Paper-Based Bipolar Electrode Electrochemiluminescence Switch for Label-Free and Sensitive Genetic Detection of Pathogenic Bacteria

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Supporting Information

ABSTRACT: Genetic analysis is of great importance for the detection of pathogenic bacteria. Bacterial identification must become simpler, less expensive, and more rapid than the traditional methods. In this study, a low-cost, label-free, and wireless paper-based bipolar electrode electrochemiluminescence (pBPE-ECL) analysis system was constructed for the rapid and sensitive genetic detection of pathogenic bacteria. Wax-screen printing was used to form hydrophilic channels on filter paper, and a carbon ink-based bipolar electrode and driving electrodes were screen-printed into the channels. The “light-switch” molecule [Ru(phen),dppz] (phen = 1,10-phenanthroline; dppz = dipyridophenazine) was used to intercalate into the base pairs of the double-stranded DNA PCR amplification products, and the complexes were then applied to the paper-based bipolar electrode to perform the ECL assays; the ECL of [Ru(phen),dppz]$^{2+}$ is quenched in aqueous solution, but this molecule displays intense ECL when intercalated into double-stranded DNA. Under optimized experimental conditions, as little as 10 copies/μL of the genomic DNA of Listeria monocytogenes could be detected. Additionally, the system could also specifically distinguish Listeria monocytogenes from Salmonella, Escherichia coli O157:H7, and Staphylococcus aureus. This label-free, simple, and rapid method has potential in point-of-care applications for pathogen detection.

Genetic analysis is of great importance for the detection of pathogenic bacteria. Various strategies and technologies, such as real-time polymerase chain reaction (RT-PCR), nucleic acid sequence-based amplification (NASBA), and loop-mediated isothermal amplification (LAMP) assays, have been developed to identify specific genes in target pathogens and have been used as standard protocols in central research laboratories. However, such conventional methods are usually laborious and time-consuming. With the development of biological sensing technology, certain optical analytical methods, including colorimetry, fluorescence spectrometry, chemiluminescence, and electrochemiluminescence (ECL), have been developed to address these problems. ECL as one of the most sensitive optical analytical techniques that combines electrochemical and luminescence methods has attracted considerable interest because of its distinct advantages, including a high signal-to-noise ratio (SNR), high sensitivity, and spatial controllability.1-3 Our group has been using ECL with genetic assays for nearly a decade. Unfortunately, we have found that such methods usually require complicated probe designs and wired power supplies, which limit their utility for point-of-care diagnostics in extremely resource-limited environments.

Bipolar electrodes (BPEs) are electronic conductors that are immersed in an electrolyte solution without a direct electrical connection required to activate the electrochemical reactions at its poles.17,18 When sufficient voltage is applied between the two ends of a microchannel, the potential difference between the BPE and the solution simultaneously drives redox reactions at both poles of the BPE.19 Over the past decade, the wireless aspect of BPEs has allowed for a promising new means to integrate ECL into lab-on-a-chip systems that can be controlled with just a single DC power supply. A typical bipolar electrode-based electrochemiluminescence (BPE-ECL) detection system uses tris(2, 2’-bipyridyl) ruthenium(II) (Ru(bpy)$_3$$^{2+}$) as the ECL luminophore and tripropylamine (TPrA) as the co-reactant.20 In this strategy, Ru(bpy)$_3$$^{2+}$ and TPrA are oxidized at the anodic pole of the BPE, emitting the ECL signal. Compared with other detection methods, this biosensing platform possesses advantages of easy fabrication, high sensitivity, and no need for a direct external connection to the electrode, which facilitates its integration in miniaturized devices. Due to these reasons, the BPE-ECL systems has been gradually adopted in bioanalysis for a variety of applications, including the detection of TPrA,2 cancer cells,22,23 cell surface proteins,24 H$_2$O$_2$, and ATP.25 However, conventional BPE-ECL detection systems usually employ complicated and relatively expensive microfluidic technologies to integrate the detection cell into miniaturized lab-on-a-chip devices. Furthermore, those techniques require expensive instrumentation or skilled personnel, which limits their utility for point-of-care diagnostics.26

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Microfluidic paper-based analytical devices (µPADs) are powerful tools that meet the criteria of being ASSURED (i.e., affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable to end-users) and have thus gained increasing attention in recent years.\(^7\)–\(^10\) µPADs provide spatial and quantitative control of biological fluids using capillary action within paper without requiring external switches or pumps, and µPADs can be developed using inkjet printing, wax printing, or screen printing technology.\(^5\) Put simply, these devices can be regarded as either a paper variant of conventional microfluidics or an advanced version of classical lateral flow test strips.\(^33\)\(^34\)

Therefore, we intended to develop a paper-based biosensor with ECL technology for the qualitative and quantitative analysis of specific genes of pathogenic bacteria. However, working with labeled probes on paper presents certain challenges,\(^34\) such as requiring costly probe-tagging moieties\(^35\) and the difficulty in removing nonspecific probe bands, leading to a relatively high background signal.\(^36\) Moreover, most paper-based assays are usually based on colorimetry, and the resulting quantitative or semiquantitative analysis is not adequate when the level of an analyte is important.\(^37\) For these reasons, few applications can be found for genetic assays on paper coupled with ECL.\(^38\) To overcome these challenges, more sensitive, quantitative, and label-free methods must be developed.

In this study, we fabricated for the first time a paper-based BPE-ECL (pBPE-ECL) device coupled with the “light-switch” molecule \([\text{Ru}(\text{phen})_2\text{dppz}]^{2+}\) (phen = 1,10-phenanthroline; dppz = dipyridophenazine) for the low-cost, wireless, sensitive, and label-free analysis of DNA from pathogenic bacteria. \([\text{Ru}(\text{phen})_2\text{dppz}]^{2+}\) has been found to intercalate into DNA with high affinity and to generate ECL emission via metal-to-ligand charge transfer (MLCT) involving its triplet excited state.\(^39\)–\(^43\) In aqueous solution, the ECL of \([\text{Ru}(\text{phen})_2\text{dppz}]^{2+}\) is quenched by the protonation of the phenazine N atoms in the excited state. When \([\text{Ru}(\text{phen})_2\text{dppz}]^{2+}\) binds to DNA, the N atoms are protected because of the intercalation of the planar phenazine ligand between the DNA base pairs, resulting in intense ECL emission.\(^43\)–\(^45\) This pBPE-ECL device consists of one wax-screen-printed hydrophilic cell and a carbon ink BPE as an electronic conductor plus another two carbon ink electrodes as the driving electrodes, which were fabricated through screen printing onto the paper channel. Two \textit{Listeria monocytogenes}-specific primers that target the \textit{hlyA} virulence genes were designed for a polymerase chain reaction (PCR) to generate double-stranded DNA (dsDNA) amplicons. The light-switch molecule \([\text{Ru}(\text{phen})_2\text{dppz}]^{2+}\) was used to intercalate into the base pairs of the dsDNA PCR amplification products, which were then directly applied to the pBPE-ECL device to perform the ECL assays. Under optimized experimental conditions, as little as 10 copies/µL of genomic DNA from \textit{Listeria monocytogenes} could be detected. The device was also used to specifically distinguish \textit{Listeria monocytogenes} from \textit{Salmonella}, \textit{Escherichia coli}, \textit{O157:H7} and \textit{Staphylococcus aureus}. Because the device is simple to construct, disposable, rapid, low-cost, label-free, and has high sensitivity, the pBPE-ECL molecular switch system has great potential for the point-of-care gene detection of pathogenic bacteria in developing countries and resource-limited and remote regions if integrating a battery as the power supply\(^46\) and a smartphone\(^37\) as the readout in the future.

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**EXPERIMENTAL SECTION**

**Materials.** Whatman chromatography paper (Φ = 125.0 mm, pure cellulose paper) was purchased from Hangzhou WoHua filter paper Co., Ltd. (Zhejiang, China) and used after adjustments in size. The conductive carbon ink (model number CNB-7, <60 Ω square\(^{-1}\)), which was used as a fabrication material for the driving/working electrodes, was obtained from Xuzhou Bohui New Materials Technology Co., Ltd. (Xuzhou, China). Solid wax and smooth spoon-like metal utensils were obtained from a local department store. \([\text{Ru}(\text{phen})_2\text{dppz}]^{2+}\) was synthesized and characterized by Professor Caiping Tan from Sun Yat-Sen University. The \(^1\)H NMR spectra of the compound are given in Figure S1. TPcA (≥98%, article number: 102-69-2) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

The primers were obtained from Shanghai Sangon (Shanghai, China). \textit{Salmonella enterica} (CMCC 50040), \textit{Escherichia coli} (O157:H7 GW1.0202), \textit{Listeria monocytogenes} (CMCC 54007), and \textit{Staphylococcus aureus} (CMCC 26003) were purchased from the Guangzhou Institute of Microbiology (Guangzhou, China). Chemicals for the PCR mixtures, including 10× PCR buffer (plus Mg\(^{2+}\)), the deoxynucleotide triphosphate (dNTP) mixture, and Taq polymerase, were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China) SYBR Green I dyes were obtained from SBS Genetech Co., Ltd. (Beijing, China). The DS 2000 DNA markers, which contained 2000-, 1000-, 750-, 500-, 250-, and 100-bp DNA fragments, were provided by Dongsheng Biotech Co., Ltd. (Guangzhou, China).

Deionized water was prepared with a water purification system (≥18 MΩ) and used in all of the experiments. All of the chemical reagents used were of analytical reagent grade without any further purification.

**Apparatus.** The DC power supply (Model LW-K605D) was purchased from Longwei Instrument Meter Co., Ltd. (Hong Kong, China). The voltage of the photomultiplier tube (PMT; MP-962, PerkinElmer, Wiesbaden, Germany) was set to 850 V for detection. The signal was then amplified and discriminated with transistor–transistor logic (TTL) and quantified using a multifunction acquisition card (PCI-1751, Advantech, Taiwan) controlled by a Labview-based software program that was configured in-house.

**Bacterial DNA Isolation and PCR.** The bacterial DNA was isolated using the protocol and reagents from the TIANamp Bacteria DNA Kit and then suspended in 50 µL of TE buffer. The PCR amplification reactions were performed in 25 µL of reaction mixture with final concentrations of 0.2 U µL\(^{-1}\) Taq DNA polymerase, varied amounts of target DNA, 0.2 mM each dNTP and 1X PCR buffer, and 40 nM each primer (i.e., forward and reverse). The control solution (blank) contained all of the PCR reagents except for the DNA template. The size of the PCR products of the target virulence gene, \textit{hlyA} of \textit{Listeria monocytogenes}, was 179 bp. The sequences were as follows:

Forward primer: 5′-AGGAATGACTAATCAAGACAAT-3′

Reverse primer: 5′-GGAAGGTCTTTCTAGGTTTCAT-3′

The PCR was performed in a thermocycler that was programmed with an initial step of denaturation at 95 °C for 5 min. The cycling conditions were as follows: denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and elongation at 72 °C for 40 s. In total, 35 cycles of the above program were performed. The last round of elongation was 8 min. The PCR products were analyzed with the pBPE-ECL system.

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Fabrication of the pBPE. The screen printing fabrication process for the pBPE was prepared according to previously reported methods, with slight modifications, as shown in Figure 2A. The hydrophilic channel was produced by a wax-screen printing method, whereas, the BPE and driving electrodes were fabricated on the hydrophilic channel by a screen printing technique with carbon ink. The designed shapes of the hydrophilic-channel-made screen for wax-screen printing were coupled to shapes of the electrode-made screen for screen-printing and were designed with Adobe Illustrator CS6 simultaneously. Then, the design documents were sent to the printing equipment shop (Lianchang, Guangzhou, China) to make the two kinds of 200-mesh screens.

The fabrication procedure of the pBPE chip included the following steps. First, the bare paper was cut into a uniform 100 mm × 80 mm size. Second, screen-printed the BPE and driving electrodes onto the paper simultaneously with the electrode-made screen and carbon ink. The semi-finished product were then dried for 5 min in an oven at 90 °C and cooled down to room temperature. Third, the dry semi-finished product was placed under the hydrophilic-channel-made screen, then, solid wax was squeezed onto the paper through the screen. A smooth metal spoon was used to further press and scrape the screen to ensure enough solid wax to print on the paper. Once the paper was wax-screen printed, it was placed in an oven together with the hydrophilic-channel-made screen. They were heated together at 80 °C for approximately 10 s to melt the wax into the paper through screen mesh interspace and form the hydrophobic barriers. While areas with a cross-linked photosensitive material blocked the melted wax and yielded the hydrophilic channel.

pBPE-ECL Assays. After the device fabrication was completed, the pBPE-ECL assays were performed. A detailed procedure is described below (Figure 1B). The as-prepared pBPE-ECL chip was placed on a 3D-printed substrate, and the pair of driving electrodes was connected to a DC power supply. Then, 25 μL of the assay solution containing 0.1 mM [Ru(phen)2dppz]2+ and 50 mM TPrA, which was prepared in 0.1 M PBS (pH 6.9), was dropped onto the center of the pBPE. The pBPE chip was turned over to ensure that the pBPE was facing toward the PMT, and the assembly was placed into a black box. Before the device was powered, a 10 s wait time was necessary to ensure that the entire channel was completely filled with the solution. Next, the DC power supply was turned on, and the ECL at the BPE anodic pole could be obtained. Finally, the ECL intensity was determined with a PMT.

Data Acquisition and Analysis. The ECL emission collected by the PMT was recorded with a Labview-based photon-counting computer program. The luminescence observed in the experiments was not constant and actually decayed over time. This behavior was likely caused by the irreversible decomposition of the luminophore. Regardless, it was found that the maximum luminescence signal, which was observed within 10 s, gave reproducible signals. This parameter (i.e., the maximum signal observed within 10 s) was used for all experiments.

RESULTS AND DISCUSSION

Detection Principle of pBPE-ECL. The proposed detection method was aimed at the construction of a disposable, wireless, low-cost, label-free, and sensitive system for genetic analysis of pathogenic bacteria. To achieve this goal, Listeria monocytogenes was selected as a specimen, and two specific primers that targeted the Listeria monocytogenes hlyA virulence genes were well designed to generate dsDNA amplicons via PCR. The light-switch molecule [Ru(phen),dppz]2+ was used to intercalate into the base pairs of the dsDNA of the PCR amplification products, which were then directly applied to the disposable, wireless, low-cost pBPE-ECL device to perform the ECL assays.

Initially, the label-free detection strategy was inspired by the fact that the ECL of [Ru(phen)2dppz]2+ is quenched in aqueous solution by the protonation of the phenazine N atoms in the excited state. However, when [Ru(phen)2dppz]2+ binds to DNA, the N atoms are protected by the planar phenazine ligand interacted with the major groove of DNA, which produced a change in microenvironment that favored the population of a luminescent state, resulting in intense ECL emission.

In addition, the emission intensity of the ECL is proportional to the [Ru(phen)2dppz]2+–DNA complex concentration in the presence of excess TPrA, thereby enabling quantitative genetic detection of pathogenic bacteria. The analytical principle of the pBPE-ECL molecular switch system is shown in Figure 1A. The mechanism of the [Ru(phen)2dppz]2+–DNA complex reaction with TPrA at the anode of the pBPE is depicted in the following equations:

\[
[Ru(phen)2dppz]^{2+} - DNA - e^- \rightarrow [Ru(phen)2dppz]^{3+} - DNA
\]  

(1)

\[
TPrA - e^- \rightarrow TPrA^+ - TPrA^+ + H^+
\]  

(2)

\[
[Ru(phen)2dppz]^{3+} - DNA + TPrA^+ \rightarrow [Ru(phen)2dppz]^{2+} - DNA + products
\]  

(3)

\[
[Ru(phen)2dppz]^{2+} - DNA \rightarrow [Ru(phen)2dppz]^{2+} - DNA + hv
\]  

(4)

Finally, the disposable, low-cost, and wireless characteristics of this analysis system are apparent from the workflow of the pBPE-
ECL system, which is illustrated in Figure 1B. In this study, the as-prepared pBPE was placed into two 3D-printed substrates to assemble the pBPE chip, and the pair of driving electrodes was connected to a DC power supply. Then, the assay solution containing \([\text{Ru(phen)}_2\text{dppz}]^{2+}\)–DNA complexes and TPrA was dropped onto the pBPE. The chip was turned over to ensure that the pBPE was facing toward the PMT, and the assembly was placed into a black box. When sufficient voltage is applied between the two ends of the driving electrode, the potential difference between the pBPE and the solution simultaneously drives redox reactions at both poles of the pBPE. As a result, ECL signals, which are generated at the anodic pole of the pBPE, are captured by the PMT and recorded with a Labview-based photon-counting computer program for further analysis.

Characterization of the pBPE-ECL Device. As a proof of concept, the fabrication procedure of the screen-printed pBPE is shown in Figure 2A. Briefly, wax-screen printing was employed to form hydrophilic channels on filter paper, and the carbon ink-based BPE and driving electrodes were screen-printed into the channels. The as-prepared pBPE was placed into two 3D-printed substrates to assemble the pBPE chip. Figure 2B shows that the overall length of the hydrophilic channel was designed to be 22 mm, in which the length of the BPE and driving electrodes were 3 mm and 6 mm, respectively. And the width of the BPE-containing region was 4 mm, as designed. The minimum width of the BPE that could be satisfactorily screen-printed onto paper was designed to be 1 mm. A typical screen-printed pBPE chip is displayed in Figure 2C, and the assembled pBPE chip is displayed in Figure 2D. As seen from the Tabletop Microscope TM3030 scanning electron microscope (Hitachi High Technologies, America, Inc.) image of the prepared pBPE (Figure 2E–G), the hydrophilic channel, hydrophobic region, and carbon electrodes were formed well on the paper. Figure 2E shows that the 3D porous structures and microfibers of the bare paper provided high surface area and excellent hydrophilic microenvironment for spatial and quantitative control of biological fluids using capillary action within paper. It can be also seen from Figure 2F that the wax had been well penetrated into the paper to form a hydrophobic barrier owing to the porous character of the paper. As shown in Figure 2G, the obtained continuous and dense carbon layer on the surface of the paper formed a good conductive electrode. The pBPE can be fabricated in a small batch with this method (Figure S2).

Optimization of the Driving Voltages. For this sensing platform, the electric field between the driving electrodes is a vital factor that influences the ECL intensity. Only an appropriate driving voltage can activate the sufficient simultaneous oxidation of the \([\text{Ru(phen)}_2\text{dppz}]^{2+}\)–DNA complex/TPrA and the reduction of \(\text{O}_2\). Therefore, the ECL results under driving voltages varying from 10 to 17 V were evaluated. In these experiments, it was found that the ECL intensity increased and reached a maximum value when the driving voltage was 14 V. In parallel assays with the control, weak ECL signals were always recorded regardless of the driving voltage (Figure 3A). The original ECL intensity data corresponding to each driving voltage are presented in Figure 3B. These results indicate that 14 V is the most appropriate voltage to trigger the faradaic electrochemical reactions at both poles of the BPE. Thus, 14 V was selected as the optimal voltage to supply between the two driving electrodes for the remaining experiments.

Sensitivity of the pBPE-ECL Analysis System. To validate the sensitivity of our newly developed pBPE-ECL assay system, under the optimal driving voltage, different concentrations of DNA from \(L.\) monocytogenes were employed in the PCR. The DNA was then incubated with 0.1 mM \([\text{Ru(phen)}_2\text{dppz}]^{2+}\) and 50 mM TPrA to evaluate the sensitivity of the pBPE-ECL analysis system. Figure 4A shows the electrophoretic analysis of PCR amplification reactions with different concentrations of target DNA; lanes 1–6 are \(10^6,10^5,10^4,10^3,10^2,\) and 10 copies/\(\mu\)L, respectively, and lane C is the negative control. As shown in Figure 4B, the ECL intensities increase linearly with increasing target DNA concentration from \(1 \times 10^1\) to \(1 \times 10^6\) copies/\(\mu\)L. The maximum luminescence signals corresponding to each concentration of target DNA and the negative control are presented in Figure 4C. A linear analysis of the experimental results in Figure 4D indicates a correlation coefficient (R²) of 0.9672, which suggests that this system is capable of reliably performing genetic assays. To define if a sample is \(L.\) monocytogenes-positive, a cutoff value is calculated based on the following formula

\[
V_{\text{cutoff}} = V_{\text{control}} + 3V_{\text{dev(con)}}
\]

where the \(V_{\text{control}}\) is the average light emission from the negative control without target DNA and \(V_{\text{dev(con)}}\) represents the standard deviation of the ECL reading from the negative control samples. According to this formula, the cutoff level for \(L.\) monocytogenes-positive samples was set at 372 counts/s. ECL signal less than this value should not be indicated as \(L.\) monocytogenes-positive under our conditions. With the use of our method, the limit of detection (LOD) was determined to be 10 copies/\(\mu\)L.

Similar to the SYBR Green I dyes that are used to bind to dsDNA for electrophoretic analysis, \([\text{Ru(phen)}_2\text{dppz}]^{2+}\) was used in the current assay not only for interacting with the major groove of DNA but also for the more sensitive ECL assay at the
same time. If we compare the electrophoretic analysis results in Figure 4A with the pBPE-ECL analysis results in Figure 4B, we can find that the sensitivity of the pBPE-ECL analysis system was as much as 100 times that of the electrophoretic analysis method. Overall, these results demonstrate well the extraordinary capability of our pBPE-ECL analysis system to detect Listeria monocytogenes.

**Specificity of the Current Assay.** Along with analytical sensitivity, specificity is also considered as one of the major factors that affect the analytical performance of a biosensor. To
Listeria monocytogenes and Staphylococcus aureus biological, clinical, and environmental applications. and label-free test platform also shows great potential for this simple, disposable, rapid, low-cost, highly sensitive, wireless, power supply and a smartphone as the readout in the future. ECL chip holds great promise for point-of-care and in-

Further verify the specificity of the pBPE-ECL analysis system, 10^6 copies/μL of target DNA from Listeria monocytogenes (Lis.), Salmonella (Sal.), Escherichia coli O157:H7 (E. coli.), and Staphylococcus aureus (Sta.) were subjected to PCR with the same Listeria monocytogenes-specific primers used previously. The gel electrophoresis results depicted in Figure 5A indicate that no amplification reactions occurred for Salmonella, Escherichia coli O157:H7, Staphylococcus aureus, or the control. However, a distinct band was observed in the assays with the Listeria monocytogenes bacterial DNA. The results with the pBPE-ECL analysis system in Figure 5B are consistent with the gel electrophoresis results. Only Listeria monocytogenes-specific PCR products coupled with [Ru(phen)2dppz]^2+ exhibited significant ECL intensity. These results confirm that the designed pBPE-ECL analysis system exhibits good specificity in distinguishing Listeria monocytogenes from Salmonella, Escherichia coli O157:H7, and Staphylococcus aureus due to the well-designed specific primers.

**CONCLUSION**

In summary, we have demonstrated for the first time a low-cost and disposable pBPE-ECL device coupled with the light-switch molecule [Ru(phen)2dppz]^2+ for wireless, label-free, and sensitive analysis of DNA from pathogenic bacteria. The optimal voltage used to drive the pBPE-ECL device was experimentally determined. The assay takes advantage of the inherent character of the light-switch molecule, which can intercalate into the base pairs of DNA for label-free detection, and the wireless pBPE-ECL platform to yield low-cost, disposable, and sensitive analysis. The device has a LOD of 10 copies/μL of genomic DNA for Listeria monocytogenes. Additionally, we showed that the device can be used to distinguish Listeria monocytogenes from Salmonella, Escherichia coli O157:H7, and Staphylococcus aureus. The pBPE-ECL chip holds great promise for point-of-care and in-field analyses of other pathogenic bacteria by integrating a battery as the power supply and a smartphone as the readout in the future. This simple, disposable, rapid, low-cost, highly sensitive, wireless, and label-free test platform also shows great potential for biological, clinical, and environmental applications.

**ASSOCIATED CONTENT**

**Supporting Information**

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Further experimental details including synthesis and characterization of [Ru(phen)2dppz]^2+ and a depiction of a small batch of the fabricated pBPE (PDF).

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**Notes**

The authors declare no competing financial interest.

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