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Supplemental Information

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The Characterization of Cationic Bolaamphiphile Vesicles for siRNA Delivery into Tumors and Brain
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Supplementary Information

Molecular dynamics (MD) simulations:

The preparation of Force fields (FF) and topology for GLH-19, GLH-20, cholesterol (CHOL), and cholesteryl hemisuccinate (CHEMS): The Amber antechamber package was used to assign general amber force field (GAFF) and topology to GLH-19, GLH-20, CHOL and CHEMS, respectively. Gaussian package was used to perform *ab initio* calculation to obtain electrostatic potential (eps) at each molecular surface. The partial charges of each molecule were obtained by fitting esp of each molecule using the Restrained Electrostatic Potential (RESP) method. Prepared FF, partial charges, and topologies were stored in prep and frcmod files for each molecule. Both files and molecular structure in pdb format were loaded into Amber Leap module to generate topology files for MD simulations.

MD simulation protocols: Each membrane was solvated with the aid of the Amber Leap module. TIP3P water was filled 3.0 nm above and below of the membrane surfaces. Neutralization ions (Cl^-) and extra ions (Na^+ and Cl^-) were added to produce 0.15 M salt concentration. Energy minimization and MD simulations were performed by NAMD package. The solvated system was minimized to remove atomic clashes between the bolas. After minimization, to arrange the hydrophobic atoms properly, a short MD simulation (0.5 ns) was applied to the hydrophobic atoms of the bola, CHOL, and CHEMS while all other atoms were fixed. Additional short MD simulation (0.5 ns) was followed by applying constant pressure on the sides of the membrane patch to remove any empty space in the membrane. The constant pressure was also applied following the 0.5 ns equilibration step. From these simulations, the equilibrated surface areas of each membrane patch was obtained and used for a 100 ns production NPAT (constant numbers of atoms, pressure, surface area, and temperature) simulations. From the 100 ns MD trajectory, two snapshots of the membrane were selected between 80 and 100 ns range. Waters and ions were removed from the selected membranes and an siRNA was placed 12 Å above the membrane surface. These systems were solvated with neutralizing Cl- ions and 0.15 M salt concentration using Amber Leap module. Each system was initially minimized and equilibrated before the 100 ns NPAT simulation was performed. For the data analysis, the last 30 ns (70-100 ns) trajectory was used.

In silico data analysis: The behavior of the bola head groups on the membrane surface was measured in terms of the length variation between the positively charged head group (N atom) and hydrophobic membrane surface (C1 atom) and the angular variation between three atoms, N, C1, and C2 (see Figure. S2). The population density of Cl- ions on the membrane surface was measured by the resident time of Cl- ions within 12 Å cutoff distance from N atoms of the bolas at every 5 ns interval from 50 ns to 100 ns (Figure S2).

The population density of Cl- ions was normalized by the number of N and Cl- atoms in each system. The population density of the bola head groups in the vicinity of a phosphate group (P_i) of the siRNA was calculated by using the equation:

$$P_i = \sum_{n=1}^{N} \frac{T_n}{D_n} \qquad (1)$$

where T_n = time duration of a bola head group located within a cutoff distance (12 Å) from a phosphate P_i, D_n = average distance between the bola head group and the phosphate group (P_i) calculated over last 30 ns, and N is total number of bola head groups within the cutoff distance (Figure. S2 (c)).

Preparation of membrane patch: Prior to building a bola membrane patch, the behavior of single bola of each GLH-19 and GLH-20 was tested using MD simulations. During MD simulation, two different folding patterns were observed. One folding pattern was linear "I" shape which hydrophobic chain was folded while two positively charged hydrophilic head groups were positioned at the ends of hydrophobic chain. The other folding pattern was "U" shape which two hydrophilic head groups were positioned at the same side of the folded hydrophobic chain. Multiple "I" and "U" shapes of structures from MD trajectory were selected and randomly displaced to build membrane patches. For example, "I" shape of bolas were used to build a monolayer membrane patches which hydrophilic head groups were positioned on the top and bottom of membrane surfaces. The "U" shape of bola were used to build bilayer membranes by placing one "U" shape and one reversed "U" shape ("∩") in vertically aligned in pair. This way, hydrophobic chains filled the space between top and bottom surfaces where hydrophilic head groups covered. The mixture of mono- and bilayer was built by placing "I" and U shape of bolas alternatively. The mixture of GLH-19 and GLH-20 was also built by similar manner. According to the experimental conditions, one CHOL and one CHEMS molecules were placed between two bolas. The negatively charged oxygen atom of CHEMS was placed on the membrane surface, while hydrophobic body was aligned along the hydrophobic chains of bolas. CHOL was also placed between bolas in similar manner. The prepared membrane patches were equilibrated by the MD protocols that are described in the Method section in the main text.

Sequences used in this project:

All oligos (both non-labled and fluorescently labeled RNAs and DNAs) listed below were purchased from Integrated DNA Technologies, Inc (IDT) with HPLC purification. Duplexes were prepared by mixing the cognate partners (e.g., sense and antisense stands) at an equimolar ratio in water, heating to 95 °C for 2 min, snap cooling on ice for 2 min, adding 20% volume of 5 X assembly buffer (final concentration: 89 mM TB (pH 8.2), 50 mM KCl, 2 mM $MgCl_2$), and further incubating for 30 min at 30 °C.

DS RNA against GFP[1]

DS RNA sense

5'-pACCCUGAAGUUCAUCUGCACCACCG

DS RNA antisense

5'-CGGUGGUGCAGAUGAACUUCAGGGUCA

Fluorescently labeled Oligos

DS RNA sense 3`-end labeled with AF488

5'-pACCCUGAAGUUCAUCUGCACCACCG-Alexa488

DS RNA antisense 5`-end labeled with AF546

5'-Alexa546-CGGUGGUGCAGAUGAACUUCAGGGUCA

DNA-Sense-AF488

GGAGACCGTGACCGGTGGTGCAGATGAACTTCAGGGTCATT-Alexa488

DNA-Antisense-Iowa Black

Iowa Black Quencher-TGACCCTGAAGTTCATCTGCACCACCGGTCACGGTCTCC

DNA-Sense-IRDye700

GGAGACCGTGACCGGTGGTGCAGATGAACTTCAGGGTCATT-Alexa488

Antisense-Iowa Black

5'-TGACCCTGAAGTTCATCTGCACCACCGGTCACGGTCTCC

$BRAF^{V600E}$

siRNA 5'-GCUACAGAGAAAUCUCGAUUU

Non-targeting siRNA pool (ThermoFisher Scientific, D-001810-10-05)

References:

1. Rose, S. D.; Kim, D. H.; Amarzguioui, M.; Heidel, J. D.; Collingwood, M. A.; Davis, M. E.; Rossi, J. J.; Behlke, M. A. *Nucleic acids research* **2005,** 33, (13), 4140-56.

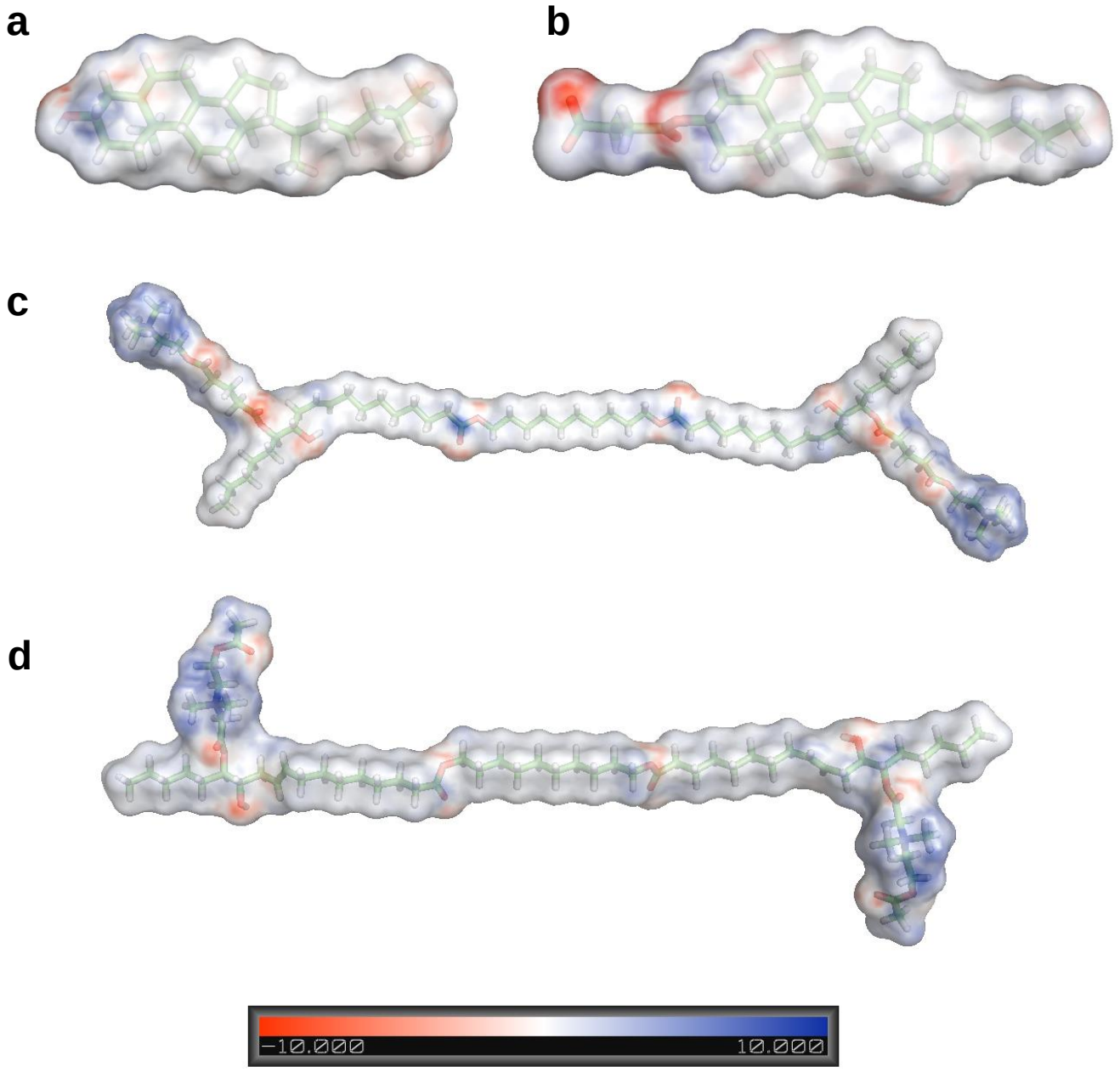
Membrane Type	GLH-19	GLH-20	GLH-19/GLH-20*
Monolayer	• Membrane without CHOL & CHEMS (100 ns) • Membrane with CHOL & CHEMS (100 ns) • Membrane with CHOL, CHEMS, & siRNA (2× 100 ns)	• Membrane without CHOL & CHEMS (100 ns) • Membrane with CHOL & CHEMS (100 ns) • Membrane with CHOL, CHEMS, & siRNA (2× 100 ns)	• Membrane with CHOL & CHEMS (100 ns) • Membrane with CHOL, CHEMS, & siRNA (2× 100 ns)
Bilayer	• Membrane without CHOL & CHEMS (100 ns) • Membrane with CHOL & CHEMS (100 ns) • Membrane with CHOL, CHEMS, & siRNA (2× 100 ns)	• Membrane without CHOL & CHEMS (100 ns) • Membrane with CHOL & CHEMS (100 ns) • Membrane with CHOL, CHEMS, & siRNA (2× 100 ns)	• Membrane with CHOL & CHEMS(100 ns) • Membrane with CHOL, CHEMS, & siRNA (2× 100 ns)
Mono/ bilayer*	• Membrane with CHOL & CHEMS (100 ns) • Membrane with CHOL, CHEMS, & siRNA (2× 100 ns)	• Membrane with CHOL & CHEMS (100 ns) • Membrane with CHOL, CHEMS, & siRNA (2× 100 ns)	• Membrane with CHOL & CHEMS (100 ns) • Membrane with CHOL, CHEMS, & siRNA (2× 100 ns)

Table S1. The list of membranes that are used for in silico studies. Each membrane is tested by 100 ns long MD simulations. Two snapshots from trajectory of membrane simulation are selected to test the association with siRNA (2 × 100 ns).

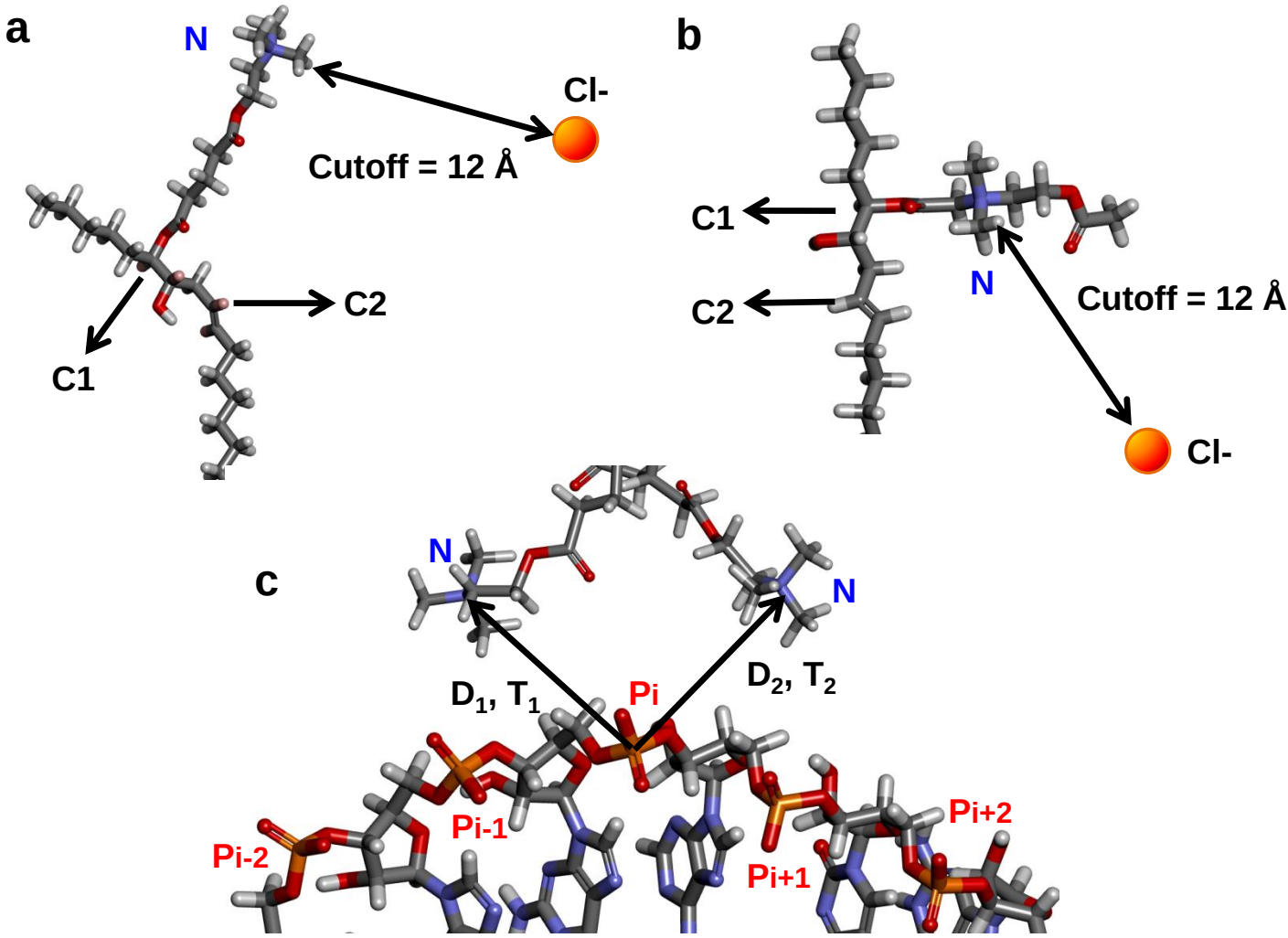
*: Since the role of CHOL and CHEMS on the stability of membrane is tested for monolayer and bilayer membranes, the effect of CHOL and CHEMS absence in the test group of mono/bilayer and GLH-19/GLH-20 are omitted.

Membrane Type		GLH-19	GLH-20	GLH-1920
Monolayer	Surface density of head group (nm^{-2})	0.82 (1.07)*****	0.85 (1.1)*****	0.86
	Head group length (Å)	10.8 ± 1.0	4.6 ± 0.2	10.8 ± 1.0 (GLH-19) 4.6 ± 0.2 (GLH-20)
	Head group bending angle (°)	131.4 ± 24.6	122.5 ± 16.7	130.8 ± 23.7 (GLH-19) 124.2 ± 15.9 (GLH-20)
Bilayer	Surface density of head group (nm^{-2})	1.3 (1.6)*****	1.1 (1.3)*****	1.2
	Head group length (Å)	10.9 ± 1.0	4.6 ± 0.2	10.9 ± 1.0 (GLH-19) 4.6 ± 0.2 (GLH-20)
	Head group bending angle (°)	133.4 ± 23.2	124.6 ± 15.5	134.5 ± 23.1 (GLH-19) 123.7 ± 15.6 (GLH-20)
Mono-/bilayer	Surface density of head group (nm^{-2})	1.1	1.0	1.0
	Head group length (Å)	10.8 ± 1.0	4.6 ± 0.2	10.8 ± 1.0 (GLH-19) 4.6 ± 0.2 (GLH-20)
	Head group bending angle (°)	132.8 ± 23.2	125.8 ± 15.9	134.1 ± 22.6 (GLH-19) 124.7 ± 15.5 (GLH-20)

Table S2. Surface properties of bola membranes. Surface density of head group was obtained by number of bola head groups per unit area (nm^{-2}). The variation of head group length and angle , which are defined in Fig. S2. were monitored for the last 30 ns of each membrane MD trajectory. All these data were obtained from membranes which contained CHOL and CHEMS, except those marked with *****.

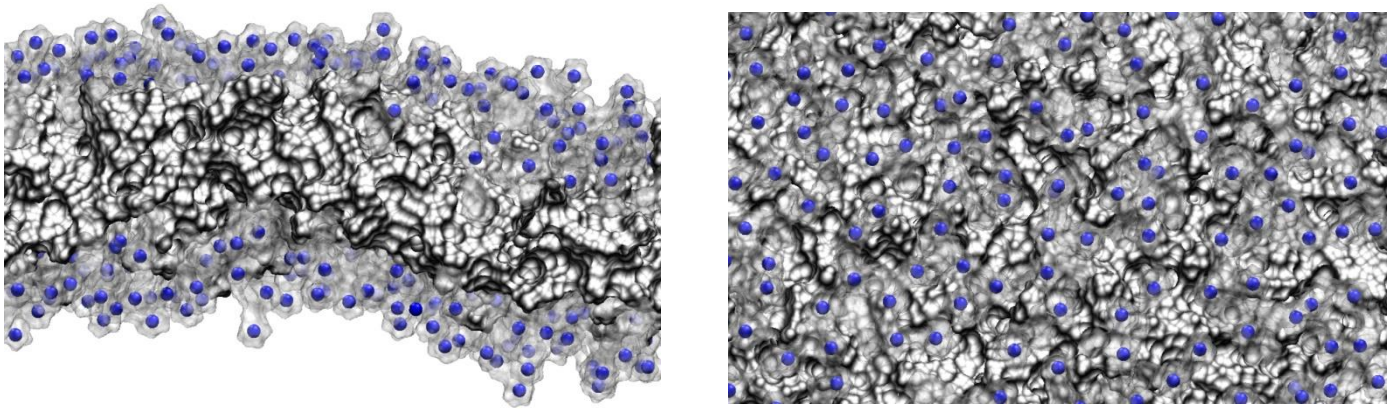


Supporting Figure S1. Atomic structures of **a** cholesterol (CHOL), **b** cholesteryl hemisuccinate (CHEMS), **c** GLH-19, and **d** GLH-20. Electrostatic potential surface of each structure is represented by transparent surface.

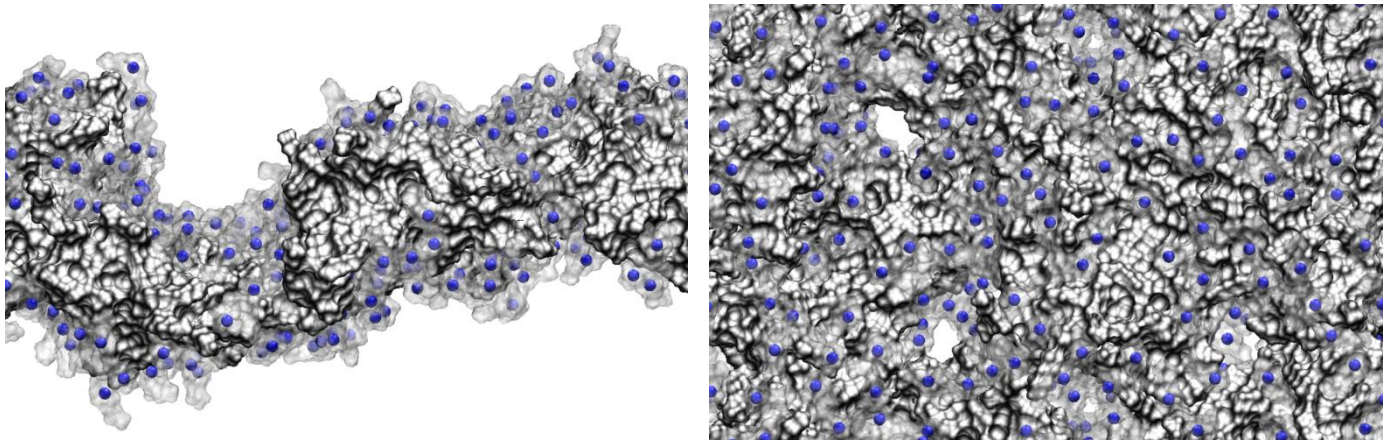


Supporting Figure S2. The atomic structure of **a** GLH19 and **b** GLH20 head group. The mobility of each head group was measured by the distance between N and C1 atoms and the angle between N, C1, and C2 atoms. The population density of Cl- ions on the membrane surface was measured by the resident time of Cl- ions around N atoms of bola within 12 Å for every 5 ns interval from 50 ns to 100 ns. The population density of Cl- ions was normalized by the number of N and Cl- atoms in each system. **c** The population density of bola head group near siRNA phosphate group was given by equation (1). Tn is the resident time (ns) of bola head group stayed within 12 Å from phosphate group, Pi and Dn = average distance between Pi and N atoms (Å). The population density of bola head groups was monitored for the last 30 ns of each simulation (70 – 100 ns).

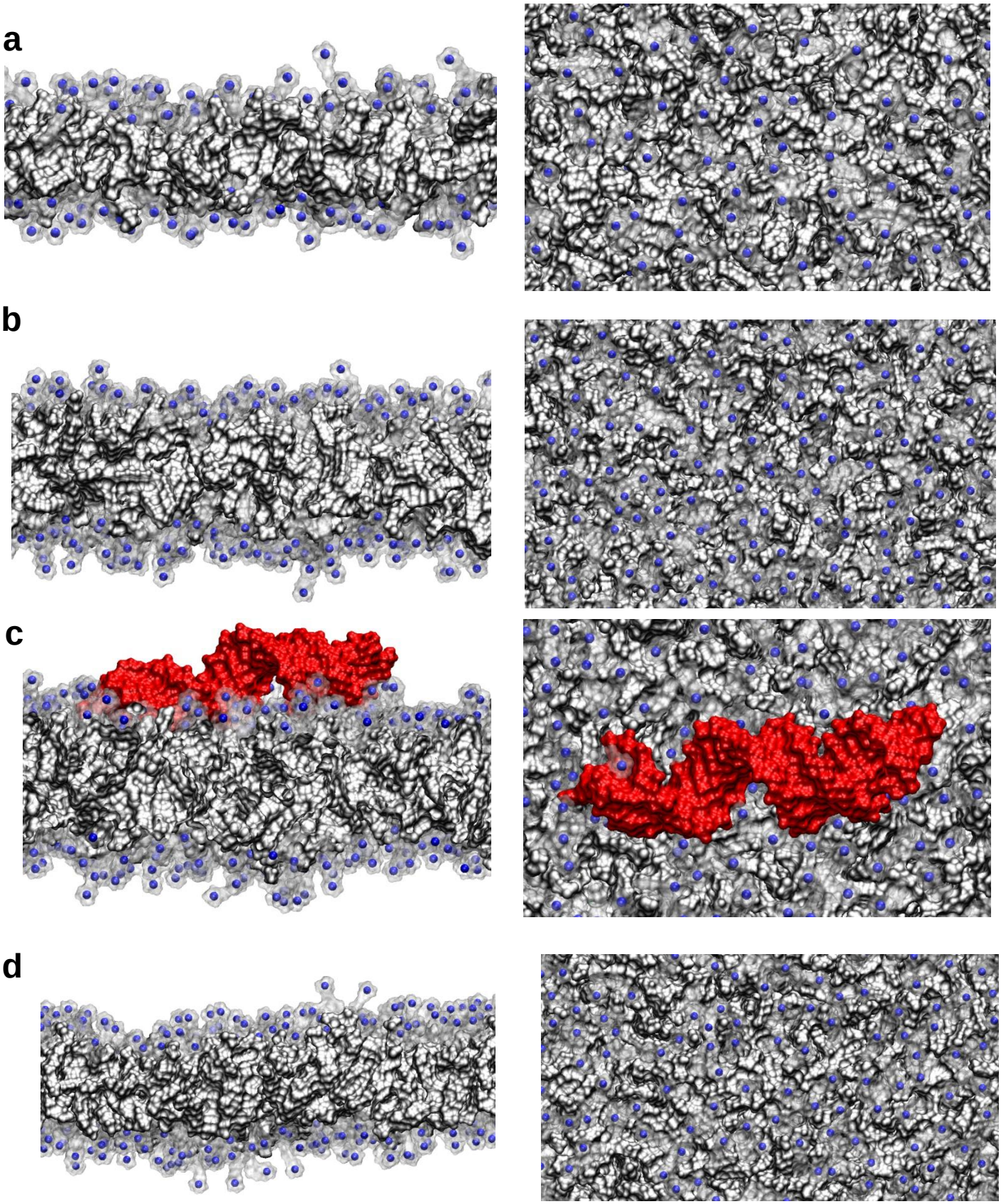
a



b



Supporting Figure S3. The side (left) and top (right) view of membrane snapshots of **a** GLH-19 bilayer and **b** GLH-20 bilayer. These membranes do not contain CHOL and CHEMS. Gray region, transparent surface, blue sphere represent hydrophobic area, head group, and positively charged nitrogen atoms (N) respectively.



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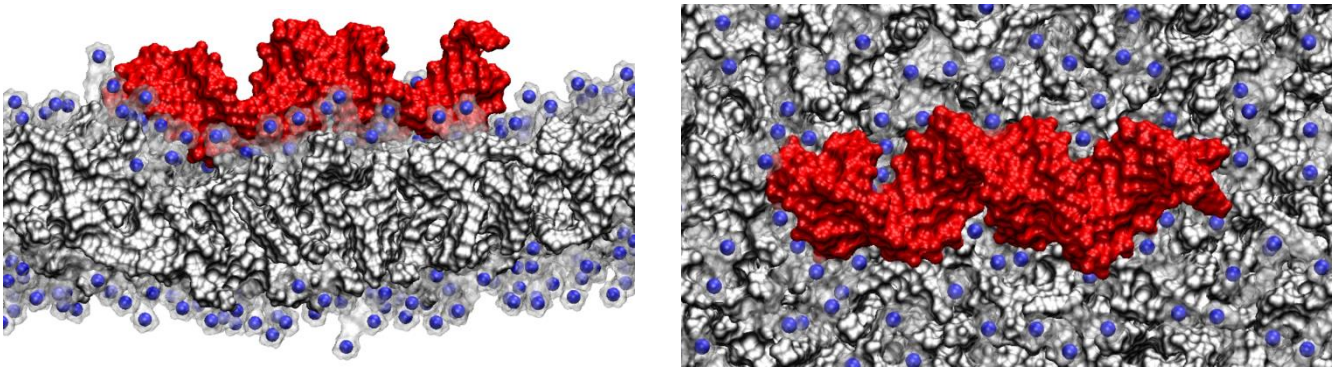
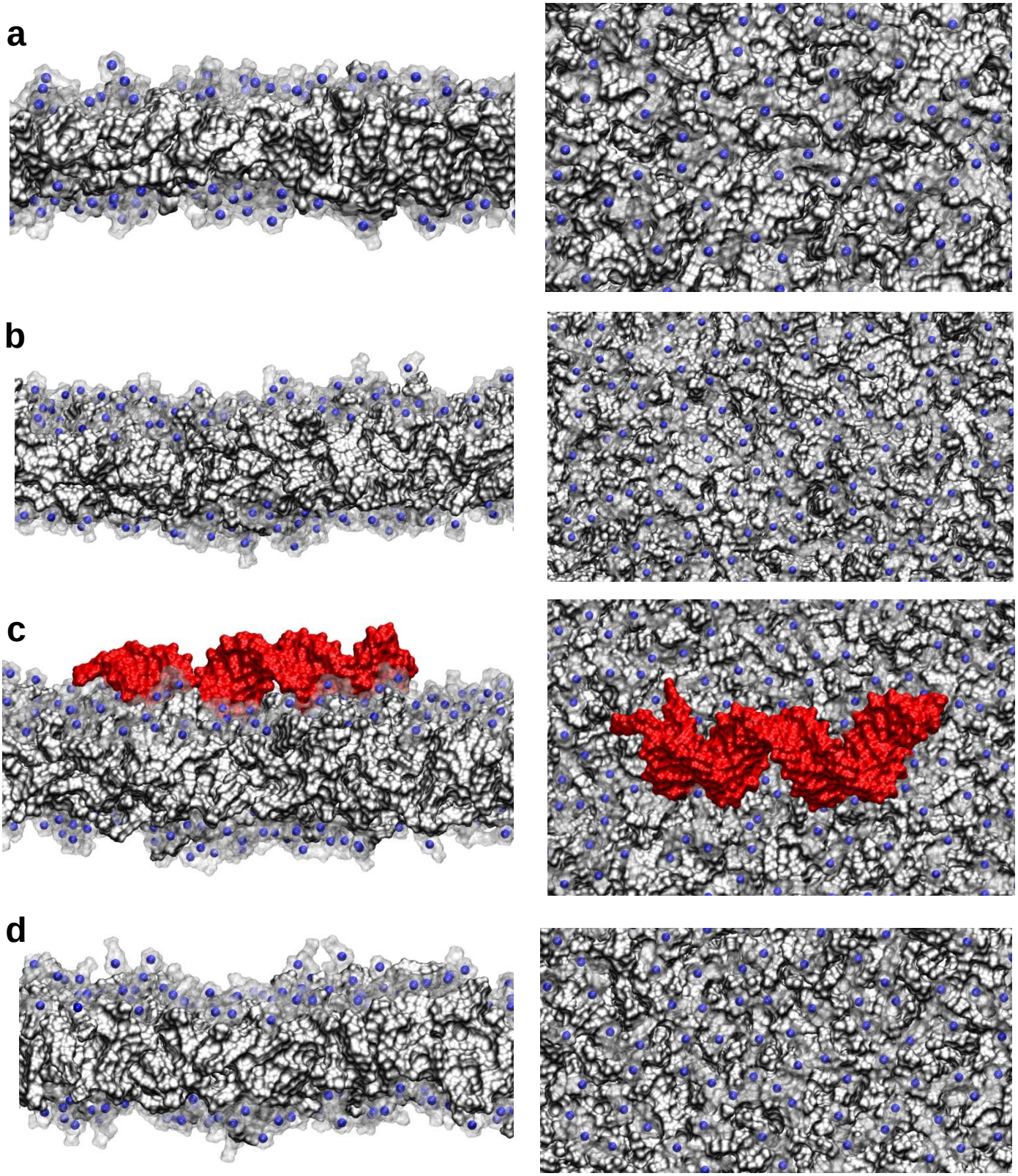


Supporting Figure S4. The side (left) and top (right) view of GLH-19 membrane snapshots of **a** monolayer, **b** monolayer with siRNA, **c** bilayer, **d** bilayer with siRNA, **e** mono/bilayer mixture, and **f** mono/bilayer mixture with siRNA. Membranes contain CHOL and CHEMS. Gray region, transparent surface, blue sphere represent hydrophobic area, head group, and positively charged nitrogen atoms (N) respectively. siRNA is represented by red surface.



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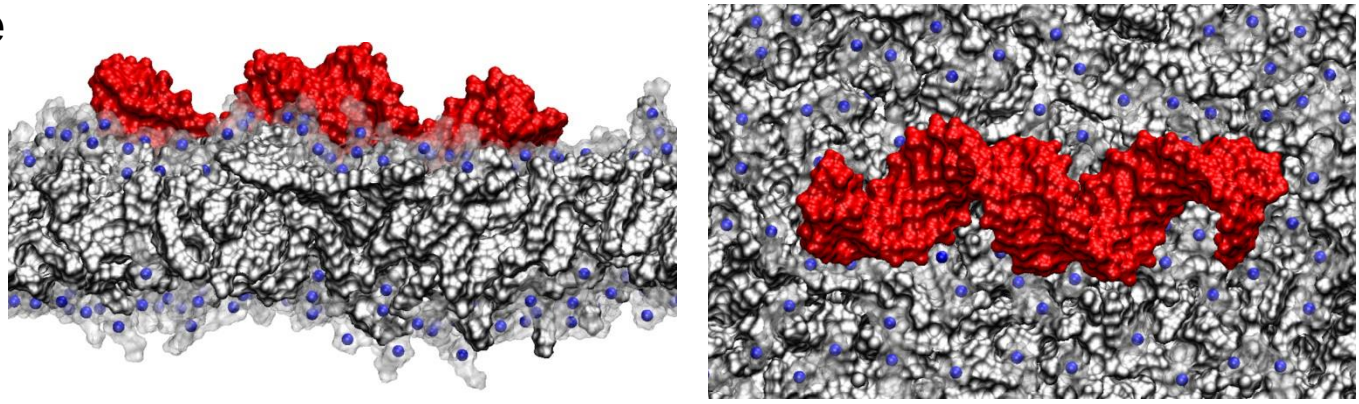
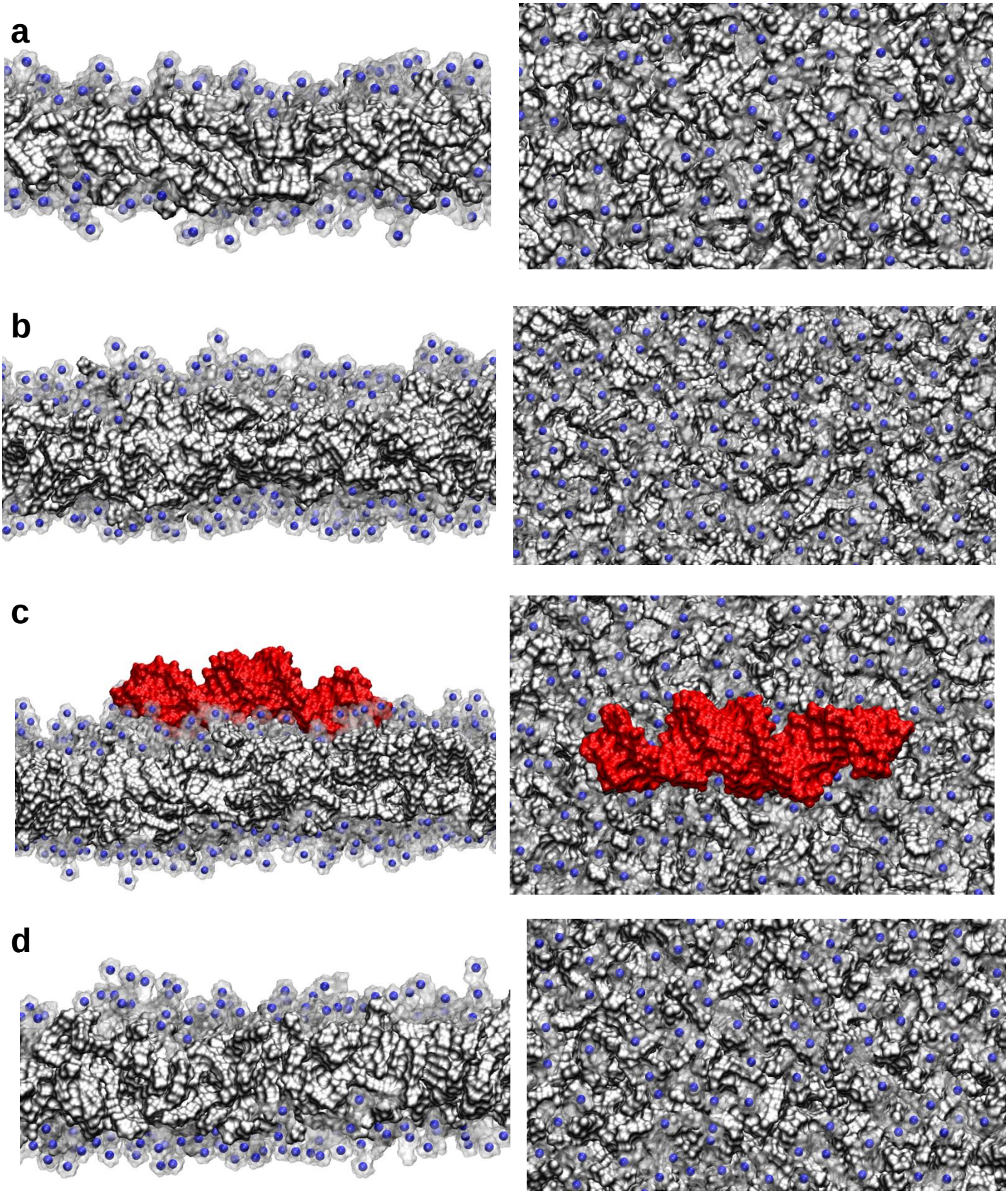
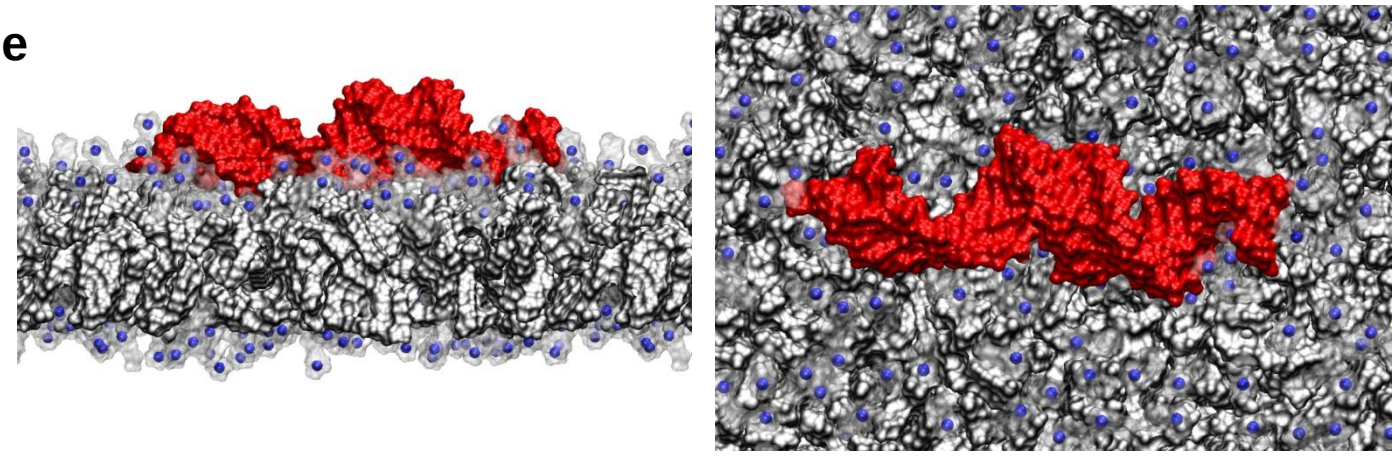


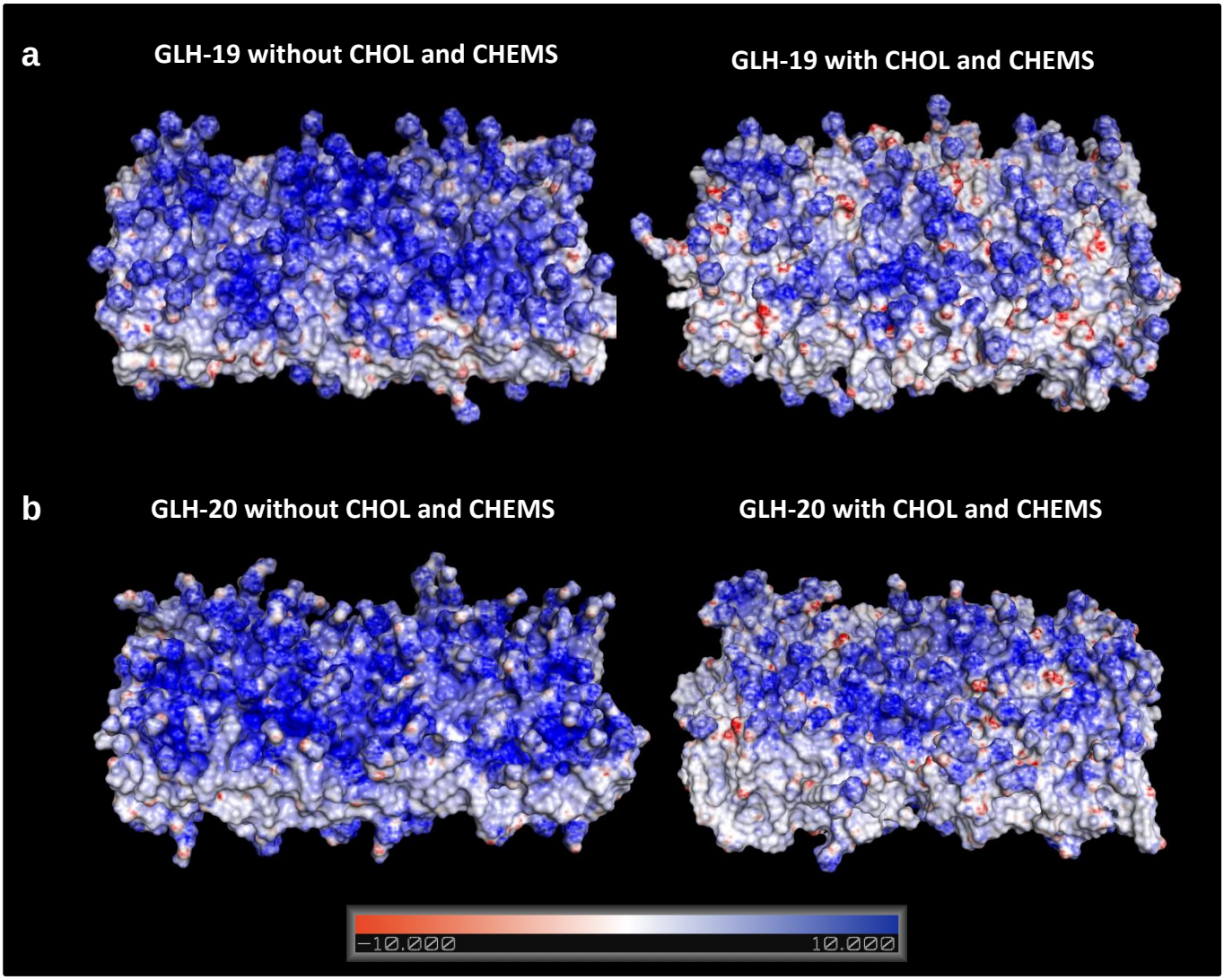
Supporting Figure S5. The side (left) and top (right) view of GLH-20 membrane snapshots of **a** monolayer, **b** monolayer with siRNA, **c** bilayer, **d** bilayer with siRNA, **e** mono/bilayer, and **f** mono/bilayer with siRNA. Membranes contain CHOL and CHEMS. Gray region, transparent surface, blue sphere represent hydrophobic area, head group, and positively charged nitrogen atoms (N) respectively. siRNA is represented by red surface.


a
b
c
d

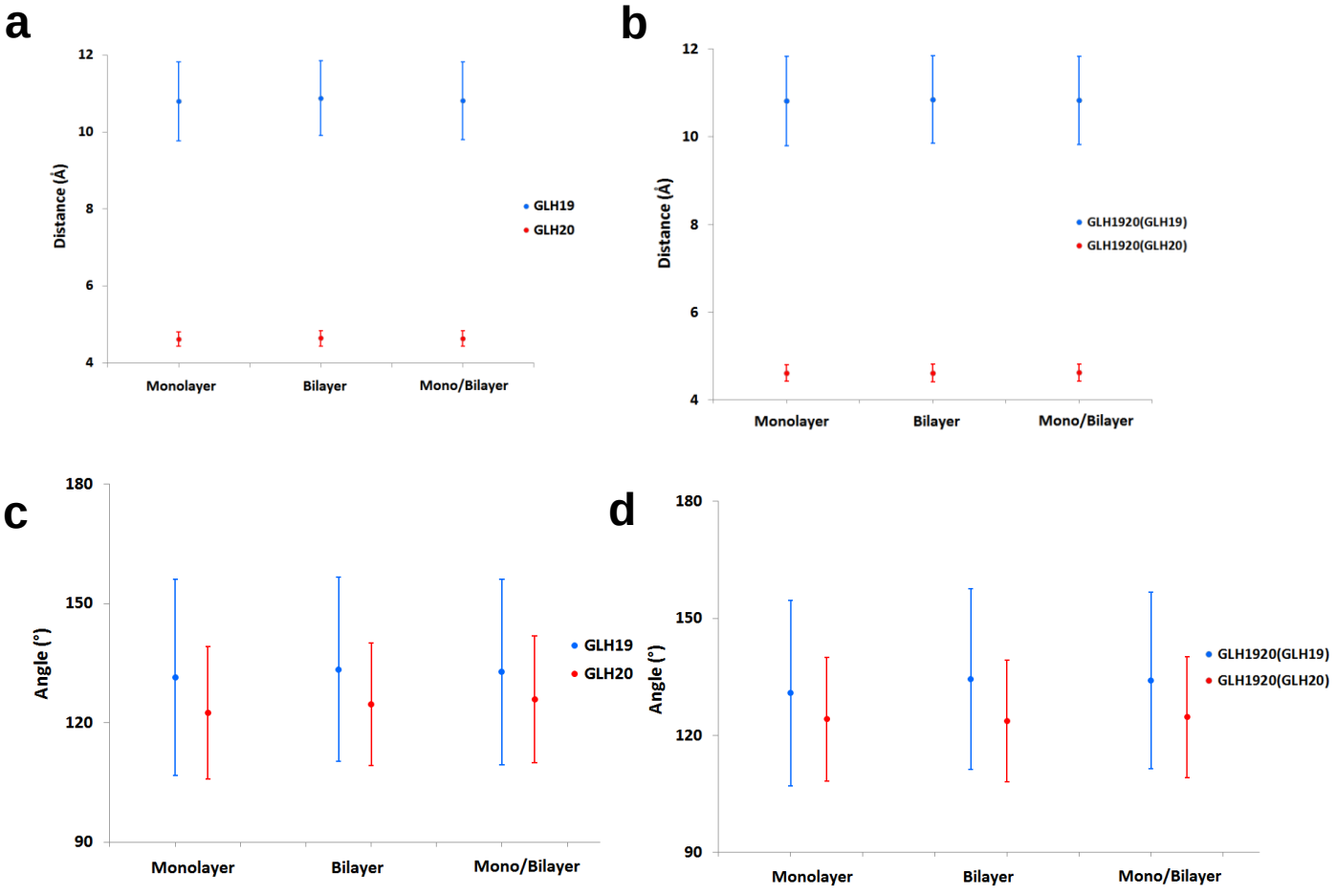
e



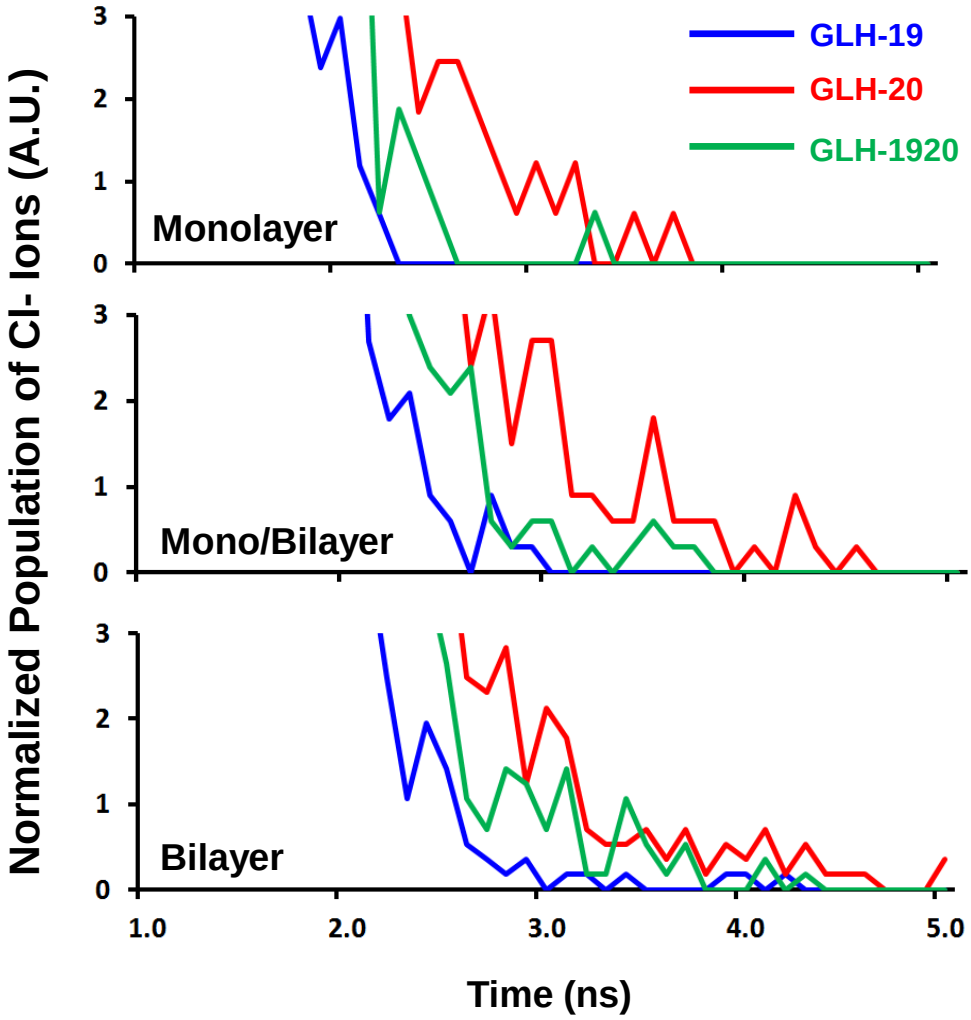
Supporting Figure S6. The side (left) and top (right) view of GLH-1920 membrane snapshots of **a** monolayer, **b** monolayer with siRNA, **c** bilayer, **d** bilayer with siRNA, **e** mono/bilayer, and **f** mono/bilayer with siRNA. Membranes contain CHOL and CHEMS. Gray region, transparent surface, blue sphere represent hydrophobic area, head group, and positively charged nitrogen atoms (N) respectively. siRNA is represented by red surface.



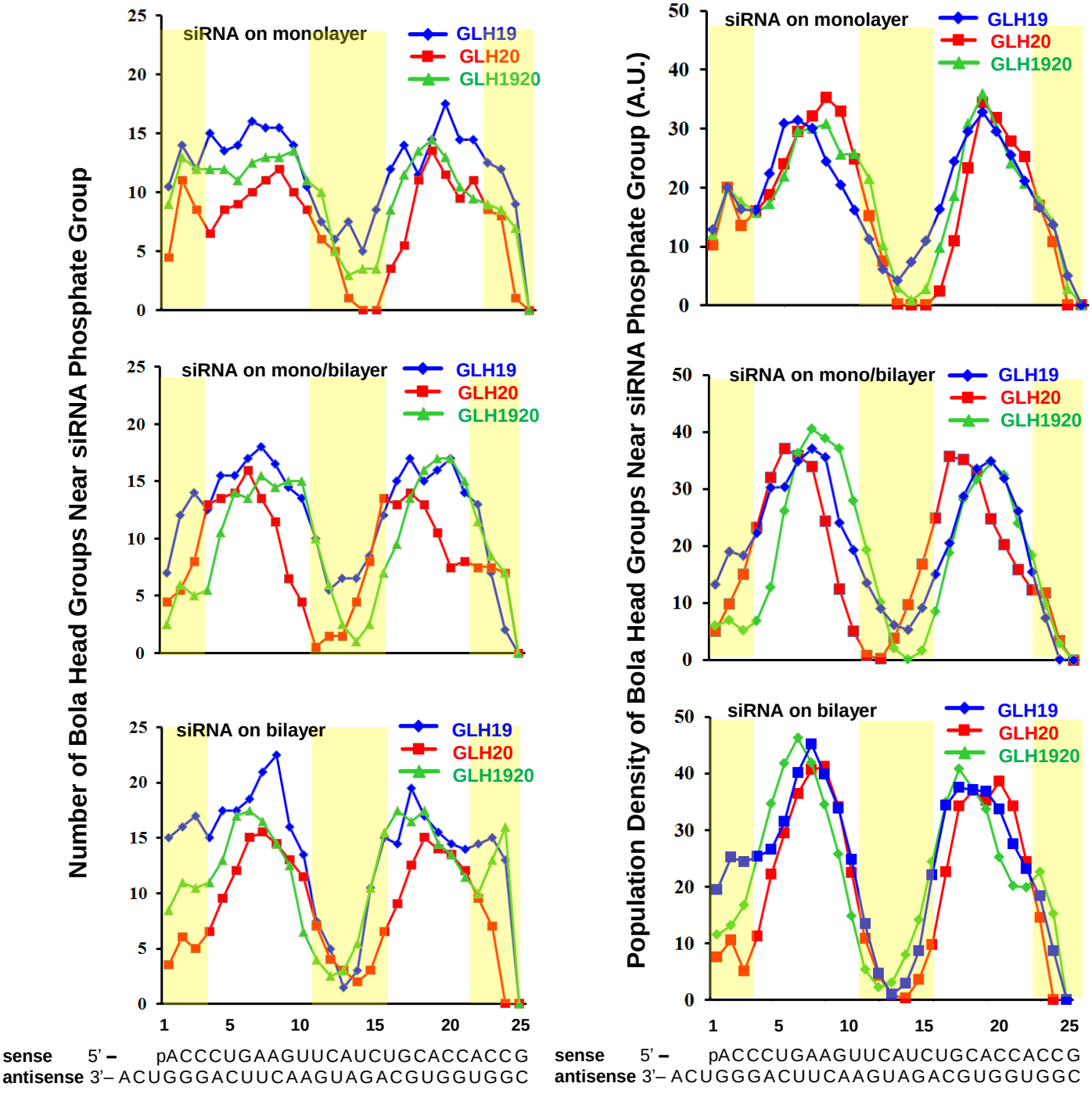
Supporting Figure S7 Electrostatic surface potential of **a** GLH-19 and **b** GLH-20 monolayer membranes. There is no CHOL and CHEMS in membranes in the left panel, while CHOL and CHEMS are mixed with each membrane in the right panel. Red and blue colors indicate negatively charged and positively charged regions, respectively. The CHOL and CHEMS in each membrane significantly reduce the electrostatic repulsion between bola head groups.



Supporting Figure S8. The distribution of bola head groups on membrane surfaces. The distance distributions of N atom above hydrophobic surface in **a** GLH-19 and GLH-20 membranes, **b** GLH-1920 mixture membranes, angular distribution of N atoms in **c** GLH-19 and GLH-20 membranes, and **d** GLH-1920 mixture membranes.



Supporting Figure S9. The population density of Cl- ions in the vicinity of membrane surfaces, monolayer (top), mono/bilayer (middle), and bottom (bilayer). Blue, red, and purple colors indicate GLH-19, -20, and the mixture of two, GLH-1920.

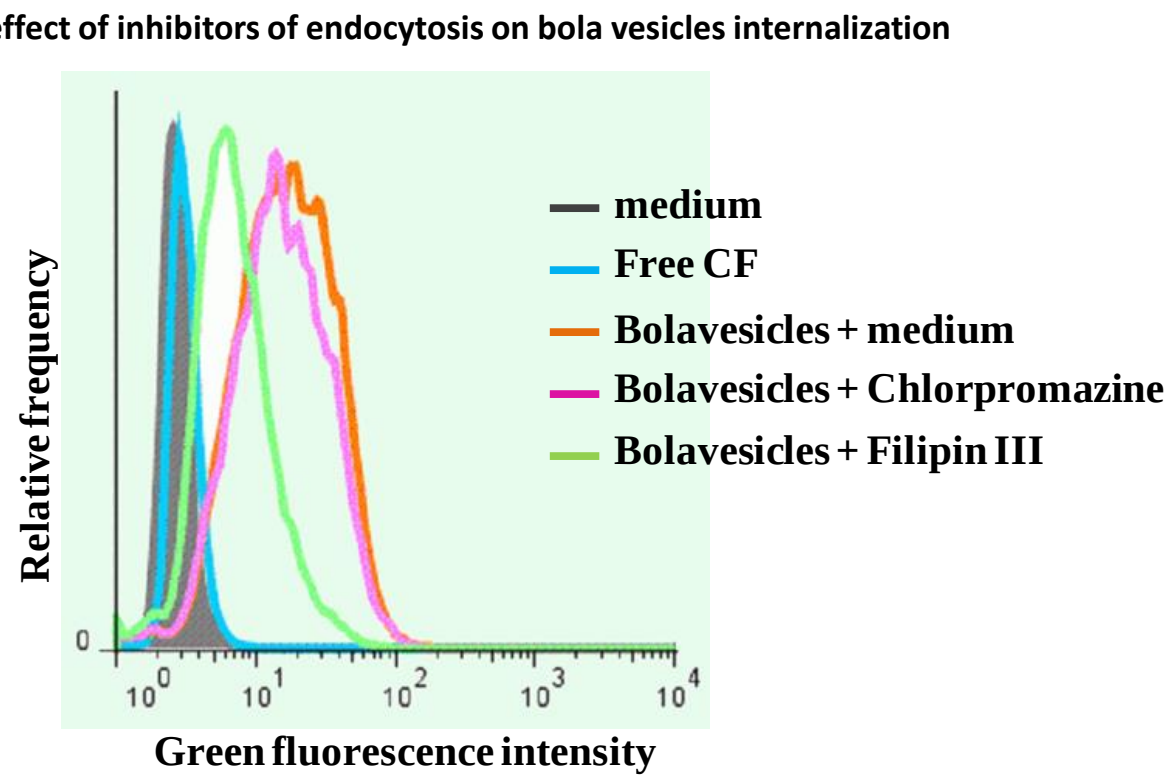


Supporting Figure S10. The number of bola head groups (left panel) and the density of bola head group (right panel) in the vicinity of siRNA phosphate groups. Yellow and white colored regions represent major and minor grooves, respectively. The density of bola head groups were calculated by equation (1) using the last 30 ns of each MD simulation trajectory (70 -100 ns range).

Vesicle size and zeta potential measured by DLS (3 measurements for each vesicle preparation

Vesicle preparation	Diameter in nm (mean ± SEM)	Zeta potential in mV (mean ± SEM)
GLH-19:GLH-20 (2:1) (empty vesicles)	113.1±10.6	37.30±2.90
GLH-19:GLH-20 (2:1) loaded with siRNA	123.2±13.2	32.15±3.79

Supporting Figure S11. Diameter and Zeta potential measured for empty and siRNA loaded bola vesicles.



Supporting Figure S12. Cultured cells were pre-incubated for 1 h with either growth medium alone, or growth medium containing 10 µg/mL chlorpromazine or 1 µg/mL Filipin III. Then cells were incubated for additional 3 hours with the same medium as used for the pre-incubation but this time the medium contained vesicles into which we loaded 0.1 mg/mL carboxyfluorescein (CF). At the end of the incubation the cells were collected and examined by FACS. A shift to the right indicates the degree of vesicle internalization (uptake).