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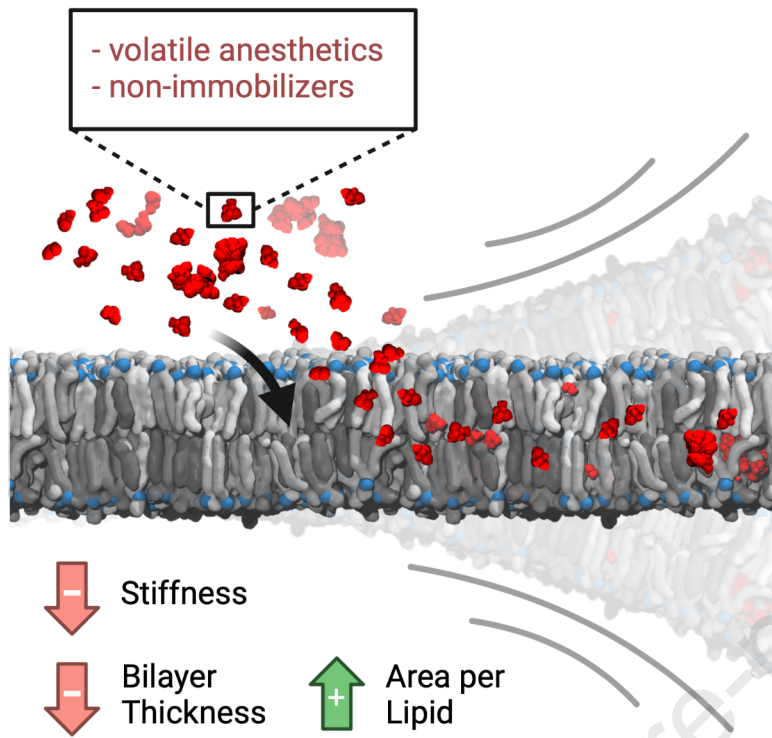
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Alteration of lipid bilayer mechanics by volatile anesthetics: insights from μ -long molecular dynamics simulations

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Summary

Very few drugs in clinical practice feature the chemical diversity, narrow therapeutic window, unique route of administration and reversible cognitive effects of volatile anesthetics. The correlation between their hydrophobicity and their potency and the increasing amount of evidence suggesting that anesthetics exert their action on transmembrane proteins, justifies the investigation of their effects on phospholipid bilayers at the molecular level, given the strong functional and structural link between transmembrane proteins and the surrounding lipid matrix. Molecular dynamics simulations of a model lipid bilayer in the presence of ethylene, desflurane, methoxyflurane and the non-immobilizer 1,2-dichlorohexafluorocyclobutane (also called F6 or 2N) at different concentrations highlight the structural consequences of VA partitioning in the lipid phase, with a decrease of lipid order and bilayer thickness, an increase in overall lipid lateral mobility and area-per-lipid, and a marked reduction in the mechanical stiffness of the membrane, that strongly correlates with the compounds' hydrophobicity.

Introduction

Volatile anesthetics (VAs) are a diverse set of compounds routinely used in medical practice to induce and/or sustain a reversible state of suspended consciousness, analgesia and amnesia. Despite the fact that modern surgery would hardly be imaginable without such compounds, little is known about their mechanism of action, especially at the molecular level. This is partly due to the high chemical and physical diversity of available VAs, which range from single-atom gases such as Xenon, to more complex molecules such as halogen-substituted ethers and even steroids. In the past decades, several different theories of anesthetic action have been proposed with the aim of explaining anesthetic behavior despite this lack of structural similarity, starting from the Meyer-Overton correlation between the lipid solubility of VAs and their clinical potency (in terms of Minimum Alveolar Concentration, MAC). This theory paved the way towards what is known as the lipid theory, which postulates that the main mechanism of action of anesthetics lies in the alteration of the structure

35 of lipid bilayers – in particular cell membranes – in a non-specific fashion (Meyer, 1937). Some shortcomings
36 of this hypothesis, including the lack of any anesthetic effect of other lipid-altering factors, e.g. temperature,
37 steered the interest of research around anesthesia towards finding specific molecular targets – i.e. proteins –
38 which could explain the clinical effects of VAs. Indeed, an increasing amount of evidence points toward ion
39 channels located in the Central Nervous System as relevant targets for anesthetics, starting from the works of
40 Franks and Lieb (Franks and Lieb, 1994, 1984). A detailed review of molecular targets of anesthetics can be
41 found in Campagna et al. (Campagna et al., 2003). Interestingly, despite the increasing evidence of interactions
42 with ion channels, the exact mechanism of action remains unclear, and researchers failed to agree on the most-
43 relevant effectors of anesthesia at clinically relevant VA concentrations. In most works, the two
44 aforementioned approaches to explaining anesthesia – the lipid theory and the receptor theory – are largely
45 regarded as irreconcilable. What seems often overlooked however, is the intimate connection between
46 transmembrane receptors such as ion channels and their surrounding lipid environment, which highlights the
47 duality, rather than the contrast, of the two theories. Indeed, the membrane-spanning portions of integral
48 membrane proteins are known to be affected by the surrounding lipids, so that the conformational
49 characteristics of specific sections of the transmembrane regions may change in response to alterations of the
50 lipid bilayer. It has been shown for example that bilayer thickness can directly influence protein activity (De
51 Planque and Killian, 2003; Mouritsen and Bloom, 1993). Conversely, there is increasing experimental
52 evidence that the presence of proteins embedded in the membrane has profound effects on the latter's
53 stabilization, mediated mainly by hydrophobic interactions (Dumas et al., 1999). At higher scales, the
54 reciprocal interaction of the membrane's lipid environment and embedded proteins has also been shown to be
55 mediated by so-called lipid rafts (Lingwood and Simons, 2010), which are sub- μm domains of spatially
56 organized lipids, typically sphingomyelin and cholesterol (Allen et al., 2006; Levental et al., 2011; Moon et
57 al., 2017).

58 It appears thus entirely reasonable that an interaction of increasingly hydrophobic compounds, such as VAs,
59 within biological membranes might have significant effects on membrane organization and structure, but at
60 the same time this cannot happen without altering the energetic landscape of the interactions between
61 membranes and transmembrane receptors. The idea that small solutes such as VAs bear the potential of altering
62 the mechanics and thermodynamics of the lipid bilayer, with possible consequences on the dynamics of
63 embedded proteins, was already introduced in the work of Cantor (Cantor, 1999), who elegantly discussed the
64 possible relevance of lateral pressure profiles within the lipid bilayer and suggested the mechanistic link
65 between anesthetics, the lipid bilayer and embedded ion channels (Cantor, 1997). Indeed, earlier molecular
66 dynamics simulations by Huang et al. had predicted a possible structural effect of anesthetics within the
67 phospholipid bilayer, in the form of an increased lateral diffusion of lipids and an increase in the overall fluidity
68 of the bilayer (Huang and Bertaccini, 1995). More recently, following earlier speculations suggesting a role of
69 lipid rafts in anesthesia (Gray et al., 2013; Lee, 1976; Lerner, 1997), Pavel et al. demonstrated a membrane-
70 mediated effect of anesthetics, whereby the anesthetic-induced alteration of lipid raft organization is able to
71 modulate the sensitivity of channel proteins to anesthetics (Pavel et al., 2020). In addition to these

72 considerations, the direct effect of anesthetics on transmembrane receptors might be exerted within the
73 transmembrane portion of the receptors rather than on the intracellular or extracellular domains alone and
74 might thus be connected to the ability of compounds to partition inside the membrane and laterally diffuse
75 within the lipid phase prior to interacting directly with cryptic, hydrophobic sites on the target. As a matter of
76 fact, compounds that are more soluble in oil-like media, as is the case for VAs as shown by the Meyer-Overton
77 correlation, tend to partition inside the membrane rather than in aqueous solutions, and vice versa.

78 In the context of investigating the properties of lipid bilayers, a vast literature exists exploring the behavior of
79 model phospholipid membranes in different physical contexts and the structural and functional link between
80 membranes and embedded proteins and peptides. Indeed, it is well-known that the structure of phospholipid
81 bilayers has strong functional consequences (Zhuang et al., 2014). The structural parameters usually reported
82 in both experimental and computational studies include (a) the Area-per-Lipid (APL), which can be calculated
83 from molecular densities or geometrically from the membrane patch surface; (b) the bilayer thickness δ , which
84 is directly related to the APL; (c) deuterium order parameters (S_{CD}), which provide quantitative evidence of
85 lipid chain order and the membrane rigidity resulting from this; (d) direct measures of the mechanical
86 characteristics of the membrane, such as the bilayer bending modulus (K_c). Due to the limitations, both
87 methodological and economical, of experimental settings aimed at investigating such properties for a vast array
88 of model membranes in different physical and biochemical contexts, computational approaches such as
89 molecular dynamics (MD) have proven a valuable tool for exploring and rationalizing the structural
90 characteristics and interaction phenomena within model bilayers at the molecular level. While a great number
91 of computational investigations employed single-component lipid patches (Grasso et al., 2018; Huang and
92 Bertaccini, 1995; Tang and Xu, 2002), mostly of phosphatidylcholines (PCs) or phosphatidylethanolamines
93 (PEs), recent advances in lipid force fields (Dickson et al., 2014, 2012; Jämbeck and Lyubartsev, 2012; Klaua
94 et al., 2010; Pluhackova et al., 2016) and the increasing power of computational resources have paved the way
95 for the simulation of complex, composite bilayers formed by multiple lipid species and varying cholesterol
96 concentrations, both at all-atom (AA) and coarse-grained (CG) resolutions (Ingólfsson et al., 2017 and
97 references therein).

98 With this in mind, the present work focuses on investigating the interaction between volatile anesthetics and a
99 composite model mammalian cell membrane through the use of computational molecular modelling, to explore
100 the effects of VAs on lipid bilayers. With the goal of exploring the effect of a chemically and physically diverse
101 set of hydrophobic compounds spanning a wide range of clinical potencies, we carried out simulations with
102 desflurane (2-(difluoromethoxy)-1,1,1,2-tetrafluoroethane), a fluorinated ether with a MAC of 6% (Riazi and
103 Ibarra Moreno, 2013), methoxyflurane (2,2-dichloro-1,1-difluoro-1-methoxyethane), a potent halogenated
104 methyl ethyl ether with a MAC value of 0.16%, now largely abandoned in the light of its nephrotoxicity
105 (Mazze, 1971), and ethylene, which is only mildly anesthetic with a MAC value of 67% (Miller et al., 1969).
106 Simulations were also carried out with F6 (1,2-dichlorohexafluorocyclobutane), a widely investigated
107 nonimmobilizer which does not follow the Meyer-Overton correlation in that it does not induce complete

108 anesthesia as would be expected from its lipophilicity, but it has been demonstrated to induce amnesia (Eger
109 et al., 2001; Perouansky et al., 2007; Taylor et al., 1999).

110 A graphical summary of the model membrane and of the simulated VAs is reported in Figure 1.

111 **Results**

112 **Potent VAs alter the membrane structure upon partitioning**

113 To quantitatively assess both the quality of the membrane model itself and the effect of volatile anesthetics on
114 overall membrane structure, the geometric Area per Lipid (gAPL) and Bilayer Thickness (δ) were evaluated
115 and are reported for all systems in detail in Table 1. The former is a crucial parameter influencing lipid diffusion
116 profiles, lipid chain order and overall membrane elastic properties. It also represents a metric to assess the
117 reached equilibrium of membrane simulations, along with the closely related bilayer thickness. The control
118 simulation without any ligands yielded an average gAPL of 42.89 \AA^2 (95% CI: $42.83 - 42.95 \text{ \AA}^2$) and an
119 average bilayer thickness of 46.85 \AA (95% CI: $46.81 - 46.89 \text{ \AA}$), and proved consistent both with previous
120 computational studies of membranes with similar lipid composition and comparable cholesterol content (Klähn
121 and Zacharias, 2013; Saeedimazine et al., 2019; Shahane et al., 2019b) and with experimental data on
122 cholesterol-enriched membranes (Maulik and Shipley, 1996), although it is to be noted that bilayer thickness
123 heavily depends on the specific bilayer composition (Li et al., 2012) and experimental data of membranes with
124 the exact lipid composition of the present model is, to the best of our knowledge, not available. Nevertheless,
125 the reduced gAPL and δ values are consistent with the high cholesterol content ($\sim 34\%$) inducing membrane
126 condensation, as demonstrated in earlier literature (Hofsäß et al., 2003; Leftin et al., 2014; Meyer and Smit,
127 2009; TJ, 1978).

128 Figure 2 shows the effect of increasing ligand concentrations on both gAPL and thickness. In the case of
129 ethylene (Figure 2A) no significant effect of ligand concentration on bilayer thickness is observed (from 46.85
130 \AA to 46.52 \AA), with only a mild increase in area per lipid, which reaches 45.16 \AA^2 with 50% ethylene.
131 Desflurane (Figure 2B) and methoxyflurane (Figure 2C) on the other hand induce a marked reduction in bilayer
132 thickness down to 45.67 \AA with 50% desflurane and 45.16 \AA with 50% methoxyflurane, despite the steric
133 hindrance of the high number of ligand molecules partitioned within the membrane. At the same time, these
134 two anesthetics induce a marked increase in gAPL, up to 48.18 \AA^2 and 48.53 \AA^2 for systems with 50%
135 desflurane and methoxyflurane, respectively. Overall, the latter two ligands induce a progressive reduction of
136 membrane thickness, along with a lateral spreading of the lipids on the xy plane, both in a fashion proportional
137 to ligand concentration. This effect is totally absent for ethylene concentrations up to 25%, with only a mild
138 increase in gAPL induced at 50% and no measurable thickness reduction effect. These results are in agreement
139 with earlier computational studies reporting a significant lateral expansion and simultaneous thickness
140 contraction induced in lipid membranes by halothane, another VA, over a wide range of molar fractions (Koubi
141 et al., 2000; Pickholz et al., 2005; Tu et al., 1998). Lastly, simulations with the nonimmobilizer F6 (Figure 2D)
142 highlight a reduction in bilayer thickness (from 46.85 \AA to 45.52 \AA with 50% F6) comparable to the simulations

143 with desflurane and methoxyflurane, whereas the increase in gAPL is more subdued at higher concentrations,
144 reaching at most 46.44 \AA^2 with 50% F6.

145 The increase in gAPL induced by ligand partitioning came alongside an increase in spontaneous water
146 permeation through the membrane, reported as the number of water molecules crossing the bilayer per
147 microsecond in Table 1: throughout the control simulation a water permeation frequency of 16 water
148 molecules/ μs was observed, whereas this frequency increased to up to 285 molecules/ μs and 391 molecules/ μs
149 in the case of 50% desflurane and 50% methoxyflurane, respectively. Conversely, just as for gAPL and bilayer
150 thickness, more subdued differences were observed with ethylene, with at most 112 molecules/ μs at the highest
151 concentration of 50%. Throughout the simulations with F6, a permeation frequency of up to 206 molecules/ μs
152 was observed at 25% simulated fraction, with a slightly lower frequency of 155 molecules/ μs at 50%
153 concentration, consistent with the trends of gAPL and bilayer thickness. Despite the increase in spontaneous
154 permeation frequency with increasing ligand concentrations, no pore formation was observed throughout the
155 whole set of simulations, with no disruption of the overall structural integrity of the bilayer.

156 **Anesthetics and nonimmobilizers are predicted to have specific localization areas within the bilayer**

157 The partitioning of ligands inside the lipid bilayer not only plays a crucial role in ligand-receptor interaction
158 with transmembrane proteins (Vauquelin and Packeu, 2009), but can also significantly alter the bilayer's
159 structural and mechanical properties (Koubi et al., 2000; Tsuchiya and Mizogami, 2013; Tu et al., 1998;
160 Yamamoto et al., 2012). The analysis of the density distributions of the different membrane components and
161 of the ligands along the z coordinate highlights a marked tendency of the four ligands to partition inside the
162 bilayer in specific hydrophobic regions.

163 **Error! Reference source not found.** reports the density distributions for the control simulation (Figure 3A)
164 and the simulations at the highest concentration of ethylene (Figure 3B), desflurane (Figure 3C),
165 methoxyflurane (Figure 3D) and F6 (Figure 3E). The corresponding plots for 12.5% and 25% ligand
166 concentrations, which highlight the same qualitative distribution pattern, are reported in Supplementary Figure
167 S1 and S2, respectively. For desflurane and methoxyflurane, three main areas of localization clearly emerge:
168 the main peak is located at the bilayer center, corresponding to the minimum of lipid tail density. This is
169 consistent with the hydrophobic nature of these compounds, and explains why the massive ligand partitioning
170 inside the membrane does not result in a simultaneous increase in bilayer thickness, as would be expected by
171 the effect of steric hindrance and molecular volume alone. Indeed, due to the low lipid tail density in the
172 membrane core, resulting in less occupied molecular volume, many freely diffusing hydrophobic species are
173 known to temporarily localize in this region, including cholesterol during flip-flop transitions (Bennett et al.,
174 2009). The secondary peaks on the other hand are located near the membrane-water interface, immediately
175 below the glycerol groups. This is in agreement with earlier computational findings by Pohorille et al., who
176 predicted this very area of localization to be involved in the molecular mechanism of anesthesia
177 (Christophe Chipot et al., 1997; Pohorille et al., 1998, 1996). Interactions of volatile anesthetics near the
178 water-lipid interface region have also been reported in the past by Tang and Xu, who employed MD

179 simulations to evaluate the effect of halothane on a gramicidin A channel protein embedded in a DMPC bilayer
180 (Tang and Xu, 2002). While these earlier simulations employed more simplistic membrane models composed
181 of a single lipid type, and investigated remarkably lower timescales, the localization near the water/lipid
182 interface is herein predicted to partially occur also in our composite, cholesterol-enriched membrane model,
183 albeit not as predominantly as the localization at the membrane core. On the contrary, in the case of F6, the
184 localization at the interface appeared comparable to that at the membrane core, resulting in a different density
185 pattern with respect to the other compounds, with no predominant peak at the membrane core. These findings
186 are consistent with the different effects observed for F6 on gAPL with respect to the VAs.

187 Quantitative measures of the tendency of ligands to reside inside the lipid bilayer with respect to the aqueous
188 solvent are reported in literature in the form of either ligand equilibrium partition coefficients (Vauquelin and
189 Packeu, 2009) – usually calculated as the ratio between the ligand concentration in the solvent and the
190 concentration within the membrane – or directly as molar (Herold et al., 2017) or molal (Seeman, 1972) ligand
191 concentration inside the membrane. Whatever the metric, these quantities depend, among others, on the
192 chemical and physical nature of the ligand itself, in particular its hydrophobicity and the presence of
193 hydrophilic moieties, on the temperature of the membrane, i.e. its phase state, and on the membrane cholesterol
194 concentration (Vauquelin and Packeu, 2009).

195 To provide a direct quantitative measurement of the amount of ligand able to dissolve into the membrane,
196 Figure 4 reports the molal concentration reached by the four simulated ligands within the lipid bilayer. Results
197 confirm that the concentration of ligands inside the membrane increases with increasing amounts of simulated
198 ligand molecules, as expected by the physical characteristics of these compounds. One notable exception is
199 represented by F6 (dotted bars in Figure 4), whose concentration inside the bilayer is comparable to that of the
200 other compounds at 12.5% and 25% simulations, reaching up to 0.35 mol/kg (at 25% simulated molar fraction,
201 95% CI 0.33 – 0.37), but showing no further increase in the case of 50% simulations, plateauing at 0.30 mol/kg
202 (95% CI 0.20 – 0.40) and with a considerable amount of ligand aggregating in the water phase without entering
203 the membrane. Also, the analysis of ligand concentration inside the membrane highlights that ethylene (white
204 bars in Figure 4) also partitioned inside the bilayer, albeit at lower rates in the 12.5% and 25% simulations.
205 Conversely, when simulated at 50% molar fraction, the reached concentration (0.535 mol/kg, 95% CI: 0.529-
206 0.541) is comparable to the one of desflurane (patterned bars in Figure 4, 0.621 mol/kg, 95% CI: 0.385-0.856)
207 and methoxyflurane (shaded bars in Figure 4, 0.596 mol/kg, 95% CI: 0.355-0.837). Also, it is worth noting
208 how the considerable number of ligands present at 50% molar fraction leads to greater fluctuations in ligand
209 partitioning in the case of the latter two ligands, but not in the case of ethylene. This is a consequence of the
210 key differences in behavior between ethylene and the other simulated ligands: firstly, ethylene does not form
211 aggregates in the water phase even at 50% concentration as opposed to the other three ligands. Indeed,
212 desflurane and methoxyflurane are observed to enter the membrane in the form of aggregates of up to tens of
213 molecules, while F6 forms aggregates at 50% concentration that are partially unable to enter the bilayer and
214 remain in the water phase throughout the simulations, resulting in lower overall membrane partitioning (see
215 dotted bars in Figure 4). Secondly, ethylene did not show the secondary localization areas below the glycerol

216 groups inside the membrane (see Figure 3), which are instead present for the other three ligands, but rather
217 preferably positions itself at the membrane core, making ligand exchange between the membrane and the water
218 phase less frequent.

219 **VAs and F6 decrease lipid chain order already at 12.5% molar fraction**

220 Deuterium order parameters S_{CD} represent a quantitative measurement of lipid packing and provide insights
221 into the mobility of the hydrophobic chains. Data for POPC from the control simulation without ligands (Figure
222 5, blue lines) is in good agreement with recently published results of compositionally similar, cholesterol- and
223 sphingomyelin-enriched POPC/POPE membranes (Saeedimasine et al., 2019), and confirms the membrane-
224 ordering effect induced by cholesterol. Conversely, in the presence of desflurane (Figure 5B and F) and
225 methoxyflurane (Figure 5C and G), the mechanical consequence of ligand partitioning within the hydrophobic
226 core as well as below the glycerol groups is a reduction in acyl chain order parameters, with a trend proportional
227 to the ligand concentration (Figure 5). This behavior is also present in the simulations with F6, (Figure 5D and
228 h), with the exception of simulations at 50% molar fraction, where the effect of the ligand on lipid chain order
229 is comparable within error to that at 25% concentration. This is coherent with the finding that there are no
230 remarkable differences in the concentration reached by F6 within the bilayer at 25% and 50% simulated molar
231 fraction (see results above), hence a comparable effect on lipid packing is not unexpected.

232 The effect on lipid order is more subdued in the case of ethylene (Figure 5A and D), where the decrease in S_{CD}
233 is particularly evident only at 50% concentration, with only marginal reductions (< 0.01) at lower ligand
234 concentrations. These trends, reported in Figure 5 for POPC, are analogous for the other lipid species included
235 in the employed membrane model (see Supplementary Information), and hint at a membrane-destabilizing
236 effect of ligand partitioning, with consequences on overall bilayer mechanics.

237 **Desflurane, methoxyflurane and F6 decrease membrane bending rigidity in a concentration-dependent** 238 **manner**

239 In the light of the ligands' tendency to partition inside the lipid bilayer, and of the structural consequences
240 thereof observed by the analysis of area per lipid, bilayer thickness and acyl chain order parameters, a more
241 specific quantification of the bilayer's mechanical characteristics was carried out by directly determining the
242 bilayer bending modulus using a previously proposed methodology relying on the analysis of lipid splay.

243 The bilayer bending modulus for the control simulation is 88.80 kT (95% CI: 87.16 – 90.44), and while a direct
244 comparison with other computational and experimental studies is often not trivial due to the differences in
245 membrane composition, temperature and methodology, this result is remarkably consistent with earlier studies
246 of membranes with similar cholesterol content (around 0.3 molar fraction) which induces structural
247 condensation of the lipid phase yielding a considerable increase in membrane stiffness and a shift towards the
248 liquid-ordered phase (Khelashvili et al., 2013; Subczynski et al., 2017). Furthermore, the obtained value for
249 the control simulation agrees with earlier literature reporting experimentally determined stiffness values for

250 plasma membrane vesicles (PMVs, $K_c = 99.75$ kT), which are representative systems of the pure plasma
251 membrane *in vitro* (see (Pontes et al., 2013) and references therein).

252 The trend of reduction of bilayer bending stiffness at increasing anesthetic concentrations is visible in Figure
253 6A. At 12.5% anesthetic concentration, the presence of desflurane, methoxyflurane and F6 leads to a reduction
254 in monolayer bending stiffness by 12.01%, 19.44% and 11.78%, respectively, compared to a mere 2.10%
255 reduction with ethylene. At 25% anesthetic concentration, the bending stiffness is reduced by 20.80% and
256 26.95% by desflurane and methoxyflurane, respectively, and by 20.20 % with F6, compared to a limited 2.48%
257 reduction caused by ethylene. Lastly, in the simulations with 50% anesthetic molar fraction, the bending
258 stiffness is reduced by 28.38% with desflurane and 28.30% by methoxyflurane, while the effect of F6 remains
259 again comparable to the 25% simulation, yielding a reduction of the bilayer bending modulus of 19.98%. Only
260 at this higher concentration does ethylene lead to a noticeable reduction in bending stiffness by 15.32%. This
261 is consistent with order parameter results, which showed a decrease in lipid tail packing in the presence of
262 ethylene only at 50% concentration (see Figure 5).

263 Overall, the trends in reduction in membrane bending stiffness are consistent with the hydrophobicity of these
264 compounds. From the analysis of the data from the 12.5% concentration simulations, which is the closest to
265 clinical concentrations, a linear relationship emerges between the lipophilicity of the ligands – quantified by
266 the octanol/water partition coefficient $\log(K_{o/w})$ – and the reduction in bilayer stiffness (ΔK_c) observed in
267 simulations ($R^2 = 0.95$, Figure 6B). Interestingly this relationship seems to hold true also for F6, which is not
268 an anesthetic but a convulsant with amnesic properties, supporting the hypothesis that the alteration of bilayer
269 mechanics might not be *per se* the mechanistic cause of anesthesia, but might be implicated in some of the
270 effects caused by these compounds, especially at *supra*-clinical concentrations.

271 Discussion

272 In the present work, we employed long all-atom molecular dynamics simulations to assess the structural effects
273 of the volatile anesthetics desflurane, methoxyflurane, ethylene, a low-potency control, and the
274 nonimmobilizer F6 on a model composite lipid bilayer composed of POPC, POPE, POPS, PSM and
275 cholesterol. Anesthetics rapidly partition inside the bilayer, reaching intra-membrane concentrations of
276 approximately 0.6 molal, while F6 is unable to reach concentrations higher than 0.3 molal even when simulated
277 at 50% ligand/lipid molar fraction. Desflurane and methoxyflurane preferentially localize at the membrane
278 core region and immediately below the glycerol groups of the bilayer, with structural consequences on both
279 area per lipid and bilayer thickness. Indeed, the partitioning of ligands causes a contraction in bilayer thickness
280 while at the same time reducing lateral condensation and causing an increase in area per lipid and in
281 spontaneous water permeation, albeit with no pore formation or disruption of overall membrane integrity. The
282 convulsant F6 shows a different localization pattern within the membrane, with preferential interaction below
283 the lipid/water interface and a less prominent residency at the membrane core region, but with similar structural
284 effects with respect to the aforementioned VAs. The structural rearrangement of the membrane has direct
285 consequences on its mechanical properties, as testified by a progressive reduction in lipid hydrocarbon chain

286 packing. The reduced energetic cost of splaying adjacent lipid tails caused by ligand partitioning leads to a
287 reduction in bilayer bending rigidity in a fashion proportional to ligand concentration. These structural effects
288 are not observed for ethylene at a molar ratio of up to 0.25 with respect to the lipids, with only marginal effects
289 at 0.5 molar ratio. Consistently with these considerations, ethylene also constitutes the least hydrophobic
290 among the three studied VAs. It is to be underlined how the non-immobilizer F6 caused a comparable reduction
291 in bilayer bending rigidity despite its lack of potency as a general anesthetic. Hence, also bearing in mind that
292 the simulated concentrations are above the typical concentrations reached in clinical settings, these findings
293 shed light on important aspects of anesthetic-membrane interactions. Firstly, the two potent VAs and the
294 nonimmobilizer F6 studied herein have, even at the smallest studied concentration, the capacity to alter the
295 energetic landscape of a model mammalian lipid bilayer, which results in profound changes of its mechanical
296 characteristics in terms of a marked reduction in bending stiffness and an overall shift towards a liquid-
297 disordered phase, as shown by the reduction in thickness, the increase in APL, the increase in spontaneous
298 water permeation and the reduction of lipid chain order. This effect appears as antagonistic to the role of
299 cholesterol, which induces instead a shift towards the liquid-ordered phase and an overall increase in
300 membrane rigidity (Subczynski et al., 2017). Interestingly, VAs and cholesterol seem to have instead a similar
301 effect in the context of lipid raft microdomains, whose number and size has been recently shown to increase
302 with both anesthetics and cholesterol (Pavel et al., 2020). Given the fundamental role of the cell membrane
303 not only in overall cell mechanics and structural stability, but also in the function of several transmembrane
304 proteins, including important ion channels thought to be directly involved in anesthesia or its side effects
305 (Bertaccini, 2010; Bertaccini et al., 2013; Franks and Lieb, 1994; Herold and Hemmings, 2012; Yamakura et
306 al., 2003), it appears entirely reasonable that bilayer alterations might be, directly or indirectly, involved in
307 some of the effects exerted by VAs and F6, in the same way in which cholesterol is a crucial modulator of
308 membrane mechanics and essential for many membrane functions. As a matter of fact, a hybrid protein/lipid
309 mechanism based on the alteration of the physics of the lipid membrane has been recently proposed by Pavel
310 et al., who described and demonstrated *in vivo* the indirect effect of volatile anesthetics on membrane-
311 embedded channel proteins by means of an alteration of sphingomyelin lipid rafts (Pavel et al., 2020). Despite
312 failing to highlight any effect of VAs on pure DOPC liposomes, employed as a model system of the pure
313 membrane, the research provided further evidence for the key role of membrane biophysics in the molecular
314 mechanisms of anesthetics, and supports the speculation that anesthetics directly interact with the phospholipid
315 membrane, with diversified effects not only at different time and length scales, e.g. on local lipid arrangement
316 vs. on larger-scale lipid microdomains, but also at different concentrations, e.g. clinical vs. *supra*-clinical.
317 Indeed, not only does the alteration of the surrounding lipid environment bear the potential of altering the
318 function of channel proteins, e.g. by modifying the energetic cost of key functional motions, but the rapid
319 partitioning of VAs into the hydrophobic core might also be an essential prerequisite for anesthetics to reach
320 cryptic hydrophobic binding sites of such proteins within regions embedded in the membrane, which are
321 inaccessible from the external water phase. In this sense, the findings reported herein do not clash with earlier
322 evidence of a direct action of anesthetics on ion channels (John Mihic et al., 1997; Mascia et al., 2000), which

323 is still debated to be the final mechanism of action causative of anesthesia. Instead, the computational
324 predictions provide a quantification of the interaction between VAs and the lipid phase and the mechanical
325 alterations of the latter at increasing VA concentrations. This mechanism might thus be necessary, but arguably
326 not sufficient, for a compound to exhibit anesthetic potency, thereby explaining both the Meyer-Overton
327 correlation and outliers thereof such as nonimmobilizers, featuring considerable hydrophobicity but low to no
328 anesthetic potency. This is confirmed by analyzing the effect on bilayer mechanics of the nonimmobilizer F6,
329 which is herein predicted to alter membrane behavior in a similar manner to potent anesthetics. This further
330 suggests that the alteration of the lipid membrane *per se* is unlikely to be the sole mechanistic cause of
331 anesthesia as a whole. Rather, it might be a biophysical mechanism involved in some of the effects that are
332 exerted both by anesthetic agents and nonimmobilizers such as F6, which has been shown e.g. to induce
333 convulsions and amnesia *in vivo*. Also, the direct action on membrane mechanics might rather provide a
334 mechanistic basis to explain the side effects of anesthetics, which arise at higher concentrations and are in
335 common with convulsants (Koblin et al., 1981; Modica et al., 1990). Indeed, given the exacerbation of the
336 alteration of bilayer structure and mechanics predicted herein at such higher concentrations – 0.25 and 0.5
337 molar fractions –, it is reasonable that such a mechanism might be involved in the molecular basis of the side
338 effects of VAs at *supra*-clinical concentrations.

339 **Conclusions**

340 The molecular mechanisms of general anesthesia are to this day an unsolved medical puzzle. While recent
341 literature generally considers transmembrane proteins as the main functional target of volatile anesthetics, the
342 Meyer-Overton correlation clearly hints at the ability of these compounds to interact with the lipid bilayer of
343 cell membranes, even if the final functional action is not exerted directly on the membrane itself. Long
344 molecular dynamics simulations of the three VAs ethylene, desflurane and methoxyflurane and of the
345 nonimmobilizer F6 confirm the strong tendency of these ligands to partition within the hydrophobic
346 environment of a model membrane, and allowed to quantify the structural effects this determines: a reduction
347 in bilayer thickness, a decrease in lipid chain order and a reduction of membrane stiffness, with a trend
348 proportional to the amount of partitioned ligands. Given the strong correlation observed between the
349 compounds' lipophilicity and the reduction in the membrane bending modulus caused by their inclusion within
350 the membrane, it appears that the phospholipid membrane might be a key component in determining some of
351 the effects of anesthetics on channel proteins, by altering their structural and mechanical characteristics in the
352 presence of VAs with possible consequences on embedded protein function and on the intracellular link
353 between the membrane and the cytoskeleton. Moreover, the remarkable tendency to dissolve in the lipid phase
354 followed by lateral diffusion within the membrane, might be an essential step to reach key functional
355 hydrophobic binding pockets in transmembrane proteins, which would be inaccessible from the aqueous
356 solvent, such as some transmembrane domains which have been shown to bind anesthetics (Mascia et al.,
357 2000). These considerations are well in line not only with the strong relationship between potency and
358 hydrophobicity, but also with the most recent theories indicating ion channels as ultimate targets for general
359 anesthetics, and pointing at the lipid environment of the membrane as a first transducer of anesthetic action

360 (Pavel et al., 2020). At the same time, the functional distinction between general anesthetics and compounds
361 without any anesthetic effect but high lipophilicity, such as F6, might involve processes and molecular players
362 downstream of the interaction with the membrane. This concept highlights how the lipid-centered and the
363 protein-centered theories of anesthetic action are not, in fact, irreconcilable, but might rather be two aspects of
364 a composite mechanism, which sees the interaction with the lipid membrane as a necessary but perhaps not
365 sufficient condition. A more thorough analysis of how this occurs and to which extent, especially as to where
366 the discrimination between general anesthetics and non-anesthetic Meyer-Overton outliers takes place, as well
367 as of the effect of the membrane alteration on the cytoskeleton linked at the intracellular interface, certainly
368 warrants further computational and experimental investigations, and seems well worth pursuing further.

369 **Limitations of the Study**

- 370 • While the membrane model employed in this work is a multi-component membrane which accounts
371 for the major lipid constituents of mammalian cell membranes, it still represents a simplified
372 representation, especially in the context of neural membranes which include several types of different
373 phosphatidylcholines, phosphatidylethanolamines, sphingomyelins, phosphatidylserines, glycolipids,
374 cerebroside and phosphatidylinositols, just to name a few. Building increasingly realistic models of
375 cellular membranes is an active topic of research and requires major computational efforts, often
376 demanding the use of coarse-grained modelling and extended parameter validations to accurately
377 capture the physical and chemical characteristics of the simulated species.
- 378 • The present work focuses on the effects of three different VAs of different chemical structure and
379 spanning a wide range of clinical potencies. However, several other VAs exist that were not included
380 in the present work, and are very well worth investigating in further studies. Also, we herein included
381 a compound that would be expected to have high potency as an anesthetic based on its hydrophobicity
382 and structural similarity to actual VAs, but actually lacks any anesthetic effect, namely F6. Given the
383 comparable effect of this compound on pure membrane mechanics, further investigations are needed
384 to explore downstream events (e.g. the interaction with transmembrane proteins) that would ultimately
385 set apart potent anesthetics from hydrophobic nonimmobilizers and other similar negative controls.

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388 computing resources and support.

389 **Author Contributions**

390 Conceptualization MD, MC and JAT; Methodology, EAZ; Formal analysis, EAZ and MAD; Investigation,
391 EAZ; Writing – Original Draft, EAZ; Writing – Review & Editing, all authors; Visualization, EAZ;
392 Supervision, MD, MC and JAT;

393 **Declaration of Interests**

394 The authors declare no competing interests.

395

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396 **Main Figure Titles and Legends**

397 Figure 1. **Visual overview of the simulated systems.** Left: visualization of the three simulated VAs (1)
 398 ethylene, (2) desflurane, (3) methoxyflurane, and the nonimmobilizer (4) F6. Right: visualization of the
 399 membrane system in its explicit TIP3P water box with ions and ligands omitted for clarity. P atoms highlighted
 400 in green, POPC lipids in pink, Cholesterol in light grey, POPE in purple, POPS in dark green, PSM in bright
 401 green. Length scale in Ångstrom reported below for reference, centered at the membrane core region.

402 Figure 2. **Distribution of the bilayer thickness (δ) and geometric area per lipid (gAPL).** Control simulation
 403 vs. ethylene (A), desflurane (B), methoxyflurane (C) and F6 (D) at increasing concentrations. Marginal axes
 404 show the individual data distributions collected in the last 750 ns of the simulations. Control simulation without
 405 anesthetics shown in grey, 12.5% concentration in red, 25% in blue and 50% in green.

406 Figure 3. **Density distributions of lipid headgroups (blue), glycerol backbone (red), lipid tails (green) and**
 407 **anesthetics (black).** (A) control simulation, (B) with 50% ethylene, (C) with 50% desflurane, (D) with 50%
 408 methoxyflurane and (E) with 50% F6. Shaded colors represent 95% confidence intervals.

409 Figure 4. **Molal concentration of the four simulated ligands inside the bilayer.** Concentrations calculated
 410 as number of moles of anesthetic per kilogram of membrane. Error bars on the histograms represent the error
 411 estimate after block averaging.

412 Figure 5. **Lipid tail order parameters for POPC sn1 (top row) and sn2 (bottom row) chains, with different**
 413 **ligands.** (A) and (E) ethylene; (B) and (F) desflurane; (C) and (G) methoxyflurane; (D) and (H) F6. For the
 414 corresponding data for POPE, POPS and PSM see Supplementary Information. Shaded intervals correspond
 415 to 95% confidence intervals.

416 Figure 6. **Effect of ligands on membrane stiffness.** (A) Bilayer bending modulus in kT units for the different
 417 systems. Control system represented as 0% ligand concentration. Error bars represent the error estimate after
 418 block averaging, omitted when smaller than the datapoint for clarity. (B) Correlation between anesthetic
 419 lipophilicity (in terms of the logarithm of the octanol/water partition coefficient, $\log(K_{o/w})$) and the decrease in
 420 membrane bending modulus in kT units, ΔK_c . Error bars represent the error estimate after block averaging,
 421 omitted when smaller than the datapoint for clarity.

422 **Main Tables and corresponding titles and legends**

423 Table 1. Average geometrical Area per lipid, bilayer thickness and frequency of water permeation for all
 424 simulated systems. 95% confidence intervals are reported in square brackets for block-averaged quantities.

System	gAPL [\AA^2]	Bilayer Thickness (δ) [\AA]	Water permeation frequency [H ₂ O/ μ s]
C	42.89 [42.83 – 42.95]	46.85 [46.81 – 46.89]	16
E12.5	43.40 [43.36 – 43.44]	46.77 [46.71 – 46.83]	40
E25	43.90 [43.80 – 44.00]	46.74 [46.68 – 46.80]	37
E50	45.16 [45.12 – 45.20]	46.52 [46.46 – 46.58]	112
D12.5	44.93 [44.81 – 45.05]	46.17 [46.03 – 46.31]	75
D25	46.47 [46.41 – 46.53]	45.91 [45.89 – 45.93]	163
D50	48.18 [46.49 – 49.87]	45.67 [45.45 – 45.89]	285
M12.5	45.12 [45.08 – 45.16]	45.88 [45.87 – 45.89]	64
M25	46.86 [46.83 – 46.89]	45.40 [45.38 – 45.42]	160
M50	48.53 [46.92 – 50.14]	45.16 [44.89 – 45.43]	391
F6 12.5	44.95 [44.81 – 45.09]	45.97 [45.89 – 46.05]	93

F6 25	46.85 [46.65 – 47.05]	45.39 [45.28 – 45.50]	206
F6 50	46.44 [45.30 – 47.58]	45.52 [45.09 – 45.95]	155

425

426 Table 2. Bilayer bending modulus K_c in kT units and reduction of K_c with respect to control simulation, ΔK_c ,
 427 for each simulated system. 95% confidence intervals reported in square brackets.

System	K_c [kT]	ΔK_c
C	88.80 [87.16 – 90.44]	--
E12.5	86.94 [84.42 – 89.44]	1.86
E25	86.60 [85.14 – 88.06]	2.20
E50	75.20 [73.98 – 76.42]	13.60
D12.5	78.14 [76.94 – 79.32]	10.66
D25	70.34 [68.82 – 71.84]	18.46
D50	63.60 [59.06 – 68.14]	25.20
M12.5	71.54 [69.92 – 73.14]	17.26
M25	64.86 [64.58 – 65.14]	23.94
M50	63.66 [60.04 – 67.30]	25.14
F6 12.5	78.33 [76.85 – 79.82]	10.47
F6 25	70.87 [69.94 – 71.80]	17.93
F6 50	71.07 [66.61 – 75.52]	17.73

428

429 **STAR Methods**430 **Resource Availability**431 **Lead Contact**

432 Further information and requests for resources should be directed to Lead Contact, prof. Marco A. Deriu
 433 (marco.deri@polito.it).

434 **Materials Availability**

435 This study did not generate any novel reagents and all materials used in this study are reported either the
 436 main text or in the Supplemental Information.

437 **Data and Code Availability**

- 438
- All data reported in this paper will be shared by the lead contact upon request.
 - This paper does not report original code.
 - Any additional information required to reanalyze the data reported in this paper is available from the
 441 lead contact upon request.

442 **Method Details**443 **System Setup**

444 To overcome the intrinsic simplifications of single-component bilayers, and to account for the presence of
 445 cholesterol, which has a well-documented ordering effect on membranes (Róg et al., 2009) with profound
 446 consequences on their mechanical properties (Leftin et al., 2014; Needham and Nunn, 1990), we chose to
 447 simulate a composite asymmetrical lipid patch representative of the mammalian cell membrane, as first
 448 described by Zachowski (1993) (Zachowski, 1993) and employed in computational studies by Klähn and
 449 Zacharias (Klähn and Zacharias, 2013) and, more recently, Shahane et al. (Shahane et al., 2019b), composed
 450 of POPC (1,2-palmitoyl-oleoyl-sn-glycero-3-phosphocholine), POPE (1-Palmitoyl-2-oleoyl-sn-glycero-3-
 451 phosphoethanolamine), POPS (1,2-palmitoyl-oleoyl-sn-glycero-3-phosphoserine), PSM
 452 (palmitoylsphingomyelin) and Cholesterol (CHOL). The detailed amounts of the lipids in the two leaflets are
 453 reported in the following table:

454 *Table. Number of different lipid molecules in the two leaflets of the model mammalian membrane.*

Lipid	Inner Leaflet	Outer Leaflet	Total
POPC	40	106	146
POPE	132	34	166
POPS	82	8	90
PSM	10	116	126
CHOL	136	136	272
Total	400	400	800

455

456 Bilayer systems were assembled using the Membrane Builder (Jo et al., 2009, 2007; Wu et al., 2014) tool of
 457 CHARMM-GUI (Jo et al., 2008), with a fixed number of 50 TIP3P waters per lipid to ensure adequate lipid
 458 hydration even at higher ligand concentrations, and a physiological NaCl concentration of 0.15M. In addition
 459 to the control simulation without any anesthetic, different systems were set up by randomly inserting
 460 desflurane, methoxyflurane, ethylene and F6 (1,2-Dichlorohexafluorocyclobutane) respectively in the
 461 surrounding aqueous solvent at 12.5%, 25% and 50% anesthetic/lipid molar ratios, for a total of 10 simulated
 462 systems, using the *insert-molecules* tool of GROMACS 2020.4 (Abraham et al., 2015). The higher
 463 concentrations (25%, 50%), while not intended to be representative of clinical concentrations, were included
 464 to enhance the sampling of the lipid-anesthetic interaction and to accelerate ligand partitioning, as seen in
 465 previously published studies (Arvayo-Zatarain et al., 2019; Koubi et al., 2000; Mojumdar and Lyubartsev,
 466 2010). The 12.5% concentration on the other hand is more representative of clinical scenarios, with the molar
 467 ratio of e.g. Halothane at MAC being in the range of 5% (McCarthy et al., 2017) to 14% (Franks and Lieb,
 468 1979). The detailed composition of each simulated system is reported in the table below.

469 *Table. Components of each simulation system*

System	Short Name	Lipids	Water molecules	Cl ⁻ ions	Na ⁺ ions	VA molecules	Total Molecules
Control	C	800	40000	96	186	0	41082
Ethylene 12.5%	E12.5	800	39754	96	186	100	40936

Ethylene 25%	E25	800	39527	96	186	200	40809
Ethylene 50%	E50	800	39062	96	186	400	40544
Desflurane 12.5%	D12.5	800	39414	96	186	100	40596
Desflurane 25%	D25	800	38892	96	186	200	40174
Desflurane 50%	D50	800	37749	96	186	400	39231
Methoxyflurane 12.5%	M12.5	800	39210	96	186	100	40392
Methoxyflurane 25%	M25	800	38484	96	186	200	39766
Methoxyflurane 50%	M50	800	36755	96	186	400	38237
F6 12.5%	F6 12.5	800	39149	96	186	100	40331
F6 25%	F6 25	800	38381	96	186	200	39663
F6 50%	F6 50	800	36486	96	186	400	37968

470

471 *Simulation Protocol*

472 Simulations were carried out in GROMACS 2020.4 (Abraham et al., 2015) using the CHARMM36 force field
473 (Klauda et al., 2010), which is well-validated for membrane simulations over a wide range of lipid
474 compositions (Zhuang et al., 2014), according to the following protocol: after an initial 5000-step energy
475 minimization, systems were equilibrated stepwise with gradually decreasing harmonic restraints (from 1000
476 to $0 \text{ kJ} \times \text{mol}^{-1} \times \text{nm}^{-1}$), first in the NVT ensemble for 250 ps with a conservative timestep of 1 fs, using the
477 Berendsen thermostat with a coupling time constant of 1 ps and a reference temperature of 303.15K, which is
478 above the phase-transition temperature for the studied lipid mixture, and subsequently in the NPT ensemble
479 for 125 ps with the same 1 fs timestep, followed by a further simulation of 375 ps with a 2 fs timestep, using
480 the Berendsen thermostat with the same parameters as before and the Berendsen barostat (Berendsen et al.,
481 1984) with semi-isotropic pressure coupling at 1 atm with a coupling time constant of 5 ps. Overall, systems
482 underwent 750 ps of equilibration, and were subsequently simulated for production runs for a total of 1 μs each
483 in the NPT ensemble, using the Nosé-Hoover thermostat (Nosé, 1998) with a time constant of 1 ps and a
484 reference temperature of 303.15K, and the Parrinello-Rahman barostat (Parrinello and Rahman, 1981), with
485 semi-isotropic pressure coupling at 1 atm with a time constant of 5 ps. Bonds involving hydrogens were
486 constrained using the LINCS algorithm (Hess et al., 1997), while the Particle Mesh Ewald (PME) algorithm
487 (Ewald, 1921) was used for electrostatics, with a cutoff radius of 1.2 nm, and a cutoff of 1.2 nm was used for
488 Van der Waals interactions, with a force-switch modifier from 1.0 to 1.2 nm. The first 250 ns of the production
489 MD runs were regarded as additional structural equilibration, while the remaining 750 ns were used for the
490 subsequent analyses described below, in line with previous literature regarding the computational simulation
491 of biological lipid bilayers (Shahane et al., 2019a, 2019b). Properties were sampled every 200 ps, unless
492 otherwise specified. Molecular visualizations were generated using the VMD software package (Humphrey et
493 al., 1996).

494 *Structural Analyses*

495 Geometric Area-per-Lipid (gAPL), Bilayer Thickness (δ) and water permeation were calculated using the
496 MDAnalysis (Michaud-Agrawal et al., 2011) library for Python (Van Rossum and Drake, 2009). Briefly, the
497 gAPL was calculated as the xy area of the simulation box divided by the number of lipids in each membrane

498 leaflet (N=400) and is reported in Å². To calculate the bilayer thickness, the position of all P atoms of each
 499 leaflet was extracted and their average z coordinate calculated for each leaflet. Bilayer thickness was calculated
 500 as the distance between the avg. z coordinates the P atom cloud. Water permeation events were calculated by
 501 tracking individual water molecules throughout the simulation. Density distribution profiles along the z
 502 coordinate were calculated using the *gmx density* tool. Acyl chain deuterium order parameters, S_{CD}, for the sn1
 503 and sn2 chains of each lipid were calculated to directly quantify structural effects on the packing of the
 504 membranes' hydrophobic core. Order parameters were calculated following equation (1), using the *gmx order*
 505 tool:

$$506 \quad S_{CD} = \frac{1}{2} \langle 3 \cos^2 \theta - 1 \rangle \quad (1)$$

507 where θ is defined as the angle between the bilayer normal and the vector C-D between the given carbon atom
 508 and the bound hydrogen atom, as sampled from the equilibrium MD simulations (Piggot et al., 2017).
 509 Unsaturated lipid chains were accounted for following the methodology described in Pluhackova et al.
 510 (Pluhackova et al., 2016).

511 To quantify the tendency of ligands to partition inside the bilayer, which can bear profound consequences on
 512 protein-ligand interaction affinity and kinetics on transmembrane protein targets, the ligand molal
 513 concentration inside the lipid bilayer was calculated as follows. MDAnalysis was used to extract the number
 514 of ligand molecules whose center-of-mass z coordinate lied between the two P-atom point clouds, i.e. between
 515 the two layers delimiting each leaflet's boundary. These ligands were regarded as being embedded inside the
 516 membrane. The remainder of the ligands was considered outside of the membrane. The molality of the
 517 anesthetics inside the membrane was calculated as number of moles of embedded ligands divided by the total
 518 weight of the membrane in kg.

519 To calculate the bilayer bending modulus, K_c, for each simulated system, the methodology proposed by
 520 Khelashvili and colleagues (Khelashvili et al., 2013) was employed, leveraging on the relationship between
 521 the splay modulus, χ_{12} , and the macroscopic bending modulus: in this approach, an improved ability of adjacent
 522 lipids to change the reciprocal orientation of their hydrophobic tails with respect to the local membrane normal,
 523 which is quantified by their splay angle (α), is associated to a decreased membrane bending rigidity. Briefly,
 524 this approach first calculates the Potential of Mean Force (PMF) of the distribution of splay angles sampled
 525 during equilibrium MD simulations, normalized with respect to the probability distribution of a non-interacting
 526 particle system (Khelashvili et al., 2010), denoted here P₀(α), as shown in equation (2):

$$527 \quad PMF(\alpha) = -k_B T \ln \frac{P(\alpha)}{P_0(\alpha)} \quad (2)$$

528 where T represents the system temperature and k_B the Boltzmann constant. The overall splay modulus, which
 529 is linked to the bilayer bending modulus as:

$$530 \quad K_c = 2k_m = 2\chi_{12} \quad (3)$$

531 can be calculated by means of a quadratic fit of the PMF obtained from Eq. (2) (Fošnarič et al., 2006; Watson
532 et al., 2011).

533 In the present work, we employed the python implementation previously demonstrated by Johner et al. (Johner
534 et al., 2016) to first extend the trajectories to neighboring periodic images, followed by wrapping the trajectory
535 around the central unit cell and re-aligning. Finally, the provided python modules were used to calculate the
536 tilt and splay angle distributions for all lipids and subsequently extract the membrane elastic properties of
537 interest following the above-mentioned methodology. We refer to (Johner et al., 2016) and references therein
538 for a more complete theoretical background of the methodology and details on the python implementation
539 relying on the OpenStructure (Biasini et al., 2013) toolkit.

540 **Quantification and statistical Analysis**

541 For a more accurate estimation of the error of the sampled properties (Flyvbjerg and Petersen, 1989; Grossfield
542 et al., 2019; Grossfield and Zuckerman, 2009; Nicholls, 2014), the equilibrium part of the MD simulations (i.e.
543 the last 750 ns) was further divided into 250-ns long trajectory blocks, in line with previous literature reporting
544 findings in μ s-long MD simulations of complex lipid membranes (Shahane et al., 2019a, 2019b). First, the
545 block average of each structural property was calculated for each block as the arithmetic mean of the data
546 points of the given property p within the block:

$$547 \quad \bar{\mu}_j = \frac{1}{N_b} \sum_{i=1}^{N_b} p_i \quad (4)$$

548 where $\bar{\mu}_j$ denotes the mean within the j -th block of property p and N_b is the number of samples composing the
549 j -th block. The final estimate of the ensemble average $\langle \mu \rangle$ of the given property p is given by the arithmetic
550 mean of the block averages:

$$551 \quad \langle \mu \rangle = \frac{1}{n} \sum_{j=1}^n \bar{\mu}_j \quad (5)$$

552 where n is the total number of blocks. Then, the experimental standard deviation of the mean, $\bar{\sigma}_\mu$, of each
553 property was calculated as:

$$554 \quad \bar{\sigma}_\mu = \sqrt{\frac{\sum_{j=1}^n (\bar{\mu}_j - \langle \mu \rangle)^2}{n - 1}} \quad (6)$$

555 where $\bar{\mu}_j$ is the arithmetic mean of a given property over the j -th block and n is the number of blocks. Finally,
556 the estimate of the standard deviation is given by:

$$557 \quad \sigma_\mu = \frac{\bar{\sigma}_\mu}{n} \quad (7)$$

558 This quantity is the reported standard deviation, represented as error bars on the plots, and was also used to
559 calculate 95% confidence intervals which are reported throughout the text and in shaded colors on the plots,
560 unless where explicitly specified.

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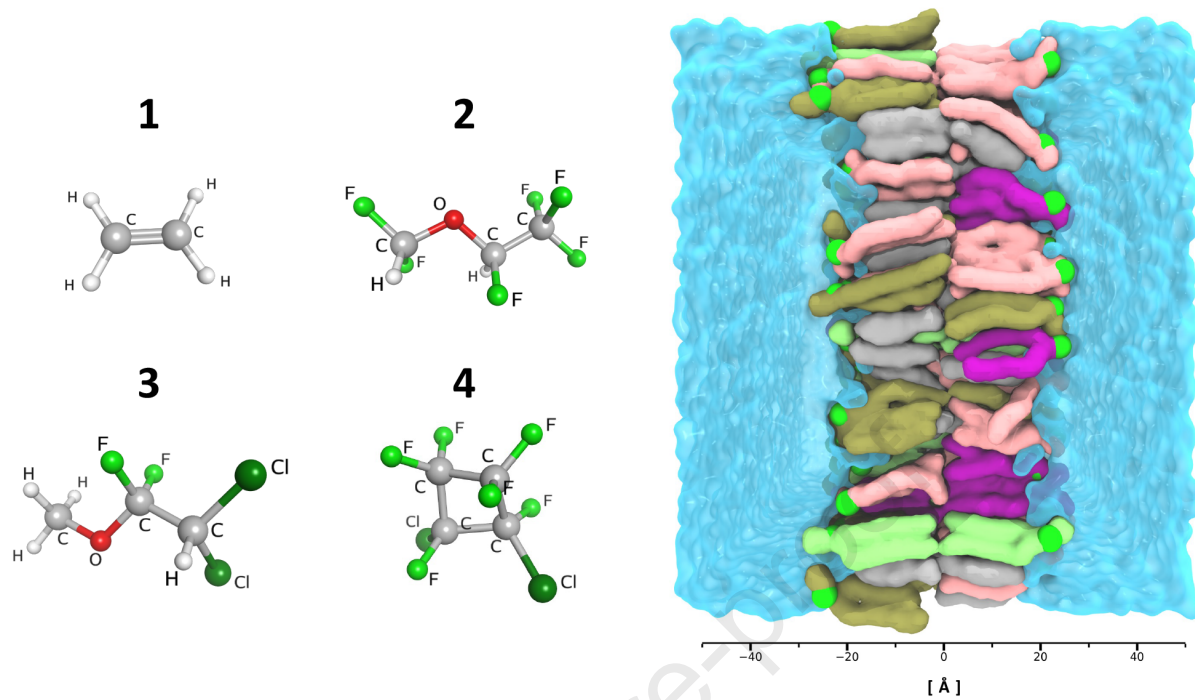
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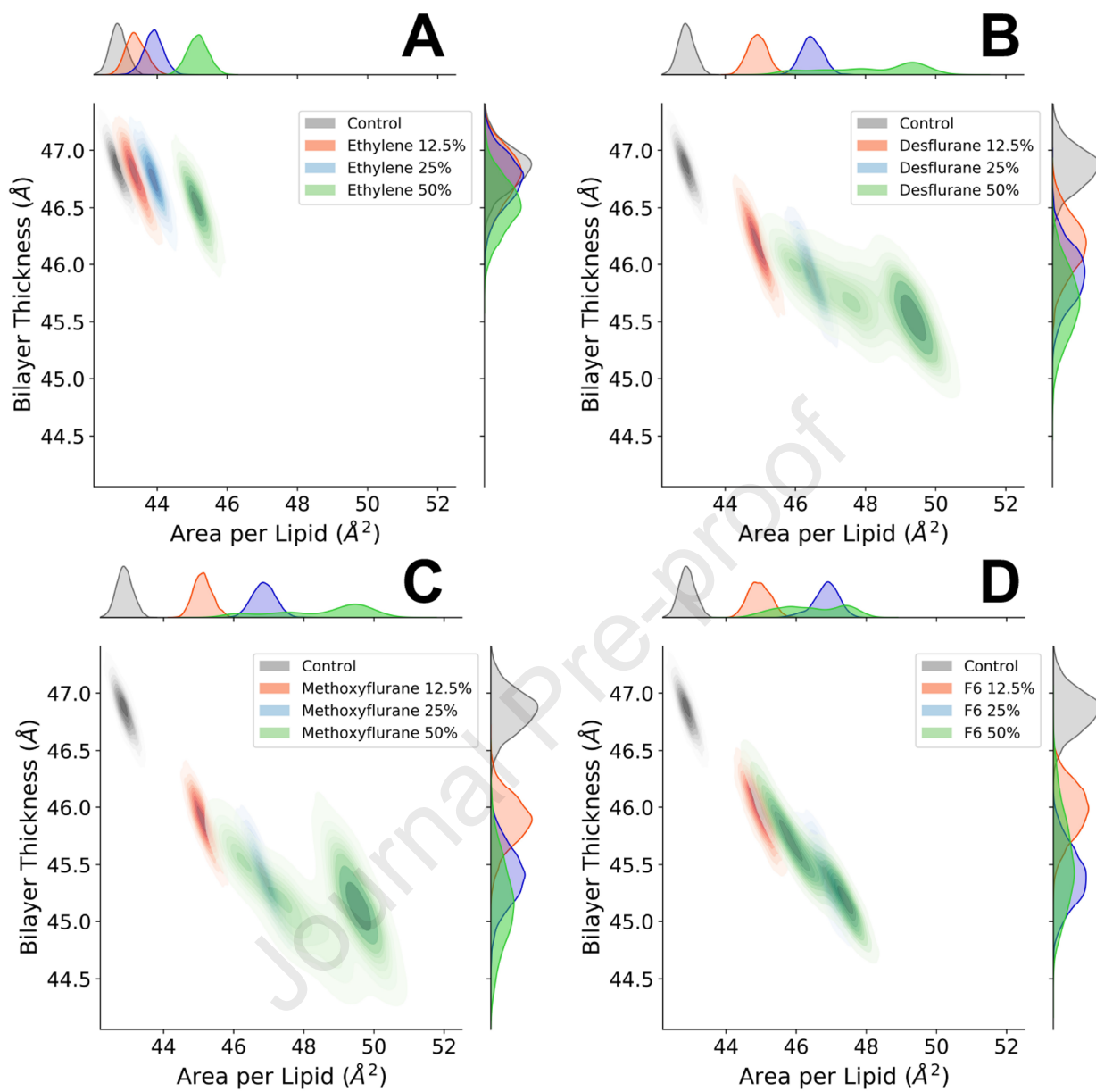
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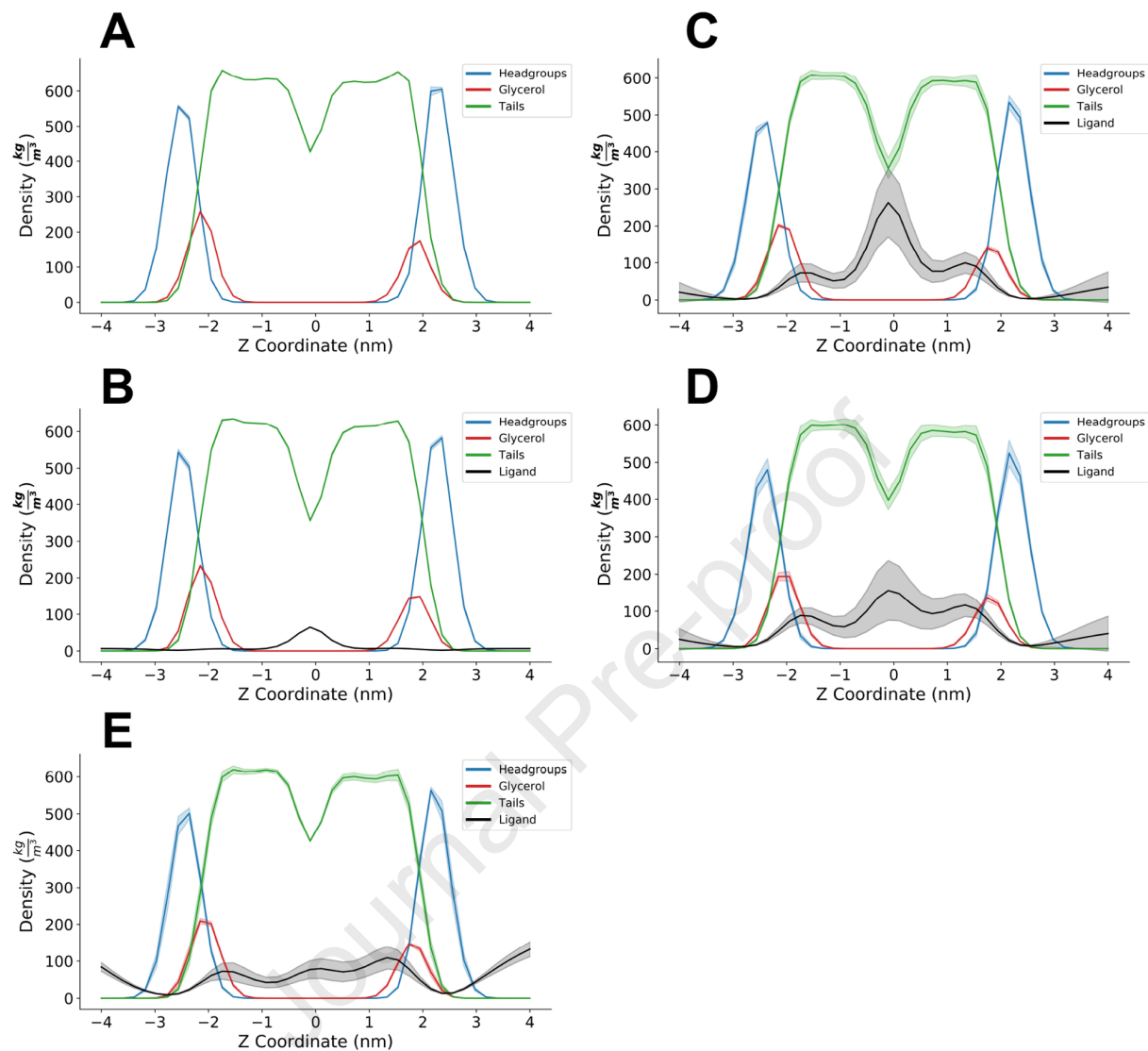
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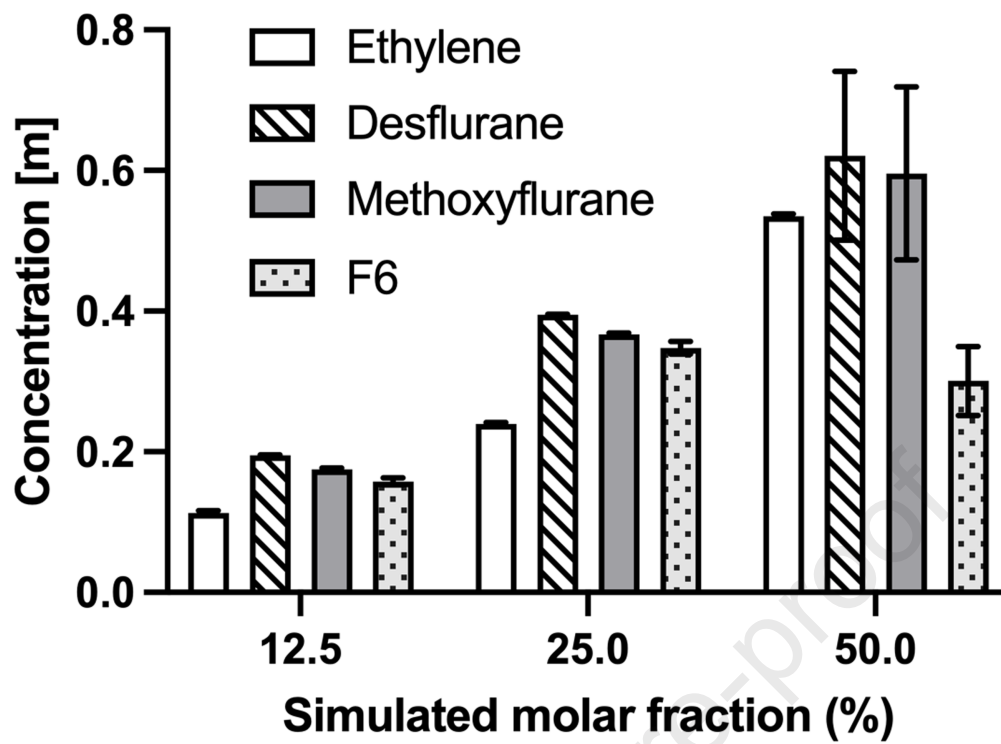
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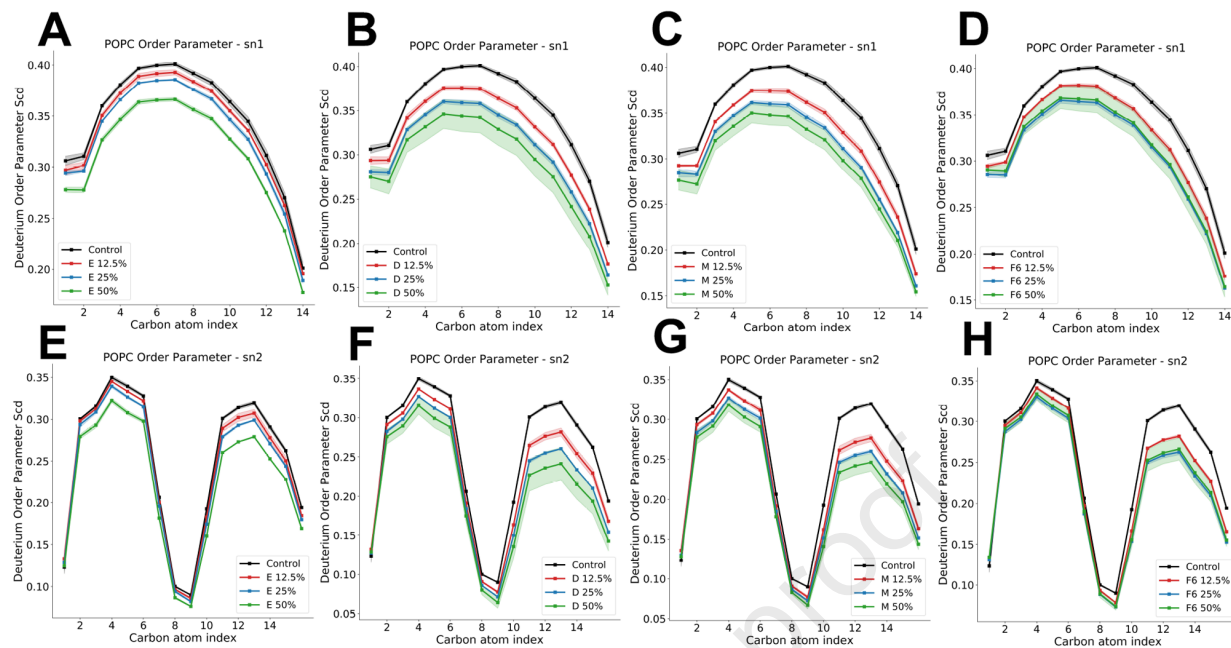
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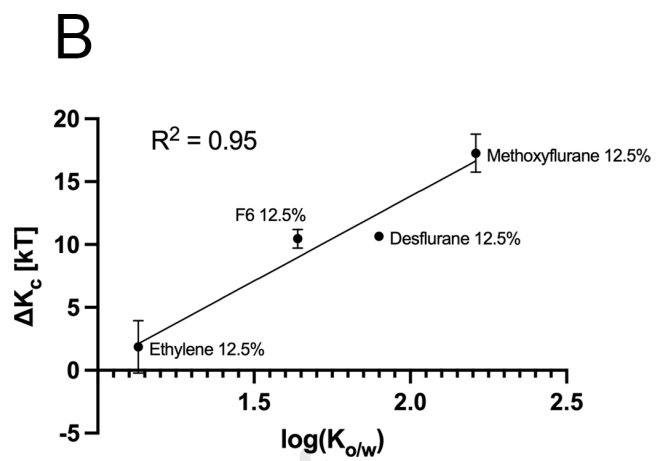
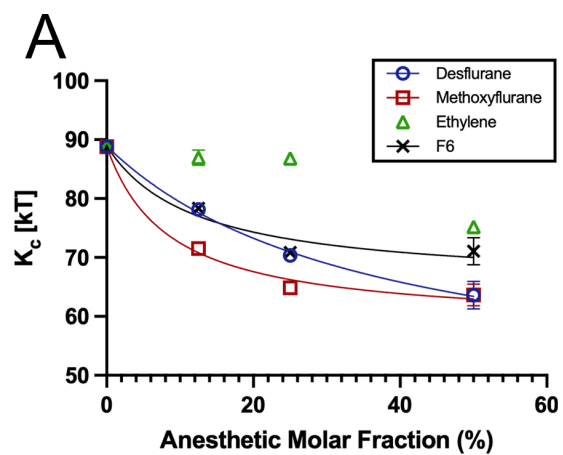












Highlights

- Molecular simulations of lipid bilayer interaction with volatile anesthetics
- Comparison of volatile anesthetics' and nonimmobilizers' effects on lipid bilayers
- Ligand-dependent partitioning of the compounds in the lipid phase
- Effects on bilayer thickness, stiffness and lipid order upon ligand partitioning

Journal Pre-proof

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
GROMACS version 2020.4	(Abraham et al., 2015)	https://manual.gromacs.org/
CHARMM-GUI Membrane Builder	(Jo et al., 2009)	https://charmm-gui.org/?doc=input
MDAnalysis version 2.0.0	Michaud-Agrawal et al., 2011)	https://www.mdanalysis.org
Python version 3.7	(Van Rossum and Drake, 2009)	https://www.python.org/downloads/release/python-370/
OpenStructure version 2.3	(Biasini et al., 2013)	https://openstructure.org
VMD Version 1.9.3	(Humphrey et al., 1996)	https://www.ks.uiuc.edu/Research/vmd/
Methodology for determining elastic properties of lipid assemblies from MD simulations	(Johner et al., 2016)	http://dx.doi.org/10.1186/s12859-016-1003-z