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The Role of Phosphaditylserine Distribution in Platelets for the Formation of Prothrombinase Complex

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UCLA CHEM 154 Section 1C

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Group 6C

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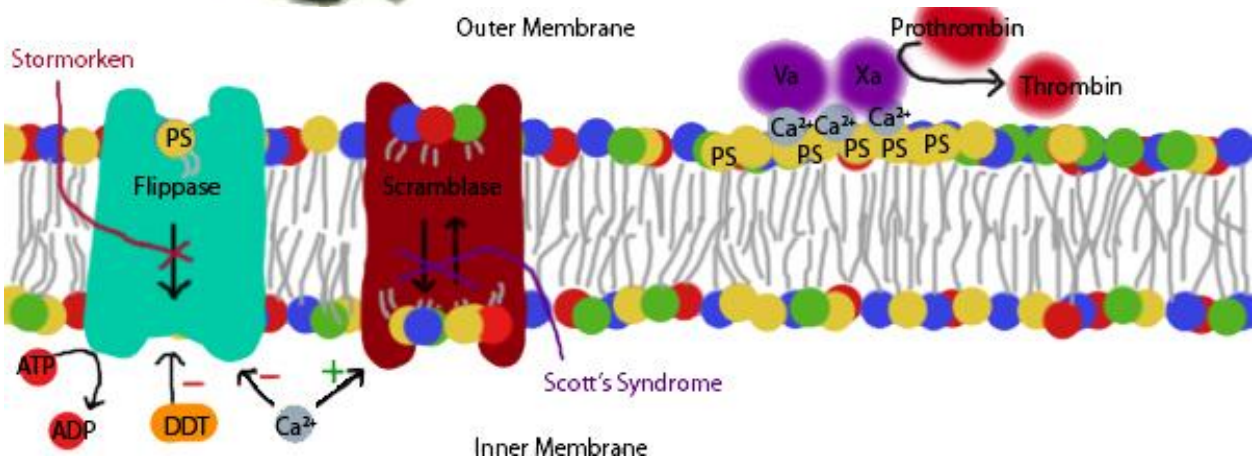

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Abstract:

Phospholipid distribution of platelets in active and resting state were compared to find Phosphatidylserine (PS) translocation to the outer membrane is crucial to the formation of prothrombinase complex. Thrombin produced by prothrombinase assays were correlated to PS present on outer membrane, so amount of translocation of PS during platelet activation is measured. Expected assay results would find PS absent on resting platelets and up to 50% PS on active results; however, premature activation on resting sample was not able to demonstrate this. The mechanism of activation was investigated to suggest that a portion of flippase on the inner layer was modified as to hinder its ability to translocate PS back to resting asymmetry. Reducing agent DDT which is capable of crossing the bilayer restored flippase resting state while glutathione that cannot cross the bilayer did not, suggesting activation by oxidation occurs inside the membrane.

Introduction:

The phospholipid bilayer is asymmetrically distributed in respect to its phospholipid (PL) content in its inner and outer membrane.[1] In healthy cells and resting platelets, phosphatidylserine (PS) and phosphatidylethanolamine (PE) are almost entirely located on the inner membrane while other phospholipids such as phosphatidylcholine (PC) and spingomyelin (SM) are predominantly located on the outer membrane.[2] The unequal distribution of PL is maintained by translocases such as flippase, floppase, and scramblase.[3] Disruption of PL distribution is observed in thrombosis, apoptosis, and cell signaling, and it explains clotting disorders such as Scott's Syndrome and Stormorken Syndrome.[3,4,5] Treatment with calcium ionophore, $CaCl_2$, diamide, or oxidation disrupts aminophospholipid translocases' ability to maintain resting asymmetry.[6,4,1,5]

When the endothelial layer of a blood vessel is damaged, platelets contact the underlying collagen which begins a clotting cascade resulting in production of thrombin.[7] Factor Xa in the cascade cleaves prothrombin (II) to thrombin (IIa) and this activity is enhanced five fold by factor Va.[7] The association of factors Xa, Va, and prothrombin, is known as the prothrombinase complex. The complex assembles with factors Xa and Va binding to negatively charged phospholipids such as PS with stabilizing divalent cation such as Ca^{2+}. Resulting thrombin (IIa) cleaves fibrinogen to clot forming fibrin[3]as well as cleaves chromogenic substrate spectrazine.[7] Many other factors exist in intrinsic and extrinsic clotting pathways, though formation of prothrombinase complex is shared and at the end of the clotting cascade and thus targeted for study.

Materials and Methods:

Experiment protocol was followed as described[5] with some modification. Isolated platelets were re-suspended in 32mL platelet suspension buffer instead of 62mL because limited porcine platelets were available. The unadjusted platelet suspensions were separated into two 15mL aliquots rather than two 30mL aliquots. Incubation with $CaCl_2$ was allowed to progress for 25 minutes at room temperature, instead of 15 minutes.

Table A: Task Responsibilities Group 6C

All six groups in the class had their own platelet samples; however, some of the more tedious tasks were shared amongst groups. Group 6C measured 1:50 and 1:55 dilutions for thrombin standard curve and 1:80, 1:90, and 1:100 dilutions for PS standard curve. Different dilutions were obtained by other groups so that standard curves could be created. Similarly, group 6C gathered assay data for only PC vesicle and flip-flop assay data with DDT with metabolic inhibitor. The remaining vesicle data and flip-flop data was provided by other groups.

Day	Part	Task	Team Member
2/18/2009	A	Solution preparation	Josh
	B	Test Prothrombinase Assay	Skipped
	C	Isolation of Platelets	TA
	C	Re-suspend Platelets	Jasmine and Heather
	D	Platelet Activation	Jasmine and Heather
	E	TNBS Labeling	Jasmine and Heather
2/20/2009	F	Adjustment of Platelet Concentration	Josh
	G	Platelet Counting	Josh
	H	Isolation of Particulate Fraction, Lipid Extraction	Heather
	I	Thrombin Standard Curve	Josh and Heather
	J	TLC of Known Phospholipids	TA
2/25/2009	K	Continued Lipid Extraction	Everyone
2/27/2009	L	Flip Flop	Jasmine and Josh
	M	TLC	Heather
	N	Preparation of Phospholipid Vesicles	TA
	N	Assay of Vesicles	Jasmine and Josh
3/4/2009	O	Phospholipid Distribution	Everyone
	P	Thrombin Assay of Age-Activated Platelets	Everyone

Adjusted platelet concentration was a 10x dilution based on the A350 of unadjusted platelets. The prothrombinase assay was not tested prior to use on resting sample. Between reading samples for prothrombinase assays, the cuvette was cleaned with HCl and rinsed 5-10 times to properly remove residual thrombin. Unilamelar phosphatidylserine (PS) vesicles were provided for thrombin assay to get PS standard curve.

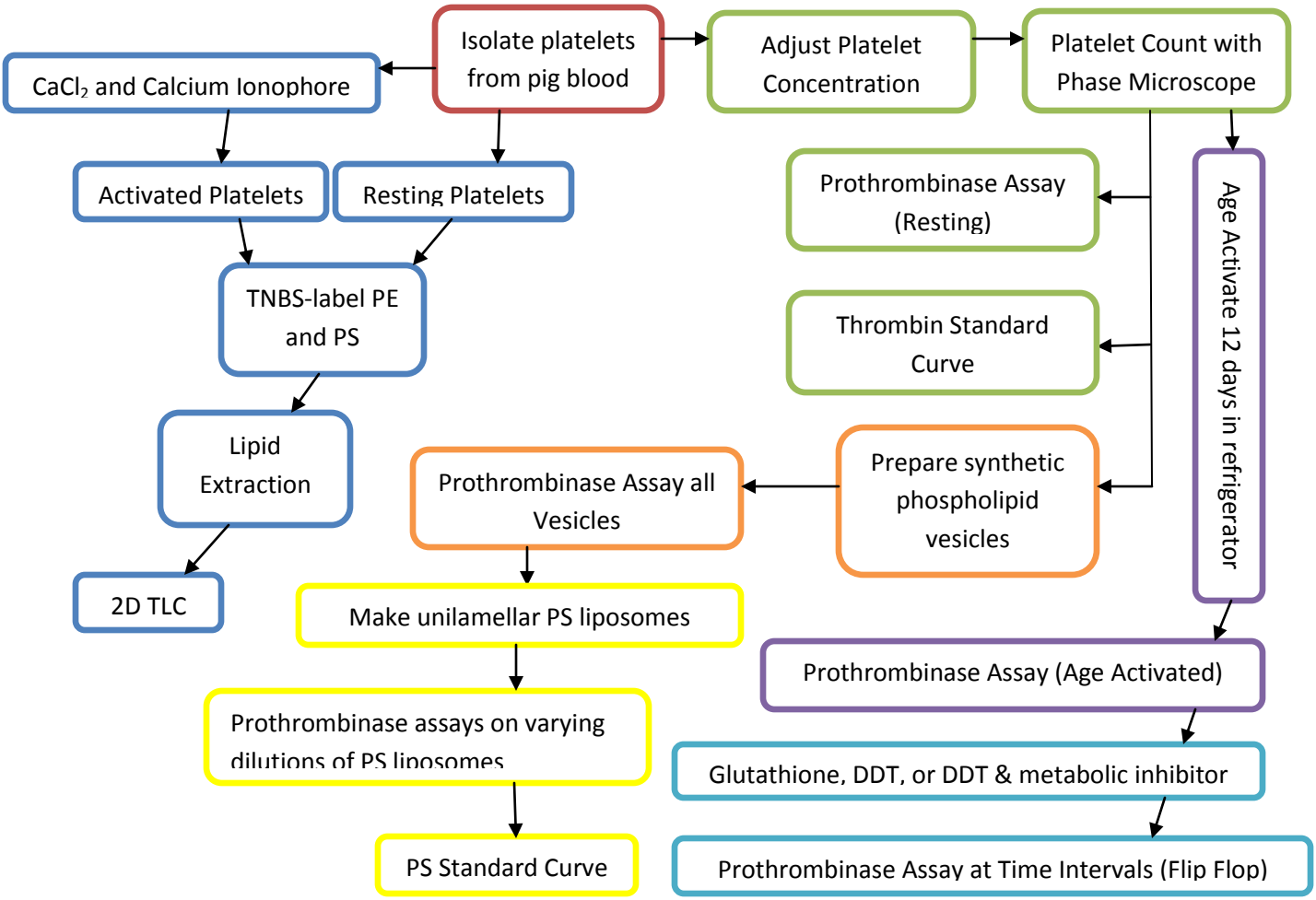
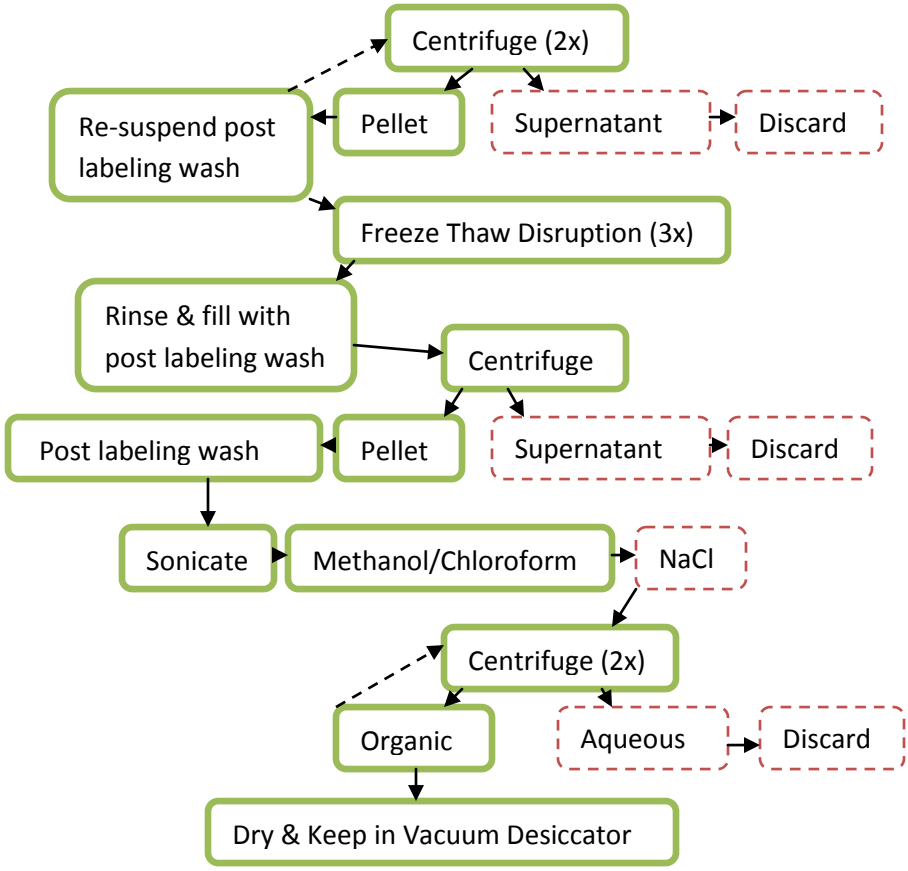


Figure 1A: Flowchart of experimental approach. Since the steps are not necessarily linear, some flexibility exists on order of performing each step. Color coordination is shown for clarity in the goal of each step, but not necessarily the order performed. It is advantageous to prioritize steps with resting platelet samples, since oxidation of flippase can activate platelets. Thus TNP-labeling and resting prothrombinase assay should be prioritized. The majority of experiment can be performed while the adjusted platelets conveniently age activate in the refrigerator.



Figuer 2B: Flowchart of Lipid Extraction: Steps outlined for lipid extraction procedure. Steps involving methanol/chloroform extractions used nitrogen gas to avoid possibility of premature oxidation of samples. Pellets containing platelets were yellow in color. Yellow color became less intense through purification steps.

Results:

Isolated platelets from porcine blood were provided and they formed yellow pellets when spun down. Re-suspension of pellets required disruption by pipette which did not intuitively seem to be gentle handling of resting platelets as to avoid activation. 2.0mL of unadjusted platelet suspension was diluted 1:4 and 1:10 and absorbance at 350nm was taken at 1.2770 and 0.81872 respectively. Adjusted platelet concentration of 1:10 was used since A350 was in linear range. Measured adjusted platelet count with phage microscope with 47 platelets per 0.004mm^3 resulting in a cell count of 117,500 platelets per µL.

The resuspended unadjusted platelets were separated into two 15mL aliquots and one aliquot was activated by $CaCl_2$ and calcium ionophore (A23187) in which aggregation was observed. TNBS was incubated in both resting and activated platelet samples. Freeze thaw cycles broke apart membranes and lipid extraction was performed. Two dimensional TLC on purified TNBS labeled phospholipids from active and resting platelets was compared to a TLC standard (figure 2). Phosphray on TLC plates resulted in blue color for unlabeled phospholipids and green color for TNBS labeled phospholipids. TLC spots were traced, measured, and RF values calculated for comparison (Table I). Green spot corresponding to PS-TNBS on standard only appeared on activated sample and not on resting sample (Figure 2).

Table I TLC of TNBS-Phosphatidylserine
2-dimensional TLC of phospholipid composition from active and resting platelet samples. Solvent system 1 consists of chloroform/methanol/water/28% ammonia (130:70:8:0.5, v/v) and solvent system 2 is chloroform/acetone/methanol/acetic acid/water (100:40:20:20:10, v/v). Plates were treated with phosphray which results in green color from blue phospholipid and yellow TNBS. See appendix Table I for more developed data.

	Standard	Active	Resting
Color	green	green	-
RF1/RF2	.633/.823	.6/.744	-

Thrombin standard curve was constructed with varying dilutions from 1:1 to 1:55 of thrombin with chromogen buffer and Spectrozyme (Figure 3A). Several rates were omitted from standard curve as they fell below

linear range (0.1Au/min) or showed poor fit to data.

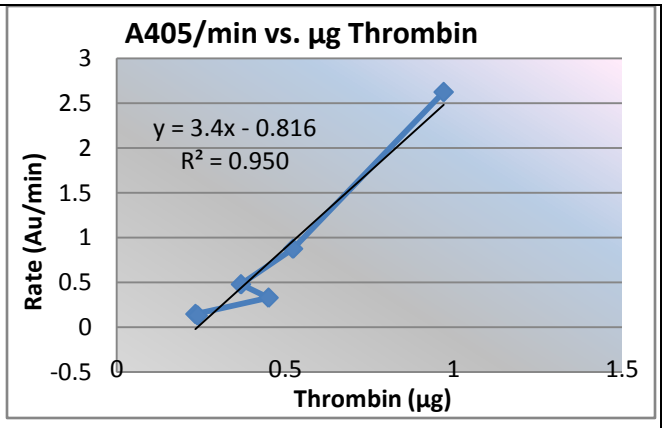


Figure 3A Thrombin Standard Curve
Varying dilutions of known thrombin concentrations from 1:1 to 1:55 were assayed with 300uL chromagen buffer and 60uL spectrozyme for absorbance at 405nm over time. Thrombin standard curve was constructed using absorbance data within linear range of 0.1-1.0 Au/min. Dilutions 2x,6x,11x,16x,36x, and 41x were used to construct a standard curve with equation Y=3.4X-0.816 where Y is Au/min and X is µg Thrombin. See appendix Figure 3A for additional data.

Prothrombinase assays were performed on adjusted resting platelet sample along with negative and positive controls (Table II). These adjusted platelets were left to age activate in refrigerator for 12 days and the prothrombinase assay was repeated on age activated platelets along with new positive and negative controls. The positive control was conveniently shared with PS vesicle controls performed in same lab period. The platelets were sonicated and prothrombinase assay was performed on this disrupted sample with a total 50x dilution to obtain absorbance in linear range. Negative controls show low A405/min in prothrombinase assay, positive controls show high activity, and active platelets had activity higher than negative control (figure 3B). By interpolation of Au/min to thrombin standard curve (Figure 3A), disrupted platelets measured 178µg thrombin, activated platelets measured 35µg thrombin, and resting platelets measured 38µg thrombin (Table II). The anomaly of high thrombin in resting platelets is likely due to accidental activation while performing prothrombinase assay, because the TLC of resting platelets had no TNBS labeled PS. This indicated resting state was maintained in unadjusted 15mL aliquot for TLC and accidental activation occurred when adjusting concentration or performing the assay.

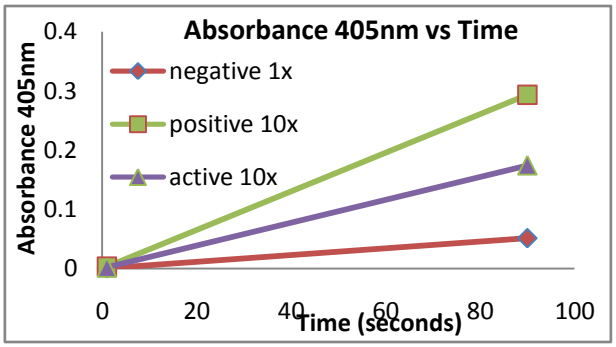


Figure 3B Active Sample Assay Result
Prothrombinase assay result of active sample as compared to controls. Positive and active samples were measured with 10x dilution to get absorbance in linear range. See appendix Figure 3B for additional data and bar charts.

Synthetic phospholipids were made to be either 100% PC or 20% SM, PE, or PS and 80% PC. Each undiluted vesicle variant was run in prothrombinase assay. Thrombin produced was interpolated by Au/min to thrombin standard curve (Figure 3A, Figure 4). Vesicles with PS had doubling of thrombin production of 6µg as compared to 3µg of negative control (Figure 4).

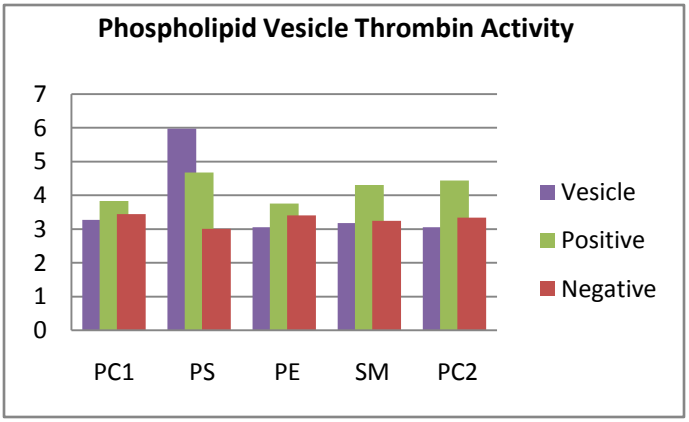


Figure 4 Phospholipid Translocation
Vesicles are either 100% phosphatidtylcholine (PC) or 20% of another phospholipid and remainder 80% with phosphatidylcholine. Rates were measured on undiluted vesicles in prothrombinase assay and interpolated to thrombin standard curve to calculate ug thrombin produced. Phospholipids with PS show double thrombin production as compared to negative control.

Since only PS vesicles showed significant increased thrombin production from negative controls, unilamellar vesicles of PS were prepared by using "LiposoFast" extrusion device. Prothrombinase assays were performed on known dilutions of unilamellar vesicles from 1:10 to 1:100 to construct a PC standard curve (Figure 5).

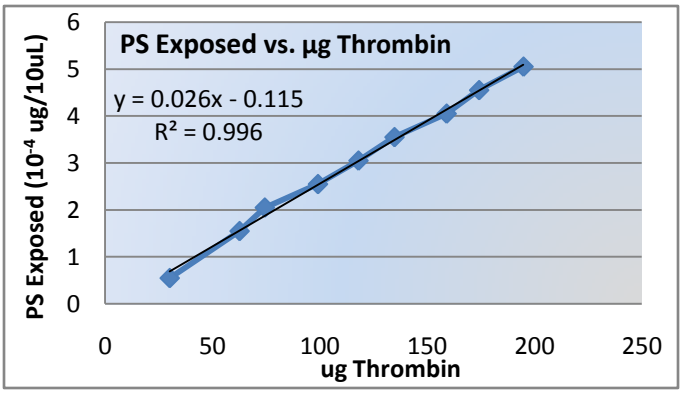


Figure 5 PS Standard Curve
Synthetic vesicles of 20% PS and 80% PC were measured in prothrombinase assay with known dilutions from 1:10 to 1:100. Using equation from thrombin standard curve, the Au/min was related to µg thrombin. Equation of Y=0.026x-0.115 where Y is PS Exposed (10^{-4}ug/10uL) and X is ug thrombin. See appendix Figure 5 for more developed data.

Disrupted, activated, and resting platelets thrombin production results previously determined having 178µg, 35µg, and 38µg respectively were interpolated to PS standard curve to determine PS exposed from each sample. Dividing by cell count, PS molecules on the surface of each platelet was determined to be $3x10^{10}$, $5x10^{9}$, and $5x10^{9}$ respectively. Disrupted sample was assumed 100% PS exposed, so by comparison resting had 20% PS exposed and activated had 18% PS exposed (Table III).

Active platelet samples were provided to test the effect of DDT, Glutathione, and DDT with metabolic inhibitor by performing prothrombinase assays every 20 minutes after addition of these reducing agents. Thrombin and PS standard curves were used to calculate changes PS exposed over time. Activated platelets exposed to glutathione showed increased PS exposed over time from 0 to 20 minutes, though there is an inconsistency since it decreased from 20 to 40 minutes. Activated platelets treated with DDT showed decrease in absorbance, thus correlated with decrease in PS exposed. DDT with metabolic inhibitor showed negligible to no change in PS exposed (Table IV).

Discussion:

In this experiment resting and active platelets were compared by two methods: TLC and prothrombinase assays. With TLC, active platelets were obtained by treating with $CaCl_2$ and ionophore (A23187). Ionophore forms complexes with divalent cations and allows Ca^{2+} to permeate cell membranes, though its role in activating platelets is not intuitive. Since scramblase is Ca^{2+} activated, increasing Ca^{2+} intracellular concentration should increase activity of scramblase to transport phospholipids to the outer membrane. This Ca^{2+} induced scramblase activity increase alone would not account for activation since properly working flippase would theoretically still maintain resting state. $CaCl_2$ and ionophore treated platelets are assumed active platelets because they have aggregation characteristics consistent with active platelets. An aliquot of untreated resting platelets and another aliquot of treated activated platelets were labeled with TNBS which is yellow in color. Since the phospholipids are similar in polarity, a two dimensional TLC with two solvent systems was used to obtain proper separation. Phospholipids PS and PE bound to TNBS and presented as green spots while unlabeled phospholipids presented as blue spots. Since TNBS labeling was done prior to sonication and

lipid extraction, only the outer layer of phospholipids was capable of labeling since TNBS cannot cross the bilayer. Only the resting sample did not have any PS-TNBS, so it can be concluded that ionophore and $CaCl_2$ treated platelets disrupt the membrane in such a way that allows TNBS to access PS. Since the treated platelets show aggregation characteristics, it is likely that activation occurred in which PS was translocated to the outer membrane. Consideration should also be considered for $CaCl_2$ and ionophore to alternatively providing a pathway for TNBS to cross to the inner the membrane, though this is unlikely.

In prothrombinase assays, activation was obtained by aging. Unfortunately the assays for resting platelets were likely mishandled to cause premature activation. Resting platelets should have similar thrombin production as the negative controls, but instead it was more consistent with positive and age activated sample absorbance. The age activated sample curiously showed a slight decrease from resting from 38µg to 35µg. This could be due to the PS in the "resting" sample was already activated and aging caused a slight degradation of the PS, which hindered the ability for the prothrombinase complex to form, produce thrombin, cleave spectrazine, and cause a higher A405. The thrombin standard curve that allowed absorbance data to be interpolated to µg thrombin was also somewhat inaccurate. Many of readings were below linear range leaving few points that could be chosen to construct a standard curve. The y-intercepts were not consistent, indicating that the timing of each assay did not have the same initial time. Even with these errors, the prothrombinase assay is superior to TLC. Repeat trials and improvement on technique could improve accuracy of prothrombinase assays while TLC has limited application and does not have potential for such quantitative analysis. TLC RF values can be calculated, though its resolution is not high.

Prothrombinase assays on synthetic vesicles showed that only vesicles with PS had increased thrombin production when compared to negative controls. This suggests that in order for the prothrombinase complex to form, PS must be present. Empowered with this information, a second standard curve is useful to relate thrombin production to the amount of PS exposed. Various known concentrations of unilamellar PS liposomes were assayed to construct the PS standard curve. The inner and outer radius of these liposomes would not be identical, though it is assumed that PS exposed on the outside for formation of prothrombinase complex is half the total PS present. Relating the platelet count per µL, PS standard curve, thrombin standard curve, and prothrombinase assays, the amount PS present on resting, active, and disrupted platelets was found. Disrupted platelets are assumed to have 100% PS exposed, though it is possible that PS aggregated together or that sonication did not entirely disrupt platelets. With this aside, a percentage of PS exposed based on the total PS present was determined which gives quantifiable amounts of PS in platelet samples. Even with the degree of error from standard curves, variance in timing, assumption of 100% PS in disrupted platelets, prothrombinase assays are superior to TLC since TLC only does not indicate the amount of PS on the outer membrane. Prothrombinase assays also have an advantage of efficiency since assays can be done in minutes while TLC required time consuming lipid extraction. Repeat trials and troubleshooting is much easier with prothrombinase assays since repeat trials are quick and assays provide an amount of PS. TLC did serve an important role in the experiment to suggest PS is translocated during activation which is further tested in prothrombinase assays. If the resting platelets for assays were not prematurely activated, the assay activity would have negligible PS activity for resting platelets.

The mechanism for age activation of platelets was investigated by flip flop experiment in which prothrombinase assays were performed over time on activated platelets treating with

several reducing agents and/or metabolic inhibitor. Scramblase continues function as it nonspecifically scrambles PL from the inner and outer membranes, disrupting the resting state of PS only on the inside. In order to maintain resting platelet symmetry ATP dependent flippase selectively transports PS back into the inner membrane. Possibilities for deactivation of flippase over time are oxidation or ATP depletion. Reactivation of flippase activity would transport PS back in the membrane and restore resting state which would be associated with a decrease in prothrombinase activity. Reducing agents DDT and glutathione differ in their permeability of the bilayer. If the inactivation of flippase is due to oxidation, then prothrombinase assays with each DDT and Glutathione suggest the site of oxidatitive deactivation since only DDT can cross the membrane. Experimentally, only DDT caused a decrease in rate suggesting that the site of oxidation takes place at the inner membrane. Glutathione initially showed an increase in prothrombinase activity suggesting that the site of oxidation does not occur on the outer membrane. There is an inconsistency however, in which the rate decreases from 20 minutes to 40 minutes, though it is still net increase from initial absorbance rate. This is likely due to spec error and a 60 minute reading could be read with an expected higher absorbance to continue the increase in absorbance by inactive flippase and active scramblase. Flippase is also ATP dependent so treatment with metabolic inhibitor would keep PS from translocating back inside the membrane and prothrombinase activity would continue without change.

The two methods of platelet activation investigated were oxidation of flippase and high intracellular Ca^{2+}. It is intuitive that high intracellular Ca^{2+} increases scramblase activity since it is a required cofactor, but properly working flippase would theoretically then restore resting state. Studies in Scott Syndrome, a bleeding disorder due to defective scramblase, show that high intracellular Ca^{2+} inhibits flippase activity, which now fully explains how high Ca^{2+} can activate

platelets. Scott syndrome RBCs and platelets aminophospholipid transocase (flippase) activity is normal, but when treated with intracellular Ca^{2+}, the activity is inhibited.[8] So in both methods of platelet activation a loss in normal flippase activity is associated with high PS on the outer membrane, assembly of prothrombinase complex, and eventually thrombosis. It can be postulated that the deactivation of flippase is similar in both methods since both Ca^{2+} and DDT are intracellular. DDT is a reducing agent for thioeester bonds, so it is tempting to say that Ca^{2+} interferes at this same site location on flippase inside the cell.

Calcium serves an additional role in blood clotting since it has positive feedback for calpain activity which activates protein tyrosine phosphatase and forms procoagulant microvesicles. The forming of these vesicles provides surface better suited for prothrombinase complex to form and increases thrombin production.[4] Microvesicle formation does not appear to be a result of redistribution of phospholipids, though they are both closely related since they are activated by Ca^{2+} and are conducive to thrombin production.[9]

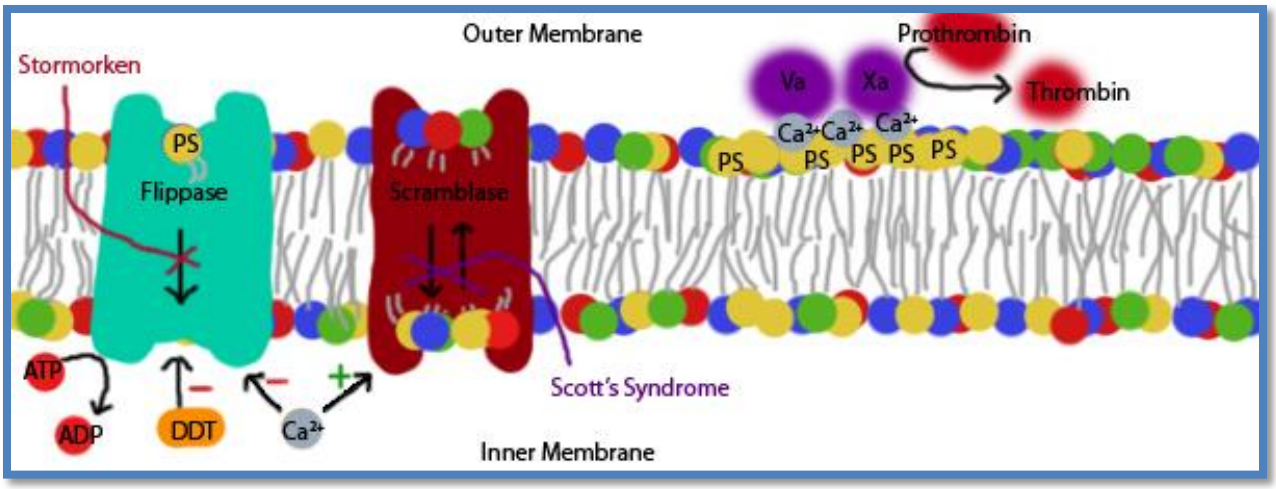


Figure 6: ATP dependent flippase transports phosphaditylserine (PS) from the outer to inner membrane and is negatively affected by intracellular DDT and Ca^{2+}. Stormorken syndrome is postulated to be a defect in flippase, though patients don't exhibit expected thrombosis due to low platelet count. Scramblase is Ca^{2+} dependent and nonselectively scrambles phospholipids. Scott's syndrome is a defect in scramblase and has symptoms of a bleeding disorder since PS does not translocate for formation of prothrombinase complex.

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Appendix:

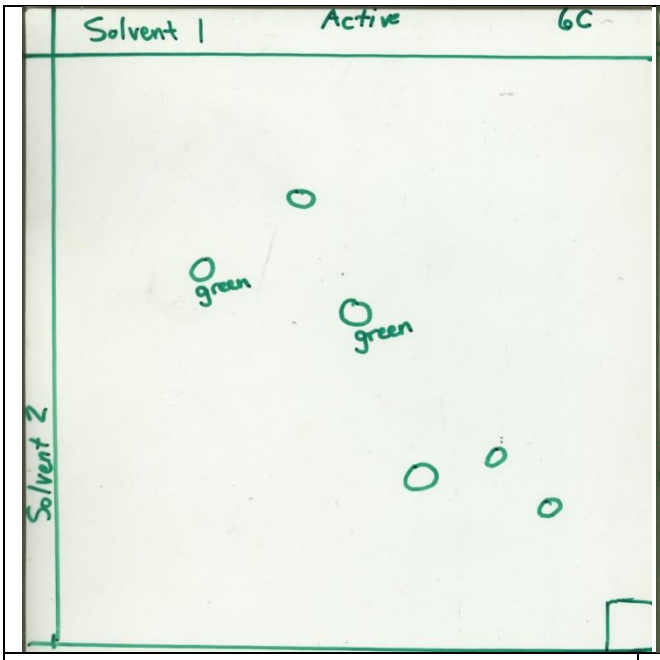


Figure 2A: TLC of TNP Labeled Phospholipids from Active Platelets

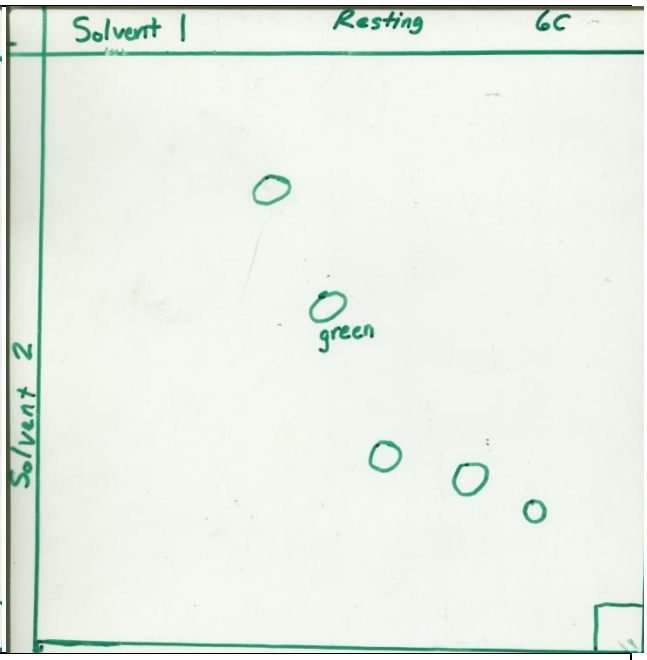


Figure 2B: TLC of TNP Labeled Phospholipids Resting from Platelets

Figure 2A-C: TLC of TNP Labeled Phospholipids

2-dimensional TLC compared phospholipid composition of active and resting platelet samples. Solvent system 1 consists of chloroform/methanol/water/28% ammonia (130:70:8:0.5, v/v) and solvent system 2 is chloroform/acetone/methanol/acetic acid/water (100:40:20:20:10, v/v). Standard was provided to assign spots to corresponding phospholipid. Trinitrophenol (TNP) binds to phosphatidylserine (PS) and phosphatidylethanolamine (PE). TNP alone is yellow color. Plates were treated with Phospray which turned phospholipids blue. Phospholipids (blue) with TNP (yellow) showed as green spots. See Table 1 for Rf values.

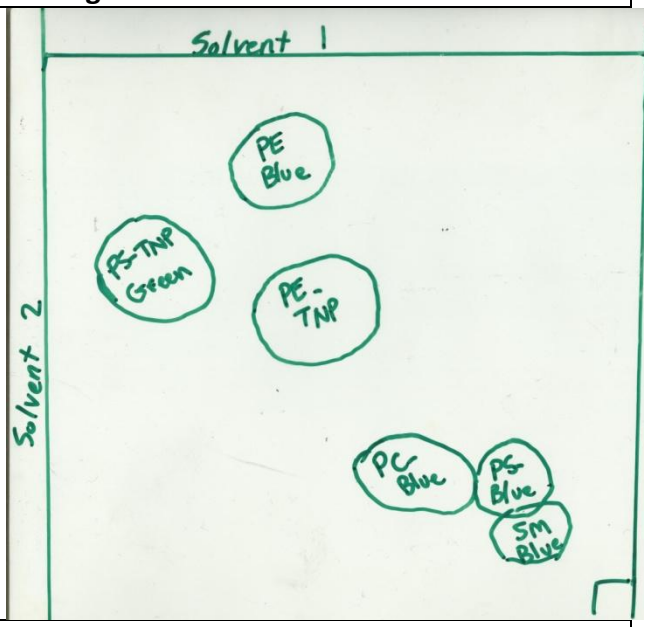


Figure 2C: TLC Standards

Table I TLC

2-dimensional TLC compared phospholipid composition of active and resting platelet samples. Solvent system 1 consists of chloroform/methanol/water/28% ammonia (130:70:8:0.5, v/v) and solvent system 2 is chloroform/acetone/methanol/acetic acid/water (100:40:20:20:10, v/v). Standard was provided to assign spots to correct phospholipid. Trinitrophenol (TNP) labeled PS and PE on the outer membrane.

Phospholipid	Plate	RF Solvent System 1	RF Solvent System 2	Spot Color
PE	standard	0.833	0.538	blue
	active	0.731	0.569	blue
	resting	0.744	0.6	blue
PS-TNP	standard	0.633	0.823	green
	active	0.6	0.744	green
	resting	no spot	no spot	no spot
PE-TNP	standard	0.533	0.506	green
	active	0.519	0.463	green
	resting	0.538	0.488	green
PC	standard	0.2	0.316	blue
	active	0.225	0.344	blue
	resting	0.256	0.381	blue
PS	standard	0.2	0.158	blue
	active	0.269	0.206	blue
	resting	0.225	0.231	blue
SM	standard	0.1	0.127	blue
	active	0.169	0.106	blue
	resting	0.163	0.113	blue

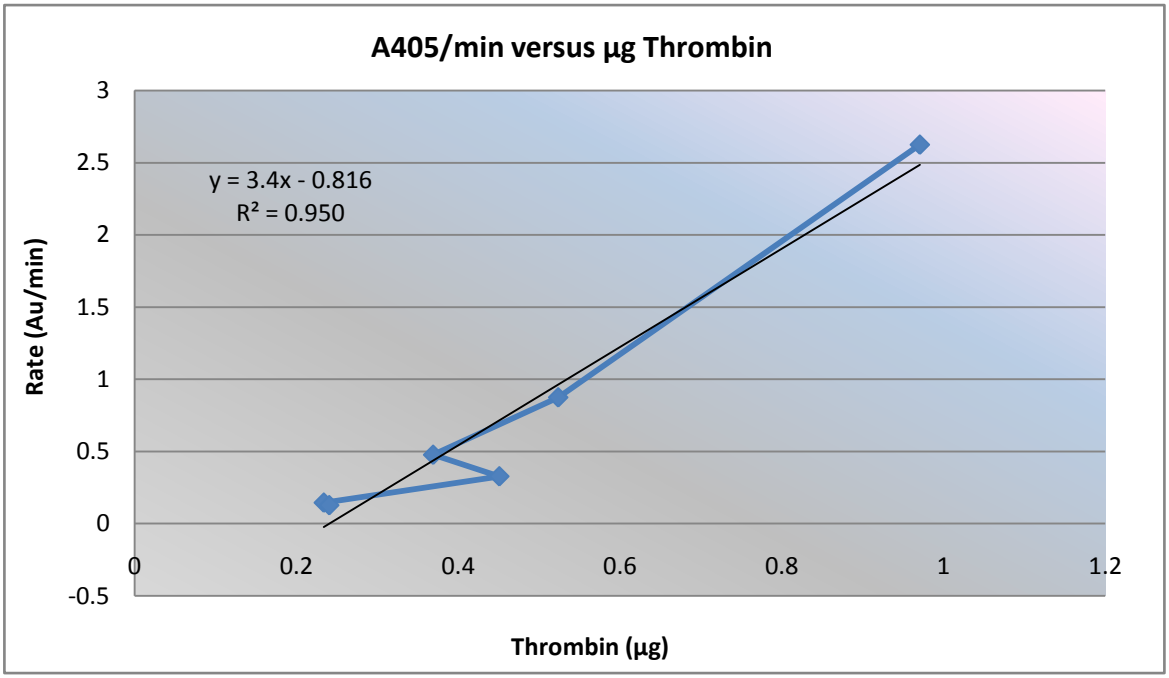
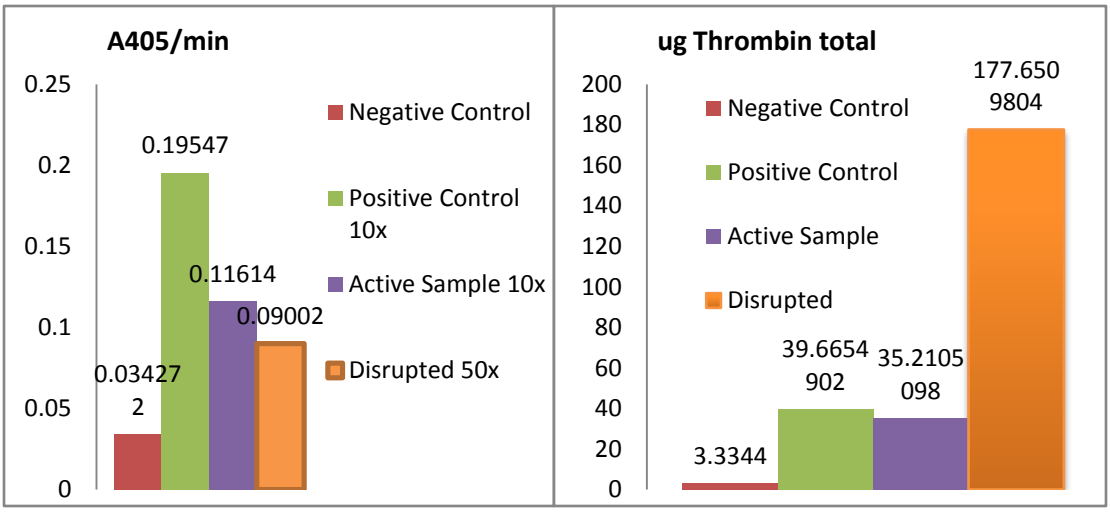
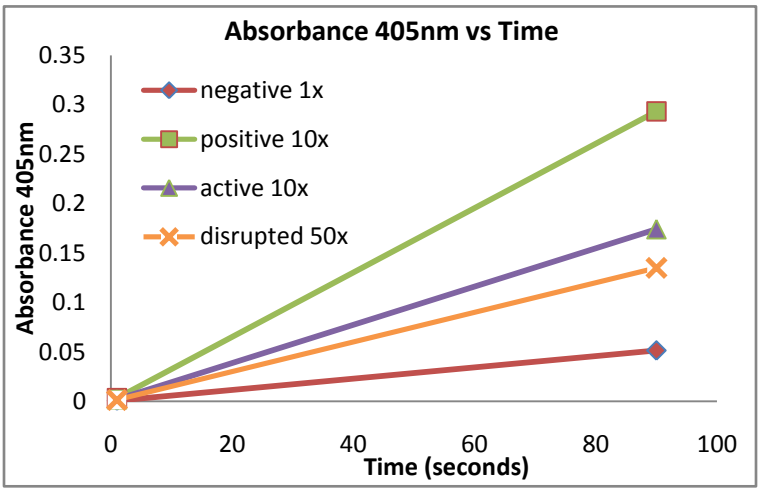


Figure 3A Thrombin Standard Curve: Varying dilutions of known thrombin concentrations from 1:1 to 1:55 were assayed with 300uL chromagen buffer and 60uL spectrozyme for absorbance at 405nm over time. Thrombin standard curve was constructed using absorbance data within linear range of 0.1-1.0 Au/min. Dilutions 2x,6x,11x,16x,36x, and 41x were used to construct a standard curve with equation Y=3.4X-0.816 where Y is Au/min and X is ug Thrombin.

Standards			
dilution (#x)	ug thrombin	Rate (Au/min)	y-intercept
1:1	2.625	0.97097	0.640126
1:5	0.875	0.5236	0.24812
1:10	0.477272727	0.36888	0.288284
1:15	0.328125	0.45077	0.292085
1:20	0.25	0.07494	0.2025
1:25	0.201923077	0.047349	0.205
1:30	0.169354839	0.098072	0.239787
1:35	0.145833333	0.23364	0.1921
1:40	0.12804878	0.24037	0.225865
1:45	0.114130435	0.26746	0.315097
1:50	0.102941176	0.026581	0.201516
1:55	0.09375	0.012818	0.1972





negative (1x)		positive (10x)	
Au/min	0.034272	Au/min	0.19547
x(time) sec	y(a405)	x(time) sec	y(a405)
1	0.000571	1	0.003258
90	0.051408	90	0.293205
active (10x dil)		**disrupted (50x)**	
Au/min	0.11614	Au/min	0.09002
x(time) sec	y(a405)	x(time) sec	y(a405)
1	0.001936	1	0.0015
90	0.17421	90	0.13503

Figure 3B Thrombin Assay Result: Thrombin assay result of active sample as compared to controls. Positive and active samples were measured with 10x dilution to get absorbance in linear range while disrupted was a 50x sample. Absorbance data of A405/min and A405/s are not multiplied by their dilution factors and is raw absorbance data. Disrupted has lower absorbance than active and positive, though when calculating ug thrombin, dilution factor is taken into account and disrupted shows much higher thrombin as expected.

Table II Prothrombinase Activity

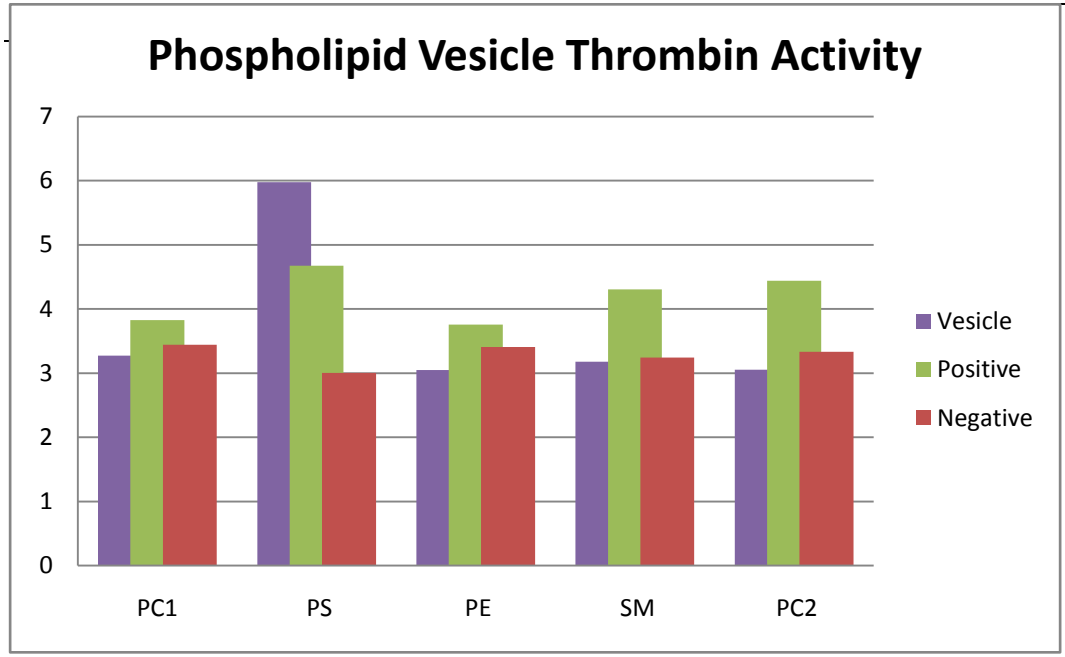
Prothrombinase assays were performed on resting, activated, and disrupted samples. Absorbances were subtracted from negative controls and rate from assays were interpolated to thrombin standard curve to get ug thrombin. Dilution factors and assay volumes were taken into account in calculations. Y intercepts ideally are consistant across samples to ensure synchronous timing of thrombin production.

		negative			positive			sample		
	Dillution Factor	**A405/min**	**y-int**	**ug thrombin**	**A405/min**	**y-int**	**ug thrombin**	**A405/min**	**y-int**	**ug thrombin**
Resting	10	0.0034923	0.170853	3.213695	0.12182	0.23318	36.77725	0.16794	0.289132	38.44893
Activated	10	0.034272	0.076359	3.3344	0.19547	0.325337	39.66549	0.11614	0.23765	35.21051
Disrupted	50			3.2			32	0.09002	0.143616	177.651

sample calculation ug thrombin: ((sample A405)-(neg control A405)-(y-int of thrombin standard curve equation))/(slope thrombin standard curve)*(400/30)*(dilution factor 10 or 50)

Suplimental Data	
Thrombin Standard Curve Equation	
y=3.4x-0.816	
m	3.4
b	-0.816

Group	PL Vesicles	y-int	rate	ug thrombin
1	PC	0.165882	0.079855	3.272176471
	+		0.22153	3.827764706
	-	0.111515	0.06145	3.440980392
2	PS	0.17353	0.65777	5.975415686
	+	0.14969	0.32664	4.676866667
	-	0.092658	-0.04996	3.00407451
3	PE	0.052917	0.01467	3.050047059
	+	0.325337	0.19547	3.759066667
	-	0.053	0.052908	3.407482353
5	SM	0.03098	0.00529	3.179372549
	+	0.193711	0.29248	4.305607843
	-	0.0302	0.01055	3.241372549
6	PC	0.281277	-0.00325	3.052869804
	+	0.1947	0.350334	4.439458824
	-	0.076359	0.034272	3.3344



Sample Calculation of ug thrombin:

(rate vesicle)-(rate negative)-(yint of ugthrombin vs rate STD curve)/(slope of ugthrombin vs rate STD curve)*(400/30)

Figure 4 Quantitation of phospholipid translocation: Vesicles are either 100% phosphatidtylcholine (PC) or 20% of another phospholipid and remainder 80% with phosphatidylcholine. Rates were measured on undiluted vesicles in prothrombinase assay and interpolated to thrombin standard curve to calculate ug thrombin produced. Phospholipids with PS show double thrombin production as compared to negative control.

PS standard curve 2 (group 4,5,6)						
dilution	**group**	**rate**	**y-int**	**PS exposed**	**ug thrombin**	**ug*df**
1:10(11x)	4	0.42866	0.438303	0.55	0.366076	30.201309
1:30	4	0.10135	0.117625	1.55	0.269809	62.730551
1:40	4	0.00755	0.068248	2.05	0.242221	74.483048
1:50	5	0.06602	0.042274	2.55	0.259418	99.22725
1:60	5	0.06162	0.049384	3.05	0.258122	118.09098
1:70	5	0.0451	0.040097	3.55	0.253265	134.86377
1:80	6	0.07463	0.064908	4.05	0.261951	159.13534
1:90	6	0.05212	0.062065	4.55	0.25533	174.26293
1:100	6	0.05896	0.044816	5.05	0.25734	194.93505
Sample Calculation PS Exposed	1ug/uL x 1/2 x 1/10 x 10uL x 1mL/uL = 5x10^-4ug/10uL					
Sample Calculation ug thrombin	solve for x with thrombin standard curve y=3.4x-0.0816 where Y is PS Exposed					
ug thrombin * df	Dilution factor (1:10 = 11x) *300/40 (for assay)					
Supplimental Data						
ug thrombin/au/min					y=3.4x-0.816	
ug thrombin/PC Exposed 10^-4ug/10ul					y=0.026x-0.115	

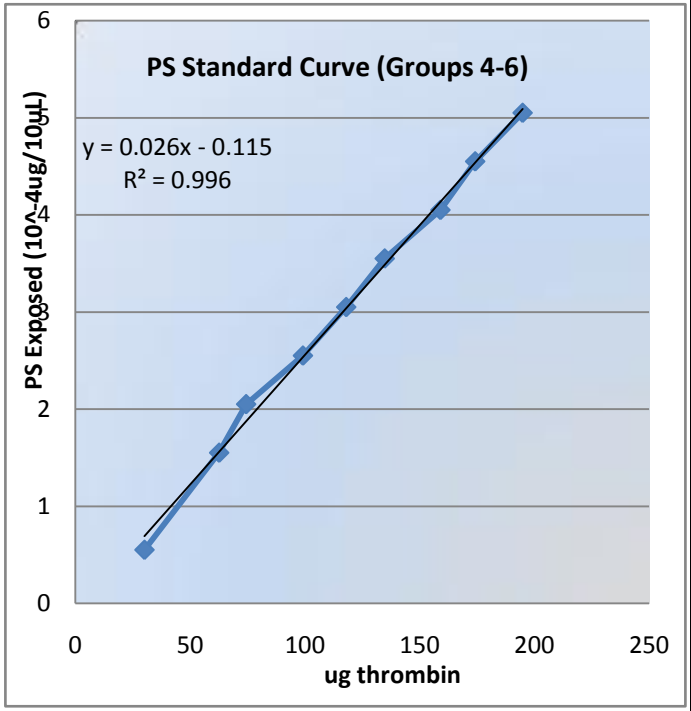


Figure 5 PS Standard Curve: Synthetic vesicles of 20% PS and 80% PC were measured in prothrombinase assay with known dilutions from 1:10 to 1:100. Using equation from thrombin standard curve, the Au/min was related to µg thrombin. Groups 1-3 data had varying absorbances that seemed prone to high error. Groups 4-6 data constructed a smooth standard curve including all dilutions relating PS exposed to ug thrombin. Groups 4-6 standard curve data is used with equation of Y=0.026x-0.115 where Y is PS Exposed (10^-4ug/10uL) and X is ug thrombin.

Table III Quantitation of PS in Resting and Activatd Platelets

Disrupted sample assumes all PS is exposed and has highest thrombin activity. The µg thrombin produced is data carried from table II. Exposed PS in each 10µL is calculated by interpolating to PS standard curve. Number of PS on outer surface of each platelet is determined by dividing by total cell count.

	ug thrombin (from table II)	exposed PS in each 10uL aliquot ug/uL	number of PS on outer surface of each platelet	percentage of total PS
resting	38.44892941	0.088467216	5959729398	19.64224689
activated	35.2105098	0.080047325	5392510559	17.77279079
disrupted	177.6509804	0.450392549	30341383193	100
Sample Calculation	From Table II	(y=0.026(ug thrombin)-0.115)/(10uL)	exposed PS ug/uL * 10uL *1g/10^6ug *1mol/758g * 6x10^23molecules/1mol	# PS molecules (rested or activated)/# PS molecules disrupted

Supplimental Data	
cell count /ul	117500
PS STD Curve Eq.	y=0.026x-.115
m	0.026
b	-0.115

Table IV Flippase Activity

Active platelets were provided and tested with reducing agents DDT or Glutathione as well as DDT and metabolic inhibitor. Prothrombinase assays were performed in 20 minute time intervals. Absorbance data was used to calculate ug thrombin from thrombin standard curve and then PS exposed from PS standard curve. Platelets treated with DDT had decrease in prothrombinase activity indicating reduction of flippase to resting state. Glutathione which cannot cross the membrane had an increase in thrombin from normal translocase. Note inconsistancy of glutathione decrease in absorbance from t=20 to t=40, so t=60 should be measured to conclude spec error. Metabolic inhibitor had expected result of same rate or increasing indicating flippase was not functioning. PS exposed in each 10uL aliquot was tabulated, though it is more useful to look at absorbance rates since PS standard curve equation has a negative y intercept making interpolation difficult in this case.

Group	Flip Flop	y int		rate	ug thrombin	PS exposed in each 10uL aliquot
1	DDT + MI	t=0	-0.00583	0.14246	3.758666667	-0.001727467
		t=20	0.20862	0.31969	4.453686275	7.95843E-05
		t=40	0.277895	0.31377	4.430470588	1.92235E-05
4	DDT	t=0	0.272958	0.33787	4.524980392	0.000264949
		t=20	0.327366	0.33329	4.507019608	0.000218251
		t=40	0.256711	0.2723	4.267843137	-0.000403608
5	Glutathione	t=0	0.117815	0.27319	4.271333333	-0.000394533
		t=20	0.4945	0.79449	6.315647059	0.004920682
		t=40	0.1935	0.36359	4.625843137	0.000527192