High Protein-Loading Silica Template for Heterogeneous Protein Crystallization

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INTRODUCTION

Because of their high potency and specificity, protein-based biologics such as monoclonal antibodies have become an important class of pharmaceutical compound over the past few decades.\(^1\) The downstream processing of proteins is much more complex and costly than traditional small molecules because of the delicate nature of proteins. For example, the downstream processing cost of monoclonal antibodies can account for up to 70% of the total production cost.\(^2\) The current purification of proteins relies on a series of chromatographic steps, which limit the throughput of the manufacturing process and are costly due to the high cost of resin.\(^3\) Crystallization is a cost-effective and scalable option for the purification of protein,\(^4\) which has been reported as an alternative technology to improve the overall productivity and to lower the manufacturing cost of protein-based biologics such as monoclonal antibodies.\(^5\)\(^,\)\(^6\)

Unlike small molecular pharmaceuticals,\(^7\)\(^,\)\(^8\) proteins are significantly more challenging to crystallize due to their complex three-dimensional configuration and hence have slow crystallization kinetics.\(^11\)\(^,\)\(^12\) To overcome this problem, numerous heterogeneous nucleants have been developed to accelerate the process, especially by tuning the surface chemistry and pore size as demonstrated in the case studies of lysozyme, catalase, and thaumatin.\(^13\)\(^,\)\(^19\)

The wide range of heterogeneous nucleants include mineral,\(^20\) nanoparticle (e.g., diamond, gold, polymer),\(^21\)\(^,\)\(^22\) membrane,\(^25\)\(^,\)\(^26\) natural material (e.g., horse hair, rat whisker, dried seaweed),\(^27\)\(^,\)\(^29\) silicate,\(^30\)\(^,\)\(^32\)\(^,\)\(^33\) surface with engineered roughness or etched pattern,\(^33\)\(^,\)\(^39\) self-assembled monolayer (e.g. fatty acid and lipid, gold, peptide and protein),\(^40\)\(^,\)\(^43\) and specific functional group.\(^46\)\(^,\)\(^47\) These materials can promote the nucleation of a wide range of proteins such as lysozyme, thaumatin, trypsin, and concanavalin A by reducing the induction time and/or inducing crystallization at low protein concentration.

Among these nucleants, various mesoporous silicas have been found to be effective nucleants for proteins.\(^15\)\(^,\)\(^16\)\(^,\)\(^48\)\(^,\)\(^50\) One promising candidate is rod-like SBA-15, which has been used for protein immobilization due to its high protein loading capacity.\(^51\)\(^−\)\(^54\) The previous studies on heterogeneous nucleants were mostly conducted in the hanging-drop mode, where the nucleants were added directly into the protein/precipitant mixture droplets without any pretreatment.\(^55\) As the nucleants interacted favorably with the proteins, sorption took place, and the protein concentration in the droplets decreased.\(^56\)

There is limited literature on the use of high protein-loading nucleants for protein crystallization, especially about how the sorption of protein affects the effectiveness of the nucleant in

Supporting Information

ABSTRACT: As a purification technology, crystallization is advantageous over chromatography and precipitation in terms of purity, cost, and scalability. In general, proteins have slow crystallization kinetics due to their complex configurations, but this problem can be overcome with heterogeneous nucleants. High protein-loading mesoporous silica is a promising nucleant due to its favorable interaction with protein. The current study employs such a template in the batch crystallization of lysozyme and thaumatin at 1 mL scale. Using lysozyme as the main model protein, the results show that the template had high protein loading (220−300 mg lysozyme/g silica) and induced significantly faster crystallization as compared to the unseeded samples over a wide range of initial protein concentration (10.0−47.5 mg/mL) at 25 °C with 0.5 M potassium sodium tartrate tetrahydrate (precipitant) concentration. It was found that the template had to be saturated with the target protein before the experiments to achieve faster kinetics, or else it could delay the crystallization process. These findings were reaffirmed by the crystallization experiments of thaumatin. The current study demonstrates the effectiveness of high protein-loading silica as a nucleant in protein crystallization and the importance of its pretreatment with the target protein.

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accelerating crystallization. The current study aims to address this knowledge gap by using a mesoporous silica with characteristics similar to rod-like SBA-15 in the crystallization of lysozyme and thaumatin at the 1 mL scale. The results show that this high protein-loading nucleant is highly effective, but must be saturated with the target protein as the pretreatment in order to expedite the crystallization process (i.e., enlarging the nucleation zone).

**EXPERIMENTAL SECTION**

**Materials.** All the chemicals were used as received from the suppliers unless otherwise stated. The details of all materials used in the experiments can be found in Table S1 in the Supporting Information.

For the synthesis of mesoporous silica, Pluronic P-123 (M₆₈ ≈ 5800) and tetraethyl orthosilicate (TEOS) (98%) were purchased from Sigma-Aldrich (Gillingham, UK). Hydrochloric acid (37% w/w) was used as the acidic catalyst, whereas potassium sodium tartrate tetrahydrate (>98%) was purchased from Sigma-Aldrich (Gillingham, UK). Sodium hydroxide (>98.5%) and sodium tartrate tetrahydrate (99%) were purchased from Sigma-Aldrich (Gillingham, UK). Hydrochloric acid (80 mL) at 40 °C overnight and then centrifuged (6000 rpm, 5 min).

For the crystallization experiments, lysozyme from chicken egg white (≥90%), thaumatin from Thaumatomoccus danielli, piperazone-1,4-bis-(2-ethanesulfonic acid) (PIPES) (≥99%), and potassium sodium tartrate tetrahydrate (99%) were purchased from Sigma-Aldrich (Gillingham, UK). Sodium hydroxide (≥98.5%) was purchased from VWR (Lutterworth, UK). Deionized water was obtained using a PURELAB Chorus (ELGA LabWater) water purification system.

For the crystallization experiments, lysozyme from chicken egg white (≥90%), thaumatin from Thaumatomoccus danielli, piperazone-1,4-bis-(2-ethanesulfonic acid) (PIPES) (≥99%), and potassium sodium tartrate tetrahydrate (99%) were purchased from Sigma-Aldrich (Gillingham, UK). Sodium hydroxide (≥98.5%) was purchased from VWR (Lutterworth, UK). Polypropylene microcentrifuge tubes were purchased from Fisher Scientific (Loughborough, UK).

For the imaging of mesoporous silica by electron microscopy, carbon-coated copper transmission electron microscope (TEM) grids were purchased from EM Resolutions (Sheffield, UK), and carbon conductive adhesive tape was purchased from Agar Scientific (Stansted, UK).

**Mesoporous Silica - Synthesis and Characterization.** The synthesis protocol was adapted from the literature.²⁷²⁸ P-123 (8 g) was completely dissolved in a mixture of deionized water (220 mL) and hydrochloric acid (80 mL) at 40 °C overnight. TEOS (18 mL) was added into this mixture at a rate of 1.8 mL/min. The mixture was stirred at 40 °C overnight and then centrifuged (6000 rpm, 5 min).

The recovered mesoporous silica was dried in a fume cupboard at room temperature and then calcined at 550 °C in air for 24 h. The calcination was conducted under normal atmosphere in a CWF 1200 laboratory chamber furnace (Carbolite Gero) with a temperature increase rate of 10 °C/min from room temperature to the target temperature (550 °C).

**For TEM analysis,** the calcined mesoporous silica was ground into fine particles and then dispersed in ethanol by sonification. A few drops of the resulting suspension were placed onto a carbon-coated copper TEM grid and dried naturally. The on-grid sample was analyzed with a 2100 Plus (JEOL) TEM.

For SEM analysis, the calcined mesoporous silica was placed onto the carbon conductive adhesive tape and then coated with chromium with a target thickness of 15 nm in a Qu150T turbomolecular-pumped coating system (Quorum Technologies) with a sputter current of 120 mA, coating factor of 2.43, and maximum sputtering time of 4 min. The chromium-coated sample was analyzed with a Leo Gemini 1525 (Carl Zeiss Microscopy) scanning electron microscope (SEM) with an accelerating voltage of 5 kV.

The calcined mesoporous silica was degassed at 120 °C overnight before the nitrogen adsorption and desorption isotherms were determined with a Micromeritics Instrument gas adsorption analyzer. The specific surface area and pore size distribution of the calcined mesoporous silica were obtained with the Brunauer–Emmett–Teller (BET) and Barrett–Joyner–Halenda (BJH) methods, respectively.

**Protein Crystallization.** Buffer and precipitant solutions were tested for pH and conductivity for quality control purposes. pH measurements were conducted with a SevenExcellence with InLab Expert Pro-ISM (Mettler Toledo) pH meter, whereas conductivity measurements were conducted with a 4330 glass bodied conductivity probe (Jenway) conductivity meter.

**PIPES buffer** (0.1 M) was prepared by dissolving PIPES (6.09 g) in a mixture of deionized water (170 mL) and aqueous sodium hydroxide solution (1.0 M) (30 mL). The pH and conductivity of the prepared buffer at room temperature were approximately 6.5 and 9 mS, respectively.

**Protein solutions** (10–100 mg/mL for lysozyme; 4–40 mg/mL for thaumatin) were prepared by dissolving the target protein in the prepared PIPES buffer. For example, lysozyme (50 mg) was added into PIPES buffer (1 mL) and then placed on a shaker overnight to prepare a lysozyme solution (50 mg/mL). Shaking was gentle to avoid the formation of foam.

The protein concentration in the protein solutions was determined by measuring the absorbance at 280 nm with a NanoDrop OneC (Thermo Fisher Scientific) microvolume UV–vis spectrophotometer with a typical sample volume of 2 μL. All the protein samples were directly analyzed without dilution. The corresponding calibration curves can be found in the Supporting Information (Figures S3 and S4).

**Precipitant solutions** (0.2–1.6 M potassium sodium tartrate tetrahydrate) were prepared by dissolving potassium sodium tartrate tetrahydrate in PIPES buffer. The pH and conductivity of the prepared precipitant solutions at room temperature were approximately 6.5 and 30–85 mS, respectively. For example, potassium sodium tartrate tetrahydrate (28.23 g) was added into PIPES buffer (100 mL) to prepare the precipitant solution (1.0 M, conductivity at room temperature = 74 mS).

All the crystallization experiments were performed at 1 mL scale in 1.5 mL polypropylene microcentrifuge tubes at 25 °C in a temperature-controlled incubator. The protein/precipitant mixture was prepared by mixing equal volumes of protein solution and precipitant solution (500 μL/500 μL). The mixing of samples was performed before the samples were put into the incubator and after they were taken out for UV–vis measurements. The samples remained static inside the incubator.

For the crystallization experiments, the calcined mesoporous silica was repeatedly washed with deionized water and then buffer/precipitant mixture (1/1, v/v) before the protein/precipitant mixture solution was added. Experiments without mesoporous silica were always conducted at the same time as controls.

For example, calcined mesoporous silica (10 mg) was added into a 1.5 mL polypropylene microcentrifuge tube, followed by deionized water (1 mL). The mixture was vortexed and centrifuged (6000 rpm, 1 min) before the supernatant was carefully removed to avoid the removal of mesoporous silica. This was repeated five times for water and another five times for buffer/precipitant mixture (500 μL/500 μL). Finally, the protein/precipitant mixture (500 μL/500 μL) was added into the microcentrifuge tube and the mixture was rotated gently and centrifuged (6000 rpm, 30 s). The protein concentration in the supernatant was analyzed with the UV–vis spectrophotometer as described above. If the protein concentration dropped due to the sorption of protein onto mesoporous silica, the conditioning of mesoporous silica with the protein/precipitant mixture was repeated until no significant drop was observed. Typically, it was repeated five times before the mesoporous silica became protein-saturated and did not take up any more protein.

During crystallization, the protein concentration was analyzed regularly. For example, a sample was taken out of the incubator every 30 min and rotated gently 20 s before a small sample (20 μL) was taken. The sample was then returned to the incubator, while the small sample was centrifuged (6000 rpm, 30 s), and the clear supernatant was analyzed by the UV–vis spectrophotometer. The centrifugation of the small sample to remove any suspended crystals and nucleation was essential for ensuring the accuracy of the UV–vis measurements as pointed out in the literature.⁶⁹

**Protein Sorption by Mesoporous Silica Template.** All sorption experiments were conducted in static mode in 1.5 mL polypropylene microcentrifuge tubes in the incubator (25 °C). Calcined mesoporous silica was washed with deionized water.
repeatedly and then dried before it was added into the micro-centrifuge tube. Lysozyme/precipitant mixtures (protein concentrations = 5.4, 7.9, 10.2, 12.8, and 15.0 mg/mL; potassium sodium tartrate tetrahydrate concentration = 0.5 M) were then added into the mesoporous silica samples. For example, the sorption study started with the washing of calcined mesoporous silica (500 mg) with deionized water (50 mL) five times. Thereafter, the washed nucleant was dried in a vacuum oven (60 °C, 200 mbar) over several days until the mass became constant. The dried mesoporous silica (10 mg) was then added into a 1.5 mL polypropylene microcentrifuge tube, followed by the addition of lysozyme (11−30 mg/mL before mixing)/precipitant mixture (1.0 M before mixing) (500 μL/500 μL). The protein concentration was analyzed regularly with NanoDrop OneC until a steady state was reached.

■ RESULTS

Characterization of Mesoporous Silica Template. The synthesized mesoporous silica had physical characteristics that were similar to the rod-like SBA-15 reported in the literature.57,58 SEM analysis (Figure 1a) confirms that the synthesized mesoporous silica had a rod-like structure with relatively high homogeneity. The typical length and width were 500 and 150 nm, respectively. TEM analysis (Figure 1b) confirms the porous structure within the synthesized mesoporous silica. The tubular pores had a lower tortuosity and were bundled tightly together within the silica tube.

The nitrogen adsorption and desorption isotherms of the calcined mesoporous silica (Figure 2) are classified as type IV (a) with H1 type of hysteresis loop according to the latest IUPAC nomenclature, which are typical for SBA-15.60 The specific surface area (BET) and the average pore size (BJH) were found to be 604 ± 2 m²/g and 6.6 nm respectively (graphs of BJH absorption and desorption dA/dD pore area in section S2 of Supporting Information showing the unimodal nature of pore size distribution).

High Protein Loading Capacity of Silica Template. It has been shown previously that the deposition of lysozyme occurs both on the external surface and inside the pores of SBA-15, as the hydrodynamic diameter of lysozyme is approximately 4.0 nm,61 allowing the protein molecules to enter the pores of SBA-15 (pore diameter = 6.6 nm in this study). Therefore, the term “sorption” is used in this article to include both the adsorption and absorption of lysozyme by the synthesized mesoporous silica template.62 The sorption of lysozyme by fresh template over time is shown in Figure 3.

Starting with a wide range of initial protein concentrations (5.4−15.0 mg/mL), the sorption reached an equilibrium between 20 and 30 days for all mesoporous silica samples. Despite the wide range of equilibrium protein concentrations (2.7−10.7 mg/mL), the sorption capacity of the mesoporous silica samples was within the range of 220−300 mg lysozyme/g mesoporous silica (Figure S5 in Supporting Information).
Similar high loading of lysozyme for rod-like SBA-15 was reported in the literature.51−54

**Pretreatment of Silica Template with Protein.** After its high protein loading capacity was confirmed, the synthesized mesoporous silica template was repeatedly loaded with lysozyme until saturation and then used as the nucleant in the crystallization of lysozyme. The loading of lysozyme was achieved by contacting the mesoporous silica template with the target protein/precipitant mixture repeatedly until no significant change in the protein concentration was detected in the supernatant. An example of the mesoporous silica conditioning is shown in Figure 4, where the conditioning solution had a protein concentration of 12.6 mg/mL, and the conditioning was repeated six times. The protein concentration in the supernatant approached the level of the conditioning solution after three repeats. All conditioning was finished within 30 min in total.

The loading of lysozyme on the synthesized mesoporous silica template was then determined from the drops in protein concentration and the volume of the protein/precipitant mixtures (i.e., 1 mL). The loaded quantity of lysozyme for various mesoporous silica samples (189−341 mg lysozyme/g mesoporous silica) was similar to the sorption test samples within the same range of protein concentrations (Figures S5 and S6 in Supporting Information). The sorption of lysozyme by the synthesized mesoporous silica template was rapid and could reach saturation within the first 10 min of the current conditioning procedure. The rapid sorption of lysozyme by similar rod-like SBA-15 has been mentioned in the literature, where more than 90% of lysozyme loading was reportedly achieved within 10 min.51

**Protein-Saturated Template for Protein Crystallization.** After the conditioning steps, the protein-loaded mesoporous silica template was immediately used as the nucleants in protein/precipitant mixtures that had the same protein concentrations as their corresponding conditioning solutions. Experiments without any mesoporous silica template were always conducted as controls. Experiments were conducted for a wide range of protein concentration in the protein/precipitant mixtures (5.0−47.5 mg/mL) to investigate the effects of protein-loaded mesoporous silica template on the crystallization kinetics and phase diagram of lysozyme (Section S5 in Supporting Information).

The crystallization kinetics was monitored with the protein concentration in the protein/precipitant/nucleant mixture, where the noticeable decrease from the initial level indicated the progress of crystallization. It was found that the protein-loaded mesoporous silica template always induced faster crystallization than the control experiments for initial protein concentrations equal and higher than 10 mg/mL, as the protein concentration curves for the seeded cases were always below the control curves (Figures S7−S13 in Supporting Information).

Using the experiment with an initial protein concentration of 10 mg/mL as an example (Figure 5), the protein concentration remained unchanged for the unseeded sample within the first 25 days, indicating the absence of crystallization. This agreed with the observation that the protein/precipitant mixture remained as a clear solution. On the other hand, the protein concentration started to decrease on the fourth day for the sample with the saturated silica template, and the decrease was gradual and consistent on the following days. Small crystals were observed under a bright-field microscope on the fifth and subsequent days, confirming that the decrease in protein concentration was mainly attributed to crystallization instead of sorption, as the mesoporous silica template was already saturated with lysozyme during the pretreatment (loading = 231 mg/g in Figure S4 in Supporting Information). This decrease in protein concentration shows clearly that the crystallization of lysozyme started much earlier than the unseeded case.

Without the current pretreatment of nucleant, it is likely that wrong conclusions are reached on the effectiveness of nucleant for inducing crystallization as the sorption of target protein by nucleant can lower the protein concentration below the nucleation zone. As shown in Figure 5, the lysozyme concentration curve for the sample with an unsaturated silica
template had an immediate drop from 10.0 mg/mL to 8.5 mg/mL when the fresh mesoporous silica template was added into the protein/precipitant mixture, indicating the rapidness of sorption as mentioned in the literature. The sorption then slowed down and reached equilibrium at a concentration of 6.1 mg/mL, which was close to the solubility point at 25 °C and 0.5 M potassium sodium tartrate tetrahydrate, and no crystallization was observed over a month. Therefore, the reasonable but wrong conclusion would be the ineffectiveness of the mesoporous silica template for inducing crystallization at this condition without the saturation of the template with lysozyme.

The problem of untreated nucleant is further complicated by the fact that the extent of sorption is proportional to the nucleant quantity, as increasing the nucleant quantity will increase the sorption quantity of protein and lower the equilibrium concentration. This problem can be eliminated by the current pretreatment method as shown in Figure 6a, where the evolution of protein concentrations of seeded samples with increasing amount of saturated template (5−20 mg/mL) and the unseeded sample over time is illustrated. Despite the large difference in template quantity, the protein curves of the seeded samples overlapped closely, showing that the crystallization kinetics was not affected by the amount of protein-loaded mesoporous silica template. Furthermore, on the basis of the observations under bright field microscope (unrecorded), no significant difference was detected for crystal size and shape for all the seeded samples. Once again, all the seeded samples had faster crystallization than the unseeded sample.

In order to demonstrate the translatability of the current methodology to other proteins, the same calcined mesoporous silica was pretreated with thaumatin/potassium sodium tartrate tetrahydrate concentration mixtures for crystallization studies. Like the case of lysozyme, the hydrodynamic diameter of thaumatin is slightly smaller than the pore diameter of the mesoporous silica synthesized in this study (6.2 nm vs 6.6 nm), allowing the sorption of protein on the silica surface and inside its pores.
The effectiveness of the saturated mesoporous silica was tested under a condition in the nucleation zone, where the unseeded sample had a latent period of approximately 2 h (Figure 6b). With the saturated mesoporous silica, the latent period was reduced to 1 h, when the protein concentration started to decrease from the initial level. As the protein level of the seeded sample overlapped closely with the one of the unseeded sample in the first hour, the subsequent rapid decrease of protein concentration in the seeded sample was most likely caused by the crystallization of protein rather than the further sorption of protein by the pretreated silica template. The subsequent protein concentrations of the seeded sample until 312 h were always lower than the ones in the unseeded sample, reaffirming faster crystallization (Figure S16 in Supporting Information).

**DISCUSSION**

Importance of Pretreatment with Target Protein. The experimental results of this study show that the protein-saturated silica template can effectively induce crystallization in the metastable zone, essentially lowering the supersolubility line (dashed lines with star and circle markers in Figure 7a), and enhancing the success chance of crystallization within a fixed period (i.e., one month in this study). Since the protein molecules are both adsorbed onto the outer surface and absorbed into the pores of the mesoporous silica, more fundamental studies should be conducted in the future to elucidate the location and the corresponding nucleation mechanism. Previous studies have demonstrated the positive effects of adsorbed molecules on a surface on protein nucleation,26,41,45 probably through the stabilization of prenucleation protein clusters.34 Other previous studies have also demonstrated the positive effects of a porous structure on protein nucleation and a close link between the pore size relative to the hydrodynamic diameter of the target protein.14,15,50,64

Conversely, the same material without any pretreatment lowers the protein concentration in the same system via sorption and expands the metastable zone by elevating the supersolubility line (dashed lines with star and square markers in Figure 7a). As a result, the success chance of crystallization within a fixed period is reduced. The lysozyme data in this study can be used to illustrate the reduction of metastable zone by protein-saturated template as shown in Figure 7b, where the x-axis represents the protein concentration at the beginning of an experiment, and the y-axis represents the final protein concentration at the end of one month at a fixed temperature and precipitant concentration (i.e., 25 °C and 0.5 M potassium sodium tartrate tetrahydrate).

For experiments without crystallization due to low initial protein concentration, the initial protein concentration was equal to the final concentration. The data points of such experiments cluster around the “uncrystallizing line”. For experiments with crystallization, the final protein concentration approached the same solubility level (i.e., the horizontal line of $c^\infty$), as the precipitant concentration and temperature were kept constant for all the experiments.

On the far right of the graph, the data points cluster around the horizontal solubility line as high initial protein concentrations led to faster crystallization and the final protein concentrations approached the solubility level (Figures S7–S10 in Supporting Information). Crystallization also occurred at lower initial protein concentrations (10−15 mg/mL), but the rate was lower, and the final protein concentrations at the end of one month were noticeably higher than the solubility level (Figures S11–S13 in Supporting Information).

With Figure 7b, the metastable zone can be identified using the data points on the uncrystallizing line above the solubility level as well as the vertical solubility line. The saturated mesoporous silica significantly reduced the width of the metastable zone and consistently induced faster crystallization as all the seeded points are below the unseeded points.

**CONCLUSION**

In this study, a mesoporous silica template with characteristics similar to rod-like SBA-15 was synthesized. The silica template had high lysozyme loading capacity (220−300 mg of lysozyme/g silica) at 25 °C over a wide range of protein concentration at equilibrium (2.7−10.7 mg/mL). Experimental results show that the template can be saturated with protein by five to six short contacts of template with the lysozyme/precipitant mixture (total time <30 min) over a similar protein concentration range (5.2−12.6 mg/mL). When applied in crystallization, the protein-saturated template led to a much shorter latent period than the unseeded samples over a wide range of initial protein concentration (10.0−47.5 mg/mL). The same approach of saturating the template was tested in the crystallization of thaumatin, and the results reaffirmed the positive effect of the saturated template on protein crystallization and demonstrated to some extent that the translatability of this approach to other proteins. This study demonstrated the use of high protein loading nucleant for efficient crystallization, as the nucleant expands the nucleation zone of the protein into the metastable zone, enhancing the success rate of crystallization within a fixed period. The findings can potentially be translated into new technologies in the downstream processes of biomanufacturing.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.cgd.9b01252.

Section S1. Summary of materials used. Section S2. Pore size determination of mesoporous silica. Section S3. Protein concentration measurements. Section S4. Lysozyme sorption by template Section S5. Lysozyme crystallisation (initial protein concentration = 5.0 − 47.5 mg/mL) (PDF)

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Notes

The authors declare no competing financial interest.

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