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AN INVESTIGATION OF PSEUDOMONAS MARINCOLA TO DETERMINE PB(II) BIOSENSOR POTENTIAL

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ABSTRACT. Biosensors are strong alternatives to conventional analytical techniques such as HPLC and spectroscopic techniques for water quality and heavy metal detection. Heavy metal contaminated waters can monitor by microbial biosensors efficiently. For this purpose, newly isolated *Pseudomonas sp.* is used to develop a highly sensitive low-cost microbial biosensor for water quality monitoring. The objective of the study is the invention of new high sensitive low-cost microbial biosensors to determine heavy metals in aqueous solutions and optimise the working conditions *Pseudomonas marincola* cells were embedded onto the Screen Printed Electrode (SPE) carbon surface and dried for 30 minutes at laminar flow cabinet. Developed microbial sensors were immersed into the Pb(II) solution for electrochemical analysis. After the exposure time, cyclic voltammetry (CV) and differential pulse voltammetry (DPV) analyses were carried out. The study shows that the sensor was found in a linear range between $1x10^{-8}$ M and $8x10^{-8}$ M, with the lowest detection limit 10^{-9} M. The optimum pre-concentration time and scan rate were measured as 10 minutes and 10 mV/s, respectively. The results support that the new isolated *Pseudomonas sp*. has significant potential to determine the trace amount of lead in aqueous solutions.

1. INTRODUCTION

Pseudomonas marincola; Aerobic, gram-negative, unpigmented, motile, rod-shaped cells, 1.8-2.5 µm long and 0.4-0.6 µm in diameter. Oxidase and catalase positive. Colonies are smooth, unpigmented, off-white, transparent, and measure 3-4 mm in diameter. Strains produce brownish diffusible pigments on peptone-enriched media. Growth is observed in 0-8% (w / v) NaCl and at 5-37 °C (optimal at 25-28 °C); weak growth occurs at 4 °C and no growth above 38 ° C is observed. The pH range is 5.5-9.5 (optimal pH 6.5-8.5). Slowly produces H_2S. *Pseudomonas*-like bacteria are reviewed as usual members of microbial communities in aquatic environments. Few species belonging to the genus *Pseudomonas* have been retrieved in seawater or marine sediments. We recently introduced a new *Pseudomonas* member from Burdur Lake, Burdur, Türkiye [1].

Heavy metal pollution significantly harms soils, water, atmosphere and human health [2]. According to the World Health Organisation (WHO), Pb poisoning is

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among the most paediatric health problems worldwide. Pb, like other heavy metals, leads to respiratory and gastric dysfunctions in the human body [3-4]. Lead ion (Pb^{+2}), one of the toxic heavy metal ions, can cause severe damage to the brain and central nervous system [5]. The World Health Organization (WHO) requires that the Pb(II) content in the drinking water should be no more than 10 $\mu g \cdot L^{-1}$. However, due to the un-degradability and extensive use of lead in paints, batteries and ceramics industry, Pb(II) has become a major environmental contaminant in water, soils and air. Therefore, considerable efforts have been devoted to the ultrasensitive and quantitative detection of Pb(II). Up to date, the conventional methods, such as atomic absorption/emission spectroscopy [6], inductively coupled plasma atomic emission spectroscopy [7], and surface plasma resonance [8] are reliable and accurate. However, the requirement of expensive instruments, professional operation and elaborated sample pre-treatment limit the application of these methods for the on-site and real-time determination. In contrast, the electrochemical method could compensate for these disadvantages with high sensitivity and sufficient accuracy, which has attracted considerable attention and progress.

Biosensors have an essential role in detecting environmental contamination, including heavy metals and hydrocarbons, human health, sample quality and many other applications [9-10]. Using biosensors for quantitative analysis as an alternative to conventional methods has many advantages, such as being fast, compact, cheap and allowing to work on-site applications [11].

In this study, *Pseudomonas marincola* was isolated from Burdur Lake, Burdur, by using Nutrient Broth, and then Pb(II) interest of the bacterium was determined by electrochemical ways. The bacterium is used to prepare a modified carbon paste electrode (MCPE) by using whole living cells. Pb interest, current changes of surface and calibration curve were studied by using MCPE. Although *Pseudomonas sp*. was studied in many biotechnological applications, including heavy metal adsorption and biosensors, this is the first report of *P. marincola* about Pb(II) interest.

2. MATERIALS AND METHODS

2.1. Chemicals

All heavy metals in nitrates form, such as Pb(II), Cu(II), Ni(II), Zn(II) and Co(II), were purchased from (Merck, Germany). Heavy metal solutions were prepared freshly from metal nitrates before experiments and then deoxygenated by passing pure nitrogen gas through each heavy metal solution. The buffer solution was prepared from Tris–HCl's analytical grade (Sigma, Germany). Graphite powder and paraffin oil (Merck, Germany) were used to prepare both modified and non-

modified carbon paste electrodes (CPE). The pH of the supporting electrolyte solution was set using 0.1 M NaOH or 0.1 M HCl (Merck, Germany).

2.2. Isolation of microorganisms and culture conditions

Aqueous samples were collected from Burdur Lake, Türkiye, then 10% inoculated into tubes containing Nutrient Broth (NB) at 30 °C and pH 6.2 for 3 days. After incubation, colonies were collected and purified on Nutrient Agar (NA) plates. The pure cultures were kept at 4 °C and were transferred to a fresh medium every 3 months. Fresh -24h incubated on NA at 30 °C, the bacterium used for experiments.

2.3. PCR and Sequencing

Whole cells from exponentially growing cultures of the isolates were used for genomic DNA isolation and amplification of internal transcribed spacer (ITS) regions of 18s rRNA by conventional PCR. Genomic DNA of bacteria strains were isolated using Qiagen DNeasy Blood and Tissue kit. ITS regions were then PCR amplified using 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 907R (5'-CCGTCAATTCMTTTGAGTTT-3') primers. PCR was carried out with using 50 µL 1x Taq Buffer (Thermo Fisher Scientific, USA), 1,5mM MgCl, 0,2 mM dNTP, 0,3 pmol/uL primers (27F and 907R) and 1.24 U Taq polymerase (Thermo Fisher Scientific, USA) for each primer. DNA sequencing was performed at ABI 3100 Genetic Analyzer device with DNA sequence analysing BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA) in an external laboratory (Refgen, Ankara, Türkiye). Strains were identified according to the assessment of the results of DNA, 18S rDNA gene sequence and PCR analyses performed.

2.4. Preparation of Designed Microbial Biosensor

The designed carbon paste electrode as a working electrode was formed using teflon tube. Electrical conductivity was established by copper wire (5 mm diameter). The designed carbon paste electrode was filled with carbon paste. Carbon paste was prepared as 10 mg of living whole-cell bacteria, 90 mg of graphite powder, and paraffin oil. The non-modified carbon paste was prepared only with graphite powder and paraffin oil. Once the designed carbon paste electrode body was filled with freshly prepared carbon paste mixture, the electrode surface was smoothed on paper to obtain more homogenous results and prevent undesired noises.

Screen Printed Electrode (DS150) is purchased from Metrohm (Switzerland). SPE was prepared freshly before using each experiment set. The bacteria culture was diluted with 0.01 M Tris-HCl buffer solution and $8x10^7$ ($5.33x10^9$ CFU/ml) bacteria cells were embedded onto the electrode surface via drying at 30 °C under laminar flow.

2.5. Electrochemical Methods and Tools

All electrochemical measurements were accomplished at room temperature with a CH Instruments 660B model potentiostat (CH Instrument, USA). The instrument was supplied with a common three-electrode cell, including buffer solution 0.01 M Tris–HCl, deoxygenated by pure nitrogen through it before the measurements. CPEs modified with *P. marincola* were used as working electrodes. The platinum wire and Ag/AgCl electrode (saturated KCl) were used as counter and reference electrodes, respectively. Electrochemical experiments were done once accumulation was finished [12].

In the accumulation step, the working electrode was immersed in 10 ml of prepared heavy metal solution at a definite concentration for a selected time at an open circuit. Voltammetric analyses were performed immediately once the accumulation was completed. Cyclic voltammetry (CV) was carried out for bare and modified working electrodes. The CV working conditions were set from -1 to +1 V (from negative to positive direction) with a 0.01 V/s scan rate.

After obtaining the optimum working conditions for Pb(II), other model heavy metal solutions were prepared with 0.01 M Tris-HCl solutions to determine their interference effect with Pb(II). For this purpose, 10 ml of heavy metal solutions ($1x10^{-8}$ M for each heavy metal) containing a particular concentration of Ni(II), Co(II), Zn(II), and Cu(II) were used.

2.6. Surface Measurements

Modified Screen-Printed Electrodes (8x107 bacteria cells and $1x10^{-5}$ M Pb) were coated with Au-Pd. The surface morphology of the microbial biosensor was examined by Environmental Scanning Electron Microscopy (FEI Quanta 200 FEG ESEM Thermo Fisher, USA) at UNAM Bilkent University, Ankara.

Modified electrode surface changes at lower Pb concentration ($1x10^{-8}$ M) were analysed at Atomic Force Microscopy (Park System XE-100 PSIA, South Korea) with the non-contact mode at UNAM Bilkent University, Ankara.

XPS analysis (Thermo Scientific K-Alpha, USA) was performed with modified electrodes (treated $1x10^{-8}$ M) at UNAM Bilkent University, Ankara.

3. RESULTS AND DISCUSSION

3.1. Electrochemical behaviour of *Pseudomonas marincola*

The electrochemical behaviour of the bacterium was shown in Figure 1. Cyclic voltammograms of both modified and bare CPE were obtained in 0.01 M Tris-HCl for $1x10^{-5}$ M Pb(II). As seen in Figure 1, the reducing peak, which was characteristic for Pb(II), around -0.6 V was observed at the modified electrode. The cathodic peak around -0.4 to -0.6 V also address to Pb(II) by other researchers [12-13]. The cathodic peak corresponds to a reduction of surface-bonded Pb(II); thus, the peak area was decreased after each cycle was completed. This result is the first time showing *P. marincola* interacted with Pb ions.

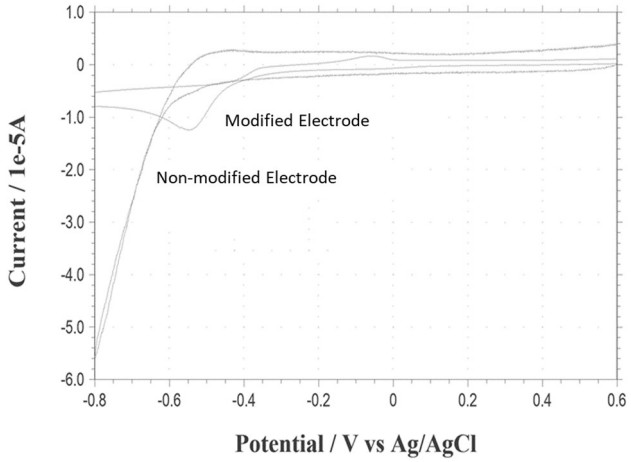

FIGURE 1. Cyclic Voltommograms of *P. marincola* ($1x10^{-5}$ M Pb(II) in 0.01 M Tris-HCl pH:7)

3.2. The effect of pre-concentration time

Pre-concentration time has significant importance in determining the bacteria response time. For this reason, modified CPE was placed in an electrochemical cell containing 10 mL $1x10^{-5}$ M Pb(II) solution at Open Circuit Potential (OCP) for 1000 seconds then-current changes were recorded in Figure 2. As seen in Figure 2, the current was increased until 10 minutes and then stabilised with minor increases-decreases. Until 10 minutes, Pb(II) ions were bound by bacteria located on the surface. After 10 minutes, the surface was saturated by ions then minor ion changes were started from surface to solution without reduction. Consequently, pre-concentration time was selected as 600 seconds and was applied for further experiments.

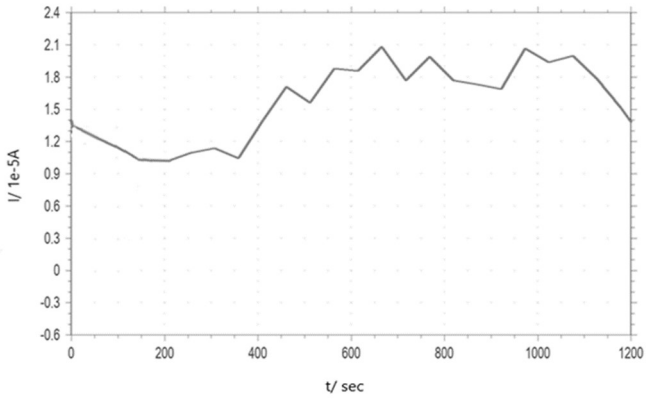

FIGURE 2. Current/ Pre-concentration time of *P. marincola*.

3.3. Detection of working limits of the designed sensor

Bacteria cells were diluted with 0.01M Tris-HCl (pH: 7) buffer solution in a sterile Eppendorf tube, and then 15 µl of the diluted solution was dropped onto the SPE surface in the sterile laminar flow cabinet. SPE was dried for 30 min at 30 °C in the laminar flow cabinet. Ligament or any mediator was not used for fixing the bacteria cells. Therefore bacteria cells were fixed on the bare SPE surface directly. All SPE were prepared freshly before the experiments. The minimum limits for Pb solutions were tested with modified SPE. Figure 3 shows the calibration range for modified SPE between $1x10^{-8}$ and $8x10^{-8}$ M Pb. The Limit of Detection (LOD) was obtained as $1x10^{-9}$ M using the formula $3\sigma/s$ [14-15].

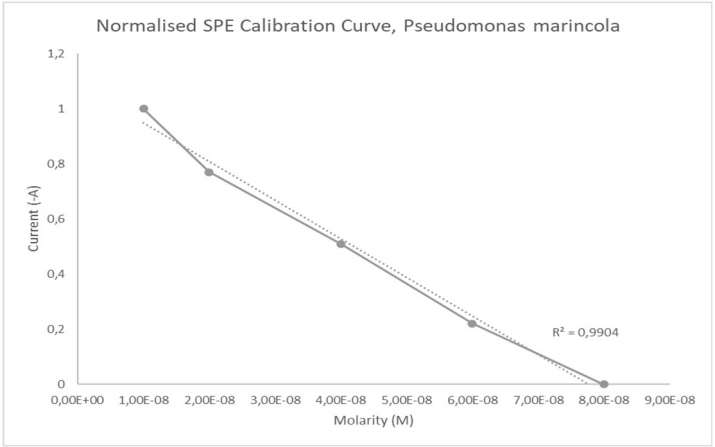

FIGURE 3. Fitting curve between $1x10^{-8}$ and $8x10^{-8}$ M for modified screen-printed electrode

3.4 Surface analysis of modified screen-printed electrode

SEM images were used to show the embedded bacteria cells and components on the electrode surface. As seen in the SEM images, bacteria cells were perfectly fixed onto the surface without using any ligament such as glutaraldehyde (Figure 4).

AFM was used for the detailed topographical analysis of modified SPE treated $1x10^{-8}$ M Pb. The topographical image shows the bacteria cells fixed in the pits on the SPE surface. Once the modified electrode was treated with $1x10^{-8}$ M Pb solution, Pb ions were accumulated on the electrode surface. While Pb ions were accumulating onto the surface, surface thickness was not changed significantly. On the other hand, the surface smoothness and homogeneity were collapsed by Pb ions, as seen in Figure 5.

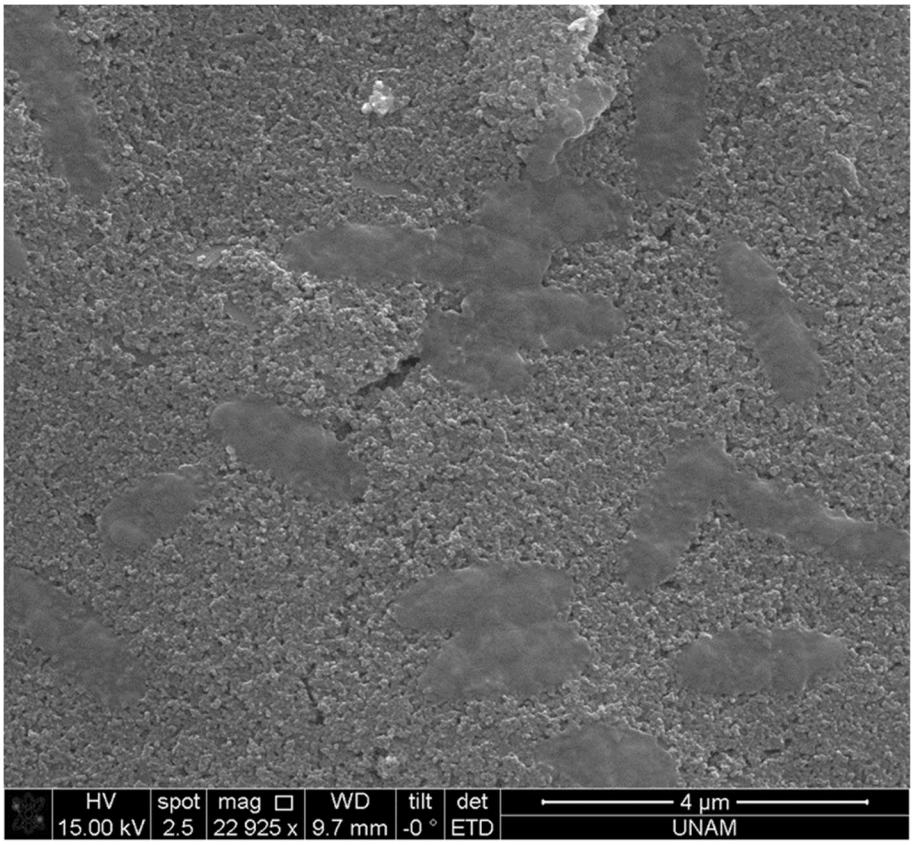

FIGURE 4. The bacteria cells onto the modified screen-printed electrode (treated $1x10^{-5}$ M)

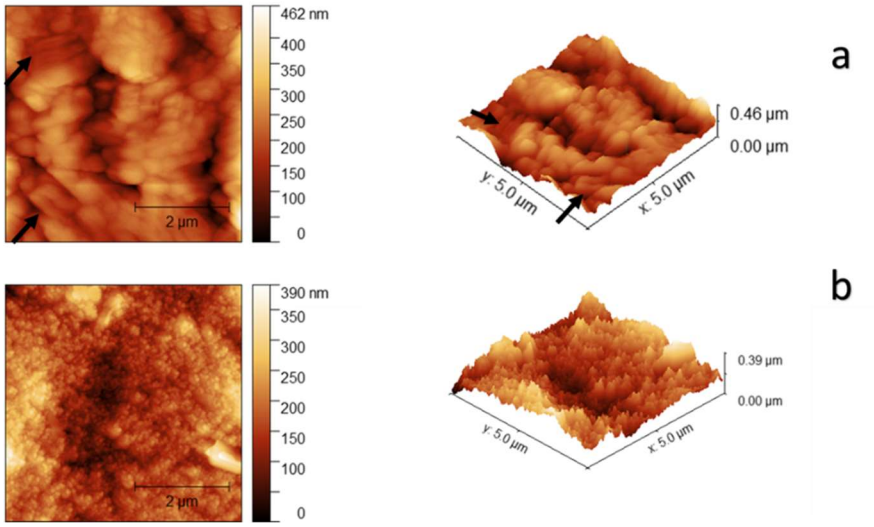

FIGURE 5. Modified electrode surface without Pb treat (a), modified electrode surface after Pb treat (b)

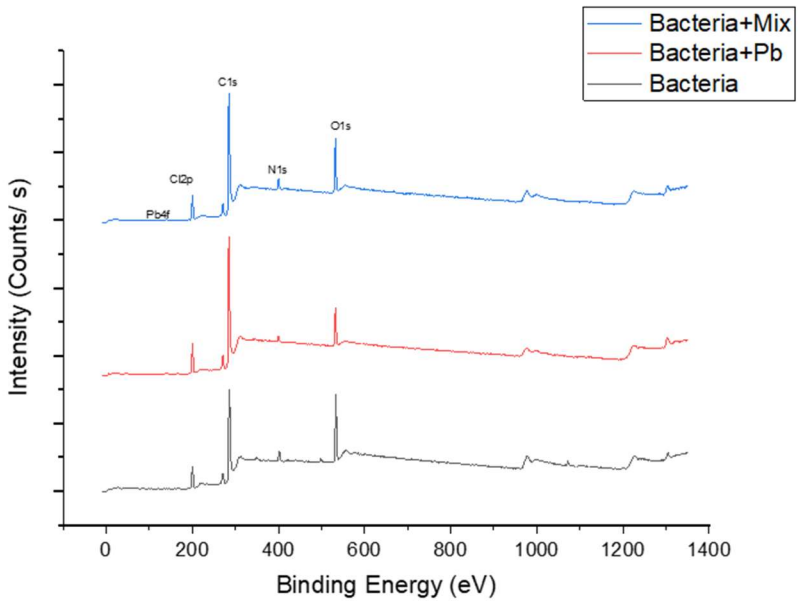

FIGURE 6. XPS scan for the non-treated electrode (bacteria), treated with $1x10^{-8}$ M Pb (bacteria+Pb) and treated with $1x10^{-8}$ M mixed heavy metal solution (bacteria+Mix)

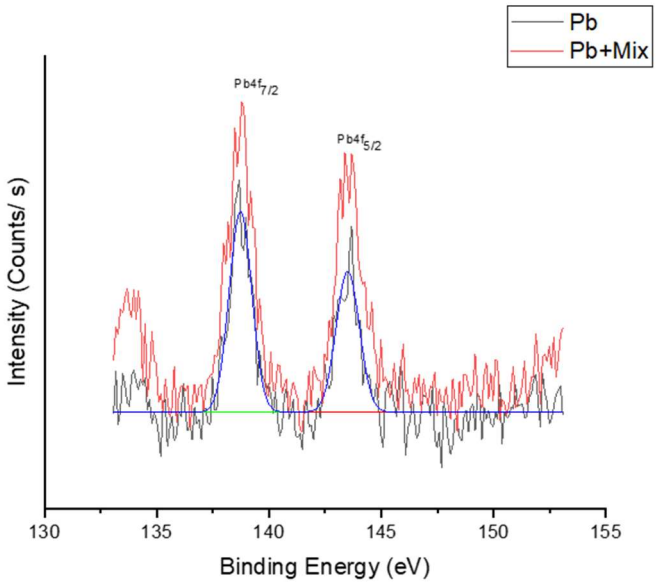

FIGURE 7. High resolution of Pb with treated $1x10^{-8}$ M Pb and $1x10^{-8}$ M mixed heavy metal solution

The whole-cell microbial biosensors have been recognised as a novel, cost-efficient replacement for conventional sensors. Another advantage of whole-cell microbial biosensors is the fast and specific detection of heavy metals. Thus whole-cell microbial biosensors can use for in-situ water monitoring [22]. On the other hand, the detection limit is a common issue for whole-cell microbial biosensors. The average LOD is 0.1 µM for whole-cell microbial biosensors; however, the operating range of the designed sensor with *P. marincola* is well beyond this limit [23]. Moreover, the developed sensor is significantly specific for Pb(II) even in 0.01 µM mixed heavy metal solution. As seen in Figure 8, the lead accumulation is not changed in mixed heavy metal solution, and the almost exact amount of lead is accumulated on the electrode surface.

4. CONCLUSIONS

In this study, newly isolated *P. marincola* from Burdur Lake, Burdur, was cultured and used as biomaterial to detect Pb(II) in aqueous solutions with a $1x10^{-9}$ M limit of detection. This study is the first report of using living whole-cell *P. marincola* as biomaterial to detect Pb(II) by electrochemical ways. Compared with other electrochemical determinations, the method presented here has a lower detection limit and a wide working range. *P. marincola*, which has biotechnological importance, may uptake Pb(II) ions on the surface in 10 minutes. Moreover, the designed low-cost biosensor does not require any

mediator for electrochemical response or fixator to embed on the SPE surface. Living cells can fix on the surface under laminar flow conditions without extra effort. The designed biosensor can be prepared and detect Pb in under 30 min from the initial step to the final stage. *P. marincola* is a promising Pb agent for potential biosensor, bioremediation and biosorption studies.

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Author Contribution Statement CB- data collection, management and manuscript writing. HN- data analysis GD- project development, manuscript writing and manuscript editing. All authors have read and approved the manuscript.

Declaration of Competing Interests The authors declare no conflict of interest.

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