Role of Ionizable Lipids in SARS-CoV-2 Vaccines As Revealed by Molecular Dynamics Simulations: From Membrane Structure to Interaction with mRNA Fragments

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1. Simulation Setup

Table S1: Composition of simulated lipid mixtures with ionizable lipids (IL, ALC-0315 for mixture P inspired by Pfizer&BioNTech, SM-102 for mixture M inspired by Moderna), PEGylated lipids (PEG-L, ALC-0159 for mixture P, DMG-PEG 2000 for mixture M) in lipid molar and mass ratios. PEG chains are not considered for calculation of mass ratios.

|       | Molar % | Mass% mixture P | Mass% mixture M |
|-------|---------|-----------------|-----------------|
| IL    | 50      | 62              | 60              |
| PEG-L | 1.5     | 1               | 1               |
| CHL   | 38.5    | 24              | 25              |
| DSPC  | 10      | 13              | 13              |
Table S2: Performed simulations for lipid systems, their composition and simulation lengths in isotropic and following anisotropic conditions with resulting lipid organization as intact bilayer (IB), porous bilayer (PB), cubic (C) or bulk phase (B).

| Lipids | Solvent | Simulation length (ns) | Anisotropic | Final phase |
|--------|---------|------------------------|-------------|-------------|
| SM-102 (+1) | 72 | 6645 | 19 | 91 | 500 |
| SM-102 (0) | 72 | 6456 | 18 | 18 | 500 |
| ALC-0315 (+1) | 72 | 6556 | 18 | 90 | 500 |
| ALC-0315 (0) | 72 | 6864 | 19 | 19 | 500 |
| DSPC | 72 | 6142 | 17 | 17 | 100 |
| CHL | 200 | 6622 | 18 | 18 | 50 |
| 4CB-M (+1)* | 100 | 20 | 76 | 18799 | 53 | 153 | 500 |
| 4CB-M (0) | 100 | 20 | 76 | 18760 | 52 | 52 | 500 |
| 4CB-P (+1)* | 100 | 20 | 76 | 18193 | 51 | 151 | 500 |
| 4CB-P (0) | 100 | 20 | 76 | 18760 | 52 | 52 | 500 |
| Mixture M (+1) – 400 lipids | 200 | 6 | 40 | 154 | 19888 | 56 | 256 | 100 | C |
| Mixture P (+1) – 400 lipids | 200 | 6 | 40 | 154 | 19888 | 56 | 256 | 100 | C |
| Mixture M (+1) – 100 lipids | 50 | 2 | 10 | 38 | 4972 | 14 | 64 | 250 | IB |
| Mixture M (+1) – no PEG | 50 | 10 | 38 | 4872 | 14 | 64 | 250 | IB |
| Mixture M (0) – 100 lipids | 50 | 2 | 10 | 38 | 4972 | 14 | 14 | 250 | IB |
| Mixture P (+1) – 100 lipids | 50 | 2 | 10 | 38 | 4972 | 14 | 64 | 250 | PB |
| Mixture P (+1) – no PEG | 50 | 10 | 38 | 4872 | 14 | 64 | 250 | C |
| Mixture P (0) – 100 lipids | 50 | 2 | 10 | 38 | 4972 | 14 | 14 | 250 | IB |
| SM-102 (+1) | 200 | 9944 | 28 | 228 | 500 | C |
| SM-102 (0) | 200 | 9944 | 28 | 28 | 500 | B |
| ALC-0315 (+1) | 200 | 9944 | 28 | 228 | 500 | C |
| ALC-0315 (0) | 200 | 9944 | 28 | 28 | 500 | B |
| DSPC | 200 | 9944 | 28 | 28 | 500 | PB |
| DSPC/CHL | 40 | 160 | 9944 | 28 | 28 | 500 | IB |

* other hydration levels: 5, 25, 50 water per lipid (WPL) simulations for 100 ns
Table S3: Performed simulations for lipid systems with RNA, their composition and simulation lengths in isotropic and following anisotropic conditions with resulting lipid organization as intact bilayer (IB), porous bilayer (PB), cubic (C) or nanodiscs (ND).

| WPL | RNA | Lipids | Solvent | Simulation length (ns) | Final phase |
|-----|-----|--------|---------|------------------------|------------|
|     |     |        |         |                        |            |
|     |     |        |         |                        |            |
|     |     |        |         |                        |            |
|     |     |        |         |                        |            |

- **Mixture M (+1) (100 lipids)**
  - 25 1 50 2 10 38 2486 7 29 500 500 PB/C
  - 50 1 50 2 10 38 4972 14 36 500 500 PB
  - 75 1 50 2 10 38 7458 21 43 500 500 ND

- **Mixture M (+1) (400 lipids)**
  - 25 1 200 6 40 154 9944 200 28 500 200 C
  - 50 1 200 6 40 154 19888 56 238 500 200 C
  - 100 1 200 6 40 154 39778 111 283 500 200 PB/ND

- **Self-assembly with RNA**
  - **Mixture M (+1) (100 lipids)**
    - 25 1 50 2 10 38 2486 7 27 500 500 IB
    - 50 1 50 2 10 38 4972 14 36 500 500 *
    - 75 1 50 2 10 38 7458 21 43 500 500 PB
  - **Mixture P (+1) (100 lipids)**
    - 25 1 200 6 40 154 9944 28 200 500 200 C
    - 50 1 200 6 40 154 19888 56 238 500 200 C
    - 100 1 200 6 40 154 39778 111 283 400 200 PB/ND

- **RNA only**
  - 1 14473 28 500

* simulation box was too narrow and RNA interacted with itself due to periodic boundary conditions

** also 5WPL and 10 WPL were simulated
Figure S1: Structures of parametrized non-standard lipid species. ALC-0315 and ALC-0159 (47 PEG units, (2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide)) are found in mixture P inspired by Pfizer&BioNTech whereas SM-102 and DMG-PEG 2000 (45 PEG units, 1,2-Dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000) are found in mixture M inspired by Moderna. Charged forms of both ILs are shown, with the protonation occurring on head group nitrogen atoms.

Lipid parameterization

To maintain compatibility among all components of the prepared systems, partial charges for non-standard lipid species were derived using the common AMBER FFs scheme. Parameters for the ionizable lipids ALC-0315 and SM-102 (in both their ionized and neutral forms; see Figure S1) and for the PEGylated lipids DMG-PEG 2000 and ALC-0159 were derived as follows. First, geometry optimization of a single lipid molecule was performed at the B3LYP/6-31G* level of theory\textsuperscript{1,2} after which the molecule’s electrostatic potential (ESP) was calculated at the HF/6-31G* level in the gas
phase. The PEGylated part of lipids was parametrized separately to obtain an equal charge distribution on the PEG chains (Figure S2). Individual partial charges were then fitted using the restrained electrostatic potential (RESP) approach. All quantum mechanical calculations were performed using the Gaussian 09 software package and the RESP fitting procedure was performed using the Antechamber tool.\textsuperscript{5}

For individual parametrized lipids, all bond, angle, and torsion terms and Lennard-Jones non-bonded terms were described using a combination of the LIPID\textsuperscript{16} and General Amber Force Field 2 (GAFF2) parameters included in the Amber16 software package.\textsuperscript{7} Parameters for lipid aliphatic tails were taken from the LIPID 17 parameter set whereas the lipid headgroups and PEG chains were described using GAFF2 parameters. Parameters defining geometrical terms at the GAFF2/LIPID 17 interface were taken from the appropriate GAFF2 terms. Topologies for the studied lipids are available at the following Zenodo repository: https://doi.org/10.5281/zenodo.5595675.

**Molecular dynamics**

We studied both single-component lipid systems and lipid mixtures\textsuperscript{8,9} representing those used in the COVID-19 mRNA vaccines developed by Pfizer&BioNTech\textsuperscript{10} and Moderna.\textsuperscript{11} For a full list of our simulated systems and their compositions, see Tables S1, S2 and S3, in the text, we call the mixture containing ALC-0315 mixture P and that containing SM-102 mixture M. The LNPs studied experimentally have complex internal structures with multiple layers having different levels of access to water or the bulk solution.\textsuperscript{12-14} To evaluate the potential effect of hydration on the lipid systems, multiple setups were simulated with between 5 and 100 water molecules per lipid molecule (WPL). Low hydration levels affected the properties of resulting lipid mixture, but no significant changes with increasing hydration were observed above ~50 WPL. We therefore discuss only the fully hydrated (>50 WPL) cases in the main text.

For simulations of RNA, the structure of stem loop 1 (SL1) from the 5\textsuperscript{'} untranslated region (UTR) of the SARS-CoV-2 genome, which was studied in detail by Bottaro et al. (2021), was used. The structure from the most populated cluster (the center_SL1_0.pdb file from ref\textsuperscript{15} dataset) was taken as an initial structure.

All systems were prepared using our in-house membrane builder via a two-step process. First, the lipids were placed in a cubic box of fixed dimensions. For bilayer simulations, both pure lipids and lipid mixtures were preassembled into a lipid bilayer structure. For studies on self-assembly, the initial positions of individual lipids within the simulation box were set randomly, with or without the presence of an RNA fragment. The systems were then solvated at the desired level of hydration, including K\textsuperscript{+} and Cl\textsuperscript{-} ions at their physiological concentration (Tables S2 and S3). For bilayer simulations, water molecules in the hydrophobic membrane region were removed.

All molecular dynamics simulations were performed using the AMBER 16 software package.\textsuperscript{7} Systems were described using the Amber ff99SB\textsuperscript{16} force field with the OL3\textsuperscript{17} and parmbsc0\textsuperscript{18} refinements for RNA. Additionally, the external interaction-specified gHBfix\textsuperscript{19} potential for improvement of pair-wise H-bond interactions was applied with potentials of 0.5 kcal/mol for -NH‧‧‧N- and -NH‧‧‧O- interactions and -0.5 kcal/mol for -OH‧‧‧nbO/bO interactions. The modified vdW radii for phosphate oxygens proposed by Steinbrecher et al.\textsuperscript{20} were also used. Systems were solvated using the OPC\textsuperscript{21} water model with KCl salt added to mimic the physiological concentration of 0.154 M and to maintain the systems’ electroneutrality. The following Joung–Cheatham\textsuperscript{22} parameters for ions were used: K\textsuperscript{+} (r = 1.590 Å, ε = 0.2795 kcal/mol) and Cl\textsuperscript{-} (r = 2.760 Å, ε = 0.1684 kcal/mol). Before each production run, the systems were relaxed as follows. Separate energy minimizations were performed for hydrogen atoms of RNA, water and the co-solvent, and lipids while restraining the RNA molecule. The RNA molecule was then subjected to energy minimization with progressively weaker constraints (having force constants of 1000, 500, 125, and 25 kcal/mol) on the sugar-phosphate backbone atoms. The resulting fully relaxed
systems were then heated in two separate thermalization steps. First, each system was heated for 100 ps under NVT conditions followed by density equilibration for another 100 ps in the NpT ensemble. Production runs were conducted under NpT conditions at a constant pressure of 1 atm. The temperature was kept constant at 298.16 K using the weak-coupling algorithm. Long range electrostatics were described using the Particle Mesh Ewald (PME) summation scheme with a cutoff distance of 10.0 Å. Bonds involving hydrogen were constrained using the SHAKE algorithm with a geometric tolerance of $10^{-5}$ Å. Periodic boundary conditions were applied in all directions. All simulations had an integration time step of 2 fs. The total length of the production runs ranged from 0.2 µs to 0.5 µs depending on the simulated system (Tables S2, S3). This simulation procedure was applied to all systems; for systems not containing RNA, steps involving treatment of RNA were omitted.

Analysis

We analyzed the structural parameters of lipid bilayers using cpptraj from AmberTools 18. The bilayers were oriented in the xy plane, with the z-axis aligned to the membrane normal. The origin of coordinates was chosen to be the center of mass of the bilayer, so the numerical position of a point on the z-axis corresponds to the distance from the middle of the membrane. All mixtures were equilibrated and average structural parameters were determined by analyzing the last 200 ns of each simulation. The area per lipid (APL) was calculated as the xy surface area divided by the number of lipids in one leaflet. Mass density profiles were calculated with respect to the center of the bilayer along the z-axis and the membrane thickness was determined based on the highest peaks in the density of the whole systems. Deuterium order parameters ($S_{<CD>}$) were calculated for all hydrocarbon chains in all lipids and displayed as functions of the most populated positions (density peak maximum) of the corresponding carbons. The orientation of individual lipid fragments was calculated based on the distributions of the angle between the z-axis and the vector defined by terminal atoms of the relevant chains.

Analysis of the interaction surfaces between individual species within each system were performed using the trjVoronoi trajectory analysis tool built on the framework of the Voro++ software. The trajectories were batch processed on frame-by-frame basis (due to the incompatibility of the cpptraj-generated .trr trajectories with trjVoronoi) using homemade bash and python scripts. Group-by-group interaction surfaces were obtained by renaming different lipid species/parts to make them fit into separate groups as defined in trjVoronoi data files. The voronoi analyses were performed on the last 200 ns of the anisotropic simulations, evaluating the interactions between molecules in different groups in blocks of 100 frames (corresponding to 20 ns each). The voronoi analysis of pure single-lipid bilayers were performed on the entire simulation length, in blocks of 100 frames (corresponding to 50 ns). For the IL headgroup/tail interaction analysis, headgroups were defined as reaching one carbon past the ionizable nitrogen in the tail direction.

The RMSD, RMSF and helical parameters of RNA were calculated using AmberTools’ cpptraj module. The eRMSD and clustering analysis of the RNA molecule was performed using Barnaba.

2. Results

2.1. Pure Bilayers

Structural parameters of pure IL membranes differ significantly from phospholipid bilayers. Due to the presence of three/four branched aliphatic tails in lipid structure, preassembled bilayers are highly disordered (Figure S4). This results in higher values of area per lipid for IL membranes (Figure 1A, Figure S3) and decreased membrane thicknesses (Figure S4) compared to DOPC membrane that we take as a phospholipid reference. The density distribution plots of the IL bilayers also differed from that for DOPC: the DOPC bilayer had a high-density region containing the lipids’ head groups in which both the head groups and water contributed to the system’s overall density (Figure 1A), in accordance
with the four region model. No such peak for the total density of the system was observed for the IL systems (Figure 1A). The lack of the high density peak originates from the different hydration of head groups regions. In case of the IL, the density profile shows proportionally similar increase of the IL density to the decrease of the water density, summing up to minimal increase in the overall density of the membrane. In DOPC membrane, we observed an increased hydration of the head groups region. We analyzed number of hydrogen bonds with water molecules and while DOPC had 5.9 hydrogen bonds with water per lipid molecule, ILs had 5.0 and 4.7 water molecules per lipid for ALC-0315 and SM-102, respectively (Figure S3). The different hydration level needs to be considered with different APLs, which are significantly larger for ILs than for DOPC. This effect of enhanced water permeation to/below the head group region significantly adds to the mass density of DOPC, corresponding to the region of high density of head groups, based on the four region model, lacking in ILs.

The unionized forms of ILs were unstable, differing with the rate of lipid translocation to bulk phase (Figure 1A). In the case of SM-102(0), some of the IL molecules flipped during the first 50 ns of the simulation and moved to the lipid bilayer core. The bilayer remained stable for another ~100 ns after which more IL molecules gradually flipped to the middle of the bilayer, forming an unstructured layer surrounded by two lipid leaflets (Figure 1B). In contrast, the ALC-0315(0) lipid bilayer exhibited negligible stability even at the start of the simulation and rapidly formed a bulk unstructured lipid layer (Figures 1B, S5). Apart from the differences in the amount of lipid tails between SM-102 and ALC-0315 described in the main text, we should highlight also the differences in the effective molecular shape in ionized and unionized form. Because SM102 has three acyl tails and ALC-0315 has four (Figure 1C), the ILs have conical molecular shapes that cannot be accommodated in a lamellar phase (Figure 1B) and thus destabilize the bilayer structure. As seem from Figure S3, ionized form of ILs has a higher APL than unionized form in the conformation of a bilayer (notice the APL value at the start of a simulation of a preassembled bilayer), therefore its headgroup is effectively larger, partially due to mutual electrostatic repulsion of the headgroups (see the radial distribution functions plotted in Figure S6), partially due to higher hydration of ILs (Figure S3), causing the molecules to adopt less conical shapes that are more compatible with the bilayer structure.

To assess the properties of single ILs, a Voronoi surface analysis was performed on the single-lipid bilayers. Interaction surfaces between the IL headgroups (including the first C atom in the tail direction from the ionizable nitrogen), tail groups and water were analyzed during the simulations (Figure S7). The ratios remain stable in the case of the charged lipids – with roughly a quarter of the lipid’s surface in touch with water (the relative difference between the length ALC-0315 and SM-102 headgroup chains accounts for larger fraction of ALC-0315 in contact with water), while only <5% of total lipid surface is in contact with headgroups. In the simulations of uncharged lipids, the ratio of lipid surface in contact with water is lower from the start of the simulation and decreases even further during its run (corroborating the hydrogen bond number between lipid and water molecules, Figure S3). Moreover, the fraction of lipid surface interacting with headgroups is larger in the uncharged systems than in the charged ones, and increases during the simulation, as the bilayer structure collapsed into a bulk phase.
**Figure S3:** The evolution of area per lipid (left panel) and number of hydrogen bonds with water per lipid molecule (right panel) in pure membranes.

**Figure S4:** Deuterium order parameters in pure lipid membranes with structure of ALC-0315 defining color codes for individual chains used in SM-102 in the same logic.
Figure S5: Orientation of individual chains in terms of an angle between z-axis (membrane normal) and vector defined by terminal atoms of individual chains.

Figure S6: Radial distribution functions of water and chlorides around ionizable lipids nitrogens (IL-N – WAT and IL-N - CL), ionizable lipids nitrogens mutually (IL-N – IL-N) and hydroxy groups of ionizable lipids (IL-OH – IL-OH).
Figure S7: Ratios of interaction surfaces between different parts of the system of pure IL bilayers.

Table S4: Structural parameters of pure bilayers (area per lipid (APL), thickness (D_{HH}))

|                | APL (nm$^2$) | D_{HH}(nm) |
|----------------|--------------|------------|
| ALC-0315 (+1)  | 1.197        | 2.45       |
| SM-102 (+1)    | 1.041        | 2.65       |
| DSPC           | 0.50         | 3.0**      |
| CHL            | 0.38         | 2.4        |
| DOPC           | 0.685        | 3.8        |

**ripple phase

2.2. Bilayer Mixtures

Order Parameter in Bilayer Mixtures.

The structural differences between mixtures P and M are caused by the different structures of ILs hydrophobic tails. ALC-0315 has two short acyl tails going out of its head groups, that branch into two short tails each (Figure S1). SM-102 on the other hand, is more asymmetric – one tail is branched into two other short tails, while the other tail is long and saturated, only with ester group creating a kink similar to the one induced by double bond in e.g. DOPC. The difference in IL tail structure is then depicted in the ordering of both lipid mixtures. While all short tails are highly disordered, the long tail of SM-102 is ordered ($S_{<CD>}$ up to 0.2, Figure S9) and resembles a regular phospholipid acyl chain behavior and is oriented with a similar distribution as DSPC tails (Figure S10). Generally, in lipid bilayers, mixture M is more ordered than mixture P, which is caused by the long acyl chain in ionizable SM-102 lipid.
Stability and Morphology of Un-ionized Bilayer Mixtures.

Similarly to the situation in single-component bilayers, lipid mixtures containing un-ionized ILs are less stable than their protonated forms. In both studied mixtures we observed some IL molecules flipping their head group to the middle of the bilayer. In case of un-ionized mixture M, we observed only few (~3) flipped molecules, but in case of mixture P the membrane lost its stability. The membrane seemed to be stable when lipids are initially randomly distributed, but during the simulation, DSPC separated from ILs (Figure S11). We observed regions rich in DSPC and CHL and other regions rich in ILs with a fraction of CHL (Table S5, Figure S13). DSPC is well ordered in its DSPC-rich phase (even more than in ionized mixture, see Figure S9), but IL-rich phase is unstable as ILs flipped and started to create a disordered layer inside the membrane. Generally, simulations of both pure and mixed bilayers showed that the lipid phase loses its stability with deprotonation, e.g. with an increase of pH. The membranes with ILs in its de-ionized state mimic the state of RNA-containing lipid nanoparticle in physiological pH, above pKa of ILs.

The Role of PEGylated Lipids in Bilayer Mixtures.

As PEGylated lipids made only a negligible fraction of lipid molecules in the system (Tables S2 and S3), they did not play a crucial role in the structure of lipid mixtures. Lipid anchors of both PEGylated lipids make only a small part of the lipid phase and therefore they do not significantly affect its stability or other structural properties. While incorporated into lipid matrix, PEGylated lipid tails behaved as ordered lipids in case of mixture M, similarly to DSPC, whereas in mixture P they were more disordered (Figure S9). It should be noted that DMG-PEG 2000 in mixture M bears a glycerol headgroup (as DSPC), while ALC-0159 from mixture P has amine nitrogen as a headgroup (Figure S1) and the ordering of their tails could be affected by both, i.e. the ordering of other lipids in the system and the chemical composition of the headgroup.

We observed that the PEG chains were mostly dissolved in water, interacting with water molecules and other PEG chains (Figure S11) and occasionally interacting with the lipid leaflet via periodic boundary conditions. The interactions with water made for more than 50 % of PEG surface, another ~ 40 % was a mutual interaction of PEG chains (Table S6). In the solvent, PEG chains were interacting with potassium ions, surrounding them in a form of a ring-like structures with negatively charged oxygens interacting with positively charged ions, as described by Stepniewski et al31 (Figure S12). The interactions of the PEG chains with either RNA or lipids were very random and varying between different simulations. The level of mutual interaction depended on the hydration level of the system – the more water in the system, the more space for PEG chains to avoid interactions with lipids or RNA and vice versa. We did not observe statistically significantly the PEG chains interactions with lipid tails (below lipid head groups) as observed in e.g. Mahmoudzadeh et al 2021.32 Also, to exclude an artifact in the formed lipid phases caused by the presence of PEG, we performed self-assembly simulations without the presence of PEGylated lipids and ended in the same set of resulting phases (Table S2). Concludingly, we did not observe any significant effect of PEG chains on the behavior of our systems and their effect could be expected in larger scale.
Figure S8: Area per lipid evolution in mixed membranes

Figure S9: Deuterium order parameters of four-component bilayers.
Figure S10: Orientation of lipid chains in terms of an angle between z-axis and vector defined by terminal atoms of individual chains in mixed bilayers. Left panel shows the orientation of IL chains in respective mixtures, right panels show orientation of cholesterol, acyl chain of DSPC, acyl chains in PEGylated ALC-0159 or DMG-PEG 2000 and a headgroup P-N vector of DSPC in all four mixtures.

Figure S11: Final snapshots of lipid mixtures in respective protonation states from top and side views. IL is shown in yellow, cholesterol dark red, DSPC blue and PEG chains in orange. In top view, PEGylated lipids are omitted. In side view, nitrogens and hydroxyl oxygens of IL are shown as blue and red balls, respectively. Water is omitted for clarity.
Figure S12: PEG chain (carbons depicted in orange, hydrogens white and oxygens red sticks) surrounding potassium ions (violet balls). PEG oxygens create rings around potassium ions. Other parts of the system are omitted for clarity.

Figure S13: Relative ratios of shared Voronoi surface/interactions between selected species and the ratios of total surface of all groups in the system (left column of each panel) in the bilayer systems. A preferable interaction between ILs and IL-rich phase formation is visible in uncharged bilayers (right panel). The lipid-water interaction surface is given with respect to the total surface of the molecule.
Table S5: The percentage of an average surface contact between different groups and selected lipid species, averaged over the last 200ns of the systems together. The values for ‘System ratios’ correspond to the system composition and are calculated as the fraction of the total surface of the given lipid, out of the total surface of all lipids in the simulation box. The standard deviation of the system fraction reflects the fact that the lipids from mixture M usually make up 61% of the total lipid surface, while the more branched lipids from mixture P make up 62% of the total lipid surface.

| Bilayers | Surface in contact (%) |          |          |          |
|----------|------------------------|----------|----------|----------|
|          |                        | IL       | CHL      | DSPC     | System   |
| Charged  | with IL                | 61 ± 1   | 53 ± 1   | 51 ± 2   | 61 ± 1   |
|          | with CHL               | 25 ± 1   | 33 ± 1   | 23 ± 2   | 23 ± 0   |
|          | with DSPC              | 13 ± 0   | 12 ± 1   | 24 ± 1   | 14 ± 0   |
| Uncharged| with IL                | 70 ± 2   | 54 ± 1   | 42 ± 2   | 61 ± 1   |
|          | with CHL               | 20 ± 1   | 29 ± 1   | 29 ± 3   | 23 ± 0   |
|          | with DSPC              | 9 ± 1    | 15 ± 0   | 28 ± 0   | 14 ± 0   |

Table S6: The percentage of an average surface contact between the PEG chain and other components in the system, averaged over the last 200ns of the systems together.

|                      | Surface contacts % of total PEG surface |
|----------------------|----------------------------------------|
|                      | self-assemblies | charged bilayers | uncharged bilayers |
| with WAT             | 54±4           | 58±1             | 54±2               |
| with PEG             | 40±1           | 39±0             | 39±1               |
| with all lipids      | 3±3            | 1±0              | 6±2                |
| with RNA             | 2±2            | ---              | ---                |
| with ions            | 1±0            | 2±0              | 1±0                |

2.3. Lipid Self-assembly

During the self-assembly process we observed that the initially randomly distributed lipids coagulated to minimize their contact with water. While smaller systems containing 100 lipid molecules usually preferred porous or intact bilayers, the cubic phase was more common in larger systems containing 400 lipid molecules (Table S3). It should be noted that the periodic boundary conditions may affect the observed morphological size-dependence. The level of hydration also affected the organization of the lipids; nanodisc formation was observed at higher levels of hydration (Table S3). In general, we can conclude that ILs preferentially form various non-lamellar phases rather than bilayers on experimentally relevant scales.

Apart from the self-assembly studies of lipid mixtures, we studied also self-assembly of single-component ILs, DSPC and binary mixture of DSPC and CHL. The uncharged ILs created unstructured bulk lipid phase. The charged form of ILs created a disordered cubic phase with a network of thin lipid tubes. On the other hand, DSPC (simulated far below its phase transition temperature) created a gel-phased porous bilayer. The binary mixture of DSPC and CHL created a well-ordered intact bilayer. We can therefore identify ILs as a clear cause for non-lamellar phases formation.
Figure S14: Phase separation during self-assembly simulations. ILs are concentrated at the edges of pores (A), in the bridges of cubic phase (B) or at the edges of nanodiscs (C). On the other hand, DSPC and CHL form thicker ordered phase in the cubic phase knots or are surrounded by ILs in the core of nanodics. IL is shown in yellow, cholesterol dark red, DSPC blue and PEG chains in orange. Nitrogens and hydroxyl oxygens of IL are shown as blue and red balls, respectively. Water and RNA are omitted for clarity.

2.4. **RNA simulations**

*RNA Simulation in Water*

In order to study RNA interactions with IL containing mixtures, we took a stem-loop 1 (SL1) from 5’ untranslated region of SARS-CoV-2 genome from Bottaro et al.\textsuperscript{15} comprising of two helices with internal asymmetrical loop segment. First, to assess the stability of the RNA molecule in our simulations, SL1 fragment alone was subjected to free MD simulation in bulk water. SL1 kept its secondary structure, eRMSD did not exceed 0.7 nm with respect to the original structure (Figure S18). Naturally, the largest conformational changes were observed within the U18-C21 loop (Figures S15, S19 and S20). In simulation of SL1 fragment in water, the major fluctuations were found within the apical loop region (U18-C21) (Figure S20 panel A). As the most fluctuating part of SL1 fragment we identified the C20 base that was observed to flank out from the RNA itself and into the solvent environment (Figures S15 panel C and Figure S20). It is worth mentioning that structure of our most populated cluster (Figure S15 panel B) differs slightly within the flexible apical loop from structure described by Bottaro et al 2021.

Figure S15: Close up on apical loop of SL1 (shown as sticks). A) The most populated cluster from Bottaro et al.\textsuperscript{15} was used as our starting structure B) The structure of the most populated cluster from our simulations of SL1 in water C) The displayed structure demonstrates the flexibility of apical loop – flanked cytosine 20 (C20) exposed to solvent environment. Structures were aligned by residue C16 and G23.
Further, we analyzed the interaction of the SL1 fragment with the potassium ions. As assumed, the accumulation of positively charged ions was prone to negatively charge phosphates in SL1 backbone. The large number of ions concentrated within the major groove of the RNA (Figure S16, panel A). Moreover, we identified preferential binding side for the potassium ion strongly coupled with the Sugar-Hoogsteen base-pairs – namely U11, A12, C28 and A29 bases near the internal triplex (Figure S16, panel B).

![Figure S16: Final snapshot of SL1 in water showing A) the accumulation of potassium ions (pink balls) around the major groove of RNA B) the specific interaction of potassium ion with U11, A12, C28 and A29. This potassium binding side around highlighted residues was observed repeatedly in most of the independent simulations. Water molecules are omitted for clarity.](image)

**RNA in Lipid Self-Assemblies**

Even though the hydration level varied between simulations, with the total surface of water molecules taking up 57-85% of the total surface of all molecules in the box, the RNA-water surface contacts did not differ dramatically between systems, suggesting that RNA always retains a layer of minimal hydration (Table S7). Therefore, the values of interaction surfaces of RNA and different lipid species were averaged over all the self-assembly simulations (Table S8).

**Table S7: The percentage of an average surface contact between RNA and water, itself (RNA) and all lipids, as an average for the last 200 ns of each simulation. The interactions with the PEG-chain and ions are not included in the table.**

| Hydration level (WPL) | 100 lipids | 400 lipids | 100 lipids | 400 lipids |
|-----------------------|------------|------------|------------|------------|
| Mixture M | 25 | 50 | 75 | 25 | 50 | 100 | 25 | 50 | 75 | 25 | 50 | 100 |
| Mixture P | 25 | 50 | 75 | 25 | 50 | 100 | 25 | 50 | 75 | 25 | 50 | 100 |
| with WAT | 46±0 | 43±1 | 46±1 | 40±2 | 48±1 | 46±1 | 44±1 | 52±1 | 41±1 | 44±1 | 43±1 | 48±1 |
| with RNA | 42±0 | 43±0 | 41±0 | 43±0 | 43±0 | 42±0 | 43±0 | 42±0 | 42±0 | 43±0 | 43±0 | 43±0 |
| with lipids | 8±1 | 13±1 | 10±1 | 13±1 | 7±0 | 11±1 | 10±1 | 5±0 | 12±1 | 11±1 | 10±1 | 8±1 |
Table S8: The percentage of an average surface contact between different groups and selected lipid species during self-assembly simulations, averaged over the last 200 ns of the systems together. The values for ‘System ratios’ correspond to the system composition and are calculated as the fraction of the total surface of the given lipid, out of the total surface of all lipids in the simulation box. The standard deviation of the system fraction reflects the fact that the M lipids usually make up 61% of the total lipid surface, while the more branched P lipids make up 62% of the total lipid surface.

| Surface in contact (%) | RNA   | IL    | CHL   | DSPC  | System ratios |
|------------------------|-------|-------|-------|-------|---------------|
| with IL                | 86 ± 8| 68 ± 1| 44 ± 1| 38 ± 2| 61 ± 1        |
| with CHL               | 5 ± 5 | 21 ± 1| 37 ± 1| 32 ± 2| 23 ± 0        |
| with DSPC              | 7 ± 5 | 10 ± 0| 17 ± 1| 29 ± 1| 14 ± 0        |

Figure S17: Radial distribution function of RNA phosphorus to IL hydroxyl oxygens (left panel) and IL nitrogen (right panel). The curves are averaged over all simulations of mixture M (blue) and mixture P (red).

To describe the global structural changes of the RNA molecules, two different methods were used. The eRMSD metric describes the similarity of nucleic acids based on the similarity of their base-pairing by considering their relative positions and orientations. Unlike the RMSD, which takes into the account only the atom coordinates (with threshold of sufficient structure similarity considered 4 Å), eRMSD calculates the relative spatial arrangement of nucleobases (near-native base–base contacts are considered having eRMSD values <0.8).

Figure S18 shows that both SL1 alone in water, and most of the other simulations fall within the thresholds for a sufficient similarity to the starting structure based on both eRMSD and RMSD scores. The exceptions are depicted in a greater detail in Figure S19 and described below.
Figure S18: A) The eRMSD values with respect to the most populated cluster of SL1 structure as discussed in Bottaro et al.\textsuperscript{15} for the whole simulation of RNA in water, B) the last 200ns of all the anisotropic systems. The high value of eRMSD in mixture P containing 100 lipids and hydration of 50 WPL is caused by the deformation of the box in the anisotropically pressure coupled environment. The RMSD values for corresponding simulations in water (C) and in lipid mixtures (D).
Figure S19: Structures of RNA in our simulations with highlighted U18-C21 region (nucleotide planes in blue (U) and red (C)) and the A12-C28 Sugar-Hoogsteen base-pairs (as highlighted by Bottaro with A17 as yellow nucleotide planes). The RNA residues with the highest eRMSD deviations are highlighted in purple rectangle. The structures depicted are: comparison of the starting structure (light gray cartoon) with A) the center structure of the largest cluster of the whole 500ns simulation of SL1 in water, B) the center structures of the largest cluster of the simulations with eRMSD < 0.8, color coded with accordance to Fig S18. C) the center structure of the largest cluster of the simulation P 400 lipids 50 WPL (magenta) – with eRMSD value around 0.8, D) the center structure of the largest cluster of the simulation P 100 lipids 25 WPL (orange) – with eRMSD value around 0.8, E) the center structure of the largest cluster of the simulation P 100 lipids 50 WPL (black) – with eRMSD value around 1, F) the center structures of the largest cluster of the simulation M 100 lipids 25 WPL (light blue) – with eRMSD around 0.8, but with the RMSD value significantly higher than all other simulations.

The structures with higher than usual eRMSD are P 100 lipids 50 WPL, M 100 lipids 25 WPL, P 100 lipids 25 WPL and P 400 lipids 50 WPL, while unusually high RMSD was observed only in the case of M 100 lipids 25 WPL; with P 100 lipids 25 WPL and P 100 lipids 50 WPL crossing the 4Å threshold only for a fraction of the simulation. In the case of the P 100 lipids 25 WPL and P 400 lipids 50 WPL simulations, the increase of eRMSD stems from parts of the structure that have been identified as prone to changes of base pairing – namely the Sugar-Hoogsteen pair at A12-C28 (P 100 lipids 25 WPL) and the apical loop (P 400 lipids 50 WPL). In the mixture P 100 lipids 50 WPL, the eRMSD increase is also related to the base-pairing changes in the same section (A12-C28), however, as the simulation box deformed under the anisotropic conditions, causing the RNA to self-interact via the periodic boundary condition, it is impossible to pinpoint exact source of any of the system’s behavior and we consider this simulation as an artifact of periodic boundary conditions. In the mixture M 100 lipids 25 WPL, the large
backbone deformation occurred gradually in the 200-250 ns part of the initial anisotropic simulation, as RNA stretched into a pore in the lipid structure at low hydration.

Figure S20: The per-residue atomic fluctuations of RNA. Color code and selected simulations in accord with the previous Figure S18, namely: A) SL1 in water, B) simulations with eRMSD < 0.8, C) P 400 lipids 50 WPL (magenta) and SL1 in water (green), D) P 100 lipids 25 WPL (orange) and SL1 in water (green), E) P 100 lipids 50 WPL (black) and SL1 in water (green), F M 100 lipids 25 WPL (light blue) and SL1 in water (green).

The RMSF further describes the SL1 flexibility per-residue. Similarly to the RMSD analysis, the RMSF show large cluster of simulations (S20, panel B) with the alike fluctuations in structure compared to the SL1 in water (S20 panel, A). Apart from this cluster, we identified simulations possessing some local irregularities in residue fluctuations. Those are mainly within the apical loop region (U18-C21) or in the vicinity of the Sugar-Hoogsteen pair (A12-C28). Those simulations (S20, panels C to F) correspond to those with higher than usual RMSD values (Figure S18, panel B). Additionally, the simulation M 400 lipids 25 WPL also exhibits a peak of RMSF, corresponding to higher local movements, at the triplex area (A14-U25-A26), suggesting a change in the base pair interactions.

To analyze the structural dynamics of RNA, we calculated helical parameters of the RNA fragment. We observed, that in most of our simulations, the helical parameters of simulations of SL1 in the presence of lipids did not differ significantly from those calculated in the simulation of SL1 in water (see Tables S9-S12). We did not take into account the simulations discarded previously, P 100 lipids 50 WPL (due to error in periodic boundary treatment) and M 100 lipids 25 WPL (due to its ladder-like structure caused by SL1 been intercalated a lipid pore). In other simulations we closely analyzed the inclination, roll and the sizes of major and minor groove. Lipids interacting with RNA induce local structural changes of RNA molecule, which are also evident in changes of helical parameters. We
observed missing base pairs in the proximity of the triplex in case of simulations M 100 lipids 50 WPL, M 400 lipids 25 WPL. These simulations were also with the highest level of RNA-lipids interactions (RNA structure interacting with lipids with 13 % of its surface, see table S7). Further, we observed a significantly higher inclination in simulation P 100 lipids 75 WPL, that interacted with lipids with 12 % of its surface. From all the performed analyses we can conclude that the more interaction of RNA with ILs, the more likely ILs cause local changes in the RNA structure.
Table S9: Mean values of inclination for the last 200 ns of each simulation. A dash means that the corresponding base step was not defined and the inclination could not be calculated.

| Base-pair step | SLI alone | Mixture M | | | Mixture P | | |
|----------------|-----------|-----------|---|---|-----------|---|---|
|                | 100 lipids | 400 lipids | 100 lipids | 400 lipids | 100 lipids | 400 lipids |
|                | 25 | 50 | 75 | 25 | 50 | 100 | 25 | 50 | 75 | 25 | 50 | 100 |
| 6-34 7-33      | 12.5 | 10.1 | 10.2 | 10.5 | 6.5 | 11.4 | 9.2 | 9.8 | 13.2 | 15.3 | 12.8 | 11.8 | 13.0 |
| 7-33 8-32      | 17.1 | 15.2 | 16.2 | 16.4 | 11.2 | 16.1 | 16.3 | 16.5 | 18.0 | 21.6 | 16.6 | 15.9 | 17.6 |
| 8-32 9-31      | 9.0 | 8.3 | 10.6 | 9.2 | 9.6 | 8.1 | 8.4 | 10.4 | 9.4 | 18.3 | 6.9 | 7.9 | 9.9 |
| 9-31 10-30     | 13.7 | 12.1 | - | 15.8 | - | 13.1 | 13.0 | 10.9 | 13.1 | 25.1 | 8.8 | 12.0 | 17.8 |
| 13-27 14-25    | 15.2 | - | 15.1 | 13.6 | 18.6 | 15.6 | 12.3 | 19.8 | - | 9.4 | 23.4 | 23.9 | 13.1 |
| 14-25 15-24    | 6.5 | 3.2 | 4.1 | 4.8 | 10.7 | 5.7 | 2.5 | 2.6 | 5.4 | 6.3 | 17.8 | 11.7 | 3.3 |
| 15-24 16-23    | 11.6 | 7.3 | 8.7 | 5.0 | 16.2 | 12.4 | 1.1 | 7.2 | 11.9 | 10.4 | 19.9 | 16.0 | 8.7 |
| 16-23 17-22    | - | 8.1 | - | - | 12.7 | - | - | 8.1 | 3.9 | - | - | - |

Table S10: Mean values of roll for the last 200 ns of each simulation. A dash means that the corresponding base step was not defined and the roll could not be calculated.

| Base-pair step | SLI alone | Mixture M | | | Mixture P | | |
|----------------|-----------|-----------|---|---|-----------|---|---|
|                | 100 lipids | 400 lipids | 100 lipids | 400 lipids | 100 lipids | 400 lipids |
|                | 25 | 50 | 75 | 25 | 50 | 100 | 25 | 50 | 75 | 25 | 50 | 100 |
| 6-34 7-33      | 30.8 | 30.2 | 30.8 | 30.3 | 30.6 | 30.8 | 29.3 | 31.2 | 30.2 | 30.1 | 30.5 | 29.6 | 30.5 |
| 7-33 8-32      | 30.7 | 30.2 | 31.1 | 31.0 | 32.7 | 31.4 | 31.1 | 30.3 | 30.8 | 29.4 | 30.4 | 30.7 | 31.0 |
| 8-32 9-31      | 28.5 | 27.4 | 28.7 | 27.9 | 29.0 | 28.3 | 29.4 | 28.5 | 28.5 | 28.9 | 28.7 | 27.4 | 28.0 |
| 9-31 10-30     | 28.0 | 27.6 | - | 27.2 | - | 27.8 | 27.6 | 27.0 | 28.1 | 28.0 | 27.0 | 27.2 | 27.1 |
| 13-27 14-25    | 52.6 | - | 52.2 | 52.3 | 52.6 | 53.0 | 53.7 | 52.1 | - | 50.2 | 50.3 | 55.4 | 53.1 |
| 14-25 15-24    | 29.5 | 25.2 | 29.1 | 27.8 | 31.0 | 28.1 | 28.0 | 26.9 | 28.5 | 29.7 | 32.5 | 29.4 | 29.1 |
| 15-24 16-23    | 30.5 | 27.4 | 29.0 | 28.4 | 32.2 | 28.6 | 27.6 | 29.6 | 29.6 | 31.5 | 29.5 | 26.7 | 29.3 |
| 16-23 17-22    | - | 27.4 | - | - | 26.5 | - | - | 26.7 | 35.1 | - | - | - |
Table S11: Mean values of size of major groove for the last 200 ns of each simulation. A dash means that the corresponding base step was not defined and the value could not be calculated.

| Base-pair | SL1 alone | Mixture M |  | Mixture P |  |
|-----------|-----------|-----------|-------------|-----------|-------------|
|           |  | 100 lipids | 400 lipids | 100 lipids | 400 lipids |
| 7-33      | 19.0      | 19.1      | 18.9      | 18.7      | 18.9      | 19.0      | 19.1      | 19.1      | 18.9      | 19.0      |
| 8-32      | 19.1      | 19.3      | 18.9      | 18.6      | 19.0      | 19.1      | 19.3      | 19.3      | 19.2      | 19.4      |
| 9-31      | 19.2      | 19.4      | 19.3      | 19.2      | 19.3      | 19.3      | 19.3      | 19.3      | 19.3      | 19.4      |
| 10-30     | 18.8      | 19.0      | 18.9      | 18.6      | 18.9      | 19.5      | 19.3      | 18.9      | 18.8      | 19.0      |
| 13-27     | 18.5      | 18.7      | 18.5      | 18.4      | 18.4      | 18.4      | 18.7      | 18.6      | 18.3      | 18.5      |
| 14-25     | 18.8      | 18.7      | 18.9      | 18.8      | 18.9      | 19.2      | 19.0      | 19.1      | 19.0      | 18.9      |
| 15-24     | 18.9      | 19.2      | 19.1      | 18.8      | 19.3      | 19.1      | 19.1      | 18.9      | 19.0      | 19.5      |
| 16-23     | 18.7      | 19.2      | 18.8      | 18.7      | 19.2      | 18.7      | 18.9      | 19.3      | 18.9      | 18.8      |
| 17-22     | 19.0      | 19.2      | 19.2      | 19.2      | 19.2      | 19.0      | 18.1      | 18.1      | 18.1      | 18.1      |

Table S12: Mean values of size of minor groove for the last 200 ns of each simulation. A dash means that the corresponding base step was not defined and the value could not be calculated.

| Base-pair | SL1 alone | Mixture M |  | Mixture P |  |
|-----------|-----------|-----------|-------------|-----------|-------------|
|           |  | 100 lipids | 400 lipids | 100 lipids | 400 lipids |
| 6-34      | 13.4      | 13.4      | 13.4      | 13.4      | 13.4      | 13.4      | 13.4      | 13.4      | 13.4      | 13.4      |
| 7-33      | 13.4      | 13.4      | 13.4      | 13.4      | 13.4      | 13.4      | 13.4      | 13.4      | 13.4      | 13.4      |
| 8-32      | 13.4      | 13.4      | 13.4      | 13.4      | 13.4      | 13.4      | 13.4      | 13.4      | 13.4      | 13.4      |
| 9-31      | 13.4      | 13.3      | 13.4      | 13.4      | 13.4      | 13.4      | 13.4      | 13.4      | 13.4      | 13.4      |
| 10-30     | 13.5      | 13.4      | 13.4      | 13.5      | 13.4      | 13.3      | 13.3      | 13.4      | 13.4      | 13.5      |
| 13-27     | 13.4      | 13.2      | 13.3      | 13.4      | 13.4      | 13.3      | 13.3      | 13.4      | 13.4      | 13.4      |
| 14-25     | 13.3      | 13.2      | 13.3      | 13.4      | 13.3      | 13.2      | 13.3      | 13.4      | 13.3      | 13.4      |
| 15-24     | 13.4      | 13.4      | 13.4      | 13.4      | 13.4      | 13.4      | 13.4      | 13.4      | 13.4      | 13.4      |
| 16-23     | 13.4      | 13.5      | 13.4      | 13.4      | 13.5      | 13.4      | 13.4      | 13.4      | 13.4      | 13.4      |
| 17-22     | 13.2      | 13.3      | 13.3      | 13.3      | 13.2      | 13.3      | 13.3      | 13.4      | 12.9      | 13.4      |
Abbreviations

mRNA, messenger RNA; IL ionizable lipid; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; DSPC, 1,2-distearoyl-sn-glycero-3-phosphocholine; LPN, lipid nanoparticle; DOPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; SM-102, 8-[(2-hydroxyethyl)6-oxo-6-(undecyloxy)hexyl]amino-octanoic acid, 1-octynonyl ester; ALC-0315, [(4-hydroxybutyl)azanediyl]di(hexane-6,1-diyl) bis(2-hexyldecanoate); APL, area per lipid; CHL, cholesterol; 4CB, four-component bilayer; PME, Particle Mesh Ewald; S_{CD}, deuterium order parameters

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