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Materials Advances

ARTICLE

Leaf-like copper oxide mesocrystals by collagen-assisted biomineralization show attractive biofunctional and electrochemical performance

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The development of high-performance biocompatible batteries is pivotal in personalized health-care devices such as pacemakers and neuro-stimulators. We have for the first time developed a one-pot collagen-assisted biomineralization strategy to create hierarchical CuO nanostructures. Recombinant collagen has been demonstrated as an excellent biotemplate to delicately tune the morphologies of copper oxide mesocrystals. The initial formation of Cu(OH)₂ nanoneedles gradually turned into leaf-like CuO nanostructures with branched edges and a compact middle during the biomineralization process. The as-prepared leaf-like CuO mesocrystals exhibited attractive electrochemical performance, which may have great potential as a promising anode material for lithium ion batteries. Notably, collagen also plays as a fabulous functional agent to endow the CuO nanomaterials with high biocompatibility and bioactivity. The environmentally amiable method assisted by collagen provides novel opportunities for the construction of advanced CuO nanomaterials with well-controlled morphology, function and electrochemical performance, which may have promising applications in implantable health-care electronics.

1. Introduction

Biomineralization is a ubiquitous process in nature to create inorganic mesocrystals with delicately controlled morphologies and polymorphs. Bones, fish scales and shells are classical products of biomineralization in various living organisms. The biomolecular templates such as proteins have been discovered to play an essential role in the growth and nucleation of these inorganic crystals. The exploration of biomolecular building blocks to synthesize hierarchically mesocrystals has gained increasing attention due to their fabulous features such as mild reaction conditions and precise tuning of sizes and shapes. Proteins have remarkable advantages such as abundant natural resources and pronounced structural diversity, and they have been extensively investigated to assist the formation of nanostructured materials. 

Metal oxide nanoparticles have been widely used in a range of applications such as energy storage, catalysis and antimicrobics. There are many materials used for lithium battery anodes. Copper oxide (CuO) has attracted particular interests due to its environmental amiability and high chemical stability of copper oxide, which exhibits good electrochemical performance and great potential as lithium ion battery anodes. Furthermore, collagen functionalizes CuO nanomaterials with high biocompatibility and bioactivity. These novel CuO nanomaterials with well-controlled morphology, function and electrochemical performance may open promising opportunities in implantable electronics.

Extensive efforts have been performed to synthesize well-ordered copper oxide nanomaterials with a variety of morphologies ranging from nanorods and nanowires to dumbbells and honeycombs. Organic ligands such as acetic, citric, and tartaric acids have been reported to regulate the crystal growth of copper oxide nanostructures through their coordination with copper ions. Recently, Chen’s group has developed a silk fibroin-assisted biomineralization route to synthesize almond-like copper oxide mesocrystals. However, the potential toxicity of the reported copper oxide nanomaterials may not meet the biocompatibility requirements of the rising demand of implantable electrical devices in health-care applications.

2. Experimental section

Preparation of recombinant collagen
Recombinant collagen was expressed in E. coli BL21 strain following previously reported protocols. Briefly, cells were grown in 50 ml LB medium overnight at 37 °C, and transferred to 1 L LB media. When OD600nm of the culture reached 0.8, 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to induce protein expression at 25 °C. Cells were harvested, and then re-suspended in the binding buffer (20 mM sodium phosphate buffer, pH 7.4, 500 mM NaCl, 20 mM imidazole). The supernatant fraction was collected after cell disruption. Raw proteins were purified using a Ni-NTA-Sepharose column with the elution buffer (20 mM sodium phosphate buffer, pH 7.4, 500 mM NaCl, 500 mM imidazole), and dialyzed against glycine buffer (30 mM, pH 8.6). Recombinant collagen was produced by trypsin digestion of the purified protein as previously described. The purity of recombinant collagen was confirmed by SDS-PAGE. The pure collagen was dialyzed, lyophilized and stored at -20 °C for future use.

Synthesis of copper oxide nanostructures
120 mg Cu(CH$_3$COO)$_2$ $\cdot$ H$_2$O and 15 ml 10 mg/ml collagen solution were added to 12 ml water, and the mixture was under stirring for 1 hour to obtain a blue transparent solution. 3 mL 1.6 mol/ L NaOH solution was added to the blue solution, and stirred for 30 min. The mixture was then transferred into a 50 ml Teflon-lined stainless-steel autoclave. The autoclave was heated to 80 °C and maintained for 12 hrs. The reaction mixture was estimated to contain 0.5wt% collagen and 0.02 mol/L Cu(CH$_3$COO)$_2$ $\cdot$ H$_2$O ($\text{Cu(II)}$) = 0.02 mol/L). After the autoclave was cooled to the room temperature, the precipitates were collected by centrifugation, washed with distilled water and ethanol in turn three times, and dried in air at room temperature. To investigate the effect of collagen on the morphology of nanoparticles, the concentration of collagen was varied from 0.01 to 0.5wt% while [Cu(II)] was set as 0.02 mol/L.

Characterization of copper oxide nanoparticles
Powder X-ray diffraction (XRD) patterns were measured on a Rigaku D/max-2400 X-ray diffractometer (Japan) with Cu Ka radiation (40 kV, 40 mA) at a scanning rate of 0.02 °/s in the 2θ range from 10 to 80°. X-ray photoelectron spectroscopy (XPS) experiments were performed on a Kratos Axis UltraDLD X-ray photoelectron spectrometer (England) with a monochromer X-ray source using Al Kα (1486.6 eV) radiation. The binding energies measured by XPS were corrected by referencing the C 1s line to 284.5 eV. FESEM (Field-emission scanning electron microscopy) images were acquired on a Hitachi S-4800 field emission scanning electron microscope (Hitachi Limited, Japan) with an operating voltage of 5.0 kV. The dried samples were sputter-coated with Au for 25 sec prior to imaging. TEM (Transmission electron microscopy), HRTEM (High-resolution transmission electron microscopy), SAED (Selected area electron diffraction), and electron diffraction (EDX) measurements were carried out using a JEM-2100 transmission electron microscope (JEOL, Japan) at 200 kV. FT-IR (Fourier transform infrared spectroscopy) spectra were recorded on a Nicolet NEXUS 670 infrared spectrophotometer. TGA (Thermogravimetric analysis) experiments were performed on a TGA/NETSCH STA449 F3 instrument under a nitrogen atmosphere, employing a heating rate of 10 °C/min from 25 °C to 800 °C.

Electrochemical properties of CuO nanostructures
Copper oxide nanomaterials prepared with 0.5wt% collagen and 0.02 mol/L [Cu(II)] were used for further characterization of electrochemical performance. 60wt% of active material (copper oxide), 20wt% super P carbon, and 20wt% binder polyacryl acid (PAA) were mixed to form a uniform slurry material. Electrodes for electrochemical testing were fabricated by loading the mixed slurry at a rate of 1.5 mg/cm$^2$ with a doctor-blade on a Cu foil current collector. The liquid electrolyte was 1.0 M LiPF$_6$ in acetonitrile, (EC) and dimethyl carbonate (DMC) at 1:1 volume ratio and separator was a Celgard 2400. A half-coin cell was assembled in a glove box with lithium foil as another electrode. The galvanostatic discharge/charge cycles were measured by using an electrochemical workstation (CHI 660E, Shanghai China) in the voltage between 0.01 and 3 V (vs Li+ /Li) at different current densities at a 0.1 mA s$^{-1}$ scan rate.

Cytotoxicity of leaf-like CuO
Cell counting kit-8 (CCK-8) was used to evaluate the cytotoxicity of the leaf-like CuO nanomaterials using HFF-1 cells. HFF-1 cells were incubated in DMEM culture medium supplemented with 15% bovine serum solution in a humidified atmosphere of 5% CO$_2$ at 37 °C. 100 ul of HFF-1 cell suspension was added in a 96-well cell-culture plate at a density of 5x10$^3$ cells per well, and incubated for 24 hrs to allow attachment. 100 μL leaf-like CuO materials with four different final concentrations (0.1, 1, 10, and 100 μg/mL) was then added into the wells. Equal volume of DMEM medium was added as in other wells as control groups. After the incubation of 24 hrs, 10 ul CCK-8 solution (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-Disulfobenzene)-2H-tetrazole monosodium salt) was added into each well. The 96-well plate was incubated for 2 hrs (5% CO$_2$ at 37 °C). Tecxan Infinite F200/M200 multifunctional microplate reader (Tecan, Mannedorf, Switzerland) was used to measure the optical density at 450 nm. Cell viability was calculated as the mean absorption value of four measurements of each condition divided by the mean absorption value of the control group.

Cell adhesion assay
Nunclon Delta TC Microwell plates were coated with one thin layer of leaf-like CuO. The plates coated with heat-denatured BSA were used as control groups. The plates were washed with PBS three times. Then 100 μL HFF-1 cell suspension (1x10$^5$ cells/ml) in serum-free DMEM medium was added, and incubated for 6 hrs at 37 °C. Unattached cells was washed off with PBS buffer (10 mM). The adhered cells were measured by a total deoxyribonucleic acid (DNA) quantification assay (Hoechst 33258, Solarbio). The cells were lysed by repeated freezing and thawing in ultrapure water. Hoechst 33258 with a final concentration of 5 μg/mL was added to the cell lysates, and the mixtures were incubated in the dark for 1 hr. The fluorescence intensity was recorded on a microplate reader (Tecan Infinite M200) at 360 nm excitation wavelength and 465 nm emission wavelength. The measurements were repeated three times.

Immunofluorescence Staining
Fluorescent confocal dishes (Nontreated) were coated with one thin layer of leaf-like CuO. The coverslips were washed 3 times with 10 mM, pH 7.4 PBS buffer. Then the HFF-1 cells were added on the coverslips at a density of 400 cells/mm$^2$, and incubated at 37 °C for 24 hrs. The attached cells were fixed in cold 4% paraformaldehyde for 10 min, and permeabilized with 0.1% Triton X-100 for 5 min. 1% BSA in PBS buffer (10 mM, pH 7.2-7.4) was used as a blocking agent for 0.5 hrs at room temperature. The cells were incubated with phallolidin-tetramethylrhodamine isothiocyanate for 1 hour at 37 °C for the actin cytoskeleton staining. Then DAPI was added for nuclear staining at 37°C for 10 minutes. The images were acquired on Leica (Leica Microsystems Inc., Wetzlar, Germany) fluorescence microscope.

3. Results and discussion
Synthesis and characterization of copper oxide nanostructures
Pure recombinant collagen was produced following previously reported procedures. Collagen was explored as biotemplates to synthesize copper oxide nanoparticles. Briefly, the mixture of collagen, copper acetate and sodium hydroxide was prepared and
incubated at 80 °C for 12 hrs to initiate the crystal transformation and assembly of primary copper oxide mesocrystals. The final product was collected for further characterization.

X-ray diffraction (XRD) and X-ray photoelectron spectroscopy (XPS) techniques were employed to investigate the crystal type and elemental composition of the as-prepared products via collagen-templated biomineralization (Figure 1, S1). The diffraction peaks followed the same pattern as the standard XRD spectrum of pure CuO crystals (copper oxide, JCPDS No. 05-0661) (Figure 1). The XPS spectra of the as-prepared samples showed characteristic peaks corresponding to Cu 2p1/2, Cu 2p 3/2 and O 1s, further confirming the presence of CuO (Figure S1). These results indicated that the nanomaterials generated by collagen-templated biominalization were pure CuO without any other phases such as Cu$_2$O.

Field-emission scanning electron microscopy (FESEM) and transmission electron microscopy (TEM) techniques were applied to characterize the morphology and detailed structure of the synthesized copper oxide mesocrystals (Figure 2). Copper oxide particles were produced via collagen-templated biomineralization with a concentration of collagen as 0.5 wt % and Cu(II) as 0.02 mol/L. The SEM images displayed a uniform three-dimensional leaf-like geometry with approximately 1 um length, 429 nm width and 35 nm thickness (Figure 2a-b). The magnified SEM image of the leaf-like particles showed that each particle was hierarchical and composed of primary nanoparticle units (Figure 2b).

The TEM images indicated that the copper oxide mesocrystals were assembled through an oriented growth with primary nanoparticles (Figure 2c-d). Notably, the leaf-like mesocrystals showed a much more compact middle region than the edges (Figure 2c-d). The HRTEM image of the middle section showed that most crystalline lattice planes were well aligned, and there were clear gaps between the primary nanoparticles in the selected area electron diffraction (SAED) pattern (Figure 2e). The single-crystal diffraction pattern with relatively short diffraction arcs indicated that the primary copper oxide nanoparticles were arranged in a highly oriented way in the middle region (Figure 2g). The inter-fringe distance was measured as 0.26 nm, corresponding to the (-111) plane of monoclinic CuO (Figure 2e). Meanwhile, the HRTEM image of the edges of leaf-like mesocrystals displayed a more random pattern, and elongated diffraction arcs were observed in the corresponding SAED pattern (Figure 2f, 2h). These results demonstrated that the lattice planes were less orderly stacked at the edges of leaf-like CuO mesocrystals.

The EDX (Energy dispersive X-ray spectroscopy) spectrum of the as-prepared particles indicated the presence of elements C, Cu, and O, demonstrating the inclusion of collagen in the CuO mesocrystals (Figure S2). In addition, the FT-IR spectrum showed vibration peaks corresponding to the N-H, C=C and Cu-O bonds, again confirming the presence of collagen within the CuO particles (Figure S3). All the results indicated that the collagen-templated biominalization process can generate copper oxide mesocrystals with well-ordered hierarchical structures.
The critical role of collagen in biomineralized CuO nanostructures

The morphologies of CuO mesocrystals obtained under various concentrations of collagen were compared in order to evaluate the critical role of collagen in the biomineralization (Figure 5). In the absence of collagen, only disordered CuO particles were observed (Figure 5a). When the concentration of collagen was increased to 0.05 wt %, the CuO particles formed uniform five-star leaf-like structure (Figure 5b). When the collagen concentration was increased to 0.2 and 0.5 wt %, the CuO particles turned to be ficus leaf-like and eucalyptus leaf-like, respectively (Figure 5c-d). The pronounced dependence of the morphologies of copper oxide mesocrystals on the concentration of collagen demonstrated that recombinant collagen could handily tune the CuO nanostructures.

Collagen has been reported as the critical biotemplate for the synthesis of hydroxyapatite with hierarchical nanostructures.18 Previous studies have shown that silk fibroin provided a nice template to modulate the shape of CuO nanoparticles.34 These results indicated that collagen likely shared a similar mechanism, and played a key templating role in the production of CuO mesocrystals.

Thermogravimetric analysis (TGA) was carried out to measure the weight loss of CuO samples synthesized under different collagen concentrations in order to evaluate the collagen participation in the formation of the CuO nanostructures (Figure 6). In the absence of collagen, CuO particles displayed a constant weight loss of approximately 2.2 wt %. When the collagen concentration was increased, the weight loss of CuO samples became larger accordingly. The mass loss reached about 4.8 wt%, when the collagen concentration was 0.5 wt % (Figure 6). These results indicated that collagen played a critical role in the formation process of CuO nanostructures, and a significant amount of collagen was packed within the copper oxide mesocrystals.

Figure 3. FESEM images of the leaf-like copper oxide mesocrystals obtained by collagen-templated biomineralization ([collagen]= 0.5 wt %, [Cu(II)] = 0.02 mol/L) after different incubation time: (a) 0.5 hr; (b) 1.5 hrs; (c) 3 hrs; (d) 6 hrs; (e) 9 hrs; (f) 12 hrs.

Figure 4. XRD patterns of the leaf-like copper oxide mesocrystals obtained by collagen-templated biomineralization ([collagen]= 0.5 wt %, [Cu(II)] = 0.02 mol/L) after different incubation time: (a) 0.5 hr; (b) 1.5 hrs; (c) 3 hrs; (d) 6 hrs; (e) 9 hrs; (f) 12 hrs.
Figure 5. FESEM images of copper oxide mesocrystals obtained after 12 hrs via collagen-templated biomineralization with a constant Cu(II) concentration (0.02 mol/L) and varying concentrations of collagen: (a) 0, (b) 0.05, (c) 0.2, (d) 0.5 wt %.

Figure 6. TGA curves of CuO samples synthesized under various concentrations of collagen: (a) 0, (b) 0.05, (c) 0.1, (d) 0.2, (e) 0.5 wt %.

The effect of copper ions on CuO nanostructures
The role of copper ions in modulating the collagen-templated synthesis of CuO mesocrystals under a constant concentration of collagen (0.1wt%) was also examined (Figure S4). When the concentration of copper ions was varied in the range of 0.01 mol/L to 0.03 mol/L, the CuO mesocrystals displayed eucalyptus leaf-like shape. When the concentration of copper ions was increased to 0.1 mol/L, the CuO mesocrystals became jujube core-like. It suggested that the morphologies of CuO mesocrystals could be delicately tuned by copper ions in the biomineralization process.

Electrochemical properties of CuO nanostructures
The electrochemical performance of the prepared eucalyptus leaf-like copper oxide mesocrystals was evaluated by the galvanostatic method. The galvanostatic discharge-charge cycle curves of the CuO electrode for the first 80 times were measured at 0.2C (1C = 890 mA/g) (Figure 7a). The first discharge curve displayed three potential regions. The first region was 2.0 to 1.8V, which indicated that Li⁺ reacted with CuO to produce Cu₁₋ₓLiₓO₁₋ₓ/₂ (0<x<0.4). The second (1.2-0.9V) and third (0.9-0.001V) regions corresponded to the formation of Cu₂O and the conversion of Cu₂O to Cu and Li₂O, respectively.²², 42, 43

Figure 7. Electrochemical properties of the CuO electrode. (a) Voltage profiles of the CuO electrode for different charge–discharge cycles at a current rate at 0.2C; (b) specific discharge capacity of the CuO electrode for the first 85 cycles at 0.2C; (c) specific capacity as a function of cycle number and charging (black open circles) or discharging (red open circles) at various rates. The rate for the first discharge process was set at 0.1C, and then the rate was respectively increased to 0.5, 1, and 2C in a voltage range of 0.001–3 V.

The cycling performance of the CuO electrode was examined for 85 cycles at 0.2C rate (Figure 7b). The discharge capacity dropped at the first cycle, and gradually decreased to reach a constant value after 25 cycles. After 85 cycles, the CuO electrode maintained a high discharge capacity of 910 mAh/g, indicating a high capacity retention.
of 76%. This is one of the highest capacity retention among all the reported anode materials consisting of pure CuO nanostructures (Table S1). The specific capacity of the CuO electrode at different current densities was summarized (Figure 7c). It showed a discharge capacity of 703 mAh/g at 0.1C rate after 5 cycles. When the current density increased, the discharge capacity got decreased. After 5 cycles of high current density at 2C, there was still a discharge capacity of about 300 mAh/g. The phenomenon probably resulted from the poor conductivity of collagen, and its effects got enhanced as the current density increased.

As an anode material for lithium ion batteries, the leaf-like CuO nanomaterials showed the highest initial discharge capacity (1369 mA·h·g⁻¹) as well as reversible capacity (1041 mAh/g) at a 0.2C rate when compared with other previously reported CuO nanostructures. In addition, it showed good cycling stability, and maintained a discharge capacity as high as 910 mAh/g even after 85 cycles. This was among the highest final discharge capacity for all the reported CuO nanostructures (Table S1).

The as-prepared leaf-like CuO mesocrystals exhibited excellent electrochemical features, which may result from its unique morphology. First, the CuO mesocrystals provided better accommodation of the strain energy and proper electrode-electrolyte contact area, and decreased the polarization of the electrode during the charge-discharge cycles. Second, the CuO mesocrystals consisted of orderly-packed 2D nanoplates, leading to shorter lithium ion diffusion path and faster surface electrochemical reactions, therefore enhancing the capacity during the charge-discharge process. Meanwhile, the leaf-like CuO mesocrystals may resist particle pulverization and agglomeration during cycling. Previous studies have suggested that the extra capacity could result from the reversible transformation of polymeric gel-like films. Further studies also revealed that transition metal nanoparticles may play as efficient electrocatalysts to activate and/or promote the reversible transformation of some inorganic components in SEI films. These factors could contribute to the increasing capacity for lithium storage.

**Biocompatibility and bioactivity of the leaf-like CuO**

The biocompatibility and bioactivity of the as-prepared leaf-like CuO nanomaterials was further investigated (Figure 8). In vitro cytotoxicity of leaf-like CuO nanomaterials was evaluated by examining the viability of HFF-1 cells using the CCK-8 assays (Figure 8a). The leaf-like CuO showed similarly high cell viability at four different concentrations (0.1, 1, 10, and 100 µg/mL), indicating that the as-prepared leaf-like CuO nanomaterials have nice biocompatibility (Figure 8a). It suggested that the inclusion of the natural protein collagen in the CuO nanomaterials probably endowed the high biocompatibility.

Collagen is a key structural and functional protein in human body. The bioactivity of leaf-like CuO nanomaterials was determined by cell adhesion assay using HFF-1 cells (Figure 8b). HFF-1 cells were cultured on the plate wells coated with leaf-like CuO and heat-denatured bovine serum albumin (BSA). The fluorescence intensity of the wells coated with leaf-like CuO was much higher than those coated with the control BSA, demonstrating that HFF-1 cells could efficiently attach to the as-prepared leaf-like CuO nanomaterials (Figure 8b). It indicated that collagen can nicely functionalize CuO nanomaterials with cell adhesion bioactivity.

Cell adhesion and spreading features were examined by confocal fluorescence microscopy (Figure 8c-d). HFF-1 cells were fixed and stained for actin stress fibers and nuclei with phalloidin-tetramethylrhodamine isothiocyanate and DAPI, respectively. The HFF-1 cells on the CuO-coated substrates showed well-developed actin cytoskeletal structure (Figure 8c-d). The extensive cell adhesion and spreading demonstrated that collagen facilitated CuO nanomaterials with superior biofunction. These results indicated that collagen not only provides a robust template to modulate the nanostructures of CuO, but also plays as a functional agent to empower the prepared nanomaterials with attractive bioactivity.

**4. Conclusion**

In conclusion, copper oxide mesocrystals with novel hierarchical structures have been successfully created using a biomineralization approach with recombinant collagen as the template. We have demonstrated that the nanostructures of copper oxide particles can be finely modulated by the collagen concentration (Scheme 1). Collagen-templated biomineralization probably displays a similar mechanism that has been reported for silk fibroin. First, Cu(II) ions form a complex with collagen by interaction with polar amino acids when copper acetate is added in the collagen solution. Then, the addition of sodium hydroxide drives Cu(II) to form Cu(OH)₂, which possesses a nanoneedle-like structure due to the interaction of Cu²⁺ and the biotemplate collagen. Thirdly, the Cu(OH)₂ is further assembled into a primary nanostructure, which is dehydrated into CuO at the meantime. Finally, the free CuO in the reaction solution gets crystallized on
the primary nanostructure, leading to the formation of larger leaf-like mesocrystals. The primary nanostructure is formed using collagen as the template, therefore leading to a more ordered, compact middle region. In contrast, the later crystallization of free CuO lacks the assistance of collagen template, and results in loose ends. During the collagen-templated biominalarization process of CuO nanoparticles, the formation of the complex between Cu(II) and collagen seems to play a determinant role.

Collagen provides a superior biotemplate for the production of copper oxide mesocrystals. Compared with silk fibroin, collagen is able to regulate the hierarchical structures of copper oxide mesocrystals with a much lower concentration, and collagen can produce a much richer diversity of hierarchical nanostructures.\(^3\) Compared with previously reported proteins, collagen contains a large number of charged amino acids, which may promote the interaction of copper ions and collagen. Furthermore, the distinct (Gly-X-Y)\(_n\) amino acid sequence pattern and triple helix structure may provide unique capability for collagen in protein-templated biominalarization. In addition, recombinant technology can produce collagen of single-size and high purity, and it can be used to easily modify the amino acid sequences of collagen.\(^9, 10\) Therefore, recombinant collagen may provide a powerful strategy to create superior biotemplates.

Notably, the novel leaf-like CuO mesocrystals prepared by the collagen-assisted biominalarization show excellent biofunctional and electrochemical performance. Advanced health-care devices such as cardiac pacemakers, neuro-stimulators and hearing aids have for the first time discovered that the unique protein collagen with exquisite morphology, but also functionalizes them with high biocompatibility and bioactivity. The newly developed environmentally friendly method assisted by collagen provides fabulous opportunities for the construction of advanced high-performance biofunctional CuO nanomaterials, which may have great potential in implantable health-care electronic systems.

Conflicts of interest
The authors declare no competing conflict of interests.

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