Biophysical properties of membrane lipids of anammox bacteria: II. Impact of temperature and bacteriohopanoids

Henry A. Boumann a, Pieter Stroeve b, Marjorie L. Longo b, Bert Poolman c, Johanna M. Kuiper c, Ellen C. Hopmans a, Mike S.M. Jetten d, Jaap S. Sinninghe Damsté a, Stefan Schouten a,⁎

a Dept. of Marine Organic Biogeochemistry, NIOZ Royal Netherlands Institute for Sea Research, PO Box 59, 1790 AB, Den Burg, Texel, The Netherlands
b Dept. of Chemical Engineering and Materials Science, University of California Davis, 1 Shields Ave, Davis, CA 95616, USA
c Dept. of Membrane Biochemistry, University of Groningen, Nijenborgh 4, 9747 AG, Groningen, The Netherlands
d Dept. of Microbiology, Radboud University Nijmegen, Toernooiveld 1, 6525 ED, Nijmegen, The Netherlands

Abstract

Anammox bacteria possess unique membranes that are mainly comprised of phospholipids with extraordinary “ladderane” hydrocarbon chains containing 3 to 5 linearly concatenated cyclobutane moieties that have been postulated to form relatively impermeable membranes. In a previous study, we demonstrated that purified ladderane phospholipids form fluid-like mono- and bilayers that are tightly packed and relatively rigid. Here we studied the impact of temperature and the presence of bacteriohopanoids on the lipid density and acyl chain ordering in anammox membranes using Langmuir monolayer and fluorescence depolarization experiments on total lipid extracts. We showed that anammox membrane lipids of representatives of Candidatus “Kuenenia stuttgartiensis”, Candidatus “Brocadia fulgida” and Candidatus “Scalindua” were closely packed and formed membranes with a relatively high acyl chain ordering at the temperatures at which the cells were grown. Our findings suggest that bacteriohopanoids might play a role in maintaining the membrane fluidity in anammox cells.

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1. Introduction

The discovery of microorganisms responsible for anaerobic ammonium oxidation (anammox) about a decade ago has significantly contributed to our understanding of the global nitrogen cycle [1]. In the anammox reaction, ammonium (NH4+) is anaerobically utilized as an electron donor for reduction of nitrite (NO2−), yielding dinitrogen gas (N2) as the final product [2]. The bacteria capable of performing this biochemical reaction were initially identified in wastewater treatment systems, but have now also been detected in various oxygen-limited fresh water and marine ecosystems [13–6]. Today, anammox bacteria play a key role in the low cost and environmental-friendly removal of ammonium in wastewater treatment systems, but have now also been detected in various oxygen-limited fresh water and marine ecosystems [13–6]. Anammox bacteria belong to the order of the Planctomycetales and form a distinct phylogenetic cluster composed of the genera Candidatus “Kuenenia”, Candidatus “Brocadia”, Candidatus “Scalindua”, Candidatus “Jettienia” and Candidatus “Anammoxoglobus propionicus” [3–12]. Although these prokaryotes are widespread, each ecosystem is dominated by a single anammox genus, suggesting that the genera have adapted to distinct niches [10]. At present, pure cultures of anammox bacteria are still lacking mainly because the enrichment of these microbes in reactor batches is challenging and yields only low amounts of biomass as the cells divide only every 10–20 days [8,9,13]. Anammox bacteria contain an intracytoplasmic compartment, the anammoxosome, which accounts for a voluminous fraction of the cell [14]. It has been proposed that the anammox process takes place in this specialized organelle, since the volatile intermediate hydrazine together with hydroxylamine/hydrazine oxidoreductase are exclusively localized in that compartment [14–18]. The latter implies that the anammoxosome necessitates a membrane that facilitates the preservation of an electrochemical proton gradient during the exceptionally slow anammox catalysis and prevents diffusion of the mutagenic anammox intermediates to the exterior of the anammoxosome [17,19,20]. Indeed, studies on the membrane lipid composition of the anammoxosome of Kuenenia stuttgartiensis revealed the presence of so-called “ladderane” lipids which contain hydrocarbon chains comprised of three or five linearly condensed cyclobutane rings forming highly strained moieties (Fig. 1) [19]. These lipids are unique in nature and have, thus far, only been reported for anammox bacteria. The majority of the intact membrane lipids have either one or two of these ‘ladderane’ hydrocarbon chains linked to...
the glycerol backbone with a phosphocholine, phosphoethanolamine or phosphoglycerol headgroup [21,22]. Whereas the sn-1 position of the ladderane phospholipids is usually linked to a C20-hydrocarbon chain with three cyclobutane rings, the sn-2 position has a more diverse repertoire of hydrocarbon chains that includes etherified or esterified ladderane, straight or methyl branched hydrocarbon chains (Fig. 1). In addition to the ladderane phospholipids, the cholesterol-like C35 bacteriohopanetetrol has been identified in the different anammox genera [20,21].

At present, relatively little is known about membranes composed of these extraordinary ladderane lipids. Since the permeability of fluorophores is significantly lower across anammoxosomal membranes relative to conventional membranes, it has been proposed that the ladderane barrier may preserve concentration gradients of other relative small molecules like hydrazine or protons [17,19]. Molecular modeling experiments, in which a bilayer composed of an ether-ester lipid with one C20-[3]- and one C20-[5]-ladderane unit was modeled, suggested that such a membrane was exceptionally dense [19]. Recently, we have demonstrated that both a PC and a mixed PE/PG ladderane lipid fractions form tightly packed but still fluid mono- and bilayer systems [23]. In another study, we showed that anammox species cultured at different temperatures change their lipid composition: with increasing temperature the relative amount of C18-ladderane hydrocarbon chains was decreasing, while the longer C20-ladderane hydrocarbon chains were increasing [24]. A similar phenomenon was noted for ladderane lipids in environmental samples from a range of temperatures and has been associated with maintaining the optimal membrane viscosity for cell viability [24,25]. Furthermore, the membrane lipid composition of anammox bacteria also contains bacteriohopanetetrol [20,21]. These bacteriohopanoids have been observed to play a role as a rigidifier in membrane

**Fig. 1.** (A) Abundant lipid hydrocarbon chains in anammox bacteria: (a) C18-[3]-ladderane, (b) C18-[5]-ladderane, (c) C20-[3]-ladderane, (d) C20-[5]-ladderane, (e) hexadecane, (f) 14-methylpentadecane, (g) 10-methylhexadecane, (h) 9,14-dimethylpentadecane. (B) Examples of the ladderane lipid classes PC, PE and PG: (i) 1-hexadecanyl-2-(8-[3]-ladderane-octanyl)-sn-glycerophosphocholine, (ii) 1-(8-[3]-ladderane-octanoyl)-2-(8-[3]-ladderane-octanyl)-sn-glycerophosphoethanolamine and (iii) 1-(6-[5]-ladderane-hexanyl)-2-(8-[3]-ladderane-octanyl)-sn-glycero-3-phospho-(1′-sn-glycerol), respectively. (C) Molecular structure of bacteriohopanetetrol.
environments [26,27]. However, the significance of bacteriohopano- 
oids in membranes composed of ladderene lipids is currently 
unknown.

To investigate the adaptation of anammox membranes to tem- 
perature, we examined the total lipid extracts from representatives 
of three different anammox genera, using Langmuir monolayers experi- 
ments. In addition, large unilamellar vesicles (LUVs) have been 
constructed to gain insight into the acyl chain ordering of lipid bilayers 
of the different anammox lipid extracts in fluorescence depolarization 
experiments. The results were compared to those of conventional 
phospholipid membranes of *Escherichia coli* and revealed unusual acyl 
chain ordering and highly packed lipid membranes for different 
anammox genera at physiological temperatures.

### 2. Material and methods

#### 2.1. Lipid material derived from anammox bacteria and *E. coli*

Three different anammox genera were incubated in individual 
sequencing batch reactors for 5 months under anoxic conditions 
[7,9,12,13]. The microbial population of the enrichment cultures was 
monitored by fluorescence *in situ* hybridization [21,28]. The reactor 
for *Candidatus* "B. fulgida" was maintained at ± 35 °C and ~75% the 
obtained cell material was composed of this anammox bacterium [10]. 
For *Candidatus* "*K. stuttgartiensis*", the reactor was set at 35 °C and 
~74% of the biomass was composed of this anammox bacterium, whereas the *Scalindua* spp. biomass was maintained at 20 °C and 
yielded cell material composed for 90% of this anammox bacterium [12,29]. Furthermore, *Candidatus* "K. stuttgartiensis" biomass was 
obtained from a suspension of anammox biomass in an oxygen-
limited wastewater treatment plant at 25 °C from Paques B.V. (Balk, 
The Netherlands). The cell material of this wastewater bioreactor was 
composed of ~80% of *Candidatus* "*K. stuttgartiensis*" cells.

All anammox biomasses were lyophilized and the total lipids 
extract were obtained according to a slightly modified Bligh and Dyer 
method [30,31]. The composition of the total lipid extracts were 
analyzed by high performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) as 
described previously and was found to be dominated by phospholi-
pids with C<sub>18</sub>-[3], C<sub>18</sub>-[5], C<sub>20</sub>-[3] and C<sub>20</sub>-[5]-ladderane hydrocarbon 
chains (see Fig. 1 and [21,22]). For control experiments, *E. coli* total 
lipid extract was purchased from Avanti Polar Lipids (Alabaster, AL) and 
washed with acetone followed by diethylther to remove residual 
proteins.

#### 2.2. Langmuir monolayers

Surface pressure-mean area per molecule isotherms were obtained 
using a Teflon® Langmuir–Blodgett trough with dimensions 20 cm by 
30 cm (Type 611, Nima, Coventry, United Kingdom). Lipids were 
dissolved in chloroform at 1.0 mg mL<sup>-1</sup>. About 75 µL of a given lipid 
solution was gently deposited at the air–water interface using a 100 µL 
Hamilton microsyringe (Hamilton, Reno, NV) at the indicated 
temperature. The lipid films were compressed at a constant speed of 
20 cm<sup>2</sup> min<sup>-1</sup>, while the surface pressure was measured using a 
Wilhelmy plate made of filter paper. Each surface pressure-area 
curve was recorded at least three times. Langmuir data was analyzed by 
determining the isothermal compressibility, *k<sub>s</sub>* as shown in Eq. (1), 
where *A* and *π* represent the molecular occupied area and film 
pressure, respectively:

\[
\kappa_s = -\frac{1}{A} \frac{dA}{d\pi}
\]

The lipids 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 
1,2-dioleoyl-sn-glycero-3-phosphocholine (DSPC) (Avanti Polar Lipids, Alabaster, AL) were analyzed as references at room tempera-
ture. Based on the total lipid composition of anammox cells as 
determined by HPLC/MS, we estimated the mean molecular weight of 
the anammox total lipid extracts to be ~725 g mol<sup>-1</sup>.

### 2.3. Fluorescence depolarization

Fluorescence depolarization experiments were conducted for the 
anammox and *E. coli* total lipid extracts using a Fluorolog®-3 
Instrument (Horiba Jobin Yvon, Edison, NJ), according to Shinitzky 
and Barenholz [32]. For experiments using 1,6-diphenyl-1,3,5-hexa-
triene (DPH) (Invitrogen, Eugene, OR), lipids were dried by rotary 
evaporation and hydrated in 50 mM KP, buffer, pH 7.0. Five 
consecutive freezing in liquid nitrogen and thawing at room 
temperature steps, followed by extrusion through 200 nm polycarbo-
nate filter yielded LUVs with an average diameter of ~170 nm [33]. 
5.0 · 10<sup>-8</sup> M LUVs in 50 mM KP buffer, pH 7.0 were incubated in the presence of 5.0 · 10<sup>-8</sup> M DPH. Excitation and emission wavelength 
were set at 360 nm and 428 nm, respectively. For the 1-[4-
(trimethylamino)phenyl]-6-phenylhexa-1,3,4-triene (TMA-DPH) 
(Invitrogen) labeling of liposomes, 5 µL TMA-DPH stock solution 
(4 mM in dimethylsulfoxide) was diluted in 245 µL 50 mM KP buffer, 
pH 7.0. Subsequently, the liposomes were mixed with TMA-DPH 
solution in a 1:1 volume ratio resulting in a TMA-DPH/lipid ratio of 
1:125 (mol/mol). After incubation for 1 h at room temperature, the 
steady-state fluorescence depolarization was measured upon 40-fold 
dilution of the liposomes. The excitation and emission wavelength 
were recorded at 360 nm and 430 nm, respectively. All experiments 
were performed using a Lauda Model RE 106 temperature controlled 
water bath (Lauda-Könighofen, Germany).

### 3. Results and discussion

#### 3.1. Langmuir trough experiments

To obtain insight into the biophysical properties of the ladderane-
containing anammox membranes, we first carried out Langmuir 
monolayer experiments. For comparison, the well-studied DSPC and 
DOPC lipids were analyzed in parallel at a temperature of 25 °C. Fig. 2A 
depicts the surface pressure versus area per molecule curve for DSPC 
lipid with an onset pressure increase at about 57 Å<sup>2</sup> per molecule and a 
collapse pressure at 61 mN/m (closed diamonds). By extrapolating 
the slope of the solid phase isotherm to zero pressure, we observed a 
minimal packing density of ~54 Å<sup>2</sup> per DSPC molecule. For the DOPC 
lipid, an initial pressure increase was observed at 108 Å<sup>2</sup> per molecule 
and the minimal lipid packing was established at ~85 Å<sup>2</sup>/molecule 
(Fig. 2A, open diamonds). Ongoing compression of this liquid-
expanded phase monolayer resulted in instability at about 43 mN/ 
m. These findings are in agreement with previous studies and clearly 
demonstrate that minimal lipid packing in monolayer films is already 
profusely affected by a double bond (‘kink’) in the hydrocarbon 
chains of the lipids [34,35].

Fig. 2B illustrates representative curves of surface pressure-area 
per mean molecule of the total lipid extracts of the different anammox 
genera. The optimal lipid packing was determined at the temperature 
corresponding to that of cell growth and at the surface pressure of 
30 mN/m at which the lipids in a monolayer are thought to occupy a 
membrane area close to that for lipids in a biological membrane [36]. 
The monolayer of the lipid extract from *Candidatus* "*K. stuttgartiensis*" 
grown at 25 °C exhibited a first pressure increase at approximately 
77 Å<sup>2</sup>/molecule and remained stable in the liquid-expanded phase 
until it collapsed at ~41 mN/m (Fig. 2B, closed circles). Interestingly, the 
mean area per molecule for this monolayer at 30 mN/m was about 
62 Å<sup>2</sup> per molecule, suggesting a relatively high lipid density 
close to that of the DSPC lipid. Since the HPLC-ESI-MS/MS analysis 
revealed that this lipid extract was dominated by ladderane
cultured at 35 °C also revealed a mean minimum area of
membrane remained relatively
anammox cells (Fig. 2B). Hence, despite the high lipid density, the
isotherms for
62 Å² per molecule at 30 mN/m (Fig. 2). None of these isotherms
formed a
phase transition with increasing compression and that the lipids
This points to the absence of a liquid-expanded to liquid-condensed
behavior is indicative of the presence of two coexisting mixtures,
the isotherms of the two
k values ± the standard deviation of three independent experiments. Asterisk-marks
indicated growth temperature.

phospholipids, this finding suggests that the cyclobutane ring
structures of the ladderane hydrocarbon chains result in relatively
tight lipid packing. Indeed, ladderane phospholipids are capable of
forming densely packed monolayers, as we have previously observed
averaged minimal area of 65 and 59 Å²/molecule, respectively, for
purified PC- and the PE/PG ladderane fractions [23]. These results are
also in agreement with the molecular modeling experiments in which
a membrane composed of ladderane lipids showed a high acyl chain
density [19]. Similar as for DOPC, the isothermal surface compressi-
bility analysis of this ladderane lipid extract yielded
k values higher
than 0.010 m/mN between the lift-off and collapse of the monolayer.
This points to the absence of a liquid-expanded to liquid-condensed
phase transition with increasing compression and that the lipids
formed a fluid membrane at the physiological temperature of the
anammox cells (Fig. 2B). Hence, despite the high lipid density, the
membrane remained relatively fluid.

It is interesting that the isotherm for Candidatus “K. stuttgarti-
ensis” cultured at 35 °C also revealed a mean minimum area of ~62 Å²
per molecule at the above mentioned conditions (Fig. 2B). Similarly, the
isotherms for Candidatus “B. fulgida” grown at 35 °C and Candidatus
“Scalindua” cultured at 20 °C exhibited mean minimum areas of near
62 Å² per molecule at 30 mN/m (Fig. 2). None of these iso-
therms showed a transition from a liquid-expanded to a liquid-condensed
phase with increasing pressure (all
k values ≥ 0.011 m/mN), suggesting that they were all fluid under these conditions (Fig. 2B).
Near a surface pressure of 15 mN/m, we observed a slight plateau in
the isotherms of the two K. stuttgartiensis lipid extracts. This type of
behavior is indicative of the presence of two coexisting mixtures,
where each will collapse independently. The finding that all these
anammox bacteria have equivalent lipid packing under these
biological relevant conditions implies that the optimal lipid packing
under different growth (i.e. temperature) conditions for anammox
bacteria is around 62 Å² per molecule. Rattray and coworkers have
shown that the lipid composition varies strongly with temperatures,
i.e. in the cultures grown at 20 and 25 °C the relative amount of C18-
ladderanes versus C20-ladderanes was larger than for those grown at
35 °C [24]. Our results suggest that this chain length adaptation may
have contributed to reaching similar packing densities at different
physiological temperatures. Indeed, when we used the lipid extract of
Candidatus “K. stuttgartiensis” grown at 35 °C to obtain monolayers at
room temperature, thus well below the growth temperature, we
observed a packing density that was substantially different, i.e. at
about 53 compared to ~62 Å² per molecule at a pressure of 30 mN/m.
Presumably, this lipid membrane packing was “too dense” in order for
the membrane to function optimally within the anammox bacteria. It
is worth mentioning that the surface pressures of the four anammox
lipid extracts were in equilibrium near 20 mN/m, corresponding with
a lipid packing of ~53 Å²/molecule (Fig. 2B). This minimal area per
molecule resembled that of DSPC near 20 mN/m and thus supports
the notion that the monolayers of the anammox lipid extracts show a
comparable biophysical behavior to that of the tightly packed DSPC
equivalent.

3.2. Fluorescence depolarization experiments

The proposed dense anammox membrane architecture was further
investigated as part of a bilayer structure, i.e. the membrane of a large
unilamellar vesicle (LUV). First, we determined the acyl chain

Fig. 2. Surface pressure-area per molecule isotherms of (A) DSPC (closed diamonds) and
DOPC (open diamonds) determined at room temperature; and (B) the total lipid
extracts derived from Candidatus “K. stuttgartiensis” grown at 25 °C (closed circles),
Candidatus “K. stuttgartiensis” cultured at 35 °C (open circles), Candidatus “B. fulgida”
grown at 35 °C (closed squares) and Candidatus “Scalindua” cultured at 20 °C (open
squares) determined at the temperature corresponding to that of cell growth. Data
points are representative for three independent measurements. Asterisk-marks
indicated growth temperature.

![Diagram](image-url)

Fig. 3. (A) Fluorescence depolarization of DPH in LUVs prepared from the total lipid
extracts of E. coli (closed diamonds), Candidatus “K. stuttgartiensis” grown at 25 °C
(closed circles), Candidatus “K. stuttgartiensis” cultured at 35 °C (open circles), Candi-
datus “B. fulgida” grown at 35 °C (closed squares) and Candidatus “Scalindua” cultured
at 20 °C (open squares). (B) TMA-DPH fluorescence depolarization in LUVs prepared
from total lipid extracts of E. coli (closed diamonds), Candidatus “K. stuttgartiensis”
grown at 25 °C (closed circles), Candidatus “K. stuttgartiensis” cultured at 35 °C (open
circles), Candidatus “B. fulgida” grown at 35 °C (closed squares) and Candidatus
“Scalindua” cultured at 20 °C (open squares). The data points shown are the mean
values ± the standard deviation of three independent experiments. Asterisk-marks
indicated growth temperature.
ordering in the hydrophobic core region of the membranes of LUVs by employing the hydrophobic probe DPH; DPH is thought to reside in the hydrophobic core of the membrane [32,37–39]. As a reference for the anammox lipid extracts, we have used the total lipid extract derived from E. coli. The latter is mainly composed of PE, PG and cardiolipin phospholipids with saturated and mono-unsaturated hydrocarbon chains (reviewed by [40]). As shown in Fig. 3A (closed diamonds), the DPH steady-state fluorescence depolarization intensity for the E. coli membranes gradually declined from 0.27 ± 0.03 to 0.07 ± 0.01 as the temperature increased. This can be explained by an increase in the rotational freedom of the hydrocarbon chains with no apparent phase changes, which is in good agreement with earlier studies [41–43].

For the anammox lipid extracts, the DPH fluorescence intensities were markedly higher than those of the E. coli membranes. These membranes showed more acyl chain ordering, as the DPH depolarization intensities of both lipid extracts from Candidatus “K. stuttgartiensis” “K. stuttgartiensis” ranged from about 0.33 ± 0.02 to 0.12 ± 0.01 (Fig. 3, closed and open circles). Similar as for E. coli, no abrupt changes in the fluorescence depolarization intensities were observed, indicating the absence of a phase transition. For Candidatus “B. fulgida” and Candidatus “Scalindua”, the DPH fluorescence depolarization intensities showed a similar trend and gradually decreased from about 0.39 to 0.24 (Fig. 3, closed and open squares). The latter values are higher than those of Candidatus “K. stuttgartiensis” lipid extracts and hence consistent with the Langmuir monolayer results that show a higher slope, i.e. a higher elastic modulus and thus more acyl chain ordering and less viscosity, for Candidatus “B. fulgida” and Candidatus “Scalindua” (cf. Fig. 2B). As the DPH probe monitors the acyl chain ordering in the interior of the lipid bilayers, this may be caused by variation in the abundance of the membrane-rigidifying bacteriohopanetetrol.

At the biological relevant temperature, the ladderane containing LUVs have DPH fluorescence depolarization intensities ranging from about 0.24 to 0.36, whereas E. coli showed a considerably lower DPH fluorescence depolarization intensity of ~0.12 at 37 °C, corresponding to the growth temperature of E. coli (Fig. 3). This result indicates that the bilayers composed of the anammox lipids are less fluid than those composed of conventional lipids. The higher ordering of acyl chains in ladderane-containing membranes is likely reflect the dense lipid packing of the anammox membrane lipids (see Fig. 2B). Interestingly, the acyl chain ordering of the total lipid extract of Candidatus “K. stuttgartiensis” is slightly more pronounced than that of the corresponding PC- and PE/PG ladderane fractions [23]. We speculate that bacteriohopanoids might moderately reduce the anammox membrane fluidity, as this rigidifying role for bacteriohopanoids has also been observed in other membrane environments [26,27].

The use of the fluorescent probe TMA-DPH allowed us to monitor the acyl chain ordering near to the periphery of the membrane as this probe anchors with its TMA moiety in the headgroup region of the lipids [39]. As expected, the fluorescence depolarization intensities of TMA-DPH for E. coli membrane lipids decreased at higher temperatures (Fig. 3B, closed diamonds) [42]. However, both Candidatus “K. stuttgartiensis” lipid extracts had relatively higher TMA-DPH depolarization intensities. The temperature dependence of TMA-DPH depolarization intensity for the Candidatus “K. stuttgartiensis”, Candidatus “B. fulgida” and Candidatus “Scalindua” lipid extracts all showed a comparable decline (Fig. 3B open squares, closed and open circles). Whereas the TMA-DPH fluorescence intensity of E. coli was near 0.26 at 37 °C, the anammox lipid extracts had slightly higher TMA-DPH values ranging from about 0.32 to ~0.34 at their biological relevant temperatures (Fig. 3). Hence, the findings above demonstrate that the relatively high acyl chain ordering in the anammox membranes can be assigned primarily to the nature of the cyclobutane rings of the ladderane hydrocarbon chains in agreement with previous modeling results which showed the highest densities in the hydrophobic region around the cyclobutane moieties [19].

4. Conclusions

The current study provides experimental evidence for the high ordering of acyl chains in membranes composed of anammox lipids. The ladderane hydrocarbon chains are likely to play a major role in accomplishing these biophysical features, since we have observed similar properties for mono- and bilayers systems composed of pure ladderane phospholipids [23]. However, despite the high lipid density and high acyl chain ordering, both monolayer and fluorescence depolarization experiments showed that the ladderane-containing membranes are in the fluid phase at biological relevant conditions. Likely, bacteriohopanoids will facilitate in obtaining the optimal equilibrium between membrane fluidity and rigidity necessary for the insertion and function of membrane proteins in these unusual membranes [27,44–46].

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