Supplementary Information

Monitoring of the Interaction of Nucleolipoplexes with Model Membranes

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Experimental Section

POPC (1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), POPG (1-Palmitoyl-2-oleoyl-*sn*-phosphatidyl-glycerol), DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) and the fluorescently labeled lipids β-BODIPY® FL C5-HPC (2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4adiaza- s-indacene-3-pentanoyl)-1 hexadecanoyl-sn-glycero-3-phosphocholine) and Liss Rhod PE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt)) were purchased from Avanti Polar Lipids (Alabaster, AL). HCl and NH_3 (33% aqueous solution) used in the synthesis of POP-Ade (1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidyladenosine) were purchased from Fluka (Buchs, Switzerland), the same for Hepes (4-(2-Hydroxyethyl)piperazine-1-ethanesulonic-acid) and $CaCl_2$. MeOH, $CHCl_3$ and DNA (deoxyribonucleic acid sodium salt from calf thymus, type I, highly polymerized) were purchased from Sigma-Aldrich (St. Louis, MO). GFP-DNA was kindly provided by dr. Ruberti (Institute of Cell Biology, National Research Council, Roma, Italy) and oligonucleoltides 50-dA (deoxyriboadenosine) and 50-dT (deoxyribothymidine) were purchased from Atdbio (Southampton, UK) and fluorescently labeled with the Rhodamine labeling kit Label-it® (Mirus Bio Corporation, Madison, WI, USA). POP-Ade was synthesized starting from POPC, Adenosine from Fluka (Buchs, Switzerland) and the enzyme Phospholipase D from Streptomyces sp AA586, purchased from Asahi Chemical Industry (Tokyo, Japan). The synthesis of POP-Ade was carried out in a two phase system, according to a modification of the method proposed by Shuto and co-workers[1-5], and obtained as an ammonium salt. Separation from the by-products was achieved by silica-gel flash chromatography. Purity was checked by thin-layer chromatography and ^{1}H NMR.

Preparation of Vesicles: The 1:4 mol/mol anionic/zwitterionic lipid mixtures were prepared by dissolving the proper amount of POPC or DOPE and POP-Ade in chloroform/methanol 6:1 (v/v).[6, 7] The lipid films were obtained by evaporating the solvent under a stream of nitrogen and overnight vacuum drying. The films were then swollen and suspended in warm (50°C) Hepes buffer 10mM pH 7.4 by vigorous vortex mixing. To prepare Unilamellar vesicles (ULV) with narrow and reproducible size distribution, the dispersions were subjected to 10 freeze-thaw cycles and then extruded 10 times through two stacked polycarbonate membranes with a pore size of 100nm at 50°C. The extrusion was performed with The Extruder by Lipex Biomembranes, Vancouver (Canada), and Nuclepore polycarbonate membranes. Liposomes stained with the fluorescent probe Liss Rhod PE were prepared with the following methodology: Liss Rhod PE or β-BODIPY lipid films were prepared in glass vials by evaporating under N_2 stream the proper amount of a Liss Rhod PE solution in $CHCl_3$, followed by under vacuum drying for two hours. The Liss Rhod PE lipid film was then swollen with the vesicles' dispersions prepared as previously described, vortexed and then stirred overnight, in order to equilibrate the systems. In each liposomes' sample, the Liss Rhod PE amount added was $5x10^{-5}$ % (mol) with respect to total lipid quantity for FCS and the β-BODIPY amount was 0.1% (mol) with respect to total lipid quantity for LSCM measurements.

Preparation of the Complexes: For LSCM and FCS measurements, the stock solutions of DNA and $CaCl_2$ in Hepes buffer and stock solutions of ULV of POP-Ade:POPC 1:4 or POP-ADE:DOPE 1:4 mol/mol ratio in Hepes buffer (non-stained or stained with Liss Rhod PE or β-BODIPY), were diluted with Hepes 10mM (pH 7.4) in order to obtain samples containing: POP-Ade:POPC 1:4 and POP-Ade:DOPE 1:4 liposomes 0.2mg/ml, GFP-DNA (or 50-dA/dT annealed Rhodamine labeled ds-DNA) 2% w/w with

respect to total lipid quantity, $CaCl_2$ 15mM. Nucleolipoplexes were obtained by adding the proper amount of liposomes' solution to the DNA-$CaCl_2$ solution. The samples were incubated at room temperature for twenty minutes and then transferred into the chamber. Ds-50-mer DNA was prepared by annealing the two separate complementary 50-mer oligonucleotides 50-dA and 50-dT, by warming oligonucleotides solution at 60°C for 10 minutes and subsequently cooling down slowly the sample at room temperature. Rhodamine labeling of the ds-50-mer DNA was performed with the Label-it® labeling kit, according to the protocol provided by the manufacturer. For SAXS measurements, the stock solutions of Calf Thymus DNA and $CaCl_2$ in Hepes buffer and stock solutions of ULV of POP-Ade:POPC 1:4 or POP-ADE:DOPE 1:4 mol/mol in H_2O were diluted in order to obtain samples containing: POP-Ade:POPC 1:4 and POP-Ade:DOPE 1:4 liposomes 20mg/ml, DNA 2% w/w with respect to total lipid quantity, $CaCl_2$ 15mM. The samples were incubated at room temperature for twenty minutes, put in a Ø 1,5 mm diameter capillary, centrifuged for thirty minutes at 1000 rpm and kept for 24h at 4°C before measurements.

GUV preparation: Giant Unilamellar Vesicles were obtained through electroformation, as previously described.[8-10] POPC or POPG:POPC 1:1 mol/mol 2.5 mg/ml stock solutions in $CHCl_3$ were prepared and $5x10^{-4}$ % and 0.1%(with respect to the total lipid amount) of the fluorescent dye Liss Rhod PE was added, respectively for FCS and LSCM measurements. 10 µl of the stock solution was deposited on each of two ITO-coated glass slides, on the conductive side. Chloroform was dried under vacuum for two hours and a dry lipid film on each sheet was obtained. The electroformation chamber was prepared sandwiching the sheets with an O-ring separating the lipid films. The chamber was filled with an aqueous solution of Sucrose 60mM, and the electrical contact between the sheets was provided by putting on each sheet a copper tape connected to a pulse generator, set at a sinusoidal alternating voltage of 10 Hz frequency and 2 Vpp amplitude for two hours.

Small Angle X-ray Scattering experiments: SAXS measurements were carried out on a S3-MICRO SAXS/WAXS instrument (HECUS GmbH, Graz, Austria) which consists of a GeniX microfocus X-ray sealed Cu K_α source (Xenocs, Grenoble, France) power 50 W which provides a detector focused X-ray beam with λ = 0.1542 nm Cu Kα line.[5, 7] The instrument is equipped with two one-dimensional (1D) position sensitive detectors (HECUS 1D-PSD-50 M system), each detector is 50 mm long (spatial resolution 54 µm/channel, 1024 channels) and cover the SAXS Q-range ($0.003 < Q < 0.6$ Å^{-1}) and the WAXS Q-range ($1.2 < Q < 1.9$ Å^{-1}). The temperature was controlled by means of a Peltier TCCS-3 Hecus. SAXS spectra were recorded at 25°C.

Laser Scanning Confocal Microscopy and Fluorescence Correlation Spectroscopy experiments: LSCM experiments were carried out with a laser scanning confocal microscope Leica TCS SP2 (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a 63x water immersion objective instrument by using the 561-nm and 496-nm laser line and by acquiring the fluorescent emission between 571-700nm and 506-530 nm, respectively for Liss-Rhod PE and β-BODIPY. FCS measurements were carried out with a ISS module *(ISS,* Inc.1602Newton Drive*Champaign, IL, USA*) equipped with two APD with 500-530nm and 607-683nm BP. FCS measurement were carried out by exciting the fluorescent probe (Liss Rhod PE inside nucleolipoplexes' lipid structure or Rhodamine conjugated to 50-mer ds-DNA) at 561 nm and acquiring the fluorescence emission between 607 and 683nm. The calibration procedure was performed with several solutions of Alexa 568 in the 1-100nM range, considering a value of 332 $\mu m^2 s^{-1}$ for the diffusion coefficient of the fluorescent probe. The confocal volume parameters, w_0 and z_0 were evaluated according to equation 4 (SI Table 1), with <c> the average concentration of the diffusing fluorescent molecule, w_0 and z_0 respectively the lateral and the axial excitation volume parameters, D the diffusion coefficient of the fluorescent molecule. Examples of calibration curves and curve fitting are reported (SI Figure 3).

Nucleolipoplexes*:* 100µl of nucleolipoplexes' solution containing POP-Ade:DOPE 1:4 or POP-Ade:POPC 1:4 liposomes fluorescently labeled with Liss Rhod PE, $CaCl_2$ 15mM and GFP-DNA 2% w/w with respect to lipid quantity, were incubated at room temperature for twenty minutes and then added to a measurement chamber containing 70µl of glucose 60mM and 180µl of sucrose 60mM.

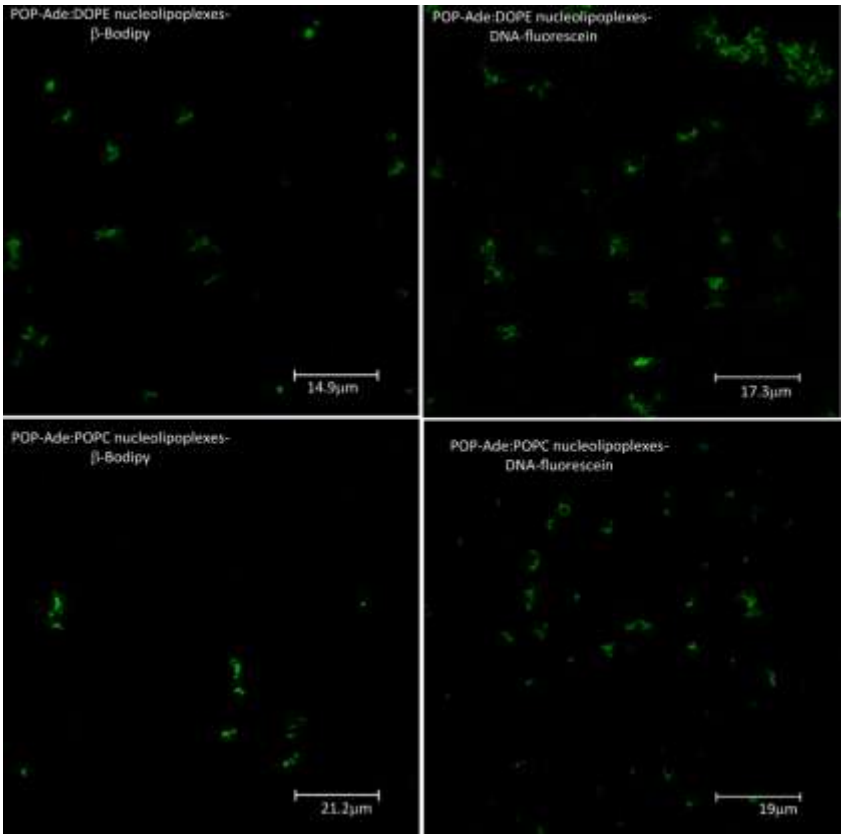
Nucleolipoplexes were allowed to settle on the chamber bottom and equilibrated for two hours at room temperature, in order to acquire the ACFs ascribable to fluorescence fluctuations due to the diffusion of the fluorescent probe within nucleolipoplexes, and to avoid diffusion, traslational or rotational movements of the whole complexes.

GUV: 70µl of GUV dispersions (respectively POPC and POPG:POPC 1:1 GUV) in Sucrose 60mM were put in the chamber (Lab-Tek® Chambered # 1.0 Borosilicate Coverglass System, Nalge Nunc International, Rochester, NY USA) and then diluted adding 180µl of Glucose 60mM. GUVs were allowed to settle on the chamber bottom and equilibrated at room temperature for two hours. FCS measurements were carried out on GUVs' top, and the diffusion coefficient of the fluorescent probe within GUVs' bilayer was estimated with a calibration dependent and a z-scan techniques.

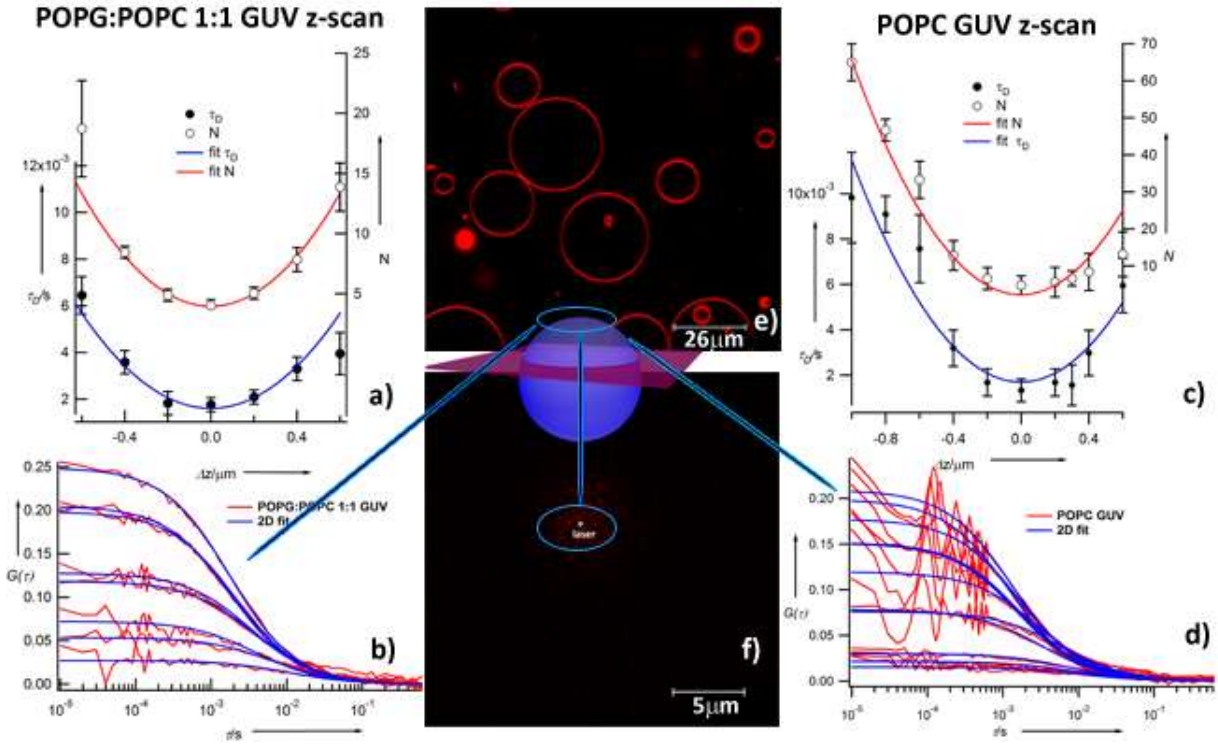
Nucleolipoplexes and GUV: 70µl of GUV dispersion in Sucrose 60 mM were transferred in the measurement chamber (Lab-Tek® Chambered # 1.0 Borosilicate Coverglass System, Nalge Nunc International, Rochester, NY USA) and then diluted adding 180µl of Glucose 60mM. 100µl of nucleolipoplexes' solution containing POP-Ade:DOPE 1:4 or POP-Ade:POPC 1:4 not-stained liposomes (for FCS measurements on Liss Rhod PE diffusion within nucleolipoplexes-GUVs interaction regions) or β-BODIPY-stained liposomes (for LSCM measurements), $CaCl_2$ 15mM and DNA 2% w/w with respect to lipid quantity (GFP-DNA or 50-dA/50-dT annealed Rhodamine-labeled ds-DNA), were incubated at room temperature for twenty minutes and then added to GUV dispersions in the chambers. The samples containing nucleolipoplexes and GUVs were incubated for two hours before acquisition of the images and of the ACFs.

	Model	Equation	Parameters
1)	1D fit	$G(\tau) = \frac{1}{N}\left[1 + \left(\frac{\tau}{\tau_D}\right)^{\alpha}\right]^{-1/2}$	N= number of diffusing particles τ_D= diffusion time of the probe (s)
2)	2D fit	$G(\tau) = \frac{1}{N}\left[1 + \left(\frac{\tau}{\tau_D}\right)^{\alpha}\right]^{-1}$	N= number of diffusing particles τ_D= diffusion time of the probe (s)
3)	2D	$G(\tau) = \frac{1}{N}\left(1 + \frac{4D\tau}{{w_0}^2}\right)^{-1}$	N= number of diffusing particles w0=lateral parameter of volume excitation (μm) D= diffusion coefficient of the probe ($\mu m^2 s^{-1}$)
4)	3D	$G(\tau) = \frac{1}{< c > \pi^{\frac{3}{2}} {w_0}^2 z_0}\left(1 + \frac{4D\tau}{{w_0}^2}\right)^{-1}\left(1 + \frac{4D\tau}{{z_0}^2}\right)^{-\frac{1}{2}}$	<c>= averaged fluorescent molecule concentration (nM) w0=lateral parameter of volume excitation (μm) z0=axial parameter of volume excitation (μm) D= diffusion coefficient of the probe ($\mu m^2 s^{-1}$)
5)	z-scan GUVs (τ_D)	$\tau_D = \frac{{w_0}^2}{4D}\left(1 + \frac{{\lambda_0}^2 \Delta z^2}{\pi^2 n^2 {w_0}^4}\right)$	τ_D= diffusion time of the probe (s) D= diffusion coefficient of the probe ($\mu m^2 s^{-1}$) λ0 =wavelength of the excitation light (μm) Δz= distance between sample position and focal plane(μm) n=refractive index of the medium
6)	z-scan GUVs (N)	$N = \pi c {w_0}^2\left(1 + \frac{{\lambda_0}^2 \Delta z^2}{\pi^2 n^2 {w_0}^4}\right)$	N= number of diffusing particles D= diffusion coefficient of the probe ($\mu m^2 s^{-1}$) λ0 =wavelength of the excitation light (μm) Δz= distance between sample position and focal plane(μm) n=refractive index of the medium c= averaged fluorescent molecule surface concentration
7)	1D-2D two components fit	$G(\tau) = \frac{f1}{N}\left[1 + \left(\frac{\tau}{\tau_{D1}}\right)\right]^{-1/2} + \frac{(1-f1)}{N}\left[1 + \left(\frac{\tau}{\tau_{D2}}\right)\right]^{-1}$	N= number of diffusing particles f_1= molar fraction of particles with 1D diffusion time τ_{D1} $(1-f_1)$= molar fraction of particles with 2D diffusion time τ_{D2} $\tau_{D1,2}$= diffusion time (s)
8)	2D-2D two components fit	$G(\tau) = \frac{f1}{N}\left[1 + \left(\frac{\tau}{\tau_{D1}}\right)\right]^{-1/2} + \frac{(1-f1)}{N}\left[1 + \left(\frac{\tau}{\tau_{D2}}\right)\right]^{-1/2}$	N= number of diffusing particles f_1= molar fraction of particles with 2D diffusion time τ_{D1} $(1-f_1)$= molar fraction of particles with 2D diffusion time τ_{D2} $\tau_{D1,2}$= diffusion time (s)
9)	3D-3D two components fit	$G(\tau) = \frac{1}{< c > \pi^{\frac{3}{2}} {w_0}^2 z_0} f_1\left[\left(1 + \frac{4D_1\tau}{{w_0}^2}\right)^{-1}\left(1 + \frac{4D_1\tau}{{z_0}^2}\right)^{-\frac{1}{2}}\right] + f_2\left[\left(1 + \frac{4D_2\tau}{{w_0}^2}\right)^{-1}\left(1 + \frac{4D_2\tau}{{z_0}^2}\right)^{-\frac{1}{2}}\right]$	<c>= averaged fluorescent molecule concentration (nM) w0=lateral parameter of volume excitation (μm) z0=axial parameter of volume excitation (μm) f1=molar fraction of the species with diffusion coefficient D1 ($\mu m^2 s^{-1}$) f2= molar fraction of the species with diffusion coefficient D2 ($\mu m^2 s^{-1}$)

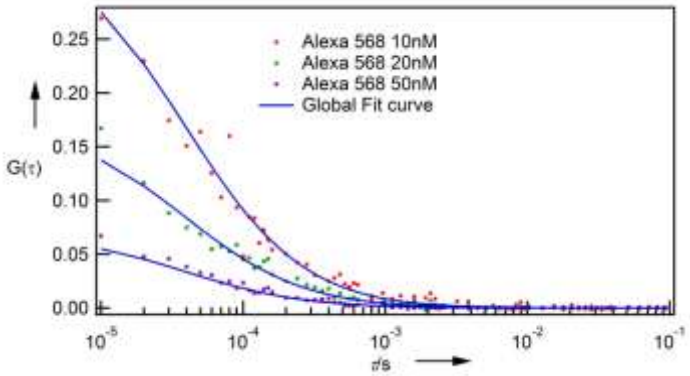
SI Table 1. Fitting models employed in the analysis of the ACFs acquired for POPC and DOPE vectors, GUVs, labeled-DNA diffusion outside-inside GUVs, nucleolipoplexes-GUVs interaction regions. [11-16]



SI Figure 1. DOPE and POPC nucleolipoplexes prepared as described in the experimental section, fluorescently labeled respectively with the fluorescent lipid β-Bodipy inserted within the liquid crystalline phase structure and with a Fluorescein tag conjugated to DNA, employing the Fluorescein labeling kit Label-it® (Mirus Bio Corporation, Madison, WI, USA).



SI Figure 2. POPC and POPG:POPC 1:1 Giant unilamellar vesicles. LSCM images (e,f). FCS Autocorrelation functions of the fluorescence intensity measured at different z-positions of the focal plan (continuous red lines) and 2D normal diffusion (α=1) fit (equation 2SI Table 1) (continuous blue line) (b,d). N (open circles) and τ_D (filled circles) values with respect to z-position, curve fit of N (continuous red line, equation 6 SI Table 1) and of τ_D (continuous blue line, equation 5 SI Table 1) (a,c). FCS measurements on GUVs were carried out on GUVs top, as represented in LSCM images (e, f) and the estimation of the diffusion coefficient *D* of the probe was carried out with a calibration-dependent and with the z-scan techniques. The first one consists in finding the right z-position of the focal plan with respect to GUVs' bilayer plan on the top of the GUVs by finding the absolute maximum fluorescence intensity along the z-axis. Z-scan technique is a calibration free methodology, that allows taking into account possible artifacts due to calibration procedure and it was carried out by measuring a set of FCS curves at various z positions of the focal plane and the bilayer spaced by 0.2μm. The obtained particle number (*N*) and the diffusion time τ_D parabolic dependence on the z position of the objective were fitted with equations 5 and 6 (SI Table 1) where *D* is the lateral diffusion coefficient, c is the average surface concentration of diffusing fluorescent molecules in the illuminated area, n is the refractive index of the medium, $\lambda 0$ is the wavelength of the excitation light, and Δz is the distance between the sample position and the focal plane. The experimental data and fitting curves obtained with z-scan methodology respectively for POPC and POPG:POPC 1:1 GUVs are reported (a, b, c, d).

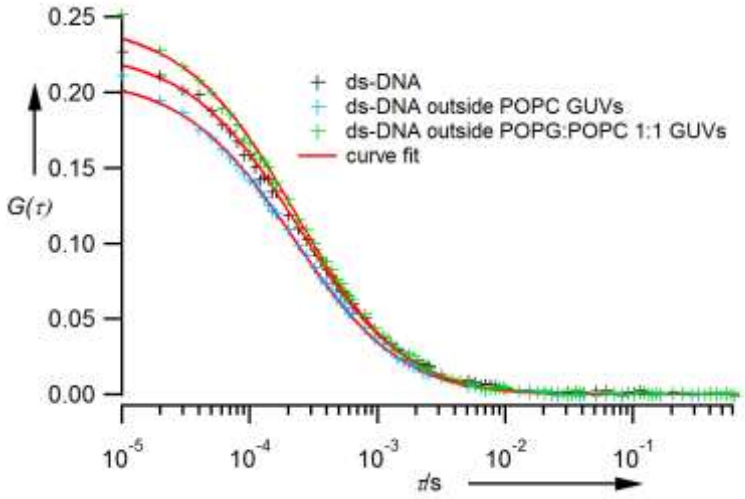


SI Figure 3. Calibration procedure carried out with three aqueous solutions of the Alexa 568 with known concentrations: 10nM; 20nM; 50nM. Alexa 568 diffusion coefficient D=332μm²s⁻¹. From the fitting procedure (equation 4 SI Table 1) excitation volume parameters were evaluated: w_0=0.2268 μm±0.006μm z_0=1.002μm±0.03 μm, being the corresponding volume evaluated as: $V_{ex} = \pi\sqrt{\pi}w_0^2 z_0 \approx 0.287\mu m^3$, which shape is approximated as a 3D Gaussian function.

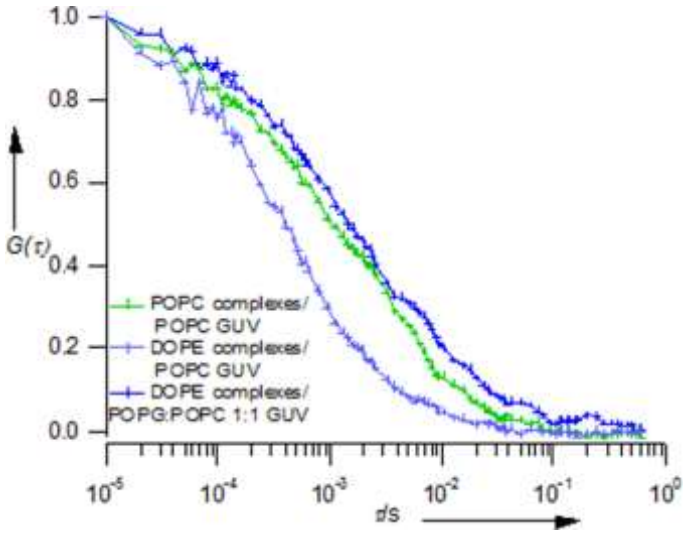
	D ($\mu m^2 s^{-1}$)	T_D (s)
POPC GUVs[a]	8.24±1	$1.32x10^{-3}\pm 8x10^{-5}$
POPC GUVs[b]	9.97±0.3	$1.26x10^{-3}\pm 4x10^{-5}$
POPG:POPC GUVs[a]	7.87±0.3	$1.75x10^{-3} \pm 9x10^{-5}$
POPG:POPC GUVs[b]	6.98±0.4	$1.80x10^{-3} \pm 1x10^{-4}$

[a] z-scan measurement [b] calibration-dependent measurement

SI Table 2. POPC and POPG:POPC 1:1 GUV 2D fit results, obtained through z-scan free-calibration methodology (equation 6 SI Table 1) and calibration-dependent methodology (equation 3 SI Table 1). Fitting results reported as an average of at least 10 measurements carried out in two independent experiments.



SI Figure 4. Rhodamine-labeled 50-mer-ds-DNA. Representative FCS curves acquired for DNA (black markers), DNA outside POPC GUVs' lumen (light blue markers) and outside POPG:POPC 1:1 GUVs' lumen (green markers) after two hours incubation at r.t. Fitting curves (red continuous lines) according to a 3D normal diffusion model (equation 4 SI Table 1), allowed after calibration with Alexa 568 (as reported in SI Figure 3) the evaluation of the diffusion coefficient of DNA, as: D (bare DNA)=46.4±3μm^{2}s^{-1}; D (DNA incubated with POPC GUVs, outside GUVs lumen)=49.2±1μm^{2}s^{-1}; D (DNA incubated with POPG:POPC 1:1 GUVs, outside GUVs lumen)=48.5±2μm^{2}s^{-1}, while fluorescence intensity inside GUVs was found negligible.

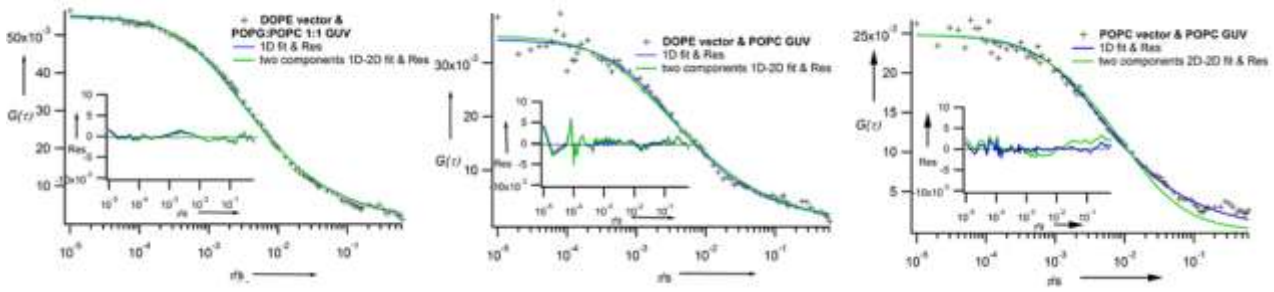


SI Figure 5. Rhodamine-labeled 50-mer-ds-DNA in POPC (green line and markers) and DOPE (light blue and dark blue lines and markers) nucleolipoplexes incubated with GUVs. Representative normalized FCS curves acquired inside POPC and POPG:POPC 1:1 GUVs.

	D_2 (μm^{2}s^{-1})	D_2 (μm^{2}s^{-1})	f_2	% In/out

	(average)	(variability)		fluorescence
DOPE complexes outside GUVs	2.6±0.7	/	0.52±0.2	/
POPC complexes outside GUVs	2.6±0.9	/	0.45±0.2	/
DOPE complexes inside POPC GUVs	2.78	0.4-46.4	0.42±0.08	40.1±3
DOPE complexes inside POPG:POPC 1:1 GUVs	17.9	0.73-46.4	0.76±0.2	23.1±15
POPC complexes inside POPC GUVs	22.5	3.03-46.4	0.90±0.2	35.6±3

SI Table 3. Rhodamine-labeled 50-mer-ds-DNA inside nucleolipoplexes. Diffusion coefficient of DNA complexed by POP-Ade:DOPE liposomes and by POP-Ade:POPC liposomes inside and outside GUVs. Experimental curves were fitted according to a two component 3D normal diffusion model (equation 9 SI Table 1), in the hypothesis of a partially bound DNA. To improve the robustness of the fitting D_1 diffusion coefficient was kept fixed to the value of free DNA (46.4 $\mu m^2 s^{-1}$;). D_2 reported values refer to the diffusion coefficient of bound DNA, being f_2 the fraction of bound DNA with respect to free DNA. The variability of DNA diffusion mode inside different GUVs incubated with nucleolipoplexes is reported as an average value and a range from minimum (large complexes) to maximum (free DNA) value. The DNA penetration degree inside GUVs is reported as the fluorescence intensity inside GUVs lumen/outside GUVs lumen percentage ratio. Data reported result from an average of twenty measurements carried out in two independent experiments.



SI Figure 5. POP-Ade:DOPE 1:4-Ca^{2+} 15mM-DNA nucleolipoplexes (DOPE vectors) and POP-Ade:POPC 1:4-Ca^{2+} 15mM-DNA nucleolipoplexes (POPC vectors) incubated for two hours with POPG:POPC 1:1 and POPC GUVs fluorescently labeled with Liss Rhod PE. ACFs acquired in the interaction region between nucleolipoplexes and GUVs (black markers), normal ($\alpha = 1$) one component 1D (continuous blue line, equation 1 SI Table 1) and two components 1D-2D and 2D-2D(continuous green line, equation 7, 8 SI Table 1)curve fit and residuals.

	DOPE complexes POPC:POPG GUVs	**DOPE complexes POPC GUVs**	**POPC complexes POPC GUVs**
1D T_D (s) [a]	$2.06x10^{-3} \pm 6x10^{-4}$	$2.00x10^{-3} \pm 1x10^{-3}$	$1.54x10^{-3} \pm 6x10^{-4}$
2D an. T_D (s)[b]	$6.4x10^{-3} \pm 3x10^{-3}$	$6.2x10^{-3} \pm 3x10^{-3}$	$3.8x10^{-3} \pm 1x10^{-3}$
2D an. α[b]	0.7± 0.1	0.8 ± 0.1	0.7 ± 0.11

[a] normal diffusion (α=1) [b] anomalous diffusion model (α≠1)

SI Table 4. POP-Ade:DOPE-Ca^{2+} 15mM-DNA nucleolipoplexes incubated with POPG:POPC 1:1 GUV and with POPC GUV ; POP-Ade:POPC-Ca^{2+} 15mM-DNA nucleolipoplexes incubated with POPC GUV. 1D (equation 1 SI Table 1) normal diffusion ($\alpha = 1$) and 2D (equation 2 SI Table 1) anomalous diffusion ($\alpha \neq 1$) fit results. Fit results reported are an average of 15 measurements carried out in three independent experiments.

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