Supporting Information

# Dissociation of Fluorescently Labeled Lipids from Liposomes in Biological Environments Challenges the Interpretation of Uptake Studies

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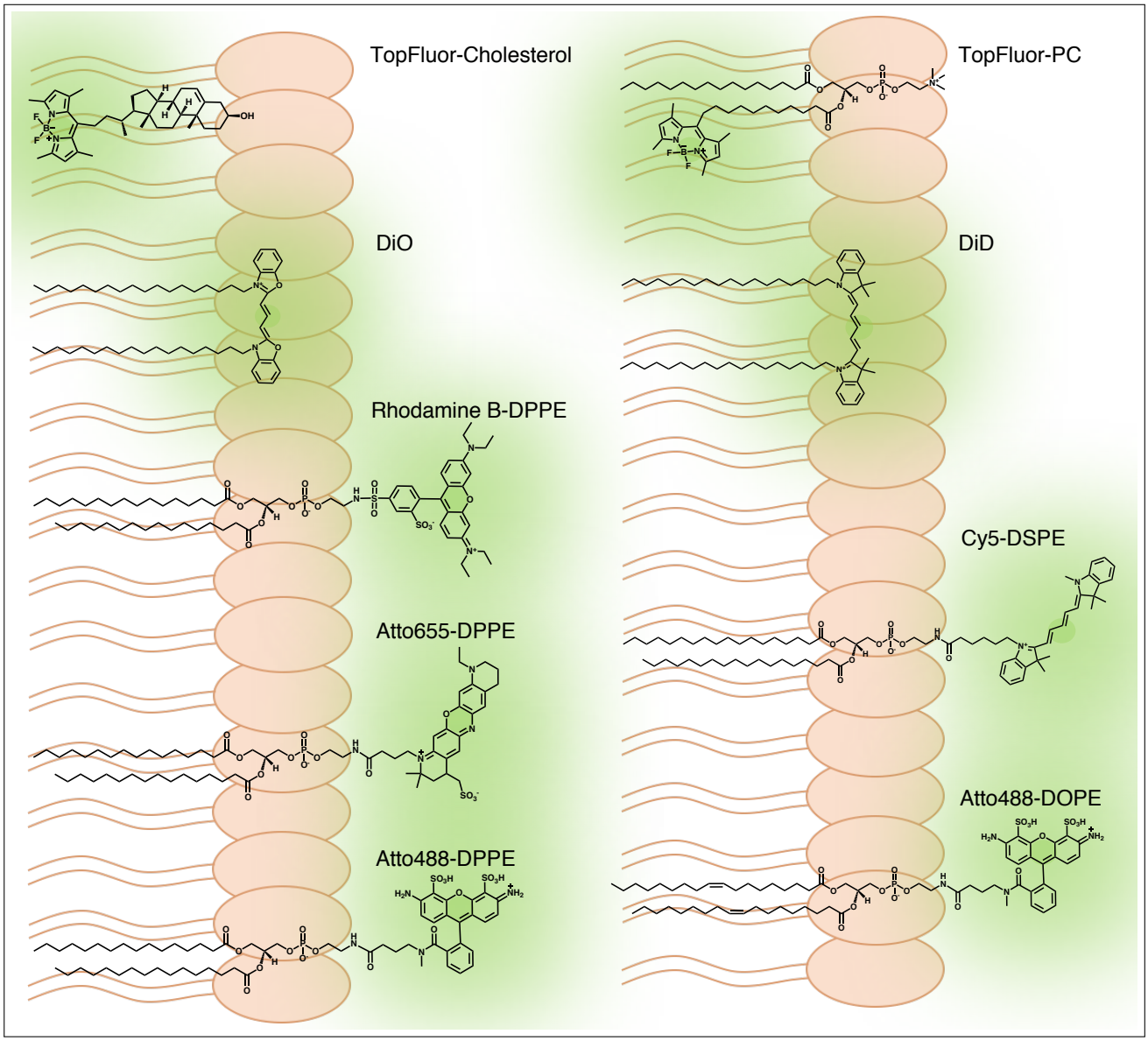


Figure S1: Structures of the FLLs included in the study. Structures were obtained from the supplier's homepage where available; structures of the Atto FLLs were inferred from the individual lipid and fluorophore structures. The fluorochrome in each structure is highlighted in green.

# SI 1 Materials and Methods

## SI 1.1 Materials

All chemicals for the HEPES buffer, inductively coupled plasma mass spectrometry (ICP-MS) diluent, and zeta-potential buffer were acquired from Sigma Aldrich (Brøndby, Denmark). 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and Cholesterol were acquired from Lipoid (Ludwigshafen, Germany). 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (DSPE-PEG2000), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000]) (DOPE-PEG2000), 1-palmitoyl-2-(dipyrromethene boron difluoride)undecanoyl-sn-glycero-3-phosphocholine) (TopFluor-PC), 23-(dipyrromethene boron difluoride)-24-norcholesterol (TopFluor-Cholesterol), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (RhodamineB-DPPE) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(Cyanine 5) (Cy5-DSPE) were acquired from Avanti Polar Lipids (Alabaster, AL, US). Atto488-DOPE, Atto488-DPPE, and Atto655-DPPE were acquired from ATTO-

TEC (Siegen, Germany). 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine (DiD) was acquired from Thermo Fisher Scientific (Waltham, MA, US). 3,3'-Dioctadecyloxacarbocyanine (DiO) was acquired from Sigma Aldrich. BSA, BSA-Biotin and Streptavidin for the single liposome assays were acquired from Sigma Aldrich.

### SI 1.2 Collection of Human Plasma

Blood was drawn by certified staff from healthy donors under signed consent. The identities of the donors were unknown to the researchers performing the experiments. Blood was collected in BD Vacutainer EDTA tubes (Becton Dickinson, Franklin Lakes, NJ, US). The blood was transferred to 2 mL Protein LoBind Eppendorf tubes (Hamburg, Germany) and centrifuged at 3000 $g$ for 15 minutes in order to separate cells from plasma. The plasma supernatant was transferred to fresh LoBind tubes and stored at 4 °C for minimum 24 h and maximum 96 h before usage.

### SI 1.3 Liposome Preparation

Lipids in powder forms were dissolved in *tert*-butanol:MQ water 9:1, mixed to the desired lipid compositions in glass vials and freeze-dried overnight. The dry lipids were re-hydrated in HEPES buffer (10 mM HEPES, 150 mM NaCl buffer, pH 7.4) to a concentration of 50 mM total lipid and put under 65 °C heating and magnet stirring for minimum 1 hour. The size of the liposomes were controlled by extruding 21 times through a 100 nm Whatman filter (GE Healthcare, Little Chalfont, UK) using an Avanti mini-extruder (Avanti Polar Lipids) on a heating block at 65 °C. The liposomes were transferred to a new glass vial and stored at 4 °C.

The composition of the saturated liposomes where DSPC:Cholesterol:DSPE-PEG2000 (molar ratio 56.6:38.2:5.2) and the composition of the unsaturated liposomes POPC:Cholesterol:DOPE-PEG2000 (molar ratio 56.6:38.2:5.2). 0.1 mol% FLL was added to these formulations.

### SI 1.4 Liposome Characterization

Total lipid concentration of the liposome stocks was determined by measuring the phosphorus concentration using ICP-MS. Samples were diluted 10,000 times in an ICP-MS diluent (2% HCl, 10 ppb Ga) to fall within a standard range of 25-100 ppb phosphorus, and the phosphorus content was measured on an ICAP-Q from Thermo Fisher Scientific. The lipid concentration was calculated based on the assumption that 61.8 % of the lipids in our formulations contain a phosphorus atom.

The hydrodynamic diameter and polydispersity index (PDI) of the liposomes were measured by dynamic light scattering (DLS) using a ZetaSizer Nano ZS from Malvern Instruments (Malvern, Worcestershire, UK), equipped with a 633 nm laser. The liposomes were diluted to about 120 µM total lipid in HEPES buffer, and the size measured as the average from 3 runs of 15 cycles. The zeta potential of the liposomes was measured using the same instrument by Mixed Measurement Mode Phase Analysis Light Scattering (M3-PALS) in glucose buffer (300 mM glucose, 10 mM HEPES, 1 mM $CaCl_2$ at pH 7.4) at 120 µM total lipid. Each measurement consisted of 3 individual runs in automatic mode (10-100 cycles).

### SI 1.5 Dependence of Liposome Fluorescence on Local Environment

The fluorescence properties of the liposomes in various environments were investigated by diluting the liposomes to a final concentration ranging from 37.5 to 300 µM total lipid in either HEPES buffer, in HEPES buffer supplemented with 20 mM sodium cholate detergent (to micellize the liposomes and, thereby, avoid self-quenching of the fluorophores) or plasma diluted 20 times (comparable to the highest measured concentration in fractions from SEC). The samples were incubated at 37 °C for 4 h to allow micellation of the liposomes in cholate. After incubation, the fluorescence emission intensity of the samples was measured in the same way as for the collected SEC fractions, as described below.

## SI 1.6 Sample Incubation and Size Exclusion Chromatography

The liposomes were diluted to a final concentration of 2 mM total lipid (a typical lipid concentration for animal *in vivo* experiments) in either HEPES buffer or plasma, and incubated in Protein LoBind tubes for 24 h at 37 °C at 1000 rpm on a plate shaker. Then, 300 µL of each sample was loaded onto a 10/200 mm C10/20 column (GE Healthcare Europe, Brøndby, Denmark) packed with Sepharose CL-4B matrix (GE Healthcare). The column was fitted onto an LC-20AD liquid chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a DGU-20A SR degassing unit and a SIL-20AC HT autosampler. The flow rate was 0.75 mL/min with HEPES buffer as eluent. The optical density (OD) was detected with an SPD-M20A Photodiode Array Detector fitted with a Deuterium Tungsten lamp (Shimadzu) located immediately after the column. Fractions of 1 mL were collected in Protein LoBind tubes using an automated FRC-10A fraction collector (Shimadzu).

For completeness, it should be mentioned that with an exclusion limit of $2\times10^7$ Da for the Sepharose CL-4B matrix, particles with a diameter larger than roughly 35 nm elute in the void fractions.

## SI 1.7 Analysis of SEC Fractions

For each collected fraction from SEC, 150 µL was loaded into a nontreated black flat-bottom 96-well plate (Nunc, Thermo Fisher Scientific). The fluorescence intensity in each well was measured on a TECAN Spark microplate reader (Tecan, Männedorf, Switzerland).

The emission and excitation wavelenghts used for detection of the FLLs in the experiments are listed in table S1. In all cases, both excitation and emission was with a bandwidth of 5 nm, except when measuring tryptophan, in which case the bandwidths were 20 nm. Gain settings were held constant for all samples, in order to make measurements in different environments and repetitions directly comparable.

Table S1: Excitation and emission wavelenghts for detection of fluorophores.

| Fluorophore | Excitation (nm) | Emission (nm) |
|---|---|---|
| Atto488 | 500 | 520 |
| Atto655 | 660 | 685 |
| Cy5 | 646 | 670 |
| DiD | 644 | 665 |
| DiO | 484 | 510 |
| RhodamineB | 560 | 583 |
| TopFluor | 495 | 520 |
| Tryptophan | 280 | 355 |

The background fluorescence from fractions containing non-labeled liposomes in the same environment (HEPES or plasma) was subtracted before calculating the dissociation. The background for non-labeled liposomes in plasma was usually below 50 A.U. for all wavelenghts relevant for measuring fluorophore content, compared to a typical maximum of 50,000 A.U. for the liposome peak.

The elution profile of the plasma proteins from the SEC column, otherwise measured using the tryptophan fluorescence, was verified using a colorimetric BCA assay. For this assay, 25 µL of the collected fractions was transferred to a non-treated transparent 96-well plate. Additionally, 25 µL of standard samples with varying concentration of bovine serum albumin between 0-2 mg/mL was also transferred to the plate. Then, 200 µL BCA working reagent (Thermo Fisher Scientific) was added to all wells, and the plate was incubated in a 37 °C oven for 30 min. Subsequently, the absorbance at 570 nm was measured in all wells using a Victor$^3$ 1420 Multilabel Counter (PerkinElmer, Waltham, MA, USA). An absorbance-concentration standard curve was established from the bovine serum albumin standard samples, and finally, the protein concentration of the collected fractions was determined by comparing their absorbance to the standard curve. If the protein concentration of a given fraction

was found to be >2 mg/mL, it was diluted in HEPES buffer and measured again following the above protocol.

The elution profile of liposomes from the SEC column was verified by measuring the phosphorus content of the fractions using ICP-MS. The collected fractions were diluted ×32 in the ICP-MS diluent to fall within a standard range of 0-100 ppb. The phosphorus and lipid concentration was then determined as described above.

## SI 1.8 Determination of the Absorption of the FLLs

The optical densities recorded in the SEC experiments were a superposition of (i) the absorbance of the FLLs, (ii) the absorbance and scattering of plasma components, and (iii) the scattering of the liposomes. The latter effect was mainly present in the void volume (retention volume 4–7 mL). A detailed overview of how the absorption of the FLLs was determined is given in figure S2. In brief, to subtract the contribution of the plasma components, the optical density of a plasma control sample was subtracted from the optical density of the sample under investigation. In the void volume, the scattering contribution of the liposomes was identified by fitting a polynomial to the scattering profile. The absorbance of the FLLs could then be determined.

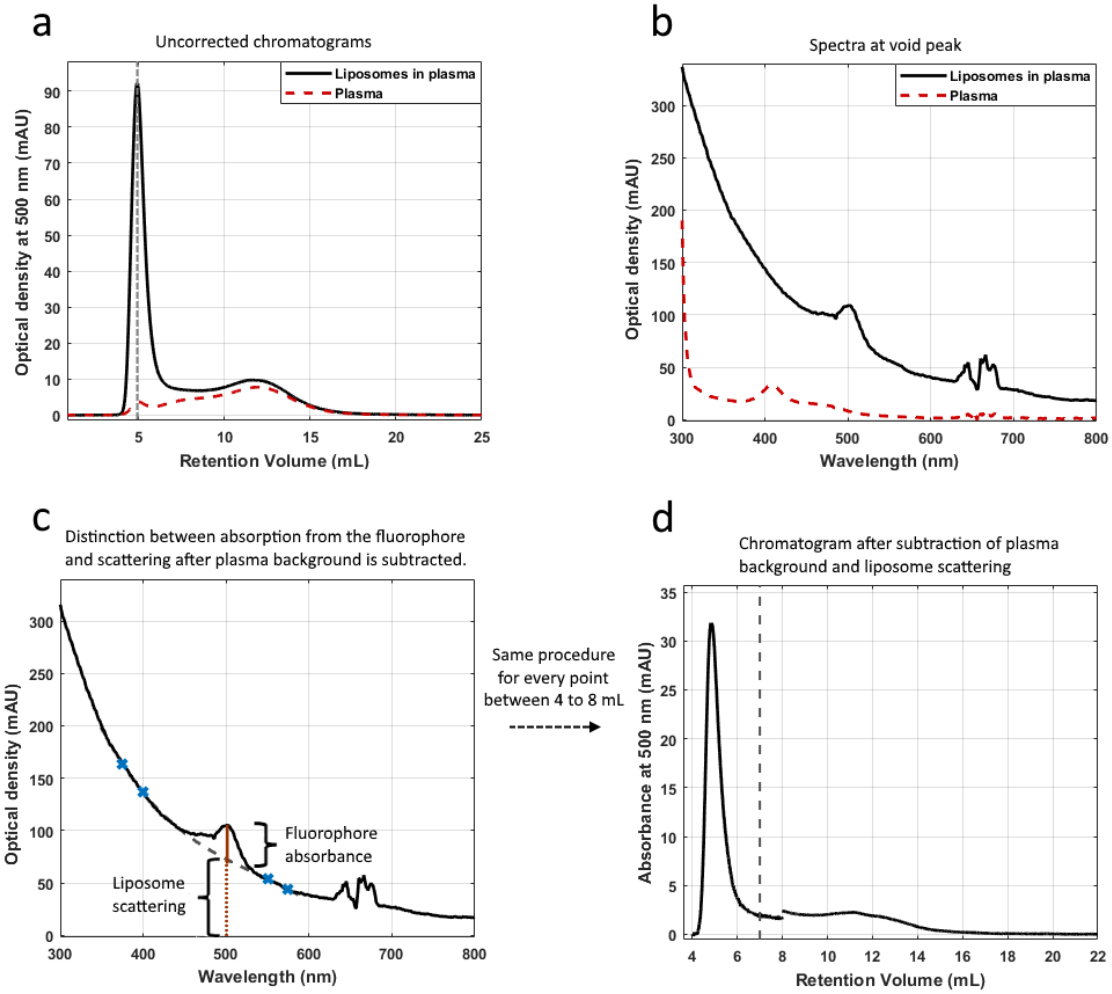


Figure S2: Illustration of the approach used to calculate the absorbance of the FLLs. (**a**) Example of SEC chromatograms obtained by measuring optical density at 500 nm for plasma and for liposomes containing Atto488-DOPE in plasma. (**b**) The spectra between 300 and 800 nm at the void peak (retention volume 4.9 mL). A plasma sample from the same donor was used to determine the plasma background. (**c**) The spectrum after plasma background subtraction was used to distinguish between absorption of the FLL and scattering of the liposomes. This was achieved by fitting an interpolating polynomial to the boundaries of the characteristic absorption peak of the FLL and points on the scattering profile 25 nm from these boundaries (blue crosses). (**d**) The chromatogram obtained after performing the same procedure at every point, thus ideally only representing the absorption of the FLL.

## SI 1.9 Calculation of FLL Dissociation

The total flourescence elution profiles were used to calculate the percentage dissociation of the FLL from the liposomes, after subtracting the background fluorescence determined from non-labeled liposomes in the same environment (plasma or buffer). The total integrated area, $A_{\text{Total}}$, of the elution profile was calculated, and the area of the non-liposome fractions, $A_{\text{Dissociated}}$, was used to calculate the FLL dissociation from the liposomes, see figure S3. The following equation was used to calculate the percentage of dissociated FLL:

$$\text{Dissociation} = 100 \times \left( \frac{A_{\text{Dissociated}}}{A_{\text{Total}}} \right)$$

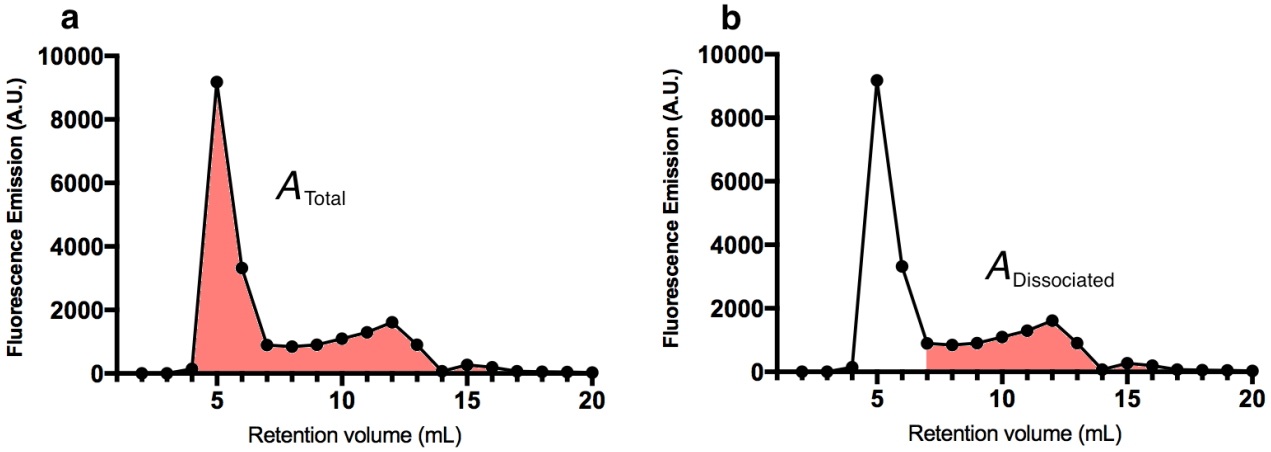


Figure S3: (**a**) Elution profile of a TopFluor-PC labeled liposome in plasma, with the area marked with red being the total eluting fluorescence $A_{\text{Total}}$. (**b**) Area of the elution profile representing fluorescence of dissociated FLL $A_{\text{Dissociated}}$.

It should be mentioned that this method does not take account for possible changes in fluorescent properties of the FLLs in the different environments (liposome-associated versus protein-associated). Self-quenching of the FLLs when packed into the liposomal membrane, could lead to overestimation of the dissociation, because quenching decreases when the FLLs leaves the liposomes. However, the data presented in section SI 2.3 indicate that this does not represent an issue. Finally, it should be mentioned that the calculation of the dissociation values based on the recorded UV-VIS absorbance elution profile (see section SI 1.8 and figure S8) was based on the same approach as the one described above.

## SI 1.10 Uptake Study in Peripheral Blood Leukocytes

Blood was drawn as for the plasma incubation studies and portioned out in 2 mL LoBind tubes. Liposomes labeled with Atto488-DPPE, DiO, or TopFluor-Cholesterol were added to a final concentration of 1 mM total lipid, resulting in a plasma concentration similar to in the dissociation studies (2 mM) when assuming a 50% haematocrit. The samples were incubated at 37 °C and 5% $CO_2$ under rotation for 5 h.

The 5 h incubation time in whole blood was chosen because the stability and metabolic activity of the blood cells may be altered at longer incubation times,[1] that is, incubation times longer than 5 h could potentially lead to unreliable uptake data. Additionally, as the circulation half-life of stealth liposomes is >45 h in humans[2,3] we expected very low liposomal uptake by peripheral blood leukocytes at 5 h, allowing us to study the uptake of dissociated FLLs.

Leukocytes were separated from blood as follows: Cells were washed twice by pelleting the cells (by centrifugation at 200$g$ for 5 minutes) and re-suspending in PBS supplemented with 1% Fetal Bovine Serum (FBS). Subsequently, the erythrocytes were lysed using BD Pharm Lyse lysis buffer (BD Biosciences) according to manufacturers procotol. Leukocytes were washed twice again in PBS with 1% FBS as described above, before blocking unspecific binding with human IgG (2 µg/$10^6$ cells) for 10 minutes on ice. The cells were then portioned out in a 96-well plate, and CD14 positive monocytes stained for 30 minutes using APC pre-conjugated specific antibodies (BD Biosciences) while keeping the plate on ice. The plate was then spun for 8 minutes at 400$g$ and washed twice with PBS, before performing flow cytometry (see section SI 1.12).

In these experiment, two independent liposome formulations were tested in two different donors, giving a total of four samples per FLL. The liposome formulations used for the uptake studies were the same batches as those used for the plasma dissociation experiments.

## SI 1.11 Uptake of Dissociated FLLs

Unsaturated TopFluor-PC labeled liposomes (2 mM) were incubated in plasma at 37 °C for 1 h at 1000 rpm. SEC was performed using the setup described in section SI 1.6. However, a pre-packed Superdex200 10/300 GL SEC column (GE Healthcare) was used instead of a Sepharose CL-4B column. The void fraction with liposomes, and the fraction with the highest fluorescence of dissociated FLL were chosen. Then, 300 µL of these fractions were added to 600 µL fresh whole human blood from the same donor who gave the plasma, and the uptake of FLL studied using the protocol described in section SI 1.10 and SI 1.12. As control, 300 µL 2 mM liposomes was added directly to 600 µL blood.

## SI 1.12 Flow Cytometry

Flow cytometry was performed using an ACCURI C6 flow cytometer from BD Biosciences. A minimum of 100,000 cells were acquired. APC fluorescence from the CD14 staining was measured by exciting at 640 nm and detecting at 675/25 nm (FL4). Single cells were gated in a FSC-A/FSC-H plot, and cell subsets were gated using APC fluorescence and a FSC-A/SSC-A plot. CD14 positive monocytes were determined using the combined morphology (according to the FSC-A/SSC-A plots) and CD14 staining (FL4-A/SSC-A plots) gates. Granulocytes and lymphocytes were gated solely according to morphology. The association of FLLs with cells was evaluated using excitation at 488 nm and emission measured at 533/30 nm (FL1) for all types of liposomes. The gating strategy is sketched in figure S4. Analysis was done in the FlowJo software (FlowJo, Ashland, OR, US), and the plots were made in GraphPad Prism v7 (GraphPad Software, La Jolla, CA, US). Statistics were based on a two-way ANOVA with multiple comparisons.

The three FLLs have differing fluorescence intensitites at the wavelenghts at which the uptake was evaluated. In order to take account for the different emission properties of the three FLLs, the fluorescence intensity of the liposome formulations used for the study was measured on a TECAN Spark microplate reader. The same filter settings as in the FL1 channel of the flow cytometer (excitation at 488nm and emission measured at 533/30 nm) were used. The measured intensities were in good agreement with the theoretical brightness of the fluorophores. The data in figure 3 of the main paper are normalized to the respective intensity measured on the plate reader.

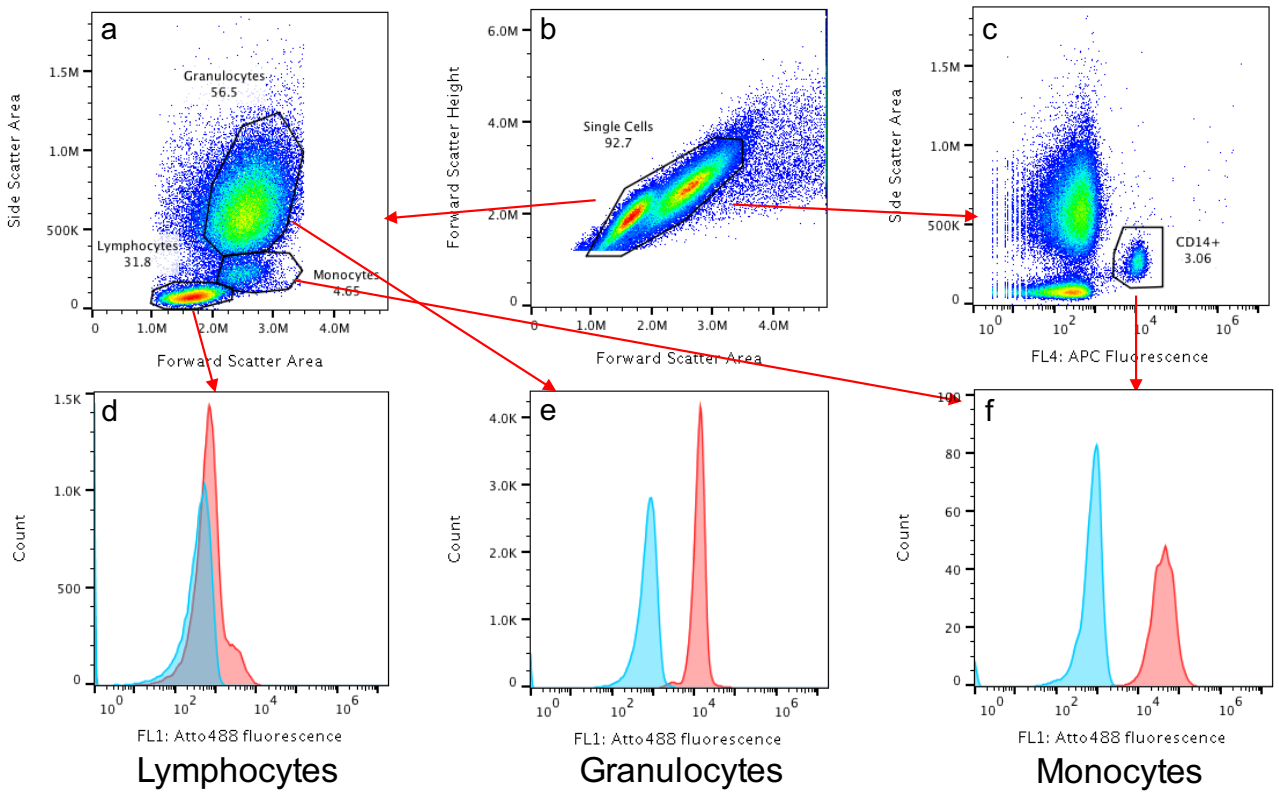


Figure S4: Gating strategy used to analyze flow cytometry data. (**a**) Monocytes, lymphocytes and granulocytes were gated according to morphology in a FSC-A/SSC-A plot after having gated single cells using a FSC-A/FSC-H plot (**b**). Monocytes were furthermore gated according to staining with APC-labeled antibodies against CD14 (FL4-A/SSC-A plot, (**c**)). Blue populations in histograms (**d, e, f**) are cells treated with unstained liposomes, red populations are cells treated with Atto488-DPPE labeled liposomes. The median fluorescence intensity from these populations (MFI) is plotted in the main text.

## SI 1.13 Single Liposome assay

Liposomes were prepared as described in section SI 1.3, but containing both 0.1% TopFluor-Cholesterol and 0.1% Cy5-DSPE, as well as 0.05% Biotin-PEG-DSPE.

To image individual liposomes we followed previously published protocols, allowing us to tether single liposomes on a passivated glass surface and image them using confocal microscopy[4,5]. In brief, each chamber in an Ibidi µ-slide 8 well glass coverslips for microscopy (Ibidi, Martinsreid, Germany) was incubated with a 1 mg/mL 1:10 mixture of BSA-Biotin:BSA for 20 minutes at room temperature, After washing with HEPES buffer, each chamber was incubated with 25 µg/mL Streptavidin for 10 minutes, followed by additional washes by HEPES buffer. Liposomes were diluted to a final concentration of 0.25 µM lipid and added to chambers on the coverslip. We imaged the liposomes both in the TopFluor and Cy5 channel in HEPES, before exchanging the solution in one of the chambers with human plasma through five washing steps and acquiring again the TopFluor and Cy5 channels. Neither the washing nor the presence of plasma changed the intensity in either of the channels. Then we incubated the liposomes for 2.5 h with either HEPES or plasma and acquired both channels for the respective chambers again.

For imaging liposomes we used a Leica TCS SP5 inverted confocal microscope and an oil immersion objective HCX PL APO CS × 100 (NA 1.4) (Leica Microsystems, Wetzlar, Germany). Detection of TopFluor was performed at 495–580 nm (exc. 488 nm); detection of Cy5 was performed at 645–800 nm (exc. 633 nm) using photo-multipliers. In all cases, sequential imaging was used to avoid cross excitation. Images had a resolution of 1024 × 1024 pixels, with a pixel size of 50.5 nm and a bit depth of 16. Temperature control (±0.5 °C) in the microscope chamber was achieved by enclosing the whole microscope within a box heated by stable air flow (The Cube 2 Temperature Controller, Life Imaging Services, Basel, Germany). All experiments were performed at 37 °C.

Image analysis and data treatment were performed using custom-made routines in Igor Pro (Wavemetrics, Lake Oswego, OR, US) and Fiji (ImageJ).

## SI 1.14 Single Liposome assay on fractions from SEC

The double-labeled liposomes used in the Single Liposome assay (see section SI 1.13) as well as similar liposomes with Atto488-DPPE instead of TopFluor-Cholesterol were incubated with plasma and run on SEC using the same procedure as described in section SI 1.6. The fraction with the highest fluorescence intensity was diluted 30 times in HEPES buffer, transferred to chambers of Ibidi slides, and immobilized and imaged as described in section SI 1.13.

# SI 2 Additional Results

## SI 2.1 Liposome Characteristics

The size and polydispersity index (PDI) of all liposome formulations are given in table S2. The biotinylated liposomes for the single liposome assay was only made in one batch. For the liposomes prepared with Atto655-DPPE, Cy5-DSPE, or DiD, the values are representing mean and standard deviation from three formulations, for all other liposomes from two formulations.

Overall, the liposomes had a size of 120–130 nm, were very monodisperse and could be made with high reproducability. An exception was the DiD liposomes, which tended to be larger, with sizes varying from 157 to 192 nm.

Table S2: Liposome Characteristics (mean and SD)

| Type | Fluorophore | Size (nm) | PDI | Zeta potential (mV) |
|---|---|---|---|---|
| Saturated | Blank | 129.8 ± 13.3 | 0.051 ± 0.036 | -2.07 ± 0.10 |
| | Atto488-DPPE | 130.6 ± 5.7 | 0.025 ± 0.001 | -2.49 ± 0.45 |
| | Atto655-DPPE | 131.2 ± 5.9 | 0.033 ± 0.012 | -2.43 ± 0.15 |
| | Cy5-DSPE | 161.8 ± 58.0 | 0.063 ± 0.060 | -2.07 ± 0.28 |
| | DiD | 162.8 ± 26.2 | 0.136 ± 0.093 | -2.02 ± 0.42 |
| | DiO | 129.8 ± 3.5 | 0.065 ± 0.025 | -2.71 ± 0.66 |
| | RhodamineB-DPPE | 129.2 ± 7.1 | 0.045 ± 0.016 | -2.64 ± 0.40 |
| | TopFluor-Chol. | 131.9 ± 13.2 | 0.036 ± 0.008 | -2.64 ± 0.66 |
| | TopFluor-PC | 135.9 ± 8.8 | 0.033 ± 0.001 | -2.27 ± 0.34 |
| Unsaturated | Blank | 122.2 ± 1.9 | 0.026 ± 0.001 | -4.09 ± 0.45 |
| | Atto488-DOPE | 123.3 ± 1.8 | 0.035 ± 0.008 | -3.94 ± 0.62 |
| | TopFluor-Chol. | 124.9 ± 6.2 | 0.041 ± 0.006 | -4.22 ± 0.79 |
| | TopFluor-PC | 124.5 ± 0.5 | 0.038 ± 0.008 | -4.40 ± 0.81 |
| Biotinylated | Atto488-DPPE / Cy5-DSPE | 145.5 | 0.171 | -2.11 |
| | TopFluor-Chol. / Cy5-DSPE | 138.4 | 0.066 | -2.05 |

## SI 2.2 Elution Profiles of Liposomes and Plasma Proteins Measured Using BCA and ICP-MS

To evaluate the ability of SEC to separate liposomes from plasma proteins, we performed a SEC experiment with non-labeled liposomes after incubation in buffer or plasma. The elution of lipids was measured using ICP-MS to detect phosphorus in the fractions. The elution of plasma proteins was evaluated using BCA.

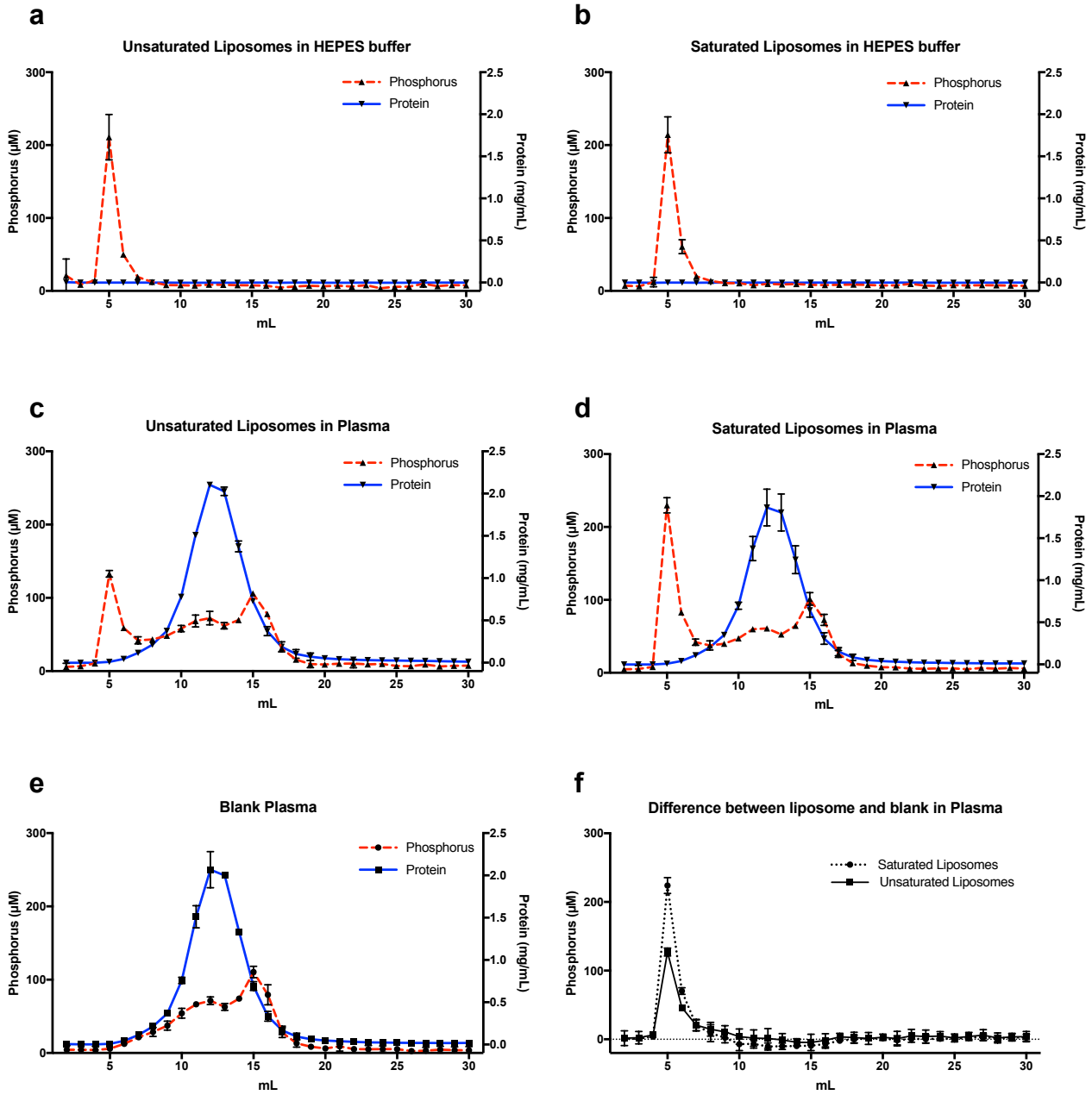


Figure S5: Elution profiles of plasma proteins measured with BCA and phospholipid by measuring phosphorus content with ICP-MS.

The results are shown in figure S5. The elution profiles are in very good agreement with the elution profile illustrated in figure 2 of the main paper. The elution profiles confirm that the liposomes elute before the 7 mL mark, and that the main plasma peak is around 12–13 mL.

Elution profiles for blank plasma without liposomes (figure S5e) show that there is a significant background of eluting phosphorus (phosphorylated proteins, salts, DNA, lipids, *etc.*). When subtracting this background from the elution profiles of the liposome-plasma samples, there is no extra phosphorus eluting in the late fractions (figure S5f), further corroborating the notion that the liposomes

elute within the first 7 mL, also after incubation plasma.

The ICP-MS measurements were also used to determine if aggregation or other factors that could induce loss of lipid was influencing the experimental setup. Thus, the total amount of phosphorus eluting in the main liposome fractions (with the blank subtracted) was normalized to the expected amount, given the concentration of the liposomes in the samples before SEC:

- Saturated liposomes in buffer: 110.2% ±7.3
- Unsaturated liposomes in buffer: 104.2% ±14.7
- Saturated liposomes in plasma: 127.3% ±9.9
- Unsaturated liposomes in plasma: 78.4% ±7.3

There is good agreement between the expected and measured amount of phosphorus eluting on the SEC column. This confirms that the liposomes are very stable in plasma and elute in the void volume.

## SI 2.3 Fluorescent Properties of Liposomes in Various Environments

It is likely that the fluorescent properties of the FLLs could change when they dissociate from the liposomes. For example, if the FLLs are self-quenching when incorporated in the liposomes, the emission intensity could potentially increase upon FLL dissociation. Alternatively, the new environment of the FLL, when associated to a protein or lipoprotein particle, could both mean increases or decreases in the intensity. Finally, plasma components in the SEC fractions could absorb/scatter photons of the excitation source and, thus, lead to less excitation of the FLLs. These factors could create a bias between the fluorescence properties in void fractions and plasma protein fractions in the elution profiles.

To evaluate whether any of these effects played a role, the fluorescence emission intensities of the liposomes were measured in both HEPES buffer, in plasma, and in HEPES buffer with 20 mM sodium cholate detergent. Results are shown in figure S6.

If self-quenching due to close packing in the liposomes were affecting the fluorescence intensity of the FLLs, addition of cholate should lead to an increase in fluorescence as the liposomes become micellized. However, we generally note a decrease in fluorescence upon incubation in cholate. For some FLLs, e.g. DiO and DiD, the manufacturer states that they are weakly fluorescent in water, but highly fluorescent in membranes. The fluorescent properties might thus be depending on the environment, while self-quenching is not an issue with the amount of FLLs added to the liposomes in our study (0.1 mol%).

For all FLLs, except Cy5-DSPE, we also observe a decrease in fluorescence intensity when incubating in plasma. This would indicate that either light absorption/scattering by other plasma components, environmental changes or quenching by plasma proteins affect the recorded fluorescence intensity in plasma. An investigation of the reason for this decrease is beyond the scope of this study.

Overall, however, it should be emphasized that the fluorescence intensity of the FLLs only change slightly in the different environments. This overall indicates that the fluorescence intensity can be used for quantifying the amount of FLLs eluting in the individual SEC fractions.

To fully confirm this notion, we compared the total fluorescence signal eluting on the SEC column for liposome samples incubated in plasma versus buffer. Table S3 shows the results of this calculation. For comparison, we performed a similar calculation based on the total absorbance signal eluting on the SEC column. For all FLLs, the signal for plasma samples versus buffer samples were fairly close to 100%, indicating no major liposome aggregation or quenching of fluorescence in plasma.

Table S3: Total elution signal in plasma versus in buffer

| Type | Fluorophore | Absorption (%) | Fluorescence (%) |
|---|---|---|---|
| Saturated | Atto488-DPPE | 95.6 ± 3.7 | 81.0 ± 10.4 |
| | Atto655-DPPE | 102.0 ± 13.5 | 142.7 ± 49.2 |
| | Cy5-DSPE | 118.5 ± 16.2 | 112.2 ± 24.3 |
| | DiD | 106.0 ± 13.9 | 106.0 ± 46.2 |
| | DiO | 116.5 ± 13.9 | 86.1 ± 16.4 |
| | RhodamineB-DPPE | 131.4 ± 1.0 | 137.1 ± 36.1 |
| | TopFluor-Chol. | 107.8 ± 21.1 | 96.5 ± 33.5 |
| | TopFluor-PC | 75.7 ± 16.8 | 69.0 ± 23.3 |
| Unsaturated | Atto488-DOPE | 109.3 ± 5.0 | 85.8 ± 10.3 |
| | TopFluor-Chol. | 85.5 ± 11.4 | 81.6 ± 11.1 |
| | TopFluor-PC | 102.0 ± 22.4 | 107.9 ± 31.7 |

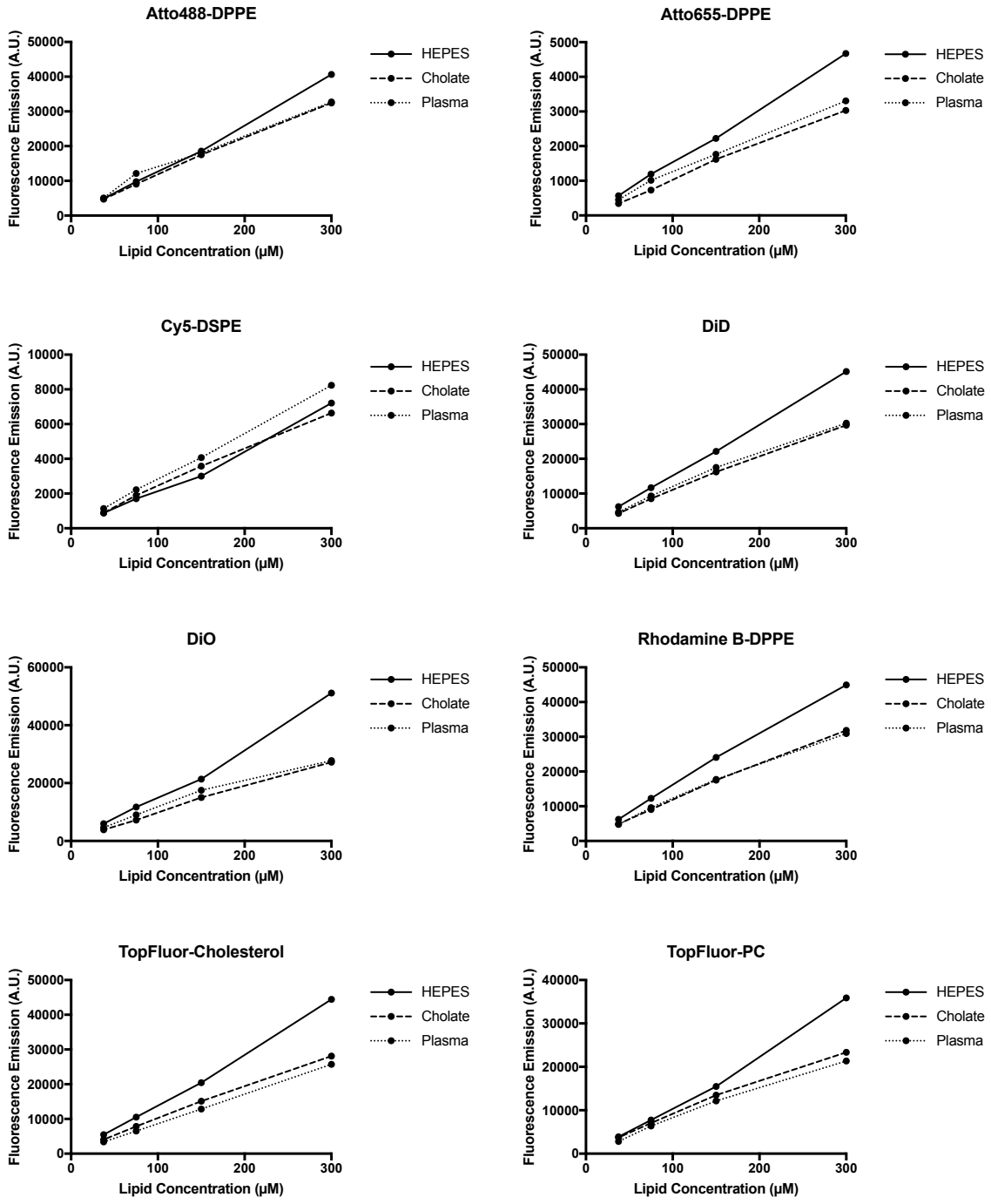


Figure S6: Fluorescence emission intensity of the tested FLLs in three different environments; HEPES buffer, plasma and HEPES buffer with 20 mM cholate.

## SI 2.4 Dissociation in HEPES buffer

A full overview of the dissociation measured in HEPES buffer after 24h incubation at 37 °C with shaking is given in figure S7. Both results from absorption and fluorescence measurements are plotted. Overall, the dissociation is very low compared to after incubation in plasma (compare with figure S8).

For Atto488-DOPE, we observe more than 20% dissociation in HEPES buffer. The dissociated compound eluted quite late on the column (15-16 mL), which might indicate incomplete attachment of the fluorophore to the anchor lipid, e.g. an impurity in the FLL batch.

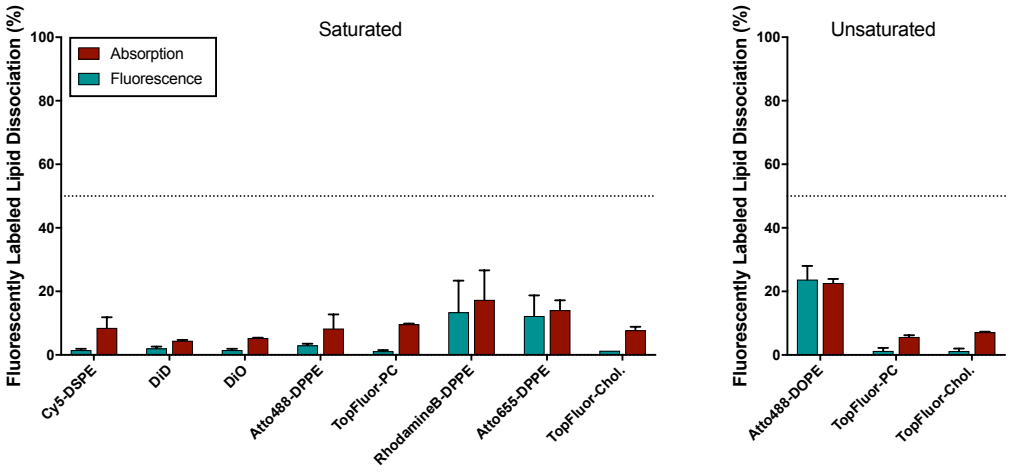


Figure S7: Dissociation of FLLs from liposomes after 24 h incubation in HEPES buffer at 37 °with shaking. Dissociation is calculated using both absorption and fluorescence. n=2–3, error bars show SEM.

## SI 2.5 Dissociation in Plasma Measured Using Absorption

In addition to the dissociation values reported in figure 2 in the main paper, based on fluorescence measurements, the dissociation of FLLs was also calculated using absorption measurements, as described in section SI 1.6 and SI 1.8. The absorption results are compared to the fluorescence results in figure S8.

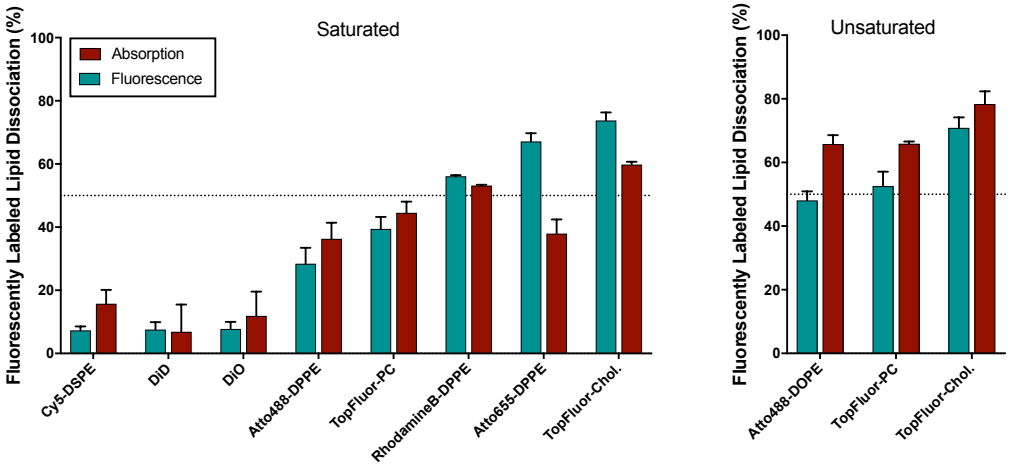


Figure S8: Dissociation of FLLs from liposomes after 24 h incubation in plasma at 37 °with shaking. Dissociation is calculated using both absorption and fluorescence. The fluorescence-based results are also shown in figure 2 of the main paper. n=2–3, error bars show SEM.

The fluorescence-based method generally gave a slightly lower dissociation value than the absorption-based method. We observed that there was a slight decrease in fluorescence intensity when the lipo-

somes were incubated in plasma (see figure S6), and the lower dissociation calculated for the fluorescence data could thus be caused by the FLL being less bright upon dissociation from the liposomes. Overall, however, there is good agreement between the two measurement methods (absorption and fluorescence).

For Atto655-DPPE, there was some disagreement between the fluorescence and absorption readouts. This disagreement may be explained by uncertainty of the absorption measurements caused by high instrument noise in the area of the absorption spectrum for this FLL ($\lambda_{max} = 663$ nm). This noise can be ascribed to the 654 nm D-alpha line of the D2W light source (see figure S2b and c).

## SI 2.6 Dissociation Kinetics of Atto488-DPPE

In order to cast light on whether the dissociation of FLLs was a burst-release as soon as the liposomes were added to the plasma, or a slow release over time, we investigated the leakage kinetics of Atto488-DPPE. Atto488-DPPE was chosen as example, as this FLL had demonstrated a medium-scale dissociation in the initial studies. The dissociation of this FLL from saturated liposomes was thus measured in human plasma after 1, 4, 12 and 24 h. Results show that the dissociation occurs gradually, and appears to be an almost linear function of time within the 24 h of the experiment.

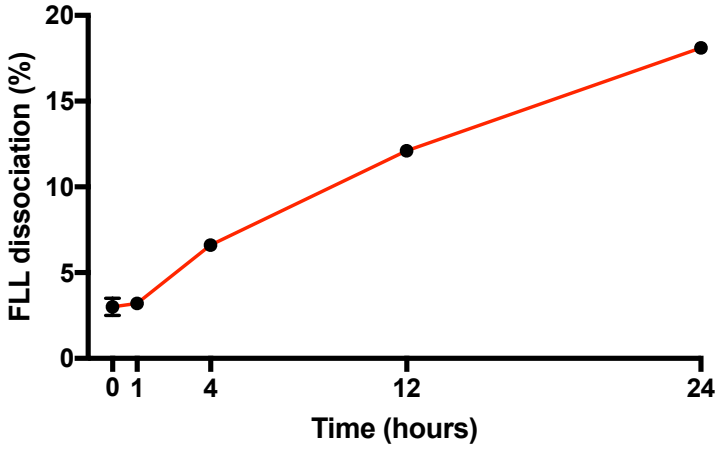


Figure S9: Dissociation kinetics of Atto488-DPPE from saturated liposomes during incubation in plasma at 37 °with shaking. $t$=0 is defined as liposomes in HEPES buffer with no incubation at 37 °C. $n$=2, error bars show SEM. For some points, the error bars would be shorter than the symbol, and these error bars are hence not shown.

The study was performed for two different liposome formulations, but with plasma from the same blood donor. We note that for all timepoints, the variations are very small. This illustrates that the main cause for the variation in figure 2 of the main paper is the donor variation, which can e.g. be caused by different concentrations of proteins and lipoprotein particles in the plasma. While being lower than the mean value for Atto488-DPPE in figure 2 of the main paper, the 24 h timepoint in the kinetic study is within the error for the dissociation of this FLL.

## SI 2.7 Single Liposome Assay

Biotinylated liposomes double-labeled with both TopFluor-Cholesterol and Cy5-DSPE were immobilized on a BSA covered surface *via* a streptavidin coupling, and the dissociation of FLLs studied on a single-liposome basis using confocal microscopy.

From the images in figure S10a, it is obvious that both Cy5-DSPE and TopFluor-Cholesterol stay associated to the liposomes during incubation in HEPES buffer for 2.5 h, as there is no change in intensity of either label when comparing to the liposomes before incubation start (0 h images). In contrast, when incubating in plasma for 2.5 h, the fluorescence for TopFluor-Cholesterol decreased remarkably when comparing to the 0 h timepoint, while there was still no decrease in fluorescence for the Cy5-DSPE label.

From the single-liposome intensity histograms in figure S10b and c, we see that there is only a small change in intensity after incubation in buffer for both labels, while in plasma, the TopFluor-Cholesterol intensity decreased to 37% of the original fluorescence intensity.

From the distribution of TopFluor-Cholesterol after 2.5 h in plasma, we furthermore note that there is only one population present. This indicates that TopFluor-Cholesterol dissociates equally from all liposomes. Hence, the dissociation is not a complete release from some liposomes while other liposomes do not loose any label at all.

The data furthermore demonstrate that the mechanism behind the dissociation is not a complete destabilization of the liposomes, as the presence of TopFluor-Cholesterol in the formulation does not induce dissociation of Cy5-DSPE.

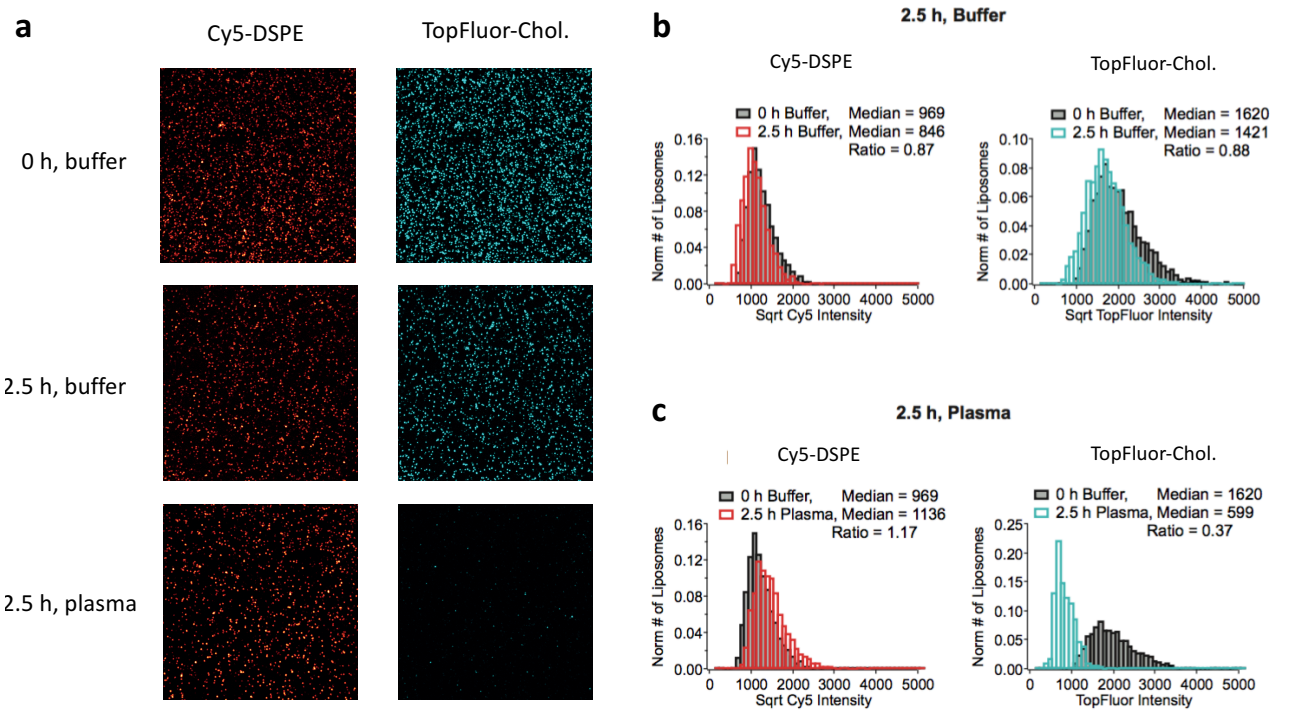


Figure S10: Single Liposome analysis of FLL dissociation by immobilizing the liposomes on a surface and performing imaging with confocal microscopy. The spots on the images in (**a**) corresponds to single liposomes. Histograms in (**b**) and (**c**) are the distributions of the intensities of the single liposomes after incubation in HEPES buffer or plasma, respectively. Incubation was done at 37 °C for 2.5 h.

## SI 2.8 Combined SEC and Single Liposome Assay

We wanted to confirm that the different style of incubation in the single liposome assay (see section SI 2.7), with liposomes immobilized on a surface rather than being in solution as in the SEC-based experiments, did not affect the outcome of the experiment. We thus performed an experiment in which the double-labeled biotinylated liposomes were incubated in tubes with plasma or buffer for 24 h at 37 °with shaking, before being loaded onto the SEC column. The void fractions, containing the liposomes, where then transferred to the microscopy slides where the liposomes were immobilized.

In table S4, the FLL dissociation is reported as in the main paper by measuring the fluorescence intensity in all fractions from the elution profile. We see that the results with the double-labeled liposomes are very similar to the results with the single-labeled liposomes, both for Cy5-DSPE, TopFluor-Cholesterol and Atto488-DPPE. The presence of two FLLs in the liposomes thus do not affect the dissociation tendencies of the individual label. Also, the presence of biotin on the liposome surface does not affect the results.

Figure S11 and S12 show the fluorescence intensities of the FLLs incorporated into the liposomes eluting in the void fractions from the SEC column. Figure S11 show liposomes labeled with Cy5-DSPE and TopFluor-Cholesterol, and figure S12 show liposomes labeled with Cy5-DSPE and Atto488-DPPE. The Cy5-DSPE emission intensity was similar when liposomes have incubated in plasma and buffer. The TopFluor-Cholesterol and Atto488-DPPE emission intensity, on the other hand, decreased by 61% and 25%, respectively, in plasma compared to buffer, similar to the results in figure S10.

The Single Liposome Assay in combination with the SEC-based setup thus gives the same results as the two methods separately, consequently underpinning the findings of both methods.

Table S4: Dissociation of FLLs from double-labeled biotinylated liposomes measured with SEC

| Liposome | Label | Dissociation in HEPES | Dissociation in plasma |
|---|---|---|---|
| Cy5-DSPE / Atto488-DPPE | Cy5-DSPE | 3.65 % | 7.42 % |
| | Atto488-DPPE | 5.72 % | 37.6 % |
| Cy5-DSPE / TopFluor-Cholesterol | Cy5-DSPE | 3.03 % | 9.04 % |
| | TopFluor-Chol. | 1.96 % | 82.5 % |

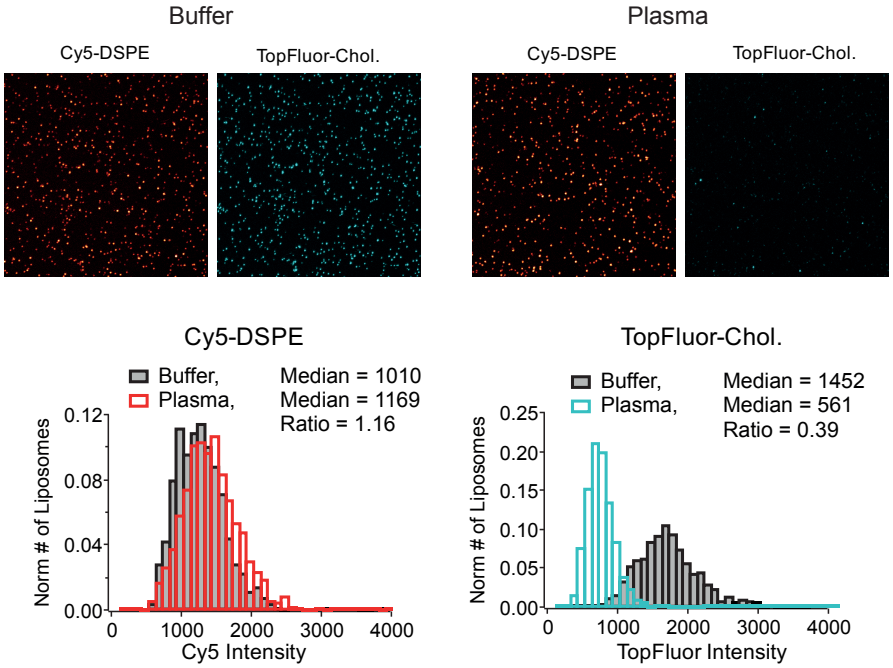


Figure S11: Combination of SEC and the single liposome assay with liposomes double-labeled with Cy-DSPE and TopFluor-Cholesterol. After 24 h incubation in either HEPES buffer or plasma at 37 °C with shaking, liposomes eluting in the void fractions from SEC were immobilized in microscopy wells and imaged.

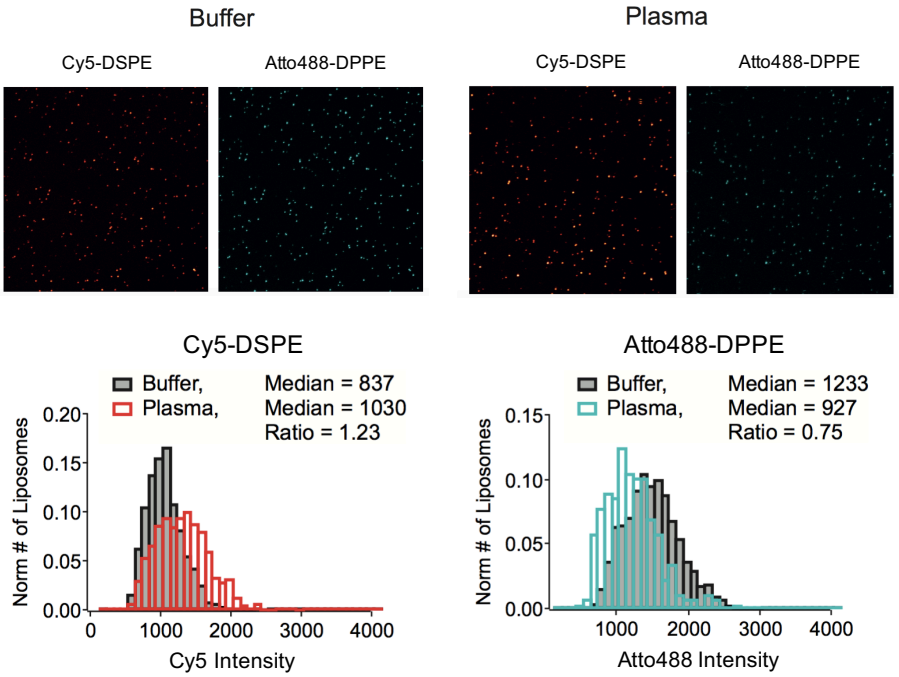


Figure S12: Combination of SEC and the single liposome assay with liposomes double-labeled with Cy-DSPE and Atto488-DPPE. After 24 h incubation in either HEPES buffer or plasma at 37 °C with shaking, liposomes eluting in the void fractions from SEC were immobilized in microscopy wells and imaged.

## SI 2.9 Uptake of Dissociated FLLs

We wanted to confirm that the uptake of FLLs in leukocytes, demonstrated in figure 3 of the main paper, was indeed dissociated FLLs that were taken up independently of the liposomes. In principle, FLL still associated to the liposomes could serve as targeting ligands, and promote uptake in the leukocytes. In order to investigate this, we performed an experiment with TopFluor-PC labeled saturated stealth liposomes. Using SEC, we first separated dissociated TopFluor-PC from TopFluor-PC still associated to liposomes after the liposomes had incubated for 1 hour in plasma. We then selected the void fraction with the highest concentration of TopFluor-PC, as well as the plasma-protein fraction with the highest concentration of TopFluor-PC. These two fractions were then applied separately to whole blood samples, and the cell uptake investigated using flow cytometry, following the same procedure as in the regular cell uptake experiment. No liposomes were present in the fraction with the dissociated FLLs. Any cell uptake of FLLs experienced in the latter fraction would thus be TopFluor-PC, either free or in complex with a protein or lipoprotein particle, being taken up by the cells. TopFluor-PC might however continue to dissociate from the liposomes during the incubation in whole blood. In the sample with the liposome fraction, there might therefore also be uptake of dissociated FLLs adding to the measured uptake.

The results are plotted in figure S13. We note an uptake for both of the fractions, also for the fraction not containing any liposomes but solely dissociated FLLs. This demonstrates that FLLs not associated to the liposomes are able to be taken up by the cells. This validates that the uptake shown in figure 3 of the main text is, to a significant extent, due to FLLs being taken up independently of the liposomes.

We furthermore note that the free FLLs have higher association with monocytes than with granulocytes and lymphocytes. This pinpoints that using certain FLLs can give misleading results in uptake experiments, as TopFluor-PC in itself is monocyte-targeting.

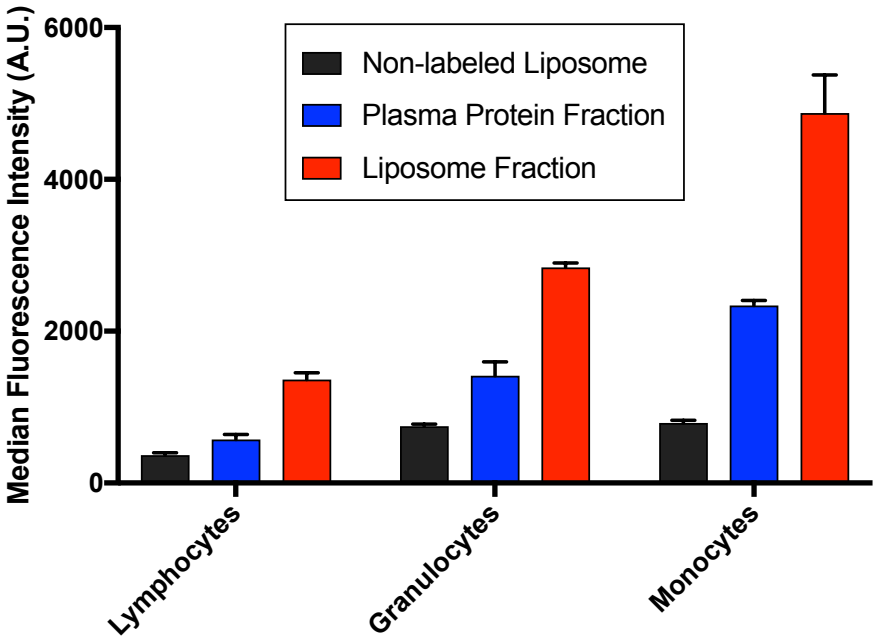


Figure S13: Uptake of dissociated FLLs in peripheral blood leukocytes investigated with flow cytometry. Saturated stealth liposomes labeled with TopFluor-PC were first incubated with fresh human plasma for 1 hour followed by SEC. Fractions containing either the (i) liposomes with associated TopFluor-PC or (ii) plasma proteins with dissociated TopFluor-PC were then added to fresh human blood from the same donor. The figure shows the association of TopFluor-PC with peripheral blood leukocytes. $n$=2, error bars show SEM.

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