNPs. At the same time, R6G is used here for its following two advantages. First, R6G (Fig. 1C) is proven to interact with Ag or Au surface easily (Fabris et al., 2007), which has been usually used to overcome the repulsion between the NPs. Therefore, the molecule here can not only be taken to block the AuNPs surface but also play a role to stabilize the AuNPs. We compare the sensing sensitivity of the AuNPs with and without R6G. It is easily observed (Fig. 5) at the same experiment condition, when the TBA/thrombin modified electrode interacts with the AuNPs functionalized only by TBA, the Ret increase is much less than the one interacted with TBA/R6G functionalized AuNPs. That illustrates the addition of R6G to the sensing system does bring more sensitive response for the sensor. It is well known that R6G is commonly chosen as SERS probes (Fabris et al., 2007). So, for its use, the second advantage is to provide a possibility of recognizing protein with SERS.

3.5. Control experiment

For testing the specific recognition to thrombin of the sensor designed, BSA, which is usually used in control experiments, is adopted. The TBA modified electrode was immersed in the

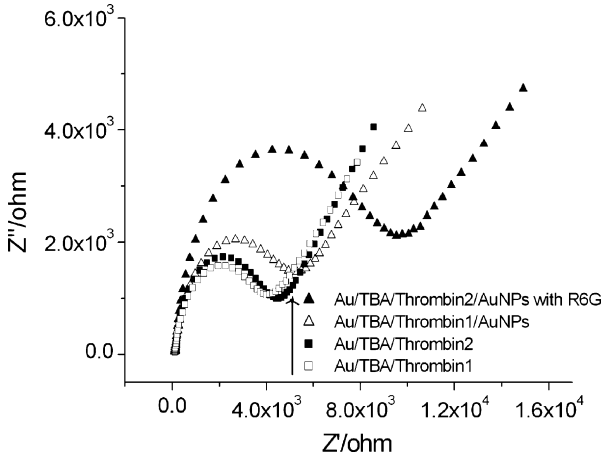

Fig. 5. Comparison of Niqiust plots between AuNPs with and without R6G. As shown, the figure with thrombin 1 represents the electrode used for binding AuNPs without R6G, and the figure with thrombin 2 represents the electrode used for binding AuNPs with R6G. The thrombin used for both electrodes was 15 nM.

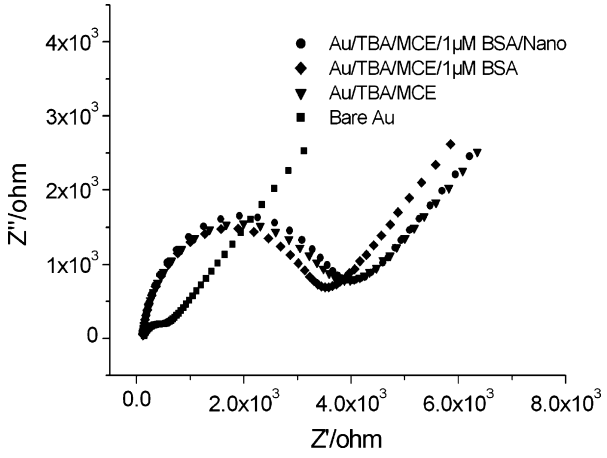

Fig. 6. Control experiment of 1 μM BSA.

solution of 1 μM BSA for 1 h, followed by immersion in the functionalized AuNPs for 2 h. As shown in Fig. 6, in both the two steps, the Ret of the electrode keeps almost the same with the one of TBA modified electrode. This means that BSA could not interact with the TBA and interfere the detection of thrombin. The control experiment demonstrates the sensor is specific to thrombin.

4. Conclusions

In summary, adopting a sandwich manner, an impedimetric aptasensor is developed based on the functionalized AuNPs, which could recognize thrombin and produce an amplified impedance signal due to the ssDNA modified on the AuNPs. Through the sensing process designed, a very low detection limit of 0.02 nM is realized, which indicates the high sensitivity of the amplified method. What's more, the sensing signal can be amplified through layer-by-layer assembly of thrombin and AuNPs, exhibiting the potential for more sensitive detection. At last, because the AuNPs can be functionalized with both aptamers and signal probes, for example R6G molecules this time, the sensor designed provides a promising signal amplified model for protein detection with even other techniques.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2007.09.019.

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