Insertion Mechanism and Stability of Boron Nitride Nanotubes in Lipid Bilayers

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ABSTRACT. We provide insight into the interaction of boron nitride nanotubes (BNNTs) with cell membranes to better understand their improved biocompatibility compared to carbon nanotubes (CNTs). Contrary to CNTs, no computational studies exist investigating the insertion mechanism and stability of BNNTs in membranes. Our molecular dynamics simulations demonstrate that BNNTs are spontaneously attracted to lipid bilayers and are stable once inserted. They insert via a lipid-mediated, passive insertion mechanism. BNNTs demonstrate similar characteristics to more biocompatible functionalized CNTs.

KEYWORDS: boron nitride nanotube, all-atom molecular dynamics, cell membrane, entry dynamics, penetration, toxicity.

Introduction

Increasing attention is being given to nanotubes for various biomedical applications, such as artificial ion channels,¹⁻⁴ drug delivery vehicles,^{5, 6} and regenerative medicine⁷. The success of these applications relies on their biocompatibility and ability to insert into and remain stable in the cell membrane. The general consensus is that, in the absence of functional groups, carbon nanotubes (CNT) are toxic to cells.⁸⁻¹⁰ For example, non-functionalized CNTs have been shown to severely damage the lipid bilayer in *E. coli*¹⁰ and it has been recently suggested that cellular membrane disruption is a potential mechanism leading to nanotoxicity.¹¹ The toxicity of CNTs can be reduced by functionalizing their surface either covalently or non-covalently, for example by the immobilization of proteins, or with carboxylic groups.¹²⁻¹⁴ Although their toxicity can be reduced by functionalization, the possibility of in situ desorption increases the risk of toxicity in biological applications and their subsequent disposal post-use.¹⁵

Boron nitride nanotubes (BNNT) are more chemically inert and structurally stable than CNTs. Although the available literature is somewhat contradictory, BNNTs are generally considered to be inherently noncytotoxic.^{7, 15-19} For example, Chen et al.¹⁵ demonstrated the ability of BNNTs to deliver DNA oligomers to the interior of cells with no apparent toxicity. In contrast, Horváth et al.²⁰ recently described how BNNTs are cytotoxic in a time- and dose-dependent manner, with their toxicity being more pronounced in cells with high endocytic ability. A possible explanation for the toxicity observed by Horváth et al.²⁰ is the considerable length of the tested BNNTs compared to other groups since it is well known that aspect ratio affects toxicity.¹⁶ As a result of their generally improved biocompatibility BNNTs are likely to be more appropriate for use in biomedical applications. Therefore, both human and environmental exposure to BNNTs is likely to increase in the near future. As a result their biological responses, such as interaction with cells, needs to be thoroughly examined.

The uptake mechanism of CNTs into cells is still under debate: some groups report energy-dependent endocytosis^{14, 21-23} whereas others describe passive energy-independent insertion and diffusion.^{13, 24, 25} Shi et al.^{26, 27} find that CNTs enter cells either by wrapping into the cell membrane or by directly piercing through the membrane depending on their radius. Studies have shown that the insertion mechanism is highly influenced by CNT dimensions.^{23, 28, 29} Larger diameter CNTs disrupt the bilayer to a larger extent and allow more interactions with the membrane during simulations, therefore requiring larger forces to move through the bilayer.^{28, 29} Moreover, CNTs shorter than the membrane thickness (~60 Å) are more likely to rotate while moving through the bilayer.²⁸ Very few studies exist on the uptake mechanism of BNNTs. Ciofani and colleagues^{7, 17, 19} found that uptake of polyethyleneimine-coated and poly-L-lysine-coated BNNTs was via energy dependent endocytosis, and attributed this to the chemical reactivity of the polyethyleneimine coating.¹⁷

A number of researchers have used computational tools, typically coarse-grained molecular dynamics (MD) simulations, to investigate the mechanisms of insertion and stability of CNTs in cell membranes.^{26, 28-35} Non-functionalized CNTs are found to insert into the membrane and tilt such that they are surrounded by the non-polar lipid tails.^{25, 32-34, 36, 37} Höfinger et al.³² observe insertion and aggregation of short non-functionalized CNTs within the hydrophobic core of cell membranes and suggest that aggregation may play a role in their toxicity. When the CNTs are modified such that their ends are hydrophilic (either through functionalization or directly applying a partial charge to the carbon atoms) the nanotubes spontaneously insert into, and align across the lipid bilayer.^{33, 34} Baoukina et al.³⁷ confirm these results with free energy calculations. Functionalized CNTs can form open pores across the bilayer, provided there is a close match between the functional groups attached to the carbon nanotube ends and the hydrophilic domain of the lipid bilayer.^{33, 34} Generally, the lipids are found to occlude the pore when there is a hydrophobic/hydrophilic mismatch.³⁴ These MD studies suggest a passive energy-independent uptake mechanism, termed by some as a “nanoneedle” mechanism.

Despite their biocompatibility advantage no studies have investigated the insertion mechanism and stability of BNNTs in cell membranes. Here we use all-atom molecular dynamics simulations to investigate two properties of BNNTs:

- (i) the stability of BNNTs in lipid bilayers by conducting standard MD simulations and,
- (ii) the insertion mechanism by using a combination of standard and steered MD simulations.

We examine (10, 0) zigzag and (10, 10) armchair BNNTs, with lengths of 2 and 3.8 nm, and 2 nm length (10, 0) and (10, 10) CNTs for comparison. Each system comprises of a POPC bilayer surrounded by a TIP3P water box; the nanotube is initially placed in the lipid bilayer for simulations that investigate stability, and in the water box when investigating the insertion mechanism. More system details and simulation protocol can be found in the supplementary material. Using computational modelling, we aim to shed light on the interaction between BNNTs and cell membranes to help understand their reduced cytotoxicity compared to CNTs.

Simulation Methods

MD simulations are conducted using NAMD and visualized in VMD.^{38, 39} The CHARMM36 force field parameters^{40, 41} and TIP3P water model are used in all simulations. All simulations are carried out using 1 fs timesteps and under a constant pressure of 1 atm using a Langevin piston and a constant temperature of 298 K using Langevin dynamics. Each simulation employs a periodic boundary condition in the x, y and z directions and Particle Mesh Ewald is used to calculate electrostatic interactions. A switch distance of 10 Å and a cutoff distance of 12 Å is used for electrostatic and van der Waals interactions. Each system was setup so that the BNNT would not see an image of itself. There is a trade-off between the size of the lipid patch and simulation time, a larger patch provides more accuracy but also increases simulation time. On average, the number of POPC molecules for standard unbiased MD and SMD simulations are 138 and 180, respectively. This simulation size

compares well with previous work on coarse-grain and all-atom MD.^{34,48} For example, Ge et al.⁴⁸ use 170 all-atom POPC molecules for investigating the insertion of nanodiamonds into lipid bilayers.

We investigate (10, 0) and (10, 10) boron nitride nanotubes with lengths of either 2 nm or 3.8 nm, so as to examine the effect of both diameter and length. The BNNTs are constructed using the basic relations governing the fundamental parameters of a CNT⁴² and a boron-nitrogen bond distance of 1.446 Å.⁴³ The Lennard-Jones constants and partial charges (± 0.4 e) for boron and nitrogen atoms of the BNNTs are taken from Won and Aluru.^{43, 44} The (10, 0) and (10, 10) BNNTs have diameters of approximately 8 and 14 Å, respectively. These diameters are similar to those used to examine the insertion and stability of CNTs,^{28,32-34} providing us the opportunity to directly compare our results.

Standard unbiased molecular dynamics simulations

Two types of standard unbiased MD simulations are conducted: four simulations with the BNNT (both the (10, 0) and (10, 10) for both lengths) initially placed in the 3-palmitoyl-2-oleoyl-DGlycero-1-phosphatidylcholine (POPC) lipid bilayers with water reservoirs added to either side, and two simulations with the BNNT (both the (10, 0) and (10, 10) for 2 nm length only) initially placed parallel to the bilayer normal and 15 Å above the lipid bilayer in the upper water reservoir. All simulations use a symmetric bilayer. Moreover, to draw a comparison with lipid blockage simulations were also conducted with a (10, 10) CNT 2 nm in length. To form the BNNT in the lipid systems, several lipid molecules are removed from a section of membrane, so that there is no overlap of the van der Waals surface of the lipid and the BNNT. The position of the lipid atoms is then minimized while the position of the BNNT is held fixed so that the gaps between the lipid and BNNT are reduced. The entire system is then minimized and the equilibrated for 1 ns. After this, standard MD simulations are conducted. Table S1 in the Supporting Information provides details of the system sizes and simulation times.

Water flow rates through each nanotube are measured during the simulation to determine whether the nanotube is fully or partially blocked by lipids. A nanotube is deemed fully blocked when no water molecules can pass through. An edge detection algorithm applied to a cylinder is used to determine if a

nanotube is blocked by a lipid molecule. Gross flow rates are determined by summing the number of crossing events between the three regions: bulk, upper half of nanotube, and lower half of nanotube. This is then averaged over a time period of typically 0.5-2 ns.

Steered molecular dynamics simulations

Steered molecular dynamics simulations were conducted on the BNNT/lipid/water/ion system for the 2 nm length (10, 10) and (10, 0) BNNTs with a NaCl concentration of 140 mM. The BNNT is initially placed in the water reservoir 50 Å from the center of the lipid bilayer. Force constants of $k = 100, 50, 10, 5, 1, 0.5$ and 0.1 kcal/mol/Å are applied over every atom in the BNNT. In other words, each atom is subject to a force of k/N , where N is the number of atoms in the nanotube. Therefore, rotation about one end of the nanotube is not prohibited under this scheme; however at small values of k we observe overall rotation of the nanotube. This force accelerates the BNNT in the direction normal to the lipid bilayer. The BNNT is then moved with respect to the center of mass of the phosphorus atoms of the bilayer. The bilayer is not restrained in any way, and no constant velocity control is applied. These simulations give an indication of the likely pathway the BNNT takes when entering the lipid bilayer.

The BNNT is placed in the water reservoir at various angles to the bilayer normal to investigate the effect of starting orientation. In particular, the (10, 10) BNNT is placed at 0 and 90 degrees to the normal of the bilayer, and the (10, 0) BNNT is placed at 0, 90 and 180 degrees to the bilayer normal. The extra initial configuration is necessary to account for the dipole of the (10, 0) BNNT (note that the (10, 10) does not have a dipole) able to change. The values given are at the end of the equilibration period.

Results and Discussion

Stability in Lipid Membranes

To examine the stability of the BNNTs in the bilayer standard MD simulations with the nanotubes initially inserted into the bilayer were conducted. The (10, 0) and (10, 10) nanotubes of both lengths (2

nm and 3.8 nm); are stable in the bilayer, and remain embedded for the entire ~200 ns simulation. Their stability is demonstrated by examining the angular fluctuations (or tilt angle, θ) between the bilayer normal and the long axis of the BNNTs, shown in Figure 1 for the 2 nm length (10, 10) BNNT only. Each BNNT exhibits similar angular fluctuations (Figure S1 in the Supporting Information for all BNNTs), remaining approximately parallel to the bilayer normal (vertical or transmembrane orientation) throughout the simulations. As shown in Figure 1, the BNNT tilt angles compare well with the hydrophobic tubes with hydrophilic caps presented by Lopez et al.^{33, 34}. Nielsen et al.³⁵ predict that tilting becomes more pronounced as the ratio of functionalized-CNT radius to length decreases. We have not examined a large enough sample of nanotube diameters and lengths to determine if this is also the case for BNNTs. For CNTs to form open pores their length and functionalization must be highly controlled so as to create a hydrophobic/hydrophilic match with the lipid bilayer. It seems that BNNTs may not need to be as strictly controlled to form open pores across membranes, which may be useful for drug delivery or antimicrobial applications.

Figure 1. Oscillations of the tilt angle (θ) of the nanotube long axis with respect to the bilayer normal. (A) 2 nm length (10, 10) BNNT, (B) and (C) purely hydrophobic tube, and hydrophobic tube with hydrophilic caps, respectively with a similar diameter to the (10, 10) BNNT. The corresponding time accumulated histograms of the angles are embedded in each graph. (B) and (C) are reprinted from Lopez et al.³³, Copyright 2005, with permission from Elsevier.

Figure 2 details how lipid molecules interact with the pore of the BNNT. We have computed water flow (lines) and examined the presence of lipid in the interior of the BNNT (gray overlay). In the (10,

0) BNNTs and the (10, 10) CNT this is indicative of a block of at least one end of the nanotube. This is demonstrated by the gross flow rate between each end of the nanotube and bulk water; diffusion is completely halted at one end for the (10, 0) BNNTs and both ends for the (10, 10) CNT. However, lipid interaction with the interior of the nanotube does not imply a block of the pore; water is still able to flow in and out of the pore albeit at a reduced rate. This is the case in (10, 10) BNNTs as the wider pores are only partially occluded by lipid molecules as demonstrated by a decrease (but not to zero) of the gross water flow rate. CNT stability in the lipid bilayer has typically been investigated using coarse-grain MD simulations, usually utilizing a 3:1 mapping.^{29, 33-35} Coarse grain MD simulations trade a more detailed description of the system for longer simulation timescales. Generally, these studies have found that an open pore is formed for functionalized CNTs embedded in lipid bilayers. However, lipids can block the pore if there is a hydrophobic/hydrophilic mismatch,³⁴ or if the nanotubes are purely hydrophobic,²⁹ as in the case with the CNTs modeled in this study.

Figure 2. Water flow rate and blockage of the nanotube upper and lower entrances by lipids over time.

(A) A schematic of the BNNT system set up. Gross water flow rate is shown for the (B) (10, 10) 2 nm CNT, (C) (10, 0) 2 nm BNNT, (D) (10, 0) 3.8 nm BNNT, (E) (10, 10) 2 nm BNNT and (F) (10, 10) 3.8

nm BNNT. The grey overlay indicates the presence of a lipid molecule inside the nanotube. Note that not all labels and scales are shown as the y -axis scale is identical in parts C and D, and parts E and F, and all x -axes are time (ns).

One stark difference between the blocking events in BNNTs and CNTs is the mode of lipid blocking. Tri-methyl amino groups 'cap' one end of the BNNT, as displayed in the Figure 3A and 3B. In contrast, lipid molecules are seen to completely insert into the (10, 10) CNT (Figure 3C), with several lipid exchange events occurring during the simulation. Coarse-grain simulations of pristine CNTs detail lipid insertion events, as well as in CNTs with hydrophilic ends that exhibit hydrophobic/hydrophilic mismatch with the lipid bilayer.^{33, 34}

Once aligned to the bilayer normal, BNNTs are at least as stable as previously investigated CNTs. Water diffusion is blocked for the narrower BNNTs, but only one end of the BNNT at a time. Water diffusion in the wider (10, 10) BNNTs is only decreased, not eliminated, due to interactions with lipids.

Figure 3. Pore occlusion of the 2 nm length (A) (10, 0) BNNT, (B) (10, 10) BNNT, and (C) (10, 10) CNT.

Insertion Mechanism

The two sets of simulations examining the insertion mechanism of BNNTs are purely exploratory in nature and will serve as a guide for further nuanced and detailed studies. Only qualitative conclusions will be drawn from this portion of the study.

The first set of simulations examines the insertion mechanism using standard MD simulations with the BNNTs initially placed in the water reservoir, 15 Å above the bilayer surface. These simulations are run for the shorter length tubes only (2 nm); two simulations in total. The (10, 0) and (10, 10) BNNTs reach the bilayer surface after 0.5 and 16 ns, respectively (Figure 4). Once the BNNTs make contact with the bilayer surface they do not return to the bulk water. After 200 ns the (10, 0) BNNT is nestled into the headgroups of the lipid bilayer and is almost perpendicular to the bilayer normal. In contrast, after 216 ns, the (10, 10) BNNT lies flat on the lipid bilayer surface (Figure 4B). The nanotubes approach the bilayer surface parallel to the bilayer normal (vertical orientation) and once they make contact with the bilayer surface they flip and lie perpendicular to the bilayer normal (horizontal orientation). In their work on amino functionalized carbon nanotubes, Kraszewski et al.²⁵ suggest this “landing and floating” process is the first step in the passive diffusion insertion mechanism.

Figure 4. Snapshots of the 2 nm length (A) (10, 0) and (B) (10, 10) BNNT embedding into the lipid bilayer via standard MD simulations starting in the water reservoir.

In the second set of simulations, steered molecular dynamics (SMD) is utilized to investigate the insertion process of the 2 nm (10, 0) and (10, 10) BNNTs in more detail. These simulations give an indication of the likely pathway the BNNT takes when entering the lipid bilayer, and enable a qualitative estimate of the energy barriers for entry.

The BNNT is initially placed in the bulk water 50 Å from the center of the lipid bilayer, ~30 Å from the bilayer surface (first panel in Figure 5A, B). Three different starting orientations for the (10, 0) BNNT (0°, 90°, 180° to the bilayer normal), and two for the (10, 10) BNNT (0°, 90°) are considered. A force constant of 1 kcal/mol/Å is applied over each BNNT to drive it into the lipid bilayer.

The BNNT undergoes a spontaneous rotation so that it is roughly 90 degrees to the bilayer normal as it interacts with the lipid head groups (add as shown in Figure 6). This rotation was also observed in

our standard MD simulations and is therefore unlikely to be an artifact of the SMD simulations. From here, the BNNT begin to insert into the bilayer, but only after a lipid head group begins interacting with the pore opening. In particular, the positively charged trimethyl amino group of one lipid interacts with exposed negatively charged nitrogen end of the (10, 0) BNNT, as shown in Figure 5A. This interaction seems to be necessary for insertion. In some instances, the negatively charged phosphate group of the lipid interacts with the positively charged boron exposed end of the (10, 0) BNNT, but always after the trimethyl amino group interaction with the nitrogen end of the nanotube is established. The tail of the assisting lipid appears to bunch up as it approaches the center of the bilayer. The tail group then inserts into the tails of the opposite lipid leaflet (as shown in the last panel of Figure 5A). We observe a similar mechanism for the (10, 10) BNNT, however numerous lipid head groups interact with the nanotube ends (Figure 5B). A similar observation is made with CNTs in which larger diameter nanotubes allow more interactions with the membrane lipids.^{23, 28, 29}

Figure 5. Insertion process of the (A) (10, 0) and (B) (10, 10) 2 nm length BNNTs from steered MD simulations with a force constant of $k = 1 \text{ kcal/mol/\AA}$ and a starting orientation of 90 degrees to the

bilayer normal. Note that cyan and red licorice representations denote one particular lipid molecule throughout the insertion process.

Figure 6. BNNT center of mass versus tilt angle of the BNNT long axis with respect to the bilayer normal as the nanotubes are pulled through the lipid bilayer in our steered MD simulations with a force constant of $k = 1 \text{ kcal/mol/Å}$. (A) (10, 0), and (B) (10, 10) 2 nm length BNNTs. Note that the bilayer/water interface is located at approximately 25 Å.

Insertion of the BNNT halts as the center of mass of the nanotube nears the center of the lipid bilayer, where it is found at an angle of 90 to 140 degrees to the bilayer normal. In each case where insertion takes place, water occupies the interior of the BNNT and is connected to the bulk water at the buried end of the BNNT by a water wire which is sometimes mediated by a lipid head group (Figure 7). The BNNT end closest to the center of the bilayer is maintaining favorable hydrophilic interactions, a phenomenon described by Allen et al as 'snorkeling'.^{45, 46} Additionally, entire lipids or lipid head groups are observed to enter into the (10, 10) BNNTs (see Figure S2). The inserted lipid may continue to participate in bringing the BNNT into the opposite leaflet, but the BNNT may never become an open pore spanning the bilayer.

Figure 7. “Snorkeling” effect. One molecule thick water wire connecting the (10, 10) BNNT to the bulk from steered MD with a force constant of $k = 1 \text{ kcal/mol/\AA}$.

Given the hydrophilic nature of the BNNTs (alternating ± 0.4 partial charges in our model), a large amount of energy would be lost from breaking BNNT-water and BNNT-lipid head groups interactions that may not be recouped by interactions with the hydrophobic lipid tails. This would limit the disturbances to the lipid bilayer (compared to a hydrophobic CNT) and may prohibit the formation of a lipid spanning pore. We can speculate that, as observed for ion translocation across membranes,⁴⁶ BNNTs may cross the membrane and enter the cell if one of the following events occurred:

- (i) the lipid molecule mediating insertion flips to the opposite leaflet (as in hydrophilic capped CNTs³⁴), or
- (ii) the interactions between the BNNT and one leaflet cease and new interactions with the distant leaflets form.

We have not observed a complete translocation/entry in steered MD most likely as a result of the rate limiting step associated with traversing a large energy barrier near the bilayer center. Further simulation using enhanced sampling techniques or coarse grain models may provide more definitive evidence. It is difficult to estimate the free energy barrier for insertion from these results as we could not simulate long enough to cross the energy barrier. It is hoped that our subsequent coarse-grain simulations will provide an estimate of this cost. As a simple test of the stability of the BNNT we

removed the constraint ($k = 1 \text{ kcal/mol/\AA}$) which was applied to the BNNT in our steered MD simulations and extended the simulation for a further 25 ns. We found that the BNNT rotates about its original release orientation $\pm 15^\circ$ but remains inside the lipid bilayer (as shown in Supplementary Figure S3).

Our steered MD simulations suggest that BNNTs only partially insert via a lipid-mediated passive diffusion mechanism (illustrated in Figure 5) with the following steps:

- 1) The BNNT contacts the lipid bilayer,
- 2) The BNNT re-orientates to roughly parallel to the bilayer surface.
- 3) An interaction between one end of the BNNT (the nitrogen end for (10,0)) and the head group of a lipid molecule is established, and
- 4) This interaction mediates the insertion of the BNNT, allowing it to slide into the bilayer at an angle of approximately 45 degrees.

This mechanism is similar to the insertion mechanism into lipid bilayers of amino functionalized carbon nanotubes proposed in MD studies by Kraszewski et al.,²⁵ besides the final realignment. They suggest the uptake of functionalized CNTs occurs via a passive diffusion mechanism with three distinct steps: (i) “landing and floating” of the CNT onto the membrane surface; (ii) penetration of the lipid head groups; and (iii) sliding through the lipid tails. Lopez et al.³³ also propose a similar insertion process in their MD simulations using their hydrophobic tubes with hydrophilic caps. Moreover, Kostarelos et al.⁴⁷ demonstrate experimentally that the cellular internalization of functionalized CNTs does not depend solely on endocytosis.

An advantage of BNNTs over CNTs for use in biomedical applications is that they are stable in model cell membranes without the need to perfectly match hydrophobic/hydrophilic domains to the lipid bilayer. It is possible that their inherent non-cytotoxicity is a result of their membrane stability, and the

improbability of them embedding into the hydrophobic core of a lipid bilayer. BNNTs may be better suited to drug delivery applications^{5, 6} such as nerve regeneration⁷ and as artificial ion channels¹⁻⁴ than CNTs.

Conclusion

We have demonstrated using all-atom MD simulations that BNNTs are stable in model cell membranes, with a similar stability to that of functionalized CNTs. Narrow (10, 0) BNNTs can be blocked at one end by lipid molecules, whereas wider (10, 10) BNNTs are only partially blocked. BNNTs partially insert into the lipid bilayer via a passive, lipid-mediated mechanism. Once the BNNT has contacted the bilayer lipid head groups interact with the pore opening, allowing the nanotube to slide into the bilayer. We do not observe the nanotubes aligning vertically through the bilayer, leading us to hypothesize the existence of a large energy barrier between the inserted and aligned states – which may be possible to describe with enhanced sampling techniques. The insertion mechanism is similar to that of amino functionalized CNTs²⁵, whereby the BNNTs “land and float” onto the lipid bilayer surface followed by lipid-mediated partial insertion into the bilayer.

The work presented here on BNNTs and previous investigations on CNTs suggest that a nanotube which is stable and less disruptive to the lipid bilayer is likely to be less toxic, regardless of whether an open pore is formed. It is unclear, however, if BNNTs will insert completely into lipid membranes. The hydrophilic nature of BNNTs makes them less amenable for interaction with the hydrophobic lipid tails. To mitigate this they create a polar environment of water molecules and lipid head groups around themselves upon insertion into the bilayer. Further work is needed to ascertain the magnitude of the barrier to insertion to determine if it is prohibitively large or not.

ASSOCIATED CONTENT

Supporting Information Available: Table with simulation parameters of the molecular dynamics simulations. Figures of the tilt angle of the BNNTs with respect to the bilayer normal, and lipid insertion into (10, 10) BNNT. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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