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Technical Note

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Aptamer Modified Organic–Inorganic Hybrid Silica Monolithic Capillary Columns for Highly Selective Recognition of Thrombin

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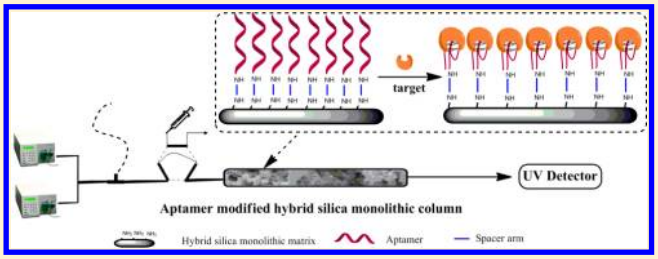
Supporting Information

ABSTRACT: A novel kind of aptamer modified organic–inorganic hybrid silica monolithic capillary column has been developed, via the covalent bonding of 5′-NH_2-modified aptamer for human α-thrombin on hybrid silica monolith, prepared by sol–gel method, with tetraethoxysilane and 3-aminopropyltriethoxysilane as precursors. Due to the large specific surface area of the hybrid matrix, the average coverage density of aptamer reached 568 pmol/μL, and the thrombin binding capacity was 1.15 μg/μL, 14 times higher than that of aptamer modified open tubular capillaries. By such an affinity capillary column, the limit of detection of thrombin was decreased to 3.4 nM with a UV detector. Furthermore, even when thrombin was mixed with 1000 times more concentrated human serum, it could be selectively enriched and detected with the signal-to-noise ratio as ca.10. These results indicate that the developed preparation strategy for aptamer based hybrid silica monolithic capillary column might provide an effective method to achieve highly selective recognition of trace targets.



Since the first discovery in 1990s,[1,2] aptamers have exhibited potentially increasing exploration in the fields of biosensing, diagnostics, and therapeutics,[3−6] due to the unique properties that are comparable or superior to antibodies, in terms of high binding affinity for small molecules, nucleic acids, proteins, cells and even tissues. Furthermore, they have the advantages of reproducible synthesis, facile modification, and good stability for storage.[4,7]

These properties make aptamers promising alternatives to antibodies as stationary phases in affinity capillary chromatography. So far, three categories of capillary columns, including packed columns,[8−11] open tubular capillaries,[12,13] and monolithic columns[14−16] have been employed for aptamer immobilization, among which monolithic columns exhibit striking features, including lower back pressure, faster mass transfer, easier preparation than packed columns, and higher loading capacity than open tubular capillaries. Although polymer monoliths possess wider pH stability, better biocompatibility, and more facile preparation than silica monoliths, shrinkage and swelling usually affect the stability and reproducibility of such columns. Furthermore, the small specific surface area of polymer monoliths limits the binding capacity for ligands, which was demonstrated by previous research.[14−18] Recently, organic–inorganic hybrid silica monoliths prepared by sol–gel method are favored, with the merits such as large specific surface area, good biocompatibility, and high mechanical stability, which have been successfully used as matrices for separation,[19] protein digestion,[20,21] and phosphopeptide enrichment.[22] Although it has been demonstrated that aptamers could be successfully immobilized on sol–gel derived materials as sensor signaling elements without activity degradation,[23−25] to the best of our knowledge, hybrid silica monoliths have not been applied as stationary phase in the field of chromatography for aptamer based affinity recognition.

Herein, aptamer modified organic–inorganic hybrid silica monolithic capillary columns were prepared by covalently bonding 5′-NH_2-modified aptamer for human α-thrombin via glutaraldehyde, by which with increased binding capacity of aptamer, the high sensitive detection of thrombin in complex samples was achieved, demonstrating the great potential of such affinity columns for trace protein detection.

EXPERIMENTAL SECTION

Chemicals and Materials. Fused-silica capillary (250 μm i.d. × 375 μm o.d.) was obtained from Sino Sumtech (Handan, China). Hexadecyltrimethyl ammonium bromide (CTAB), tetraethoxysilane (TEOS, 99%), 3-aminopropyltriethoxysilane (APTES, 99%), glutaraldehyde, sodium cyanoborohydride ($NaCNBH_3$), human serum albumin (HSA), transferrin, myoglobin, and thrombin were purchased from Sigma (St. Louis, MO). Organic solvents were all of HPLC grade. All solutions were prepared with deionized water purified by a

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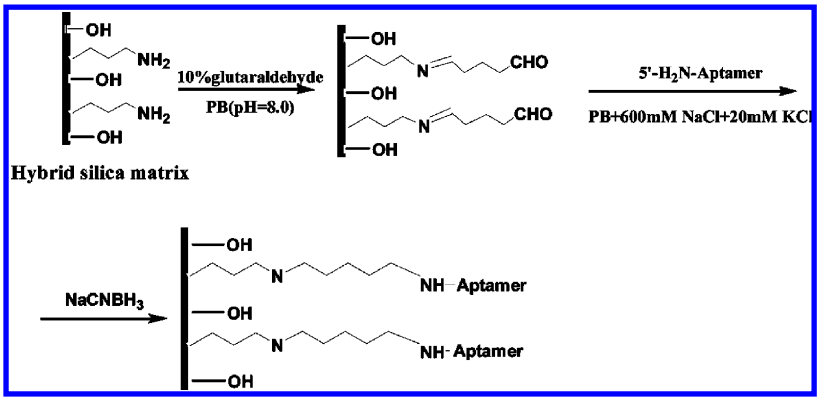

Figure 1. Reaction scheme for immobilization of huaman-α thrombin aptamer.

Milli-Q system (Millipore, Molsheim, France). The aptamer targeting human α-thrombin (5′-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3′, denoted as Apt-29) and oligonucleotide (5′-AAA AAA AAA AAA AAA AAA AAA AAA AAA AA-3′, denoted as scramble ssDNA), both with 5′-end modified by NH_2 through a C6-carbon spacer arm, were synthesized by Takara Biotechnology (Dalian).

Preparation of Aptamers Modified Organic−Inorganic Hybrid Silica Monolithic Capillary Columns. The hybrid silica monolithic capillary column was prepared according to our previous procedure,[19] but with slight modifications. Briefly, the capillary (250 μm i.d.) activated by hydrochloric acid was filled with polymerization solution, consisting of TEOS (112 μL), APTES (118 μL), anhydrous ethanol (215 μL), CTAB (8.0 mg), and water (32 μL) within 1 min and at temperature no higher than 25 °C to slow down the polymerization reaction. Then, with both ends sealed by silicon, the capillary was put in 40 °C water bath for 24 h, followed by rinsing with ethanol and water, respectively. With TEOS and APTES (molar ratio as 1:1) as precursors, and CTAB as template, beneficial to improve the homogeneity and permeability, the aminopropyl hybrid silica monolithic column was fabricated by supramolecular template-based sol−gel chemistry with one-step catalysis.

Apt-29 solution (25 μM, dissolved in 100 mM phosphate buffer, 20 mM KCl, and 600 mM NaCl, pH 7.4) was heated at 90 °C for 3 min, followed by cooling at room temperature. As shown in Figure 1, for Apt-29 immobilization, the monolithic matrix was first activated with glutaraldehyde (10%, v/v) dissolved in 100 mM phosphate buffer (pH 8.0). Then, the aptamer solution (5.7 nmol) was circulated through the column at 4 °C for 24 h, followed by flushing with 100 μL of 100 mM phosphate buffer solution (pH 8.0) to remove the unbound aptamer. Subsequently, the column was rinsed with Tris-HCl buffer solution (20 mM Tris-HCl, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$, pH 7.4) containing 5 mg/mL $NaCNBH_3$, by which unreacted aldehyde groups were capped with Tris-HCl, and C═N bounds were reduced to C—N by sodium cyanoborohydride. Finally, the column was washed by the same Tris-HCl buffer solution. The aptamer modified affinity monolithic capillary column was stored at 4 °C before usage. Unless specified otherwise, the applied affinity column length was 10 cm.

Operation Conditions for Aptamer Based Hybrid Silica Affinity Monolithic Capillary Chromatography. The aptamer based affinity capillary chromatography experiments were performed at room temperature (25 °C) with an HPLC system consisting of a P230 pump (Elite Analytical Instrument, Dalian, China), an injection valve (Valco, Houston, Texas) equipped with a 2 μL sample loop, and a K-2501 UV detector (Knauer, Berlin, Germany) set at 280 nm. To obtain microflow rate (2−3 μL/min) for separation, a flow splitter consisting of a T-union with one end connected to the monolithic column and the other end connected to a capillary (75 μm i.d., 100 cm) was introduced between the pump and injection valve. One capillary (75 μm i.d., 3.5 cm) was linked between the detector channel and monolithic column.

The separation was performed in a continuous adsorption/desorption mode by switching elutes. Before injection, the affinity column was equilibrated with loading buffer, a mixture of 20 mM Tris-HCl, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$ (pH 7.4). After sample injection, the valve was switched to loading position, and elution buffer, 2 M $NaClO_4$ solution, was introduced until the baseline was reached.

Measurement of Aptamer Coverage Density on Hybrid Silica Affinity Monolithic Capillary Column. The passed through fraction after aptamer immobilization and that after washed by 100 μL of 100 mM phosphate buffer to remove the nonspecific adsorbed aptamer were collected, and the immobilized Apt-29 amount was calculated according to the concentration decrease before and after covalent bonding, measured by a CARY 60 UV−vis spectrometer system (Agilent Technologies, Beijing) at 260 nm.

Thrombin Binding Capacity and Recovery on Aptamer Modified Hybrid Silica Monolithic Capillary Column. The binding capacity of human α-thrombin on the hybrid silica affinity monolithic capillary column was measured by dynamic frontal analysis. A 50 μg/mL thrombin sample was pumped through a 2 cm-long affinity capillary column at the flow rate of 2.6 μL/min, to saturate the aptamer binding sites. Myoglobin (0.05 mg/mL) was used to estimate the void time.

To evaluate the recovery of thrombin on aptamer modified hybrid silica monolithic capillary columns, thrombin eluted by 2 M $NaClO_4$ was collected from the affinity column, loaded on a packed microtrap column (C4, Phenomenex, 200 μm i.d. × 3.0 cm) with H_2O (0.1% trifluoroacetic acid, TFA) for 15 min, and further eluted with 80% ACN (0.1% TFA). The amount of eluted thrombin was calculated by peak area. Such a measurement was performed on affinity capillary columns prepared in three different batches.

Evaluation on Stability of Aptamer Modified Hybrid Silica Monolithic Capillary Column. To evaluate the stability, the affinity capillary column was used once a week

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for one month under the same operation condition. After thrombin eluted with $NaClO_4$ solution each time, the aptamer modified hybrid silica monolithic column was rinsed with loading buffer (20 mM Tris-HCl, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4) for 20 min and then stored at 4 °C in loading buffer before use.

RESULTS AND DISCUSSION

Characterization of Hybrid Silica Monolithic Capillary Columns. The distribution of pore size of hybrid silica monolithic capillary column was shown in Figure 2. The peak

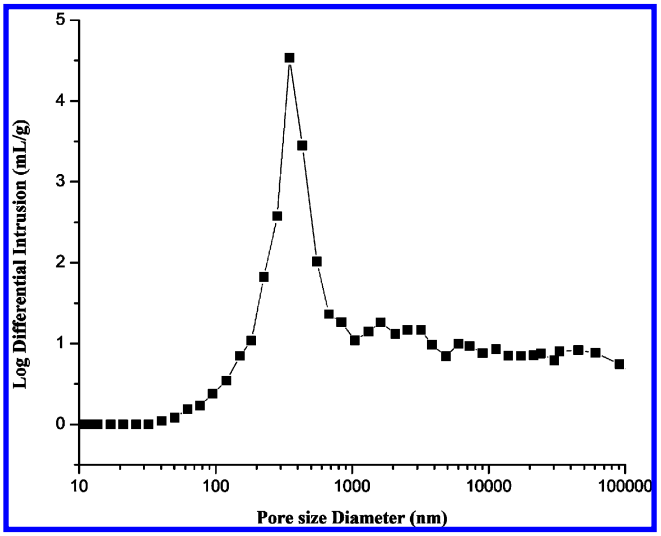

Figure 2. Pore size distribution of hybrid silica monolith, measured by mercury intrusion porosimetry.

appeared at about 380 nm demonstrated the macroporous structure of the matrix, favored for low back-pressure, high permeability, and fast mass transfer. According to Brunauer–Emmett–Teller (BET) method, the specific surface area of the hybrid monolith was determined to be 142.8 m^2/g (r^2 = 0.9999), 2 times higher than typical polymer based monolith column,[26] which was beneficial to provide large surface for aptamer immobilization.

After aptamer modification, the hybrid silica affinity monolithic capillary column was further characterized by scanning electron microscopy (FEI, Quanta 200F, Eindhoven, Holland). As shown in Figure 3, the affinity monolith was homogeneous, and tightly attached to the capillary inner wall, that would guarantee effective mass transfer and high stability of columns.

According to the previous method,[27] the aptamer density on hybrid silica affinity monolithic capillary columns was evaluated to be 568 pmol/μL (RSD = 13.5%, n = 3), much higher than that for open tubular affinity capillary columns (12 pmol/μL),[12] packed affinity capillary columns (204 pmol/μL),[11] and polymer affinity monolithic columns (254 pmol/μL, 290 pmol/μL).[14,15] This should be attributed to the high specific surface area of hybrid silica monolith, as well as the uniform distribution of active amine groups on the matrix with APTES as the functional monomer.[21]

Specificity of Aptamer Modified Hybrid Silica Monolithic Capillary Column to Thrombin. To demonstrate the selectivity of Apt-29 modified hybrid silica monolithic capillary column to human α-thrombin, myoglobin (0.4 mg/mL),

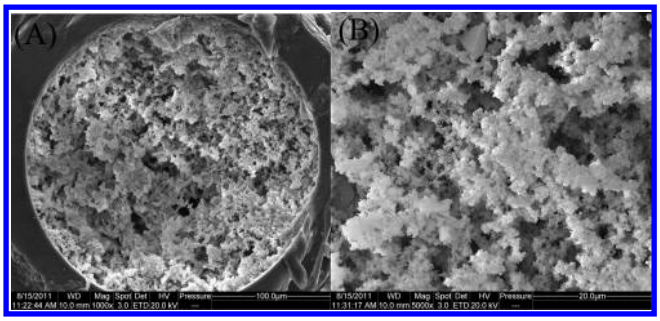

Figure 3. Scanning electron micrographs of aptamer immobilized hybrid silica monolithic capillary columns: (A) cross section of the affinity monolithic column; (B) structure of the affinity monolith inside the capillary.

transferrin (0.25 mg/mL), HSA (0.25 mg/mL), and thrombin (0.1 mg/mL) were chosen as samples, and the UV detection wavelength was set as 280 nm, due to the serious disturbance of citric sodium salt that exists in commercial thrombin lyophilized powder at 214 nm (data not shown). Herein, $NaClO_4$ was chosen as the elution reagent to release the captured thrombin because it could disrupt the stability of G-quadruplex and the water surroundings.[15] As shown in Figure 4, myoglobin, transferrin, and HSA had no retention on Apt-29 modified capillary columns, eluted at the void time, while thrombin was captured, and eluted only when washed with 2 M $NaClO_4$ solution, which was further confirmed by SDS-PAGE analysis (Figure S-1). The limit of detection of thrombin enriched by Apt-29 modified hybrid silica monolithic capillary column was further investigated to be 3.4 nM by a UV detector with signal-to-noise ratio of 3.

For comparison, the recognition capacity of bare hybrid silica monolithic column with aldehyde groups capped and hybrid silica monolithic column with 5′-end NH_2–C6 spacer modified scramble ssDNA immobilized were also studied. There was weak retention of thrombin on both columns (data not shown).

All these results elucidate that thrombin could be selectively captured on Apt-29 modified hybrid silica monolithic capillary column by the specific interaction between aptamer and human α-thrombin, instead of with the matrix or linkage arm. Meanwhile, the Apt-29 recognition ability was not altered after NH_2 modification and immobilization,.

Evaluation of Thrombin Binding Capacity, Recovery, and Stability of Aptamer Modified Hybrid Silica Monolithic Capillary Column. By dynamic frontal analysis, the dynamic binding capacity of aptamer modified monolithic column for human α-thrombin could reach 1.15 μg/μL (1.95×10^{-24} mol/nm^2, Figure S-2), ca.14 times higher than aptamer attached open tubular capillaries (1.4×10^{-25} mol/nm^2).[12] The high protein binding capacity may result from two aspects. On the one hand, glutaraldehyde was adopted here to provide $(CH_2)_5$ as a spacer arm for aptamer immobilization, to decrease the steric hindrance between ligands and targets. On the other hand, with high aptamer coverage on the affinity column, more active sites could be employed for the recognition of thrombin. Therefore, this kind of affinity column might provide the potential for the enrichment and purification of trace target proteins.

The recovery of thrombin on hybrid silica affinity monolithic columns prepared in three batches was determined to be 89.31 ± 5.6% (n = 3), similar to previous studies with polymer and silica monoliths as stationary phase for sample pretreat-

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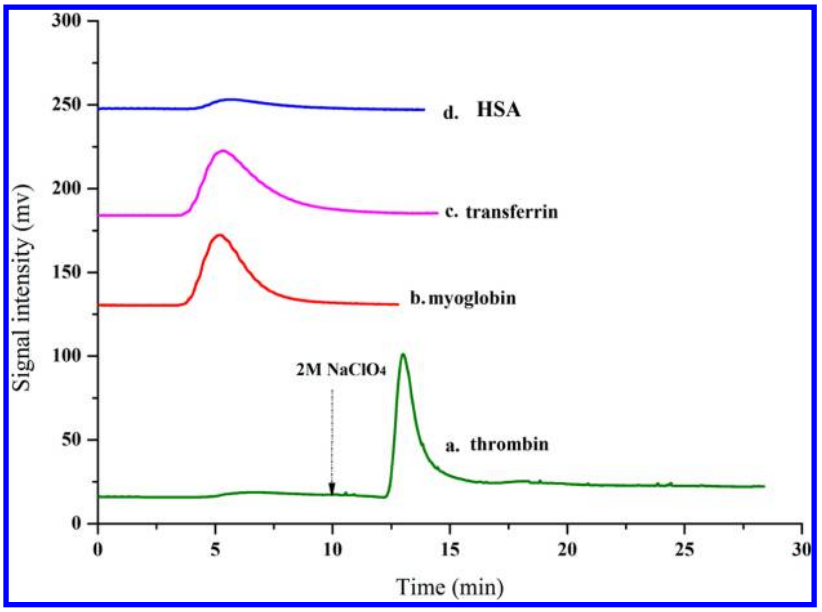

Figure 4. Chromatograms for thrombin (a) and myoglobin (b), transferrin (c), and HSA (d) on Apt-29 modified hybrid silica monolithic capillary column.

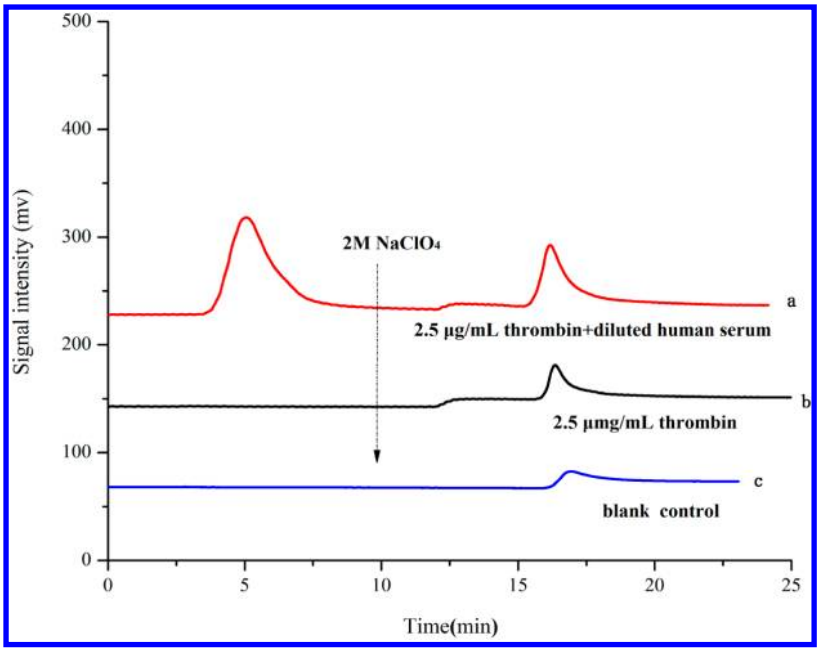

Figure 5. Chromatograms of thrombin spiked in diluted human serum and standard thrombin on Apt-29 modified hybrid silica monolithic capillary column.

ment.[28,29] With the consideration that the interpenetrating networks of hybrid silica effectively served to "cage" biomolecules, the recovery of our column was good enough.

To investigate the longevity of hybrid silica affinity monolithic column, columns (see the Experimental Section) stored at 4 °C in loading buffer were used for several hours every week for one month. The RSDs of retention time (6.12%) and peak height (2.75%) of thrombin demonstrated that the aptamer modified hybrid silica monolithic capillary column maintained good stability and reproducibility. Since the organic functional moieties provided by APTES were simultaneously covalently incorporated into hybrid silica monolithic matrices by sol–gel process, silica–carbon (Si–C) linkages were more hydrolytically stable, compared with monomeric siloxane bonds which were typically used in silica matrix surfaces.[30,31] Therefore, good mechanical stability of hybrid silica monolithic matrix would favor the high stability of the aptamer modified monolithic columns.

Application of Aptamer Modified Hybrid Silica Monolithic Capillary Column to Complex Sample. To demonstrate the feasibility of aptamer modified hybrid silica monolithic capillary columns for target enrichment in complex biological fluids, thrombin with low concentration (2.5 μg/mL) was spiked into human serum, diluted with loading buffer by 20 times. As shown in Figure 5 (curve a), a large peak appears at the void time, resulting from unreserved proteins in serum. When the mobile phase was switched to 2 M $NaClO_4$ solution, thrombin could be eluted, with the same elution time as

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standard protein, thrombin (2.5 μg/mL) (Figure 5, curve b). Although 2 M $NaClO_4$ solution also presented a peak at the same time of thrombin (Figure 5, curve c), in accordance with the recently published work,[14] the peak areas in curves a and b are much higher than that shown in curve c, illustrating that such kinds of aptamer modified hybrid silica monolithic capillary columns could be applied for specific capture of trace targets from complex biological samples without aptamer degradation via nucleases. Furthermore, due to the high affinity and high coverage density of aptamer on the hybrid silica monolith capillary columns, thrombin, as low as 67 nM (5 ng) with *S*/*N* ca.10, was detectable by such an affinity capillary column without further separation, comparable with that obtained from thrombin aptamer sensor by sandwich assay in serum matrix[32] and aptamer-conjugated gold nanorods in plasma samples.[33] This revealed the potential and feasibility of such an aptamer modified organic−inorganic hybrid silica monolithic column in future clinical analysis.

CONCLUSION

A strategy to immobilize aptamer targeting human α-thrombin on organic−inorganic hybrid silica monolithic capillary columns was proposed. Due to high surface area and uniform distribution of active groups on hybrid silica monolith, the prepared affinity columns were of high aptamer coverage density, resulting in improved binding capacity and decreased detection limit for human α-thrombin. In addition, such columns showed advantages of good stability and high recovery for target protein. All these results demonstrate that the strategy to immobilize aptamer on hybrid silica monolithic capillary columns is of great potential to achieve high sensitive detection of target proteins in therapeutics and clinical applications.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. 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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