INTRODUCTION

The ability to clearly visualize the details of human tissues represents the great progress of diagnostic technologies in modern medicine. Ultrasound imaging, as an advantageous diagnostic imaging technique, is widely used because of its favorable features, including real-time, non-invasion, low cost, and high safety. However, ultrasound imaging is more difficult to be imaged by this technique. To make low-density tissues detectable, some small particles called ultrasound contrast agents (UCAs) need to be introduced into blood.1–3

The earliest particles used as UCAs are air bubbles.4 Because of the high contrast of density and compressibility between air and water, air bubbles exhibit a very large ultrasound scattering cross-section in blood and, thus, can be detected by ultrasound. However, air bubbles without walls rapidly break and dissolve in the blood and their lifetime is too short for clinical detection. To solve this problem, microbubbles with a longer lifetime were gradually developed, where lowly diffusible gases (e.g., perfluorocarbons5–7 or sulfur hexafluoride8) and low-boiling liquid9 were used as the cores of the bubbles and solid materials (including surfactants,10 lipids,11,12 proteins,13 polymers,14 carbon nanotubes,15 Prussian blue16 and even gold17–21 and silica22–25 were used as their walls. To enable the penetration of bubbles through the lung capillaries and increase their remains in the circulatory system, the size of bubbles has to be smaller than 8 μm.26 However, fabrication of microbubbles with uniform size remains difficult for the ultrasound detection at certain frequencies. In addition, almost all of the microbubbles have single-layer structures, and a trade-off exists between their lifetime and ultrasound scattering cross-section; namely, microbubbles with a long lifetime usually possess a small scattering cross-section.27–30

In this paper, we develop a new method to fabricate multi-layer microbubbles (MLBs) with uniform size as a novel model of UCAs. In comparison to single-layer bubbles, such MLBs possess a longer lifetime and higher image sharpness (strong structure coupling effect). We apply classical acoustic theory to calculate the ultrasound scattering cross-section for the bubbles. It is found that the MLBs exhibit a larger ultrasound scattering cross-section than single-layer bubbles with the same size under certain experimental conditions. Such theoretical results agree well with the ultrasound experimental results.

EXPERIMENTAL SECTION

Materials. Vinyltriethoxysilane (VTES) was purchased from Sinopharm Chemical Reagent Co., Ltd. Azobisisbutyronitrile (AIBN) was purchased from Sinopharm Chemical Reagent Co., Ltd. and recrystallized in ethanol. Aqueous ammonia solution (28%), hydrofluoric acid (HF, 40%), sodium hydroxide, acetonitrile, and ethanol were obtained from Shanghai Qiangsheng Chemical Reagent...
Typically, VTES (3.0 mL) was added in H2O (50 mL) under on a carbon-coated copper grid. 200 kV. Samples dispersed at an appropriate concentration were cast onto a Japan) transmission electron microscope at an accelerating voltage of 75 kV. High-resolution transmission electron microscopy (TEM) images were obtained on a Czech) scanning electron microscope at an accelerating voltage of 20 kV. The samples dispersed at an appropriate concentration were cast onto a JEOL, Japan) transmission electron microscope at an accelerating voltage of 200 kV. Samples dispersed at an appropriate concentration were cast onto a carbon-coated copper grid.

**Synthesis of Vinyl-Containing Organosilica Microspheres (VOMs).** Typically, VTES (3.0 mL) was added in H2O (50 mL) under vigorous mechanical stirring (300 rpm) for 2 h. When the organic droplets were completely dissolved and the solution was transparent, diluted ammonia solution (1.0 mL; a volume ratio of ammonia/water = 1:4) was consecutively added to the reaction mixture via a microsyringe at different dripping speeds. After the addition of ammonia solution, the mixture was continued to react at room temperature for 1 h. The synthesized product was centrifuged, washed with distilled water and ethanol several times, and then freeze-dried.

**Synthesis of Single-Layer Microbubbles.** VOMs (0.1 g) were dispersed in ethanol (50 mL) and kept under a nitrogen atmosphere for 30 min. The mixture was heated to 80 °C, and then the initiator (0.15 mmol of AIBN in 5 mL of ethanol) was added to the above mixture. The mixture with mechanical stirring at 300 rpm was continued to react at 80 °C for 6 h. Finally, the obtained shell-cross-linked VOMs were centrifuged, washed with ethanol several times, and then dispersed in ethanol (50 mL) and kept under a nitrogen atmosphere for 30 min. The mixture was heated to 80 °C, and then the initiator (0.2 mmol of AIBN in 5 mL of ethanol) was added to the above mixture. The mixture with mechanical stirring at 300 rpm was continued to react at 80 °C for 6 h. The double-shell-cross-linked VOMs were centrifuged, washed with ethanol several times, and then vacuum-dried. Finally, the DLMs were obtained by etching the as-prepared double-shell-cross-linked VOMs in HF (40 mL, 2.0 wt %) for 3 h. The obtained DLMs were separated and purified 3 times by repeating centrifugation (12 000 rpm for 10 min) and the dispersion cycle in water with ultrasonic bathing and then freeze-dried.

**In Vitro Cytotoxicity and Cell Viability Study.** In vitro cytotoxicity of single-layer microbubbles was assessed on human cervical carcinoma (HeLa) cells and human embryonic kidney (HEK 293T, a normal cell line) cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Specifically, 100 μL of cells was seeded in a 96-well flat culture plate at a density of 1 × 10^4 cells/well and subsequently incubated for 24 h to allow for attachment. The samples with different concentrations (0, 50, 100, 200, and 400 μg mL\(^{-1}\)) were added to each group (three wells) for 24 and 48 h. MTT solution [20 μL, 5 μg mL\(^{-1}\) in phosphate-buffered saline (PBS)] was added to the wells and incubated for 4 h. MTT internalization was terminated by aspiration of the media, and the cells were lysed with dimethyl sulfoxide (DMSO) (150 μL). The absorbance of the suspension was measured at 490 nm on an enzyme-linked immunosorbent assay (ELISA) reader.

**In Vitro Ultrasound Imaging.** The MLBs with different layer numbers tested as UCAs were first dispersed in volatile ethanol and then freeze-dried for 48 h in a vacuum freeze-dryer. The dry powder products were stored in N\(_2\) to be filled with N\(_2\) gas. The ultrasound contrast measurement was carried out on a color digital ultrasonic diagnostic apparatus LOGIQ Book XP (GE) in conventional B mode and power Doppler mode. Typically, PBS (30 mL) with a constant concentration of MLBs (0.1 mg mL\(^{-1}\)) was filled in a dialysis bag (2.5 × 10.0 cm). The ultrasound imaging was achieved using a 8C-RS probe (B mode, convex array) and a 8L-RS probe (power Doppler mode, linear array) with a frequency of 6 MHz and mechanical indices (MIs, 0.5 for B mode and 1.1 for power Doppler mode). The transducer was coated with ultrasound gel to avoid the air background. All images were recorded as digital files for subsequent playback and analysis.

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**Characterization.** High-resolution scanning electron microscopy (HRSEM) images were performed using a TS-5136MM (TESCAN, Czech) scanning electron microscope at an accelerating voltage of 20 kV. The samples dispersed at an appropriate concentration were cast onto a JEOL, Japan) transmission electron microscope at an accelerating voltage of 75 kV.
In Vivo Ultrasound Imaging. Animal procedures were in agreement with the guidelines of the regional ethic committee for animal experiments. The animals, New Zealand white rabbits (around 3.0 kg of body weight), were fasted for 24 h before experiments, and their abdomens were shaved. The rabbits were anesthetized using 10% chloral hydrate and fixed at the ultrasound therapy system. A total of 2 mL of PBS blank and PBS of MLBs with different layer numbers from one to five (both 6.0 mg/mL) were injected via an ear vein. The livers of the rabbits were scanned before and after the administration of the as-prepared MLBs. The scanning parameter settings were optimized the same as for the in vitro experiments.

RESULTS AND DISCUSSION

The whole designed process of fabricating MLBs is illustrated in Scheme 1a. First, the vinyl groups of monodisperse vinyl-containing organosilica, which are prepared by a sol–gel reaction from VTES, are cross-linked controllably from outer to inner through a free radical polymerization using AIBN as the initiator and then can form the first layer of the MLBs.33 Second, the cross-linked VOMs as the seeds are uniformly coated with a new layer of organosilica using the VTES precursor. Then, the vinyl groups on the surface of microspheres are cross-linked with AIBN and form the second layer of the MLBs. Finally, the two cross-linked layers of VOMs are converted into DLMs through the polymer-backbone-transition method (PBTM) with an appropriate amount of aqueous HF to selectively etch off the Si–O backbone and uncross-linked organosilica layers.33 The framework of each layer of the MLBs was transformed from an inorganic Si–O backbone to an organic C–C backbone (Scheme 1a). Furthermore, the twice cross-linked VOMs can be further used as seeds with alternate coating and cross-linking processes to form multi-layer architectures. Because the functional groups Si–OH and vinyl groups in VOMs are inherited from VTES, no further surface modification or activation steps are required in the coating and cross-linking processes. In the whole process of synthesis, the organosilica not only plays the role of precursor for the layers of MLBs but also acts as the template for the cavities. In this way, the removal of the template and the formation of all layers were completed by only one etching treatment, which is very simple and well-suited for large-scale production (Scheme 1a).

Figure 1 shows HRTEM and HRSEM images of some representative MLBs with up to four layers. As seen from the electron microscopy images, all of the MLBs showed very good monodispersity in the absence of any secondary-initiated particles. The obvious contrast between the dark edge and gray interspace confirmed the multi-layer structures. Each layer exhibited well-proportioned size and uniform shape, and interdistances between the adjacent layers in the same products were almost the same. The inset in Figure 1b is a SEM image of a broken hollow sphere; the core is exposed inside the broken shell, further confirming the double-layer structure. Moreover, we noted that the position of the inner hollow core in the shell was random, showing obvious eccentric structures. It was believed that the inner core encapsulated in each layer was not fixed in the chamber and was potentially movable if the system was immersed in a liquid medium.

The spacing between the adjacent layers (the subtraction between inner and outer bubble radii) was mainly determined by the thickness of the coated organosilica layer, while the thickness of this new silica layer could be controlled by varying the concentration of VTES in the coating process. The thickness of the organosilica layer can be precisely tailored, which ensures a well-defined size and space between the adjacent layers. As shown in Figure 1a, the DLMs with diameters of 470 nm (outer) and 320 nm (inner) were prepared using 0.5 mL of VTES relative to 50 mg of VOM seeds. As the amount of VTES was increased from 1.0 to 1.5 to 2.0 mL at the constant concentration of VOMs in solution, the diameter of the outer layers increased to 625 nm (Figure 1b), 940 nm (Figure 1c), and 1180 nm (Figure 1d), respectively.

To obtain well-defined morphology of MLBs, it is important to precisely control the dripping rate of the ammonia solution. The previous paper pointed out that reducing the dripping speed of aqueous ammonia is conducive to increasing the particle size of VOMs.33 However, the change of the dripping speed of the ammonia catalyst in the coating process cannot change the size of double-layer microspheres but can control morphology. The HRTEM and HRSEM images of MLBs were prepared with varying dripping speeds of ammonia solution from one batch to 1.5 mL h−1 (see Figure S1 of the Supporting Information). The obtained VOM seeds possessed a uniformly spherical shape and a mean diameter of 320 nm (see panels a and b of Figure S1 of the Supporting Information). When the diluted ammonia solution was directly added to the VTES/water solution containing VOM seeds, the resultant double-layer microspheres showed an irregular polygon with a rough surface compared to VOM seeds (see Figure S1c of the Supporting Information). After etching with HF, the corresponding DLMs exhibited similar shape and size. The SEM image (see the inset in Figure S1d of the Supporting Information) of the broken hollow sphere further provided the evidence of the double-layer structure and rough surface. With the increase of the additional speed to 3.0 mL h−1, the shape of microspheres became ellipsoid and the corresponding DLMs looked more like an egg, which consisted of a spherical core and an ellipsoidal layer (see panels e and f of Figure S1 of the Supporting Information). When the additional speed was
decreased to 1.5 mL h$^{-1}$, highly monodisperse DLMs were obtained with the size of 470 nm and the thickness of the coated organosilica layer was estimated to be approximately 75 nm (see panels g and h of Figure S1 of the Supporting Information). The influence of the additional speed of ammonia solution on the layer morphology may be attributed to the fact that VTES undergoes hydrolysis and condensation too fast to coat evenly on the VOM seeds when the ammonia catalyst was rapidly added to the VTES/water solution. Through controlling the additional speed of ammonia solution in every coating process, we could control morphology of each layer and synthesize triple-, quadruple-, and quintuple-layer MLBs (see Figure S2 of the Supporting Information).

We have previously demonstrated, in great detail, that the shell thickness of the microbubbles is controlled by the polymerization efficiency and, in turn, by the concentrations of the initiator within the organosilica shells.$^{33}$ The more initiators that can diffuse into the shells of the VOMs, the thicker the shells of microbubbles. In the synthetic process of microbubbles, the mixture of VOMs and ethanol was first heated to 80 °C and then the ethanol solution of the initiator (AIBN) was added to the mixture. Because of the fact that the amount of AIBN was relatively little and the temperature of the mixture was high, AIBN only diffused into and polymerized the outer part of the VOMs rather than the whole VOMs. Through the outer-to-inner cross-linking radical polymerization, the shell thickness can be varied while the uniformity and integrity of the shells can be assured. Herein, we can exactly control the thickness of each shell via varying the concentration of AIBN in each cross-linking process. For example, when using 320 nm VOMs as the seeds, two different DLMs can be prepared with the same inner shell thickness of 45 nm but different outer shell thicknesses of 35 or 20 nm by decreasing the amounts of AIBN from 25 to 15 mg (panels a and b of Figure 2). As expected, the thick shell was more robust and could keep its perfect spherical shape, while the thinner shells were prone to collapse (Figure 2a) during the sample preparation under high vacuum (TEM conditions). In addition, when using 510 nm VOMs as the seeds, two other different DLMs were synthesized with the same outer shell thickness of 18 nm but with different inner shell thicknesses of 25 and 10 nm (panels c and d of Figure 2). In this case, both of the thin walls collapsed under high vacuum.

The whole etching process of DLMs with 2.0 wt % HF at room temperature was monitored by TEM. When the DLMs were etched for 30 min, multiple tiny pores were developed inside the outer layers yet the inner cores still remained solid without an obvious change, converting to a typical rattle structure (see Figure S3a of the Supporting Information). As the etching time was prolonged to 1 h, the small pores of outer layers turned into larger voids, as indicated by lower contrast; meanwhile, the inner cores appeared as multiple tiny pores (see Figure S3b of the Supporting Information). Continuous reacting for another 1 h eventually led to the formation of a hollow core, and the double-layer hollow structure became pronounced (see Figure S3c of the Supporting Information). After etching for a total of 3 h, the template was completely removed and well-developed double-layer bubble-in-bubble structures were formed (see Figure S3d of the Supporting Information). In the entire etching process, all of the microspheres showed a synchronous structure change and the outer layers and inner cores remained uniform without any change in the average diameter compared to the as-prepared double-layer microspheres and VOM seeds.

Before ultrasound imaging application, we first need to assess the cell cytotoxicity of MLBs. In vitro cell viability was performed on HeLa cells and HEK 293T cells. Single-layer microbubbles with different concentrations (50, 100, 200, and 400 μg mL$^{-1}$) were incubated with the cells for 24 and 48 h by the MTT assay. The viability of control cells was assumed to be 100%. After 24 h, there is no apparent cytotoxicity against the HeLa cells and HEK 293T cells in diverse concentrations (Figure 3a). When the incubation time was prolonged to 48 h, the microbubbles showed minimal cytotoxicity after incubation at a very high concentration up to 400 μg mL$^{-1}$ and the cell viability still remained above 85% (Figure 3b). The excellent biocompatibility of the microbubbles guarantees the practical applications for in vivo ultrasound imaging as UCAs.

To test the new concept of our bubble-in-bubble strategy (Scheme 1b), in vitro ultrasound imaging of MLBs with different layers was assessed under two-dimensional (2D) gray-scale ultrasound (B mode) and power Doppler ultrasound imaging modes. To eliminate the influence of the microbubble size on the ultrasonic signal, we accurately synthesized various microbubbles with different layer numbers but with the same diameter of about 900 nm. Figure 4 displays the evaluation results of the ultrasound contrasting behavior of the as-prepared MLBs with different layer numbers under the same ultrasound conditions. As we can see, the introduction of lyophilized microbubbles shows obvious backscatter signal enhancement under both power Doppler ultrasound imaging mode (Figure 4) and B mode (see Figure S4 of the Supporting Information) with respect to the control PBS, which is almost transparent to ultrasound. Furthermore, such contrast enhancement is found to be layer-dependent; that is, as the number of layer increases, the backscatter signals enhance linearly as well. To better characterize the quality of the microbubbles signal, we further measured the corresponding quantitative gray values in both power Doppler mode (Figure 5a) and B mode (Figure 5b). The gray values increased from 80 to 237 in power

![Figure 2. TEM images of double-shelled MLBs with different shell thicknesses: (a and b) using 320 nm VOMs as the seeds, with the same inner shell thickness of 45 nm but with a different outer shell thickness of either 35 or 20 nm and (c and d) using 510 nm VOMs as the seeds, with the same outer shell thickness of 18 nm but with a different inner shell thickness of either 25 or 10 nm. All of the bars are 500 nm.](image-url)
Doppler mode and from 36 to 97 in B mode, respectively, while the layer number increased from one to five.

The short lifetime is also an important drawback of the commercialized UCAs. The lifetime of microbubbles usually depends upon the degree of gas loss to the aqueous environment. As long as the gas pressure in the hollow cavity is greater than that in its surrounding medium, diffusion will result in the loss of gas from the microbubbles, resulting in a signal decrease and eventual disappearance. The shell is necessary to avoid fusion of adjacent bubbles and also improves stability during storage and in vivo delivery. Different shells have been proven to prevent gas from diffusion and dissolution and improve the imaging contrast.

To understand the echogenic mechanism of MLBs, we established the mathematical model of the ultrasonic process of MLBs and carried out the theoretical calculation. Herein, we consider a MLB that is composed of soft polymer matrix (SPM) and suspended in water (Figure 6a). The \( l \)th tier has an outer radius of \( r_l \), density of \( \rho_l \), and bulk modulus of \( B_l \). The tier index \( l \) = 1 and \( N + 1 \) refer to the core and background, respectively. The white, orange, and cyan regions represent air, SPM, and water, respectively.

We apply a Mie scattering method to obtain the total scattering cross-section of the microbubble. The acoustic equation can be written as

\[
V \left[ \frac{1}{\rho(r)} \nabla P(r) \right] = -\frac{\omega^2}{B(r)} P(r)
\]

The origin of spherical coordinates \( (r, \theta, \varphi) \) is at the center of the microbubble. \( \omega \) is the frequency of an acoustic plane wave.

Then, the general form of the pressure field in the microbubble can be derived as
scattering intensity of MLBs is improved with the increasing layer number of $M$, verifying the experimental results (Figure 6c). We note that the scattering intensity of bubbles is determined by whether the detection frequency (6 MHz) is close to the resonant frequencies of bubbles. The resonant frequencies are much higher than 6 MHz for current bubbles with a size of 1 μm. However, because the inner bubble can vibrate inside a MLB, a MLB has a fundamental resonant mode with a frequency lower (and closer to 6 MHz) than that of a single-layer bubble. Hence, the scattering intensity of MLBs at 6 MHz is much higher than that of single-layer bubbles.

In view of the results obtained from in vitro experiments and theoretical calculations, we assessed the acoustic contrast-enhanced behavior of MLBs in vivo. The liver ultrasound imaging was performed using New Zealand white rabbits under power Doppler imaging mode. The transducer was placed on the liver for real-time monitoring. The significant contrast enhancement of livers could be clearly observed with the increase of the layer number from one to five (Figure 7) after puncture administration of MLBs compared to the non-treated rabbit (Figure 7a), suggesting the relatively high in vivo ultrasonography efficiency of MLBs, which is consistent with the in vitro ultrasonography results. This in vivo result suggests that MLBs can act as UCAs for ultrasonography for the real-time guidance during the surgical process.

## CONCLUSION

In summary, we have synthesized MLBs with bubble-in-bubble structure, well-defined shape, and high monodispersity using a PTBM for the first time. Besides the tunable layer number and microbubble size, we could accurately control the layer-to-layer space and the thickness of each layer. More importantly, the MLBs were applied as a novel promising model of contrast agent for high-performance ultrasound imaging for the first time. According to the experimental results and theoretical calculations, a brand new concept for UCAs has been developed. The performance of the ultrasound signal showed an obvious layer number dependence and a strong structure coupling effect; as the number of layers increased, not only was the backscatter signal significantly enhanced but the lifespan was also greatly increased. Although the data reported here only represent initial work to develop a new generation of contrast agents, this work opens a new pathway for fabricating high-

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**Figure 6. (a) Schematic view of a $M$-layer microbubble with $N$ tiers ($M = N/2$ and $N = 8$ are shown here). (b) Total scattering cross-section of the sphere as shown in panel a. (c) Calculated scattering intensity $P_{sc}$ and measured reflection intensity $I_{r}$ of the MLBs with $M$ matrix layers as studied in panel b. Here, the material has a bulk modulus of $B_{\infty} = B_{m}/6000$, and $P_{sc} = \sigma_{n}Q_{sc}$, where $\sigma_{n}$ is the number of MLBs in a cubic millimeter of water. The mass of MLBs in a cubic millimeter of water is the same (0.1 mg) for the four samples of S1, S2, S3, and S4.**

$$P_{l} = P_{0} \sum_{m=0}^{\infty} \left[ a_{m}F_{n}(k_{r}l) - b_{m}h^{(1)}_{n}(k_{r}l) \right] P_{l}(\cos \theta)$$

where $l = 1, 2, ..., N + 1$ and $k_{l} = \omega^{2}(\rho(r_{l})/B(r_{l}))$. Here, the Bessel function $j_{m}$ and the first kind of Hankel function $h_{m}^{(1)}$ stand for the incident and scattering waves, respectively. Using continuities of $P_{l+1} = P_{l}$ and $(1/(i\omega \rho l_{l}+1))(i\partial P_{l+1}/\partial r) = (1/\omega \rho l_{l})(i\partial P_{l}/\partial r)$, we have

$$j_{m}(u) - D_{l+1}h_{m}^{(1)}(u) = v_{l}P_{l} j_{m}(v) - D_{l+1}h_{m}^{(1)}(v)$$

where $u = k_{l}r_{l}$, $v = k_{l}r_{l}$ and $D_{l+1} = B_{l+1}/a_{m}$. Using $D_{l+1} = 0$ and $D_{(N+1)m} = b_{m}^{(1)}(2m + 1)$, the bubble has a total scattering cross-section

$$Q_{sc} = \lambda_{N+1}^{2} \sum_{m=0}^{\infty} \left[ l_{B_{m}}^{(1)}(2m + 1) \right]^{2}$$

where $\lambda_{N+1}$ is the wavelength of the acoustic plane wave.

In the experiment, the bubbles are illuminated by ultrasonic plane waves with a frequency of 6 MHz. The air has a density of $\rho_{a} = 1.29$ kg/m$^{3}$ and bulk modulus of $B_{a} = 149.12$ kPa. The water has a density of $\rho_{w} = 1.3 \times 10^{3}$ kg/m$^{3}$ and bulk modulus of $B_{w}$. The water has a density of $\rho_{w} = 1.0 \times 10^{3}$ kg/m$^{3}$ and bulk modulus of $B_{w} = 2.2$ GPa. We study four kinds of microbubbles: namely, bubble 1 called S1 with layer number ($M = 1$), bubble 2 called S2 with $M = 2$, bubble 3 called S3 with $M = 3$, and bubble 4 called S4 with $M = 4$. The bubbles have the same values for the outer radius ($r_{o} = 0.45\mu m$), thickness in material layers (0.07 μm), and thickness in air layers (0.01 μm, except for $l = 1$). We calculate the total scattering cross-section for bubbles with different bulk modulus $B_{m}$ in the matrix (Figure 6b). When $B_{m} = B_{w}/6000$, the calculated
efficiency UCAs based on structural design and manipulation of the hollow cavity.

**ASSOCIATED CONTENT**

1. Supporting Information

TEM images of MLBs (Figures S1–S3) and in vitro ultrasound images of MLBs under B mode (Figure S4) (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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