

***Supporting Information: MARCKS-ED Peptide as a Curvature and Lipid Sensor***

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## **SUPPORTING VIDEOS**

**Supplementary Video 1:** Scatter mode of rat plasma.

**Supplementary Video 2:** Fluorescence mode of rat plasma, showing no inherent fluorescence in the absence of AlexaFluor-546<sup>TM</sup>-labeled peptides.

**Supplementary Video 3:** Fluorescence mode of rat plasma with AlexaFluor-546<sup>TM</sup>-labeled MARCKS-ED, showing fluorescence for a fraction of vesicle particles observed under scatter mode in the presence of the AlexaFluor-546<sup>TM</sup>-labeled MARCKS-ED peptide.

**Supplementary Video 4:** Fluorescence mode of rat plasma with MARCKSmut1, showing reduced fluorescence for a fraction of vesicle particles observed under scatter mode in the presence of the AlexaFluor-546<sup>TM</sup>-labeled MARCKSmut1 peptide.

**Supplementary Video 5:** Fluorescence mode of rat plasma with MARCKSmut2, showing reduced fluorescence for a fraction of vesicle particles observed under scatter mode in the presence of the AlexaFluor-546<sup>TM</sup>-labeled MARCKSmut2 peptide.

## SUPPORTING METHODS, TABLES, AND FIGURES

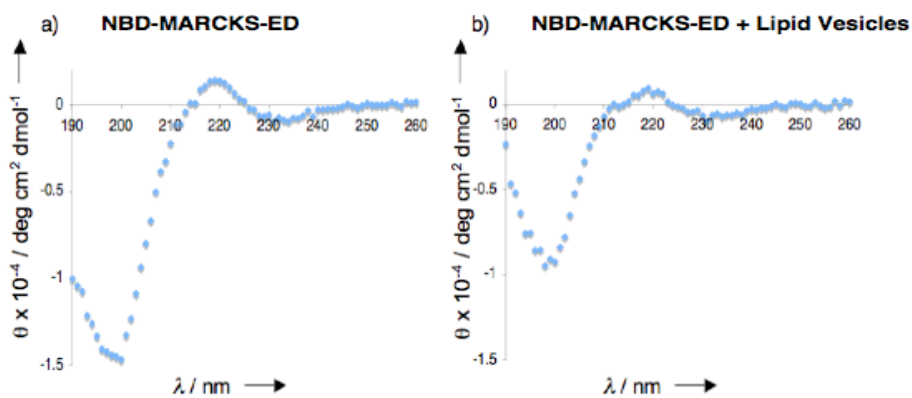
**Solid Phase Peptide Synthesis:** Peptides were synthesized using a CEM Liberty microwave-assisted solid phase peptide synthesizer (Matthews, North Carolina) following the standard Fmoc chemistry (1). At the end of the solid phase syntheses, the resin beads were washed with the following: dimethylformamide (DMF), dichloromethane (DCM), and methanol. The resin beads were then dried for one hour and the peptides cleaved using a water/trifluoroacetic acid (TFA)/triisopropylsilane (TIPS) cocktail (2.5/95/2.5) for 2-3 hours under inert conditions. The peptides were precipitated using chilled diethyl ether. The peptides were then purified using reverse phase high performance liquid chromatography (Agilent Technologies, Santa Clara, California) through a semi-prep C8 column using a linear gradient of buffer A (0.1% TFA in 95% H<sub>2</sub>O/5% acetonitrile) and buffer B (0.1% TFA in acetonitrile).

**Table 1.** Complete list of peptides synthesized, purified and characterized in these studies.

a) NBD- MARCKS -ED	NBD-Ahx-KKKKKRFSFKKSFKLSGFSFKKNNK
b) MARCKS-ED	KKKKKRFSFKKSFKLSGFSFKKNNK
c) NBD-MARCKSmut1	NBD-Ahx-KKKKKRASAKKSAKLSGASAKKNNK
d) NBD- MARCKSmut2	NBD-Ahx-AAAAAFSFAASFALSGFSFAANAA
e) NBD-C2B	NBD-GG-DYDKIGKNDA
f) Alexa Fluor 546 <sup>TM</sup> - MARCKS-ED	Alexa Fluor 546-KKKKKRFSFKKSFKLSGFSFKKNNK
g) Alexa Fluor 546 <sup>TM</sup> - MARCKSmut1	Alexa Fluor 546-KKKKKRASAKKSAKLSGASAKKNNK
h) Alexa Fluor 546 <sup>TM</sup> - MARCKSmut2	Alexa Fluor 546-AAAAAFSFAASFALSGFSFAANAA
i) MARCKS-SCRAM-scr	NBD-Ahx-KKKGKKNSSKFFFFSKFLSRKKK

Ahx =  $\epsilon$ -aminohexanoic acid

**Circular Dichroism:** Ultraviolet circular dichroism was performed to investigate the secondary structure of NBD-MARCKS-ED. These spectra yielded a non-helical, unstructured conformation consistent to previous reports for both the untreated and vesicle-treated samples (2). Five scans were obtained and averaged for each sample from 190 to 260 nm with data points taken every 1.0 nm.



**Figure 1.** Circular dichroism (CD) spectra showing an unstructured conformation of NBD-MARCKS-ED both in the presence and absence of lipid vesicles (PC:PS:PE:cholesterol/50:20:15:15) (3). NBD-MARCKS-ED in phosphate buffer in the a) absence and b) presence of 30 nm pore size vesicles (PC:PS:PE:cholesterol/50:20:15:15). [NBD-MARCKS-ED peptide] = 10  $\mu\text{M}$ . [Total lipid] = 500  $\mu\text{M}$ .

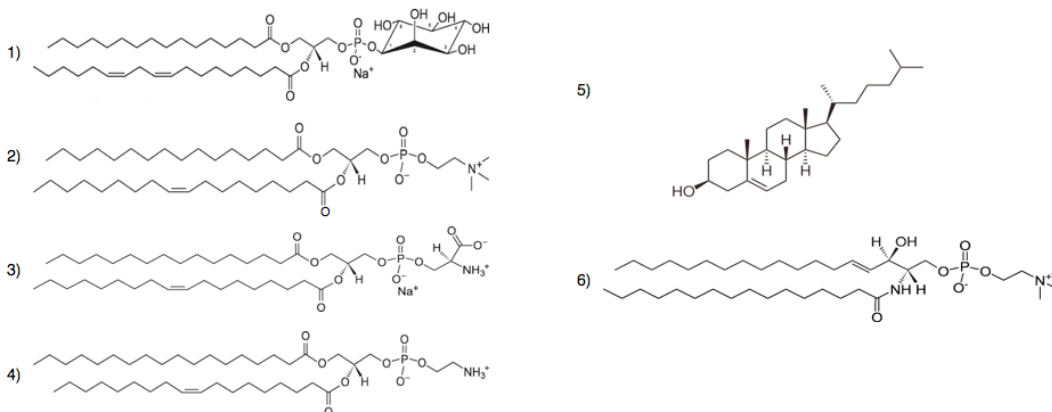
**Liposome Preparation:** To produce lipid vesicle solutions, an established protocol was followed (4). The following materials were used: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS), L- $\alpha$ -phosphatidylcholine (POPC) and cholesterol (with L- $\alpha$ -phosphatidylinositol (PI) and sphingomyelin added to samples for the Annexin-V fluorescence studies previously reported to mimic a *C. elegans* membrane model (5) at the appropriate amounts to produce 1 and 2 mM stock solutions for *in vitro* binding assays (Avanti Lipids, Alabaster, Alabama). The organic solvent was removed under a gentle stream of nitrogen gas until a thin, white film was observed at the bottom of the glass vial. The lipid mixture was placed in the dessicator in vacuo for 30 minutes. Phosphate buffer (pH= 7.40) was then added to the dry film and allowed to hydrate overnight at 4°C. Figure S1 describes all the lipid components.

**Synthetic Lipid Models:**

Model 1: 70% POPC: 15% Cholesterol: 15% POPE: 0% POPS  
Model 2: 60% POPC: 15% Cholesterol: 15% POPE: 10% POPS  
Model 3: 50% POPC: 15% Cholesterol: 15% POPE: 20% POPS

**For Annexin-V *in vitro* Lipid Models:**

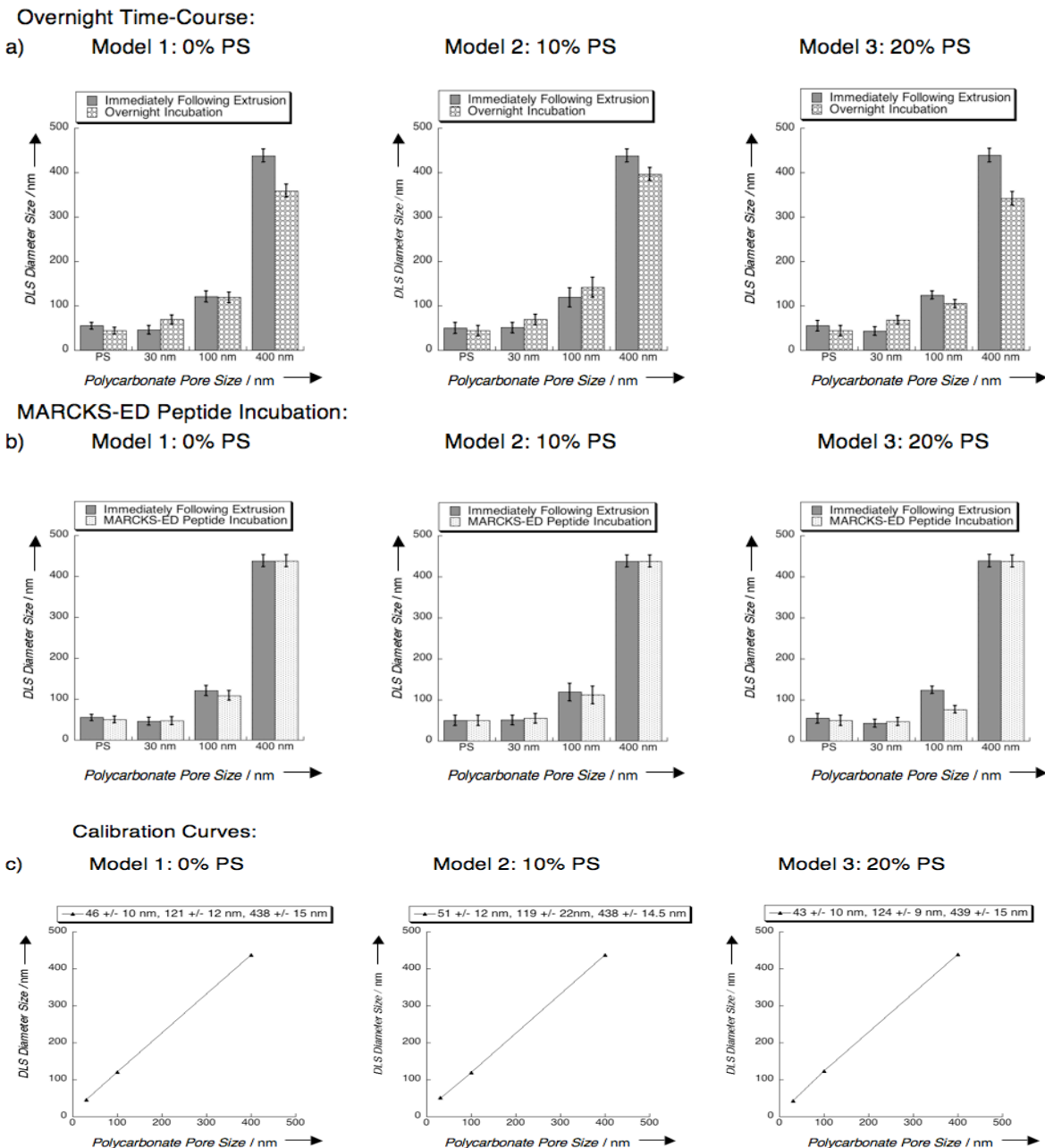
Model 4: 44% POPC: 18.5% POPE: 16.5% Sphingomyelin: 16% Cholesterol: 5% PI: 0% POPS  
Model 5: 42% POPC: 18.5% POPE: 16.5% Sphingomyelin: 16% Cholesterol: 5% PI: 2% POPS  
Model 6: 39% POPC: 18.5% POPE: 16.5% Sphingomyelin: 16% Cholesterol: 5% PI: 5% POPS  
Model 7: 34% POPC: 18.5% POPE: 16.5% Sphingomyelin: 16% Cholesterol: 5% PI: 10% POPS



**Figure 2.** Phospholipid components of all synthetic lipid models. Chemical structures of : 1) L- $\alpha$ -Phosphatidylinositol. 2) L- $\alpha$ -phosphatidylcholine. 3) Palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine. 4) 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine. 5) Cholesterol. 6) Sphingomyelin.

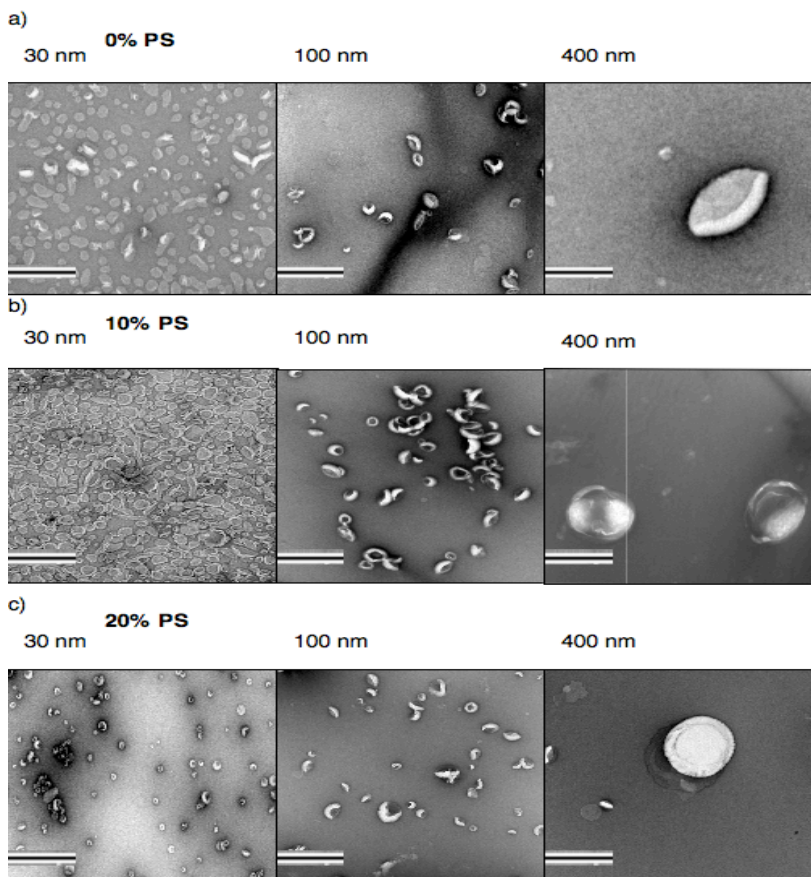
**Liposome Extrusion.** Liposome suspensions were extruded through the Avestin FL-50 pressurized extruder (Ottawa, Canada) using polycarbonate membrane filters with pore sizes of 30, 100, and 400 nm. Each liposome size was prepared by extrusion through the filters 5-6 times to increase homogeneity. A MS 800 DynaPro Dynamic Light Scattering (DLS, Dernbach, Germany) instrument was used to determine the vesicle sizes and % polydispersity for each

synthetic lipid vesicle model (Figure S2). All samples were diluted to at least 25 $\times$  prior to DLS characterization due to instrumental concentration limitations.



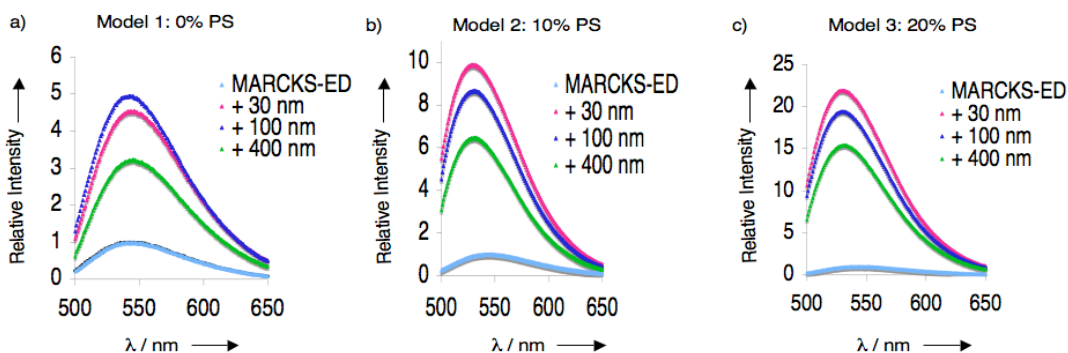
**Figure 3.** DLS plots of each vesicle pore size for all synthetic models 1, 2 and 3, including the standard 50 nm polystyrene beads (Polysciences) used for calibration. a) DLS bar plots of actual vesicle sizes for Models 1, 2 and 3 immediately following extrusion and after an untreated overnight incubation at vesicle concentrations used for the fluorescence enhancement assays. [Total Lipid] = 500  $\mu$ M. b) DLS bar plots of actual vesicle sizes for Models 1, 2 and 3 immediately following extrusion and after an overnight peptide incubation at concentrations used for the fluorescence enhancement assays. [Total Lipid] = 500  $\mu$ M. [NBD-MARCKS-ED] = 500 nM. c) Standard vesicle correction curves for Models 1, 2 and 3 to show the relationship between the polycarbonate pore sizes and the actual lipid vesicle sizes following extrusion.

**Negative stain Transmission Electron Microscopy (TEM):** Carbon formvar, mesh grids were negatively discharged prior to sample staining, where 1% uranyl-acetate in water was used to stain the samples prior to drying and imaging at  $\times 25,000$  magnification.



**Figure 4.** Negative stain transmission electron microscopic (TEM) images of untreated synthetic lipid vesicles. a) From left to right: Lipid vesicles of pore sizes 30, 100 and 400 nm containing 70% phosphatidylcholine (PC): 15% cholesterol: 15% phosphatidylethanolamine (POPE). b) From left to right: Lipid vesicles of pore sizes 30, 100 and 400 nm containing 60% phosphatidylcholine (PC): 15% cholesterol: 15% phosphatidylethanolamine (POPE): 10% phosphatidylserine (PS). c) From left to right: Lipid vesicles of pore sizes 30, 100 and 400 nm containing 50% phosphatidylcholine (PC): 15% cholesterol: 15% phosphatidylethanolamine (POPE): 20% phosphatidylserine (PS). [Total Lipid] = 500  $\mu$ M. Magnification =  $\times 25000$ . Scale bar = 0.50  $\mu$ m. Actual vesicle size measurements with standard error are observed in Supplementary Figure 2.

**Fluorescence enhancement assays:** The emission spectra for NBD-labeled MARCKS peptides were observed using the Fluorolog-3 (Horiba Jobin Yvon; Edison, New Jersey) at the  $\lambda_{\text{ex}}$  of 480 nm. Calmodulin (Sigma Aldrich; St. Louis, Missouri) was incubated with  $\text{Ca}^{2+}$  before treatment with NBD-MARCKS-ED and 400 nm pore size vesicles containing 20% PS. A non-specific linear peptide, NBD-C2B, showed remarkably reduced binding, suggesting that the curvature-sensing behavior of MARCKS peptide is sequence specific (6, 7). The NBD fluorophore showed no change in fluorescence intensity, confirming that fluorescence enhancement is based on the peptide. MARCKS-ED's binding to the membrane was reversed by the addition of CaM and  $\text{Ca}^{2+}$ , further supporting that MARCKS-ED binding is similar to the full-length MARCKS protein (Figure S5).



**Figure 5.** Emission spectra from fluorescence enhancement binding assays for MARCKS-ED. A blue shift was observed for all peptide-vesicle samples in all lipid models. Higher fluorescence enhancement was observed in vesicles with increasing PS. a) Model 1 plot of 0% PS showing the largest increase of fluorescence intensity for the 100 nm vesicles, followed by the 30 nm and then the 400 nm vesicles. b) Model 2 plot 10% PS showing the largest increase of fluorescence intensity for the 30 nm vesicles, followed by the 100 nm and then the 400 nm vesicles. c) Model 2 plot 20% PS showing the largest increase of fluorescence intensity for the 30 nm vesicles, followed by the 100 nm and then the 400 nm vesicles. [Total Lipid] = 500  $\mu\text{M}$ . [NBD-MARCKS-ED] = 500 nM.  $\lambda_{\text{ex}}$  = 480 nm.  $\lambda_{\text{em}}$  = 545 nm.

**Table 2.** ANOVA single factor analysis with  $\alpha = 0.05$ . Statistical analysis was performed to determine if the differences between the average peak maxima for each peptide-vesicle sample in the fluorescence enhancement assay were significant for MARCKS-ED. The largest significance is observed with lipid Model 2.

Model 1: 0% PS	p value	Result
MARCKS-ED vs 30 nm	0.0017	Significant
MARCKS-ED vs 100 nm	0.0020	Significant
MARCKS-ED vs 400 nm	0.0031	Significant
30 nm vs 100 nm	0.2480	Not significant
100 nm vs 400 nm	0.0149	Significant
30 nm vs 400 nm	0.0188	Significant



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<b>Model 2: 10% PS</b>	<b>p value</b>	<b>Result</b>
MARCKS-ED vs 30 nm	0.0006	Significant
MARCKS-ED vs 100 nm	0.0001	Significant
MARCKS-ED vs 400 nm	0.0023	Significant
30 nm vs 100 nm	0.0098	Significant
100 nm vs 400 nm	0.0026	Significant
30 nm vs 400 nm	0.0022	Significant

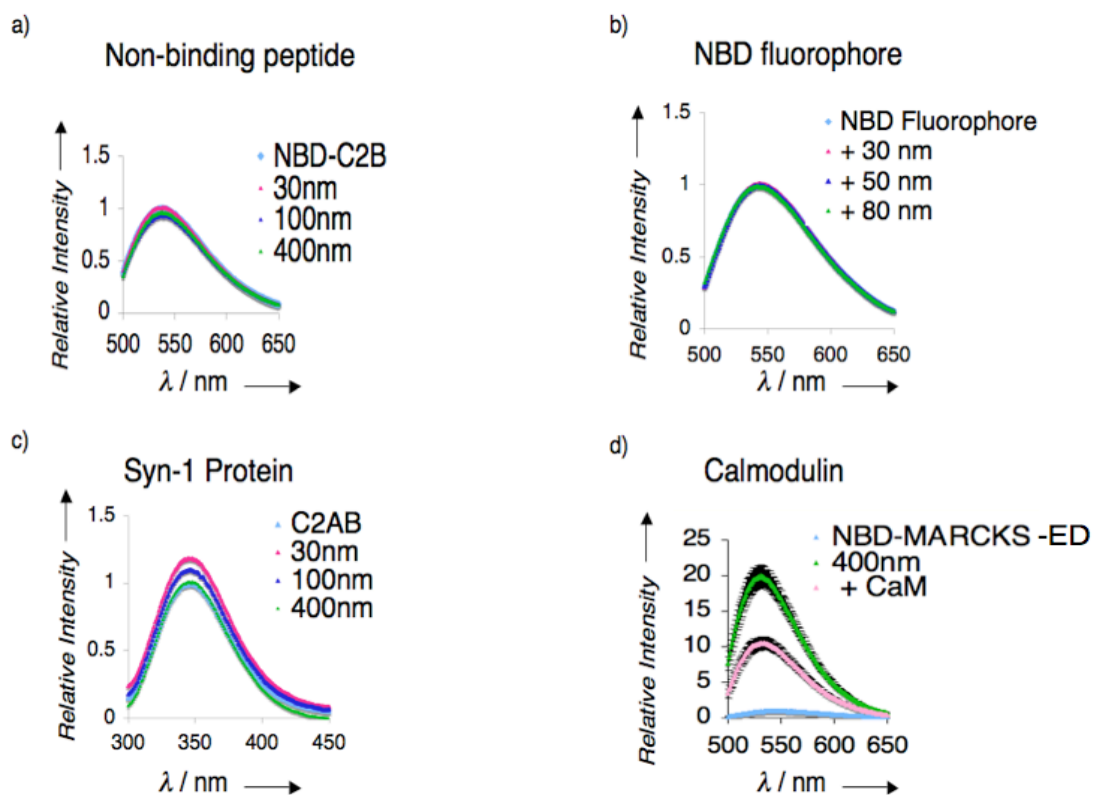
<b>Model 3: 20% PS</b>	<b>p value</b>	<b>Result</b>
MARCKS-ED vs 30 nm	0.0005	Significant
MARCKS-ED vs 100 nm	0.0004	Significant
MARCKS-ED vs 400 nm	0.0009	Significant
30 nm vs 100 nm	0.0546	Not significant
100 nm vs 400 nm	0.0195	Significant
30 nm vs 400 nm	0.0096	Significant

**Table 3.** ANOVA single factor analysis with  $\alpha= 0.05$ . Statistical analysis was performed to determine if the differences between the average peak maxima for each peptide-vesicle sample in the fluorescence enhancement assay were significant for the MARCKSmut1 peptide.

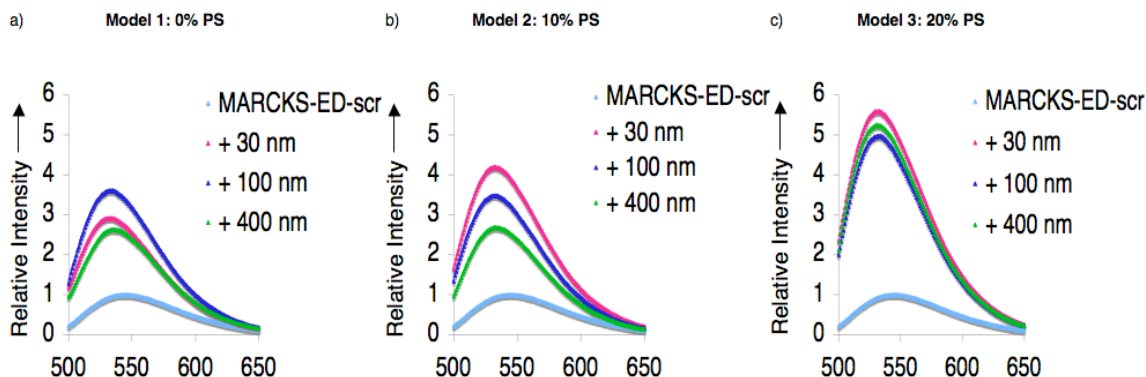
<b>Model 2: 10% PS</b>	<b>p value</b>	<b>Result</b>
MARCKSmut1 vs 30 nm	0.0037	Significant
MARCKSmut1 vs 100 nm	0.0020	Significant
MARCKSmut1 vs 400 nm	0.0120	Significant
30 nm vs 100 nm	0.0300	Significant
100 nm vs 400 nm	0.0038	Significant
30 nm vs 400 nm	0.0100	Significant

**Table 4.** ANOVA single factor analysis with  $\alpha = 0.05$ . Statistical analysis was performed to determine if the differences between the average peak maxima for each peptide-vesicle sample in the fluorescence enhancement assay were significant for the MARCKSmut2 peptide.

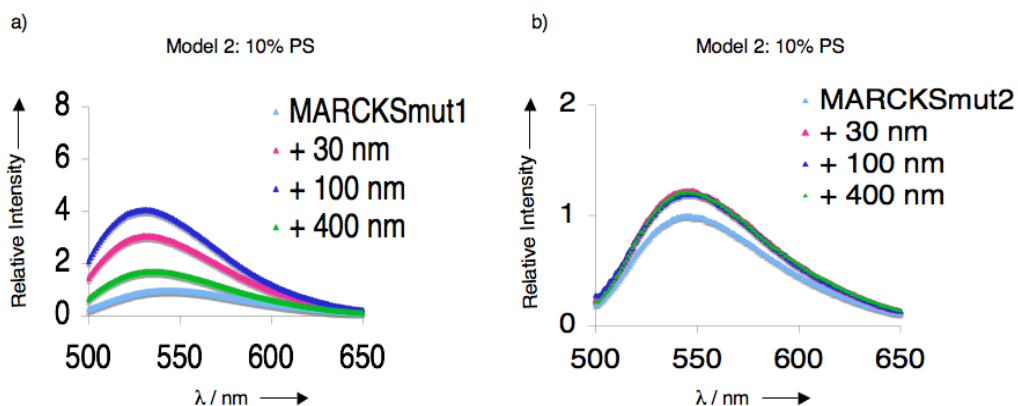
<b>Model 2: 10% PS</b>	<b>p value</b>	<b>Result</b>
MARCKSmut2 vs 30 nm	0.0036	Significant
MARCKSmut2 vs 100 nm	0.0190	Significant
MARCKSmut2 vs 400 nm	0.0300	Significant
30 nm vs 100 nm	0.1880	Not significant
100 nm vs 400 nm	0.1390	Not significant
30 nm vs 400 nm	0.3000	Not significant



**Figure 6.** Emission spectra of fluorescence enhancement binding assays. a) Emission plot showing linear, non-specific peptide, NBD-C2B with no changes in fluorescence intensity between untreated and vesicle-treated samples with vesicles of Model 3; PC (50%): PE (15%): Cholesterol (15%): PS (20%). [Peptide] = 500 nM. [Total Lipid] = 500  $\mu$ M. b) Emission plot showing the NBD fluorophore with no differences in fluorescence intensity between untreated and vesicle-treated samples with vesicles of Model 3; PC (50%): PE (15%): Cholesterol (15%): PS (20%). [NBD Label] = 500 nM. [Total Lipid] = 500  $\mu$ M. c) Emission plot showing the C2AB domain from Synaptotagmin-1 protein selectively binding to more highly curved liposomes in the presence of calcium with no difference in fluorescence intensity with the large 400 nm liposomes and the untreated C2AB protein with vesicles of Model 3; PC (50%): PE (15%): Cholesterol (15%): PS (20%). [C2AB protein] = 200 nM. [Ca<sup>2+</sup>] = 2.5 mM. [Total Lipid] = 400  $\mu$ M. d) Emission plot showing Calmodulin (CaM) competing for MARCKS-ED from the 400 nm liposome with vesicles of Model 3 (PC (50%): PE (15%): Cholesterol (15%): PS (20%). [CaM] = 1  $\mu$ M. [Ca<sup>2+</sup>] = 14  $\mu$ M. [Peptide] = 500 nM. [Total Lipid] = 500  $\mu$ M.  $\lambda_{ex}$  = 480 nm (a, b, d).  $\lambda_{ex}$  = 280 nm (c).  $\lambda_{em}$  = 545 nm (a, b, d).  $\lambda_{em}$  = 345 nm (c).



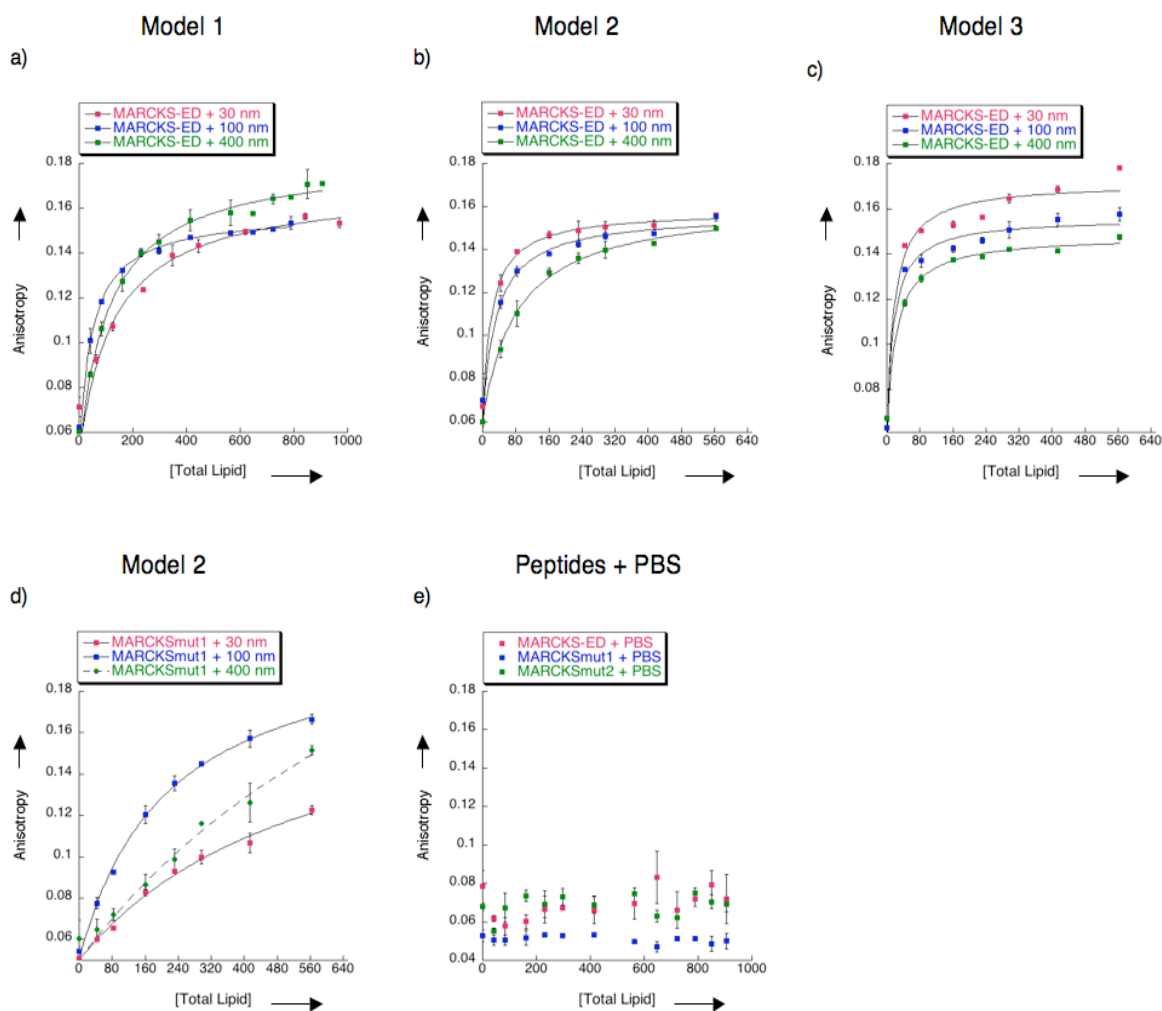
**Figure 7.** Emission spectra from fluorescence enhancement binding assays for MARCKS-ED-scr. Higher fluorescence enhancement was observed in vesicles with increasing PS. a) Model 1 plot of 0% PS showing the largest increase of fluorescence intensity for the 100 nm vesicles, followed by the 30 nm and then the 400 nm vesicles. b) Model 2 plot 10% PS showing the largest increase of fluorescence intensity for the 30 nm vesicles, followed by the 100 nm and then the 400 nm vesicles. c) Model 2 plot 20% PS showing the largest increase of fluorescence intensity for the 30 nm vesicles, followed by the 400 nm and then the 100 nm vesicles. [Total Lipid] = 500  $\mu$ M. [NBD-MARCKS-ED-scr] = 500 nM.  $\lambda_{ex}$  = 480 nm.  $\lambda_{em}$  = 545 nm.



**Figure 8.** Emission spectra from fluorescence enhancement binding assays for MARCKSmut1 and MARCKSmut2. A blue shift was observed for all peptide-incubated samples of Model 2. a) Model 2 plot with 10% PS showing the largest increase of fluorescence intensity for the 100 nm vesicles, followed by the 30 nm and then the 400 nm vesicles incubated with MARCKSmut1. b) Model 2 plot with 10% PS showing no change in fluorescence intensity among all 30, 100 and 400 nm pore size lipid vesicles incubated with MARCKSmut2 peptide. [Total Lipid] = 500  $\mu$ M. [NBD-MARCKSmut1] = 500 nM. [NBD-MARCKSmut2] = 500 nM.  $\lambda_{ex}$  = 480 nm.  $\lambda_{em}$  = 545 nm.

**Fluorescence Anisotropy:** All fluorescence anisotropy titration data were collected using a Horiba Jobin Yvon Fluorolog-3 fluorometer (Edison, New Jersey). The following equation was used to determine the dissociation constant ( $K_d$ ) values as previously reported (8).

Equation:  $F_b = K_p[L]/(1 + K_p[L])$ , where  $F_b$  expresses the fraction of peptide bound to lipids,  $K_p$  expresses the molar partition coefficient and  $[L]$  expresses the lipid concentration. The equation was fitted to the anisotropy plot where the inverse of  $K_p$  correlates to the effective dissociation constant,  $K_d$ .



**Figure 9.** Fluorescence anisotropy assays. a-c) Titration curves of 30, 100 and 400 nm pore size vesicles to NBD-MARCKS-ED with lipid models 1, 2 and 3. d) Titration curve of 30, 100 and 400 nm pore size vesicles to NBD-MARCKSmut1 with lipid model 2. e) Titration of PBS buffer to NBD-MARCKS-ED, NBD-MARCKSmut1 and NBD-MARCKSmut2. [Peptide] = 1  $\mu$ M. PBS = Phosphate-buffered saline buffer at 7.40 pH.  $\lambda_{ex}$  = 480 nm.  $\lambda_{em}$  = 545 nm.

**Table 5.** The apparent dissociation constants,  $K_d$ , of MARCKS-ED for 30, 100 and 400 nm pore size vesicles of lipid models 1, 2 and 3.

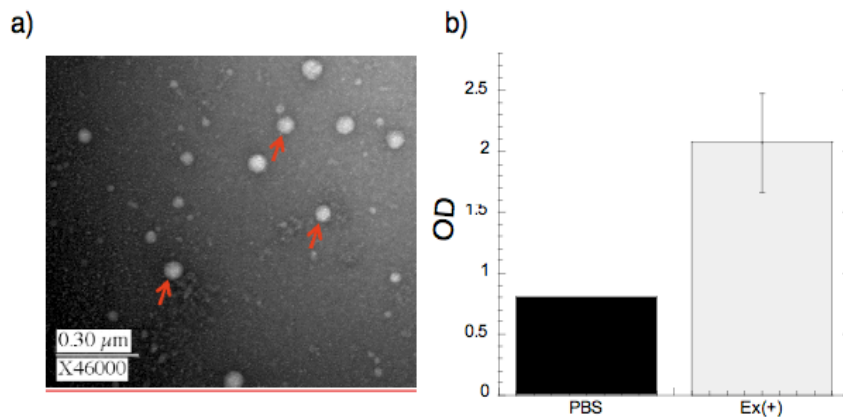
Vesicle Size (nm)	Model 1: 0% PS	Model 2: 10% PS	Model 3: 20% PS
30	141 ± 17 $\mu$ M	24 ± 3 $\mu$ M	16 ± 3 $\mu$ M
100	64 ± 5 $\mu$ M	42 ± 13 $\mu$ M	18 ± 1 $\mu$ M
400	146 ± 18 $\mu$ M	86 ± 20 $\mu$ M	26 ± 6 $\mu$ M

**Table 6.** The apparent dissociation constants,  $K_d$ , of the MARCKSmut1 and MARCKSmut2 peptides for 30, 100 and 400 nm pore size vesicles of lipid model 2.

Vesicle Size (nm)	MARCKSmut1	MARCKSmut2
30	651 ± 137 $\mu$ M	537 ± 246 $\mu$ M
100	245 ± 34 $\mu$ M	392 ± 58 $\mu$ M
400	929 ± 364 $\mu$ M	886 ± 851 $\mu$ M

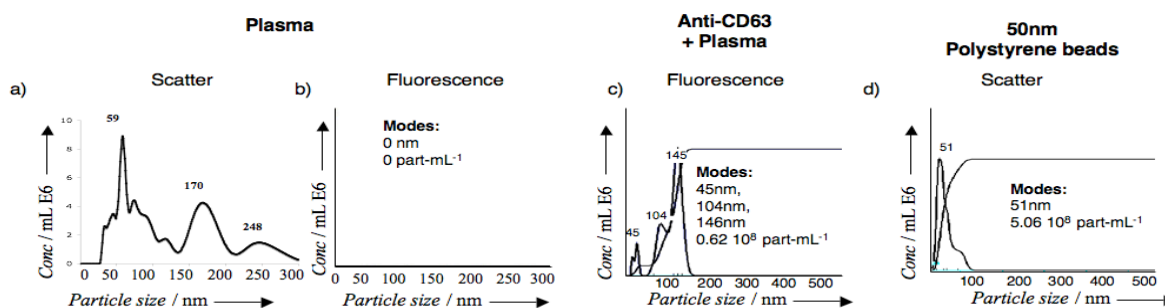
**Microvesicle Sample Preparation.** On the day of the experiment, the animals were exposed to 100, 1.5 mA, 5-second, intermittent, (average trial interval = 60 seconds  $\pm$  25 seconds) inescapable tailshocks (stress). During the stress procedure rats were placed in a plexiglass restraining tube (23.4 cm long, 7 cm diameter). Electrodes were placed across the tail, which protruded from the back of the shock tube. The shocks were administered by an automated shock system (Precision Calculated Animal Shocker; Coulbourn Instruments, Whitehall, Pennsylvania). Immediately following stressor termination, animals were sacrificed via rapid decapitation. Trunk blood was collected in EDTA-coated tubes (13 $\times$ 75 mm) and plasma was isolated via 3000  $\times$  g centrifugation at 4  $^{\circ}$ C for 15 min. Isolated microvesicles were quantified and analyzed using a scatter mode from the NTA microscope.

Individual wells in a ninety-six well plate were coated with 100  $\mu$ L of 4  $\mu$ g/mL rabbit polyclonal Rab5B antibody (clone A-20, Santa Cruz Biotechnology, Santa Cruz, California) in carbonate-bicarbonate buffer and incubated overnight at 4  $^{\circ}$ C. Following three washes with phosphate buffered saline Tween (PBST), 100  $\mu$ L of 2.5% bovine serum albumin (BSA) in PBS was added to each well and incubated overnight at 4  $^{\circ}$ C. After three washes with PBST, 50  $\mu$ L of **microvesicle** suspension or PBS was added to the wells and incubated overnight at 37  $^{\circ}$ C. Following three washes with PBST, purified mouse anti-rat CD63 antibody (clone AD1, BD Pharmingen, San Jose, California) was diluted to 8  $\mu$ g/mL in PBS and 100  $\mu$ L was added to all the wells. After incubating at 37  $^{\circ}$ C for one hour, the plate was washed three times with PBST and incubated at 37  $^{\circ}$ C with 100  $\mu$ L of goat anti-mouse Poly-HRP (Pierce, Rockford, Illinois) diluted to 1:50,000 in 1% BSA in PBS. After three washes with PBST, the plate was developed with peroxidase detection (Roche Applied Science, Milan, Italy) for 10 minutes and the reaction was stopped with 1 M H<sub>2</sub>SO<sub>4</sub>. The optical densities (OD) were recorded at 450 nm using SpectraMax Plus384 microplate reader (Molecular Devise, Sunnyvale, California). Figure S11b is the plot of the optical densities of PBS and MVs re-suspended in PBS, Ex(+).



**Figure 10.** Exosome confirmation. a) Isolated **microvesicles** from anti-CD63 rat blood plasma were suspended in PBS and observed through negative-stain transmission electron microscopy (TEM) to confirm the presence of **microvesicles**. b) Assay for CD63. Plot of the ELISA assay for CD63 confirming the presence of this protein in the **microvesicle** suspension, Ex (+).

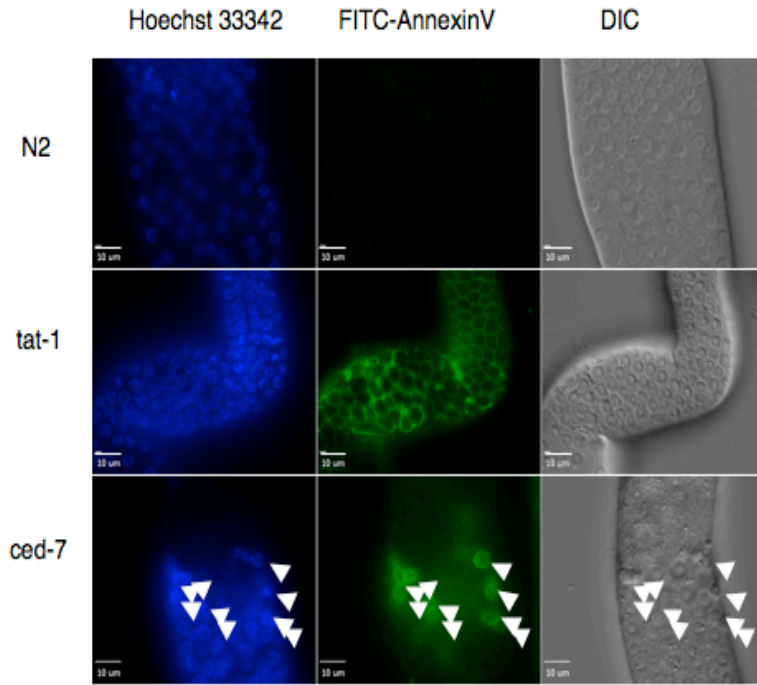
**Nanoparticle Tracking Analyses:** Resin-bound, *N*-terminal deprotected MARCKS-ED peptides (1.6 mmol) was transferred to a polypropylene tube and reacted with 500 mg Alexa Fluor 546<sup>TM</sup> (Invitrogen, Eugene, Oregon) in 75  $\mu$ L anhydrous DMF and 3.2 mmol DIPEA. The reaction was allowed to proceed for 2 hours and then washed using three cycles each of DMF, MeOH, and CHCl<sub>3</sub>. The peptide was cleaved from solid support using TFA/TIPS/H<sub>2</sub>O (95/2.5/2.5) for 2 h followed by precipitation with cold Et<sub>2</sub>O. The crude peptide was purified by reversed phase HPLC (RP-C18, 10 x 250 mm; 5 – 50% aqueous MeCN with 0.1% TFA) with detection set at 210 and 550 nm, the latter wavelength was used to detect the Alexa Fluor 546<sup>TM</sup> conjugates. Eluates were lyophilized to yield a red, fluffy solid TFA salt. A 75  $\mu$ L aliquot of MV suspension was taken and diluted to a final volume of 150  $\mu$ L using PBS and was used for the succeeding tests. Samples of 12  $\mu$ L of blood plasma were taken, treated with Alexa Fluor 546<sup>TM</sup>-labeled peptides (12  $\mu$ L, 500 nM), diluted with PBS to 300  $\mu$ L, and incubated overnight at 4 °C. To 150  $\mu$ L aliquots of these mixtures was added 150  $\mu$ L of PBS to give a final dilution of 100X with respect to the MV samples and a concentration of 10 nM for the peptide. Nanoparticle Tracking Analyses (9) were performed using the LM10-HS instrument (NanoSight Ltd., Amesbury, UK) equipped with a 638 nm laser at scatter mode and fluorescence mode (filter = 650 and 550 nm).



**Figure 11.** Nanoparticle Tracking Analysis (9) images showing the peak mode values and particle concentration in  $10^6$  particles- mL<sup>-1</sup> of rat plasma. The x-axis on the 2D plots correlates to particle size (nm). The y-axis on the 2D plots correlates to particle concentration ( $10^6$  particles-mL<sup>-1</sup>). a) Two-dimensional plot of untreated rat plasma under scatter mode. b) Two-dimensional plot of untreated rat plasma under fluorescence mode, showing no autofluorescence from the microvesicles present in the rat plasma solution. c) Two-dimensional plot of anti-CD63 treated with rat plasma under fluorescence mode. d) Two-dimensional plot of 50 nm polystyrene beads for calibration under scatter mode.



**Fluorescence staining of *C. elegans* gonads:** Gonads of wild type (N2), *tat-1* and *ced-7* *C. elegans* were stained by Hoechst 33342, NBD-MARCKS-ED peptide, NBD-MARCKSmut1 peptide and Annexin-V protein following a previously established protocol (5).



**Figure 12.** *In-vivo* fluorescence staining of *C. elegans* using Annexin-V. The exposed gonad of a wild type (**top row**) hermaphrodite animal, a *tat-1* (*tm3117*) (**middle row**) and a *ced-7* (*n2094*) (**bottom row**) animal were stained with FITC-AnnexinV and Hoechst33342. Hoechst33342 (4 µM), FITC-AnnexinV (10 nM) and Nomarski images are shown. Arrows indicate germ cell corpses stained with both Hoechst and FITC-AnnexinV protein. Scale bar = 10 µm.

## SUPPORTING REFERENCES

1. Merrifield, R. B. (1963) Merrifield Solid-Phase Peptide Synthesis, *J. Am. Chem. Soc.* **85**, 2149.
2. Arbuzova, A., Schmitz, A. A., and Vergeres, G. (2002) Cross-talk unfolded: MARCKS proteins, *Biochem. J.* **362**, 1-12.
3. Matsubara, M., Yamauchi, E., Hayashi, N., and Taniguchi, H. (1998) MARCKS, a major protein kinase C substrate, assumes non-helical conformations both in solution and in complex with Ca<sup>2+</sup>-calmodulin, *FEBS Lett.* **421**, 203-207.
4. M. B., C. L., H. M.J. (2003) Extrusion technique to generate liposomes of defined size, *Methods Enzymology* **367**, 3-14.
5. Wang, X., Wang, J., Gengyo-Ando, K., Gu, L., Sun, C. L., Yang, C., Shi, Y., Kobayashi, T., Mitani, S., Xie, X. S., and Xue, D. (2007) C. elegans mitochondrial factor WAH-1 promotes phosphatidylserine externalization in apoptotic cells through phospholipid scramblase SCRM-1, *Nat. Cell Biol.* **9**, 541-549.
6. Ellena, J. F., Burnitz, M. C., and Cafiso, D. S. (2003) Location of the myristoylated alanine-rich C-kinase substrate (MARCKS) effector domain in negatively charged phospholipid bicelles, *Biophys. J.* **85**, 2442-2448.
7. Zhang, W., Crocker, E., McLaughlin, S., and Smith, S. O. (2003) Binding of peptides with basic and aromatic residues to bilayer membranes: phenylalanine in the myristoylated alanine-rich C kinase substrate effector domain penetrates into the hydrophobic core of the bilayer, *J. Biol. Chem.* **278**, 21459-21466.
8. Vergeres, G., and Ramsden, J. J. (1998) Binding of MARCKS (myristoylated alanine-rich C kinase substrate)-related protein (MRP) to vesicular phospholipid membranes, *Biochem. J.* **330 (Pt 1)**, 5-11.
9. Camussi, G., Deregibus, M. C., Bruno, S., Cantaluppi, V., and Biancone, L. (2010) Exosomes/microvesicles as a mechanism of cell-to-cell communication, *Kidney Int.* **78**, 838-848.