Supporting Information

FRET-Based Assay for the Quantification of Extracellular Vesicles and Other Vesicles of Complex Composition

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Section 1. Theoretical model describing the behavior of the FRET signal in the FRET assay

1.1. Introductory remarks

In the experiments, liposomes containing two types of fluorophores, here called donor and acceptor fluorophores, which together form a Förster resonance energy transfer (FRET) pair, were mixed and fused with unlabeled sample vesicles to be quantified. Fusion leads to an increase in the average distance between the fluorophores which in turn lowers the rate of energy transfer between the donor and acceptor fluorophores (k_F), [1]

$$k_F = \frac{1}{\tau_D}\left(\frac{R_0}{r_j}\right)^6 \quad \text{(S1)}$$

where τ_D is the lifetime of the donor in the absence of energy transfer, R_0 is the Förster length for the FRET pair and r_j is the distance between the acceptor and donor molecules. The experiments were performed by exciting the fluorophores at a fixed wavelength around the absorption peak for the donor fluorophore, thus the emission from the acceptor fluorophore can here be approximated to exclusively originate from FRET between the fluorophores.

1.1.2. FRET and relative quantum yield on a two dimensional surface

For FRET, the relevant length scale is set by the Förster length which typically is around a few nanometers up to about ten nanometers. [2] Therefore, in a dispersed liposome solution, FRET will for the most part exclusively occur between fluorophores within the same liposome and not between neighboring liposomes. Furthermore, when the liposome size is both much larger than the Förster length and the average distance between donor and acceptor fluorophores then the effects in FRET due to curvature of the surface can be assumed to be negligible. When these assumptions are valid, FRET in liposomes can be simplified by approximating the liposomes as a flat 2D surface. This allows the use of the analytical solution proposed by Wolber and Hudson[3] describing the FRET behaviour for a two dimensional system where the donor and acceptor molecules are randomly distributed. Specifically, Wolber and Hudson[3] propose an analytical solution to describe the relative quantum yield ($q_{D,r}$) defined as the ratio between the quantum yields of the donor with ($q_{D(A)}$) and without (q_D) FRET:

$$q_{D,r} = \frac{q_{D(A)}}{q_D} \quad \text{(S2)}$$

The analytical system is based on the assumptions below, all are fulfilled in our case:

1. The steady-state concentrations of excited fluorophores are low enough so that they do not affect the effective surface concentration of fluorophores which can participate in FRET
2. The Förster length R_0 is not a function of r_j, the distance between a given donor and the jth acceptor surrounding it
3. The orientations of the fluorescent molecules are random over the timescale of the experiment
4. The closest distance between donor and acceptor is much less than R_0.

As previously detailed[3], using these assumptions $q_{D,r}$ is equal to an convergent infinite series which for simplicity can be approximated as:

$$q_{D,r} \approx A_1 e^{-k_1 C} + A_2 e^{-k_2 C} \quad \text{(S3)}$$

where $C = R_0^2 c$, c is the two-dimensional concentration of acceptor fluorophores (number of acceptors per unit area) and A_1, A_2, k_1 and k_2 are all pre-determined coefficients chosen to make the expression agree with the infinite series within 1 % [3].

1.2 Analytical expression describing the FRET signal as a function of the sample fraction

In our experiments, we exclusively used ratiometric FRET and therefore report FRET signals as the fluorescence emission intensity at two different wavelength, corresponding here to the peak emission for the acceptor λ_A (λ_A = 588 nm in our case) and donor λ_D (λ_D = 535 nm in our case). As we do not know if an emitted photon originates from a donor or acceptor fluorophore, the measured FRET signal corresponds to:

$$\text{FRET signal} = \frac{I_A(\lambda_A)+I_D(\lambda_A)}{I_A(\lambda_D)+I_D(\lambda_D)} \quad \text{(S4)}$$

where $I_i(\lambda)$ is the intensity emitted from the donor (I_D) resp. acceptor (I_A) fluorophores at the selected wavelengths. Since 535 nm is in the absorbance region of rhodamine and far from the emission peak, we can assume that $I_A(\lambda_D)$ = 0, i.e. that the rhodamine emission at 535 nm is

negligible compared to the emission from NBD at the same wavelength. Equation S4 can then be written as:

$$\text{FRET signal} = \frac{I_A(\lambda_A)}{I_D(\lambda_D)} + \frac{I_D(\lambda_A)}{I_D(\lambda_D)} = \frac{I_A(\lambda_D)}{I_D(\lambda_D)} + Y2, \quad \text{(S5)}$$

where

$$Y_2 \equiv \frac{I_D(\lambda_A)}{I_D(\lambda_D)} \quad \text{(S6)}$$

is the relative donor fluorophore emission for the two selected wavelengths and can therefore be considered independent of the presence of the acceptor fluorophores.

The FRET ratio relates the emission at two different wavelengths, but the dye's quantum yield regards the emission at all potential wavelengths. Therefore, to use the analytical model proposed by Wolber and Hudson[3] to describe the FRET behavior of our system (see section 1.1.2) the FRET ratio needs to be written as a function of the emission at all potential wavelengths for both fluorophores:

$$\frac{I_A(\lambda_A)}{I_D(\lambda_D)} = \frac{\frac{I_A(\lambda_A)d\lambda}{\int I_A(\lambda)d\lambda} \cdot \int I_A(\lambda)d\lambda}{\frac{I_D(\lambda_D)d\lambda}{\int I_D(\lambda)d\lambda} \cdot \int I_D(\lambda)d\lambda} \equiv \frac{\gamma_A}{\gamma_D} \cdot \frac{\int I_A(\lambda)d\lambda}{\int I_D(\lambda)d\lambda} \quad \text{(S7)}$$

where γ_A and γ_D is defined as

$$\gamma_A \equiv \frac{I_A(\lambda_A)d\lambda}{\int I_A(\lambda)d\lambda} \text{ and } \gamma_D \equiv \frac{I_D(\lambda_D)d\lambda}{\int I_D(\lambda)d\lambda} \quad \text{(S8)}$$

and are the ratios of relative emission around the emission peak for the two fluorophores compared to their total emission at all potential wavelengths.

The total fluorescent emission from the donor fluorophores is the number of excitations times the fluorescence quantum yield in the presence of acceptor fluorophores,

$$\int I_D(\lambda)d\lambda = q_{D(A)} I_{max} \quad \text{(S9)}$$

where I_{max} is the emission intensity if all excitations would have resulted in fluorescent emission. The total emission from the acceptor fluorophores is the number of transferred photons from donor fluorophores times the fluorescence quantum yield of the acceptor fluorophore,

$$\int I_A(\lambda)d\lambda = q_A \cdot E \cdot I_{max} = q_A(1 - q_{D,r})I_{max} \quad \text{(S10)}$$

where E is the transfer efficiency from a donor to an acceptor[3].

Combining equations E7, E8, E9 and E10 yields:

$$\frac{I_A(\lambda_A)}{I_D(\lambda_D)} = \frac{\gamma_A}{\gamma_D} \cdot \frac{\int I_A(\lambda)d\lambda}{\int I_D(\lambda)d\lambda} = \frac{\gamma_A}{\gamma_D} \cdot \frac{q_A \cdot E \cdot I_{max}}{q_{D(A)} \cdot I_{max}} = \frac{\gamma_A}{\gamma_D} \cdot \frac{q_A \cdot E}{q_D \frac{q_{D(A)}}{q_D}} = \frac{\gamma_A}{\gamma_D} \cdot \frac{q_A(1-q_{D,r})}{q_D \cdot q_{D,r}} \quad \text{(S11)}$$

By introducing the parameter Y_1,

$$Y_1 \equiv \frac{q_A}{q_D} \cdot \frac{\frac{I_A(\lambda_1)}{\int I_A(\lambda)d\lambda}}{\frac{I_D(\lambda_2)d\lambda}{\int I_D(\lambda)d\lambda}}, \quad \text{(S12)}$$

we can combine equations E5, E6, E11 and E12 to write the expression for the FRET signal as:

$$\text{FRET signal} = \frac{I_A(\lambda_A)}{I_D(\lambda_D)} = Y_1 \frac{1-q_{D,r}}{q_{D,r}} + Y_2 = Y_1 \left(-1 + \frac{1}{q_{D,r}}\right) + Y_2 \quad \text{(S13)}$$

Using E3 for $q_{D,r}$ in E13, we get:

$$FRET\ signal = Y_1 \left(-1 + \frac{1}{A_1 e^{k_1 C} + A_2 e^{k_2 C}}\right) + Y_2. \quad \text{(S14)}$$

Note here that only Y_1, Y_2 and C depend on the experimental system. To relate E14 with the sample fraction, C is linearly related to the sample fraction by

$$C = C_0(1 - x), \quad \text{(S15)}$$

where C_0 is the concentration of acceptor fluorophores before addition of unlabeled material and x is the sample fraction.

Section 2. Effect of sonication parameters on FRET liposome fluorophores

A practical aspect to be considered when establishing the FRET-based quantification method, is whether the fluorophores themselves are affected by the sonication process. In particular, we must determine whether the peak ratio between the rhodamine peak and the NBD peak (FRET signal) is affected by the sonication procedure. If so, this will have to be accounted for when comparing different sonication conditions and when establishing calibration curves. Additionally, the assay may require to be calibrated for multiple experimental procedures.

For these measurements, we sonicated a special batch of FRET liposomes with a 10-times lower fluorophore concentration than normally used in the assay (99.9 mol % POPC, 0.05 mol % Rhod-DHPE and 0.05 mol % NBD-PE in PBS). These vesicles therefore exhibit a reduced energy transfer between the fluorophores. Specifically, at this concentration, the rhodamine and NBD peaks have similar fluorescence intensity (Figure S1A and S1C), allowing us to better detect peak ratio shifts.

Our results indicate that sonication time has no discernible effect on fluorescence intensity, and accordingly the peak ratio stays constant for sonication between 10 and 30 minutes at a fixed temperature of 40°C (Figure S1A and S1B). On the other hand, the sonication temperature (at a fixed sonication time of 20 minutes) has some effect on the emitted fluorescence intensity (Figure S1C). Nevertheless, even in this case, the FRET signal peak ratio remains relatively stable with no clear trend between 20°C and 80°C (Figure S1 D).

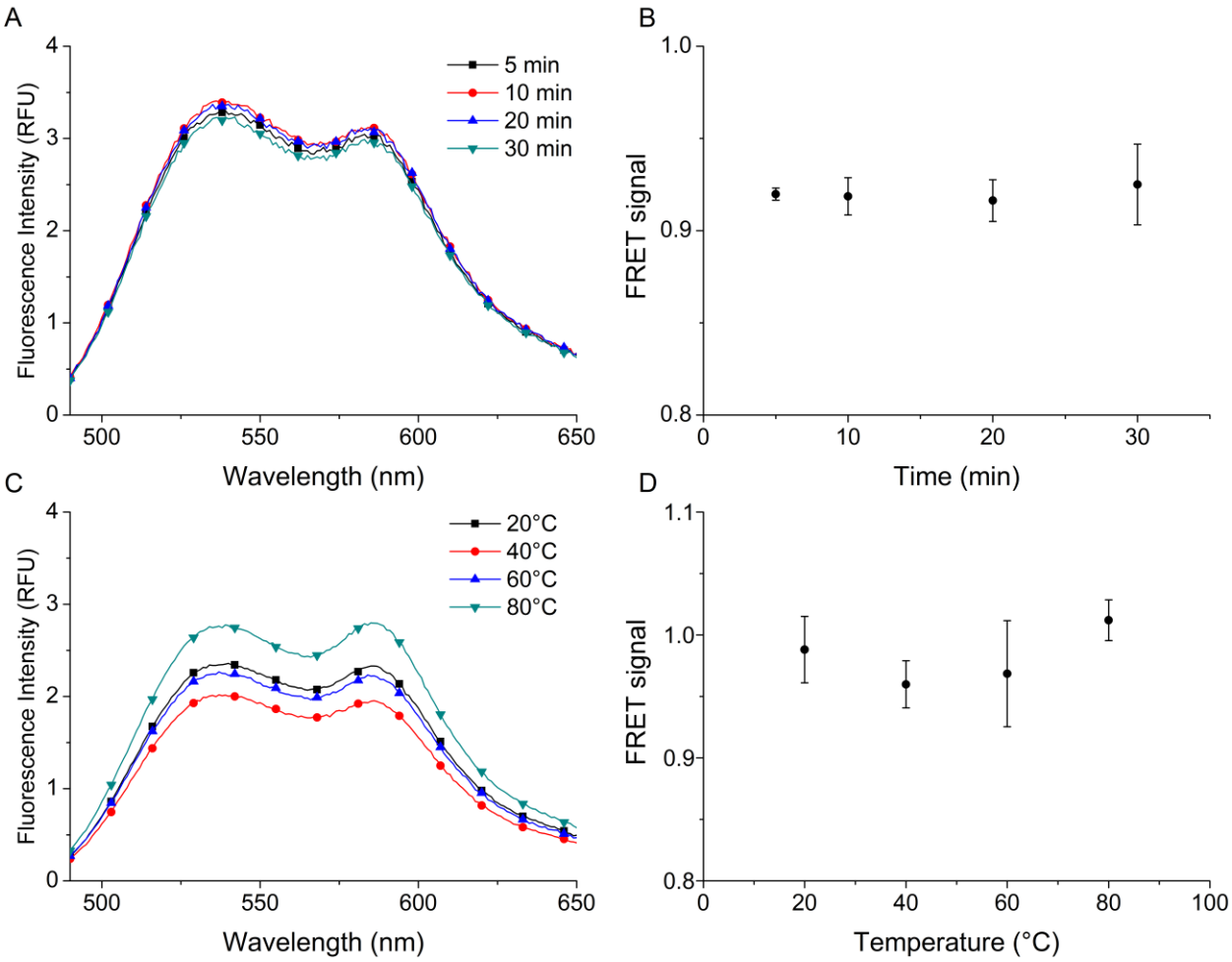

Figure S1. Influence of sonication of parameters on fluorescence emission. Control experiments were performed by sonicating vesicles containing 0.05 mol % of NBD-PE and 0.05 mol % of Rhodamine-PE. A) Fluorescence emission spectra for different sonication times, with temperature fixed at 40°C. B) Peak ratio as a function of sonication time. C) Spectra for different sonication temperatures, with sonication time fixed at 20 minutes. D) Peak ratio as a function of sonication temperature. All spectra are the average of three samples. Peak ratios are the average of the three spectra, with standard deviation as the error.

Section 3. Size distributions of POPC vesicles as measured with DLS

The size distributions shown in Figure S2, were measured using dynamic light scattering.

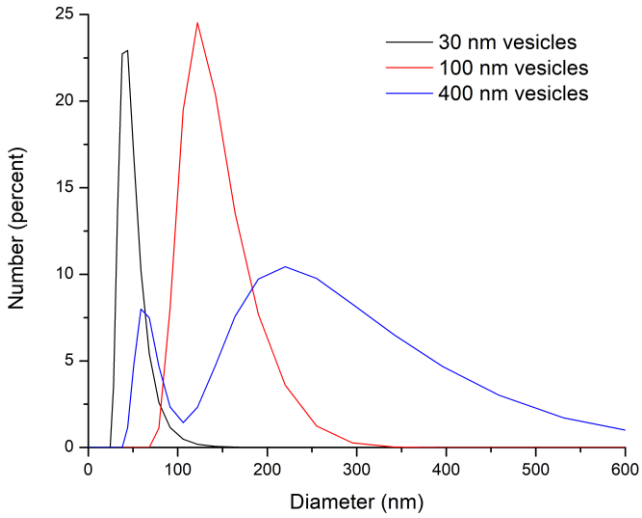

Figure S2. Vesicle size distributions as determined by DLS. Number weighted size distribution of POPC vesicles extruded with membranes of various pore size: 30 nm (black), 100 nm (red) and 400 nm (blue). The corresponding average values are: 79 ± 14 nm (black); 160.3 ± 35 nm (red) and 301 ± 108 nm (blue). The diameter values are the averages of three independent size measurements. Error bars for the diameter values are the standard deviations of the distribution.

Section 4. Parameter optimization for the quantification of OMV and HSV samples

In order to optimize the experimental conditions for each vesicle type, various ratios of FRET liposomes and sample vesicle volumes were sonicated for 20 minutes to confirm fusion and to choose a sample volume that would produce a FRET signal within the linear range of our calibration curve (Figure S3A and S3C). The role of temperature on the efficiency of vesicle fusion was then investigated (Figure S3B and S3D). Based on the results presented in Figure S3, quantification of all sample vesicles were performed at 40°C.

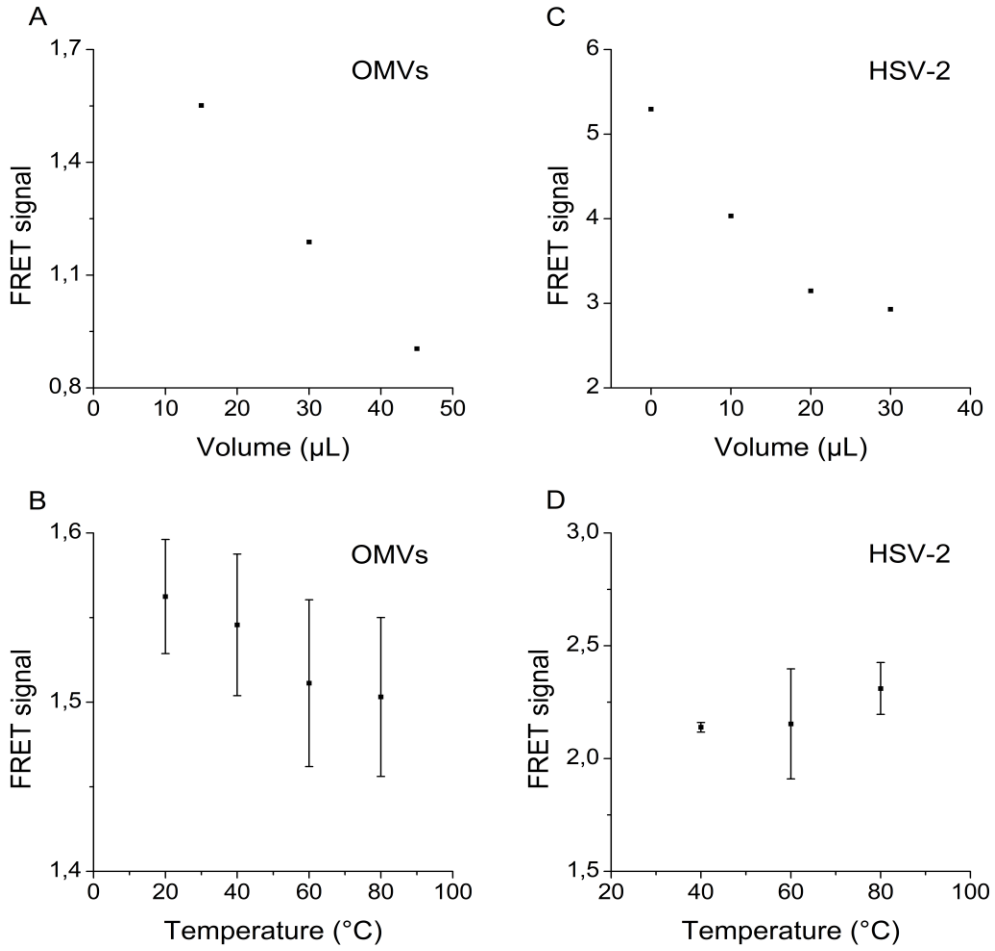

Figure S3. Optimization of the sonication parameters for the quantification of outer membrane vesicles (OMV) and Herpes Simplex Virus samples (HSV-2). A) FRET signal after addition of different volumes of OMV-samples. Sonication temperature 40°C; sonication time 20 minutes. B) FRET signal for the sonication of 20 µl of OMV-samples during 20 minutes at different temperatures. C) FRET signal after addition of different volumes of HSV-2 viral samples. Sonication temperature 40°C; sonication time 20 minutes. D) FRET signal for the sonication of 20 µl of HSV-2 viral samples during 20 min at different temperatures.

Section 5. Calibration curve for the quantification of EV, OMV, and HSV samples.

The calibration curve shown in Figure S4 focuses on the linear FRET-fraction regime and was established to quantify EV, OMV, and HSV samples.

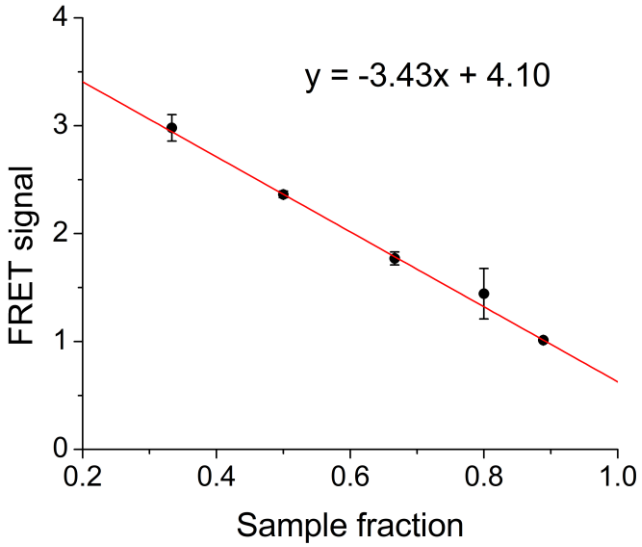

Figure S4. Calibration curve focusing on the linear regime. The calibration curve is represented as FRET signal (fluorescence intensity of the emission spectra at 588 nm divided by the fluorescence intensity at 535 nm) versus sample fraction (surface area fraction of the calibration liposomes). Each point is the average of 3 independent measurements and the error bars are their standard deviation.

Section 6. Size distribution of all vesicle samples used in the project, as measured with NTA.

The size distributions shown in Figure S5, were measured using nanoparticle tracking analysis as further detailed in the main manuscript.

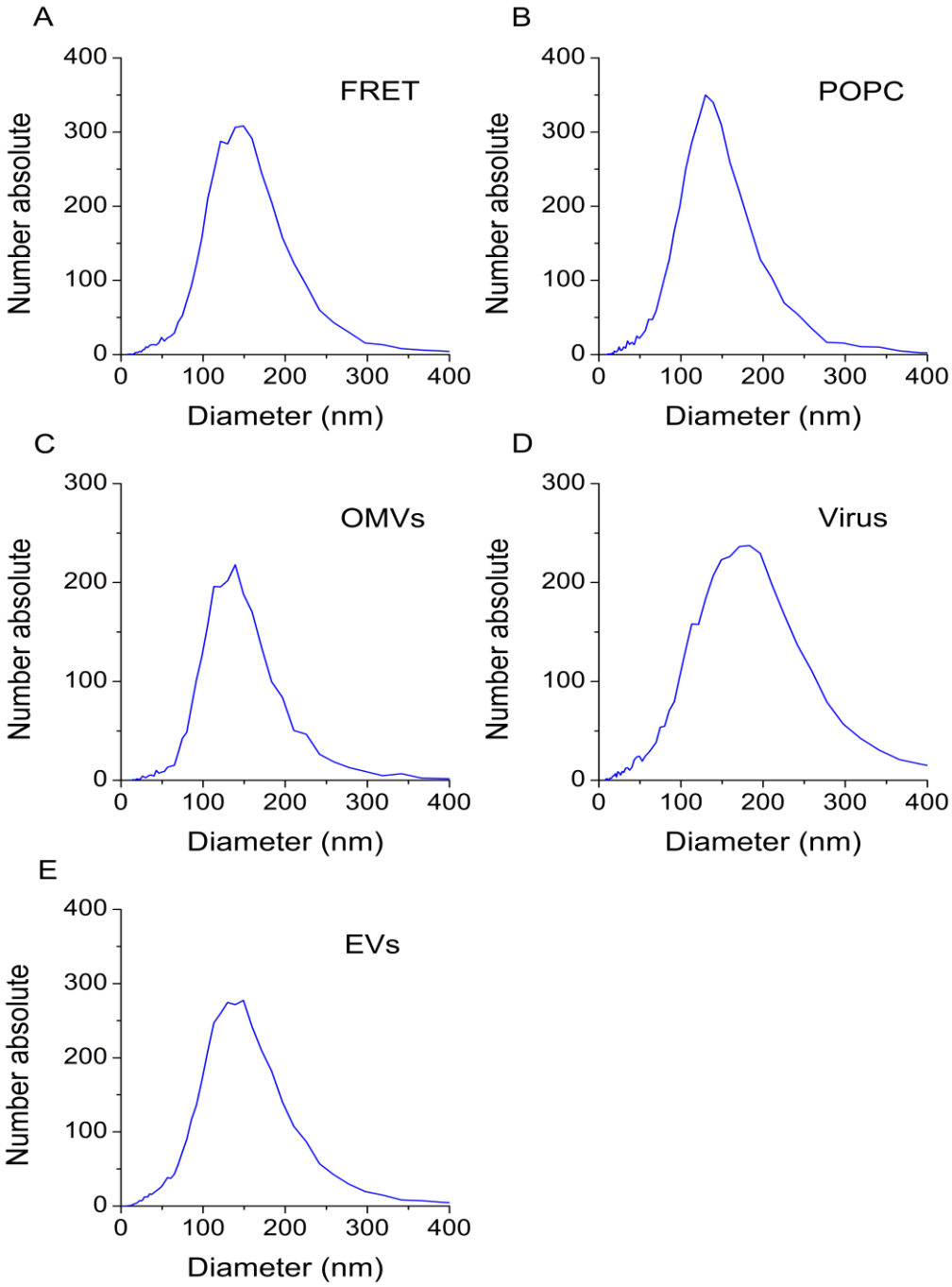

Figure S5. Vesicle size distributions as determined by NTA. A) FRET liposomes. B) POPC liposomes used for calibration curve generation. C) OMV sample. D) HSV-2 sample. E) EV sample.

Section 7: Probing the effects of contaminants on assay performance.

This assay was designed to quantify purified vesicles of complex composition from a variety of sources. While there is no evidence that a vesicle's composition or origin affects the assay's ability to accurately quantify it, there is the potential that contaminants present in the sample's diluent related to its origin and/or its purification processes could interfere with the assay. As the limit of detection (LOD) of the assay is tied to the FRET signal of a blank, i.e. the FRET vesicles alone in this case (see main manuscript), we investigated the effect of sonicating FRET vesicles in the presence of different substances (Figure S6A, black bars). Additionally we tested how different substances affected the signal of an equimolar mixture of FRET vesicles with POPC vesicles (gray bars). We found that 2 mg/mL BSA (≥98% purity) lowered the FRET signal of the blank sample, indicating that it raised the LOD. The decrease in FRET signal beyond what is expected for a known concentration of POPC in PBS, indicates that a new calibration curve needs to be prepared in a BSA background matrix, before quantification can be carried out. As a typical goal of vesicle isolation/purification protocols is to minimize free-protein contamination in the vesicle samples, it is unlikely that this effect of BSA will be detrimental to properly purified samples. However, the purification protocols themselves are likely to leave residual density gradient medium in the samples, so the effect of sucrose and Optiprep on the assay was investigated. Additionally, the commonly used cryo-protectant glycerol, which may be present in frozen aliquots, was also tested. Neither sucrose, Optiprep, nor glycerol showed a significant effect on the blank signal (black bars) or on the assays ability to quantify a vesicle sample (gray bars) (Figure S6A).

As the presence of 2 mg/mL of BSA did appear to affect both the LOD and the ability of the assay to quantify a vesicle sample, we produced a new partial calibration curve to better illustrate how

the presence of BSA affects the LOD and slope of the calibration curve (Figure S6B) in this specific case. In 2 mg/mL BSA the LOD increased by ~ a factor 4 from $9.5*10^{12}$ nm^2/mL to $4*10^{13}$ nm^2/mL. Furthermore, the decrease in calibration curve slope indicates that the assays sensitivity is decreased, although quantification remains possible under these conditions using the appropriate calibration curve. The idea that the slope of the calibration curve will be different for different concentrations of an interfering substance, provides the logical argument that if two dilutions of an unknown sample align on a calibration curve then that calibration curve is well-suited for the quantification of the said sample.

In conclusion, it is important to use proper purification techniques for vesicle isolation prior to quantification, as this assay is not meant to be used with biological fluids. Furthermore, it is strongly advised to use two dilutions in the linear region of the calibration curve for quantification. Finally, if there is ever doubt about the effect of a component present in the sample, then the creation of a new calibration curve using a background diluent that contains the suspected contaminants is of course the best strategy to take the effects of those contaminants into account.

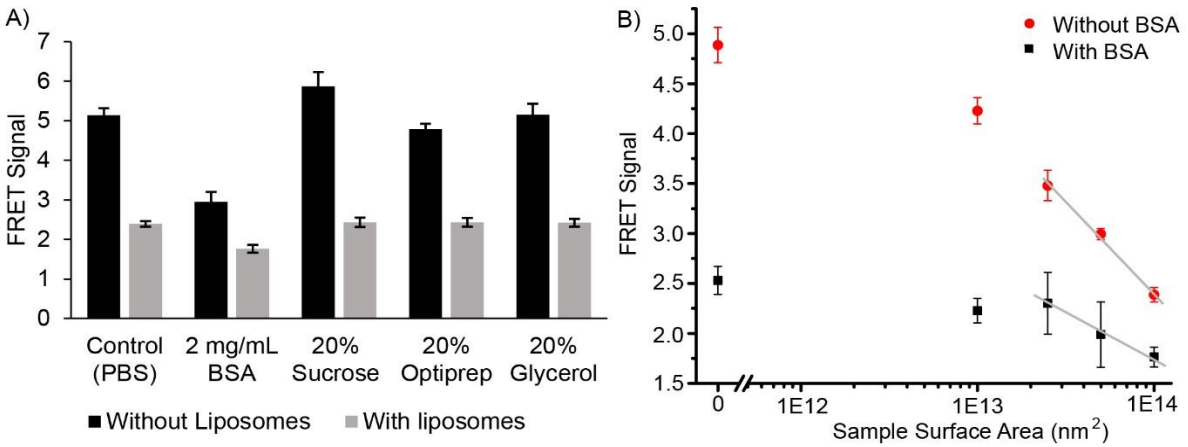

Figure S6. Effect of Contaminants on the FRET Assay. A) FRET vesicles alone (black bars) or FRET vesicles mixed 1:1 with POPC vesicles (grey bars) sonicated for 20 minutes at 40°C in different substances. B) Comparison of partial calibration curves generated with (black dots) and without (red dots) 2 mg/mL BSA present. The grey lines denote the linear regions of the calibration curves.

References

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