

# Liposomes bearing fibrinogen could potentially interfere with platelet interaction and procoagulant activity

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**Background:** The contribution of fibrinogen (FBN) to hemostasis acting on platelet aggregation and clot formation is well established. It has been suggested that FBN-coated liposomes could be useful in restoring hemostasis. In the present study, we evaluated the modifications induced by multilamellar raw liposomes (MLV) or fibrinogen-coated liposomes (MLV-FBN) on hemostatic parameters.

**Materials and methods:** Different experimental settings using whole blood or thrombocytopenic blood were used. Thromboelastometry, aggregation studies, platelet function analyzer (PFA-100®) tests and studies under flow conditions were applied to detect the effect of MLV-FBN on hemostatic parameters.

**Results:** The presence of MLV-FBN in whole blood modified its viscoelastic properties, prolonging clot formation time (CFT) ( $226.5 \pm 26.1$  mm versus  $124.1 \pm 9.4$  mm;  $P < 0.01$ ) but reducing clot firmness ( $45.4 \pm 1.8$  mm versus  $35.5 \pm 2.3$  mm;  $P < 0.05$ ). Under thrombocytopenic conditions, FIBTEM analysis revealed that MLV-FBN shortened clotting time (CT) compared to MLV ( $153.3 \pm 2.8$  s versus  $128.0 \pm 4.6$  s;  $P < 0.05$ ). Addition of either liposome decreased fibrin formation on the subendothelium (MLV  $8.1\% \pm 4.7\%$  and MLV-FBN  $0.8\% \pm 0.5\%$  versus control  $36.4\% \pm 6.7\%$ ;  $P < 0.01$ ), whereas only MLV-FBN significantly reduced fibrin deposition in thrombocytopenic blood ( $14.4\% \pm 6.3\%$  versus control  $34.5\% \pm 5.2\%$ ;  $P < 0.05$ ). MLV-FBN inhibited aggregation induced by arachidonic acid ( $52.1\% \pm 8.1\%$  versus  $88.0\% \pm 2.1\%$  in control;  $P < 0.01$ ) and ristocetin ( $40.3\% \pm 8.8\%$  versus  $94.3\% \pm 1.1\%$ ;  $P < 0.005$ ), but it did not modify closure times in PFA-100® studies. In perfusion experiments using whole blood, MLV and MLV-FBN decreased the covered surface ( $13.25\% \pm 2.4\%$  and  $9.85\% \pm 2.41\%$ , respectively, versus control  $22.0\% \pm 2.0\%$ ;  $P < 0.01$ ) and the percentage of large aggregates ( $8.4\% \pm 2.3\%$  and  $3.3\% \pm 1.01\%$ , respectively, versus control  $14.6\% \pm 1.8\%$ ;  $P < 0.01$ ).

**Conclusion:** Our results reveal that, in addition to the main contribution of fibrinogen to hemostasis, MLV-FBN inhibits platelet-mediated hemostasis and coagulation mechanisms.

**Keywords:** thrombocytopenia, hemostasis, fibrinogen, liposomes, procoagulant activity, fibrin

## Introduction

Liposomes are widely implemented for therapeutic use as drug carriers, as well as in drug targeting and other pharmacological applications, including basic research and applied technology.<sup>1,2</sup> In previous studies, our group demonstrated the development of procoagulant activity in damaged vessels of synthetic phospholipids and liposomes,<sup>3,4</sup> opening up new possibilities for using liposomes as platelet substitutes to improve hemostasis. Several groups have explored the use of modified liposomes and microcapsules as coagulants in order to improve hemostasis.<sup>3-9</sup>

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Recently, the use of nanotechnological approaches to stop bleeding has been explored.<sup>10</sup> In this context, the effects of prohemostatic molecules, such as clotting factors in liposomes, could provide many advantages in controlling hemorrhages.

Fibrinogen (FBN) is a 340 kDa protein that is present in plasma at a concentration of 2 to 3 mg/mL. It has two identical disulfide-linked subunits composed of three nonidentical polypeptide chains:  $\alpha$ ,  $\beta$ , and  $\gamma$ . In addition, a  $\gamma'$  chain is found in approximately 10% of FBN molecules. The four carboxyl-terminal amino acids found in the  $\gamma$ A chain are substituted by a highly anionic 20-amino acid sequence in the  $\gamma'$  chain.<sup>11</sup> FBN plays an important role in blood coagulation as the substrate for thrombin, which is the final step in the coagulation cascade that results in fibrin formation.<sup>12</sup> Moreover, FBN mediates platelet aggregation by binding and bridging glycoprotein IIb-IIIa (GPIIb-IIIa).<sup>13</sup> Two regions of the FBN  $\alpha$  chain that contain an arginine-glycine-aspartic acid motif, as well as the carboxyl-terminus of the FBN  $\gamma$  chain, represent potential binding sites for GPIIb-IIIa in the fibrinogen molecule.<sup>14,15</sup>

Thus, combining adequate sequences of adhesive proteins in the correct conformation on the appropriate carrier could improve primary hemostasis without enhancing fibrin formation. In the present study, we explored the effects of both raw multilamellar liposomes (MLV) and fibrinogen-coated liposomes (MLV-FBN) on platelet function by using different experimental approaches: platelet aggregation induced by different agonists; experiments under flow conditions using the platelet function analyzer PFA-100® (Siemens Healthcare Diagnostics, Deerfield, IL) device; and perfusion systems to evaluate platelet interaction and fibrin formation on the subendothelium. Moreover, viscoelastic properties and the quality of clot formation were tested by thromboelastometric techniques.

## Materials and methods

### Experimental design

The present study was designed to evaluate the possible effect of liposomes containing FBN on different hemostatic parameters. The activity of liposomes was evaluated using whole blood and mild thrombocytopenic blood (30,000 platelets/ $\mu$ L) anticoagulated with low-molecular-weight heparin (LMWH) or citrate (19 mM), depending on the test used. Blood samples were incubated with MLV or MLV-FBN liposomes and the effects on the coagulation system, platelet activation, thrombus formation, and fibrin generation were assessed by different experimental settings.

### Reagents

1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (PE) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-[4-(*p*-maleimidophenyl)butyramide] (N-MPB-PE) were purchased from Avanti Polar Lipids (Alabaster, AL); 1,2-dipalmitoyl-*rac*-glycero-3-phosphocholine (DPPC), cholesterol (CHOL), and FBN from human plasma protein from Sigma-Aldrich (St Louis, MO); and N-succinimidyl-S-acetylthioacetate (SATA) from Pierce (Rockford, IL). The buffer used was 50 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (HEPES), pH 7.5 (with and without 1 mM ethylenediaminetetraacetic acid for liposomes with and without FBN). Other reagents used were of analytical grade. The water used was ultrapure (MilliQ reverse osmosis system; Millipore, Billerica, MA) with a resistivity of 18.3 M $\Omega$ /cm. Arachidonic acid and ristocetin were purchased from Helena BioSciences Europe (Gateshead, UK), while collagen and adenosine diphosphate (ADP) were from Arkray Inc (Kyoto, Japan). ROTEM® reagents for the EXTEM and FIBTEM tests were from Pentapharm GmbH (Aesch, Switzerland).

### Liposome preparation and characterization

Phospholipids (DPPC:CHOL:N-MPB-PE and DPPC:CHOL:PE at a molar ratio of 50:20:30) were dissolved in chloroform in a round-bottomed flask and dried in a rotary evaporator under reduced pressure at 50°C to form a thin film on the flask. The film was hydrated with a solution of 10 mM HEPES buffer (pH 7.4) and 145 mM NaCl to give a lipid concentration of 20 mM. Multilamellar liposomes were formed by constant vortexing for 4 minutes on a vortex mixer followed by sonication for 4 minutes in a Transsonic Digital T460/H bath sonicator (Elma®, Singen, Germany) to guarantee the complete suspension of lipids. Vesicle size, size distribution and  $\zeta$ -potential were determined at 25°C by photon correlation spectroscopy and Doppler microelectrophoresis, using a Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK).<sup>16</sup>

### Preparation of thiolated fibrinogen and coupling to liposomes

The procedure for thiolating proteins with SATA has been previously reported.<sup>17</sup> The number of introduced thiol groups was assayed according to Ellman, using cysteine for the calibration curve.<sup>18</sup> According to this assay, the number of introduced thiol groups (expressed as mol of SH/mol of FBN) was  $1.6 \pm 0.1$ .

A volume of liposome suspension (DPPC: N-MPB-PE) was combined with an equal volume of fibrinogen solution. The resulting suspension was left to react overnight at room temperature while being centrifuged at 1,000 rpm.

The coupling reaction was stopped by adding 50  $\mu\text{L}$  of N-ethylmaleimide (8 mM in HEPES buffer) and the liposomes were separated from free protein by two centrifugation steps in Optima L-100XP (Beckman-Coulter, Fullerton, CA) for 40 minutes at 100,000  $g$  and 7°C. Liposomes (with or without FBM) were prepared in different batches. Measurements of size,  $\zeta$ -potential, and amount of thiol groups introduced on the liposomal surface served as control parameters to ensure that the physicochemical properties were the same in all batches used.

## Blood collection and preparation of thrombocytopenic blood

Blood was obtained from six healthy volunteers (4 males and 2 females, with an age range from 20 to 65 years) who had not taken drugs affecting platelets or coagulation mechanisms in the previous 10 days. Prior to the study, our local institutional ethics committee approved the protocol, which conformed to the ethical guidelines of the Helsinki Declaration. Written informed consent was obtained from all subjects. Blood was anticoagulated with LMWH (Fragmin®; Pfizer Inc, New York, NY) at a final concentration of 20 U/mL. This concentration of LMWH allows the generation of thrombin when blood is exposed to a denuded vascular segment.<sup>19–22</sup> Blood platelets and leukocytes were reduced by a filtration procedure,<sup>23</sup> using an RC100 filter (PALL Corp, Glen Cove, NY). The platelet number was adjusted to 30,000 platelets/ $\mu\text{L}$ , mixing thrombocytopenic blood and whole blood. Before each experiment, samples were preincubated with liposomes coupled or uncoupled to FBN for 2 min at 37°C. PFA-100® and aggregation experiments were performed with blood anticoagulated by citrate at a final concentration of 19 mM using standard laboratory procedures.

## Thromboelastometric studies

To examine the kinetics and quality of clot formation, we used the ROTEM® thromboelastometry analyzer.<sup>24</sup> The technique was performed according to the manufacturer's instructions. We used two different tests: EXTEM and FIBTEM. In the EXTEM test, tissue factor (TF) is used as an activator and is sensitive to changes in the extrinsic pathway of coagulation, FBN and fibrin polymerization, and platelet function. In the FIBTEM test, platelet function is inhibited by the platelet inhibitor cytochalasin D. While the clots obtained in EXTEM

were formed by platelets and fibrin, the clot obtained in the FIBTEM assay was primarily a fibrin clot.

The study was carried out with whole and thrombocytopenic blood (30,000 platelets/ $\mu\text{L}$ ) using 300  $\mu\text{L}$  of blood containing 10% liposomes coupled or uncoupled to FBN. Measurements were recorded for 30 minutes, while controls were performed simultaneously by adding 10% of the corresponding buffer to the blood.

The main effect of liposomes was measured during clot formation by the standard thrombelastometric parameters: clotting time (CT) is the period of time from the start of the analysis until the clot reached 2 mm in amplitude; clot formation time (CFT) is the period until 20 mm amplitude was reached; and clot firmness was measured at 10-minute intervals (A10).<sup>25</sup> The CT and CFT, measured in seconds, indicate the dynamics of clot formation. The clot amplitude (mm) gives information about clot strength and stability, which largely depends on FBN and platelets.

## Platelet aggregation

The experiments were performed in a four-channel aggregometer (APACT 4, Helena BioSciences) according to the classical turbidimetric technique.<sup>26</sup> Aliquots of 450  $\mu\text{L}$  of platelet-rich plasma were transferred to the aggregometer cuvette and activated with different agonists: arachidonic acid and ristocetin (Helena BioSciences) at a final concentration of 1.4 mM and 1 mg/mL, respectively, and collagen and ADP at a final concentration of 2.5  $\mu\text{g}/\text{mL}$  and 2  $\mu\text{M}$ , respectively. The values of maximal platelet aggregation were recorded and expressed as percentages.

## PFA-100® studies

PFA-100® evaluates the hemostatic capacity of platelets as a function of their ability to occlude an aperture in a membrane coated with collagen and epinephrine (EPI) or collagen and ADP under high shear stress (5000  $\text{s}^{-1}$ ).<sup>27</sup> Normality values for closure times previously established for the PFA-100® in our laboratory were  $113 \pm 24$  s (mean  $\pm$  standard deviation (SD)) with cartridges containing collagen and epinephrine (COL-EPI) and  $87 \pm 18$  s with cartridges containing collagen and adenosin diphosphate (COL-ADP).<sup>21</sup> Aliquots of blood incubated with MLV or MLV-FBN were used to study potential changes in closure time.

## Perfusion studies

Rabbit abdominal aortic segments were prepared and processed as previously described.<sup>28</sup> Everted segments were mounted on a rod and inserted into the annular chamber.

Two-mL aliquots of each liposome preparation were added to the blood samples. Control perfusions were carried out by adding equivalent amounts of the buffer used in the phospholipid preparations. The perfusates, with a total volume of 20 mL, were incubated at a constant temperature of 37°C for 2 minutes before starting the perfusion. Perfusions were conducted in annular chambers according to the method previously described.<sup>3,4,28</sup> Blood was recirculated through the chamber for 10 minutes at 37°C at a shear rate of 250 s<sup>-1</sup> using a peristaltic pump. This low shear rate facilitates fibrin deposition onto the subendothelium.

At the end of the perfusion, vessel segments were histologically processed. Platelet interaction and fibrin deposition onto perfused subendothelium were analyzed morphometrically as previously described. Platelet interaction was expressed as the percentage of covered surface by platelets and the percentage of big platelet aggregates (>5 µm) classified as % thrombi (% T). The presence of fibrin was expressed as a percentage of fibrin.<sup>20,29,30</sup>

## Data analysis

Results are expressed as mean ± standard error of the mean (SEM). The number of experiments for each preparation was at least  $n = 6$ . Student's *t*-test for paired data was used to compare differences between the control and the treated samples. The level of statistical significance was established at  $P < 0.05$ .

## Results

### Liposome characterization

The average size of liposomes, irrespective of the presence of FBN, ranged from 0.8 to 1.4 µm, and their polydispersity was close to 0.80. The incubation ratio of SATA to FBN of 10 (molar ratio) resulted in the introduction of 1.5 to 1.7 mol of thiol groups/mol of FBN. FBN was coupled at a ratio of 33 to 53 µg of FBN per µmol of phospholipid in the different FBN-MLV batches tested.

Measurements of the ζ-potential of raw liposomes in water afforded an average value of -8 mV, whereas after the coupling of fibrinogen, this value increased to -75 mV.

### Effect of liposome preparation on hemostatic parameters

#### Thromboelastometric studies

The addition of MLV-FBN to whole blood did not modify the CT in comparison with the control in the EXTEM analysis. However, the presence of MLV-FBN led to a significant increase in the CFT (226.5 s ± 26.13 versus 124.17 s ± 9.46;

$P < 0.01$ ), indicating a certain inhibition of clot formation. Moreover, MLV-FBN reduced clot firmness, measured as A10, with respect to control (35.50 ± 2.38 mm versus 45.40 ± 1.82 mm;  $P < 0.05$ ). These results are summarized in Figure 1. No differences were observed when the experiments were performed using thrombocytopenic blood.

The analysis of parameters using the FIBTEM tests indicated that the addition of MLV-FBN to whole blood did not induce differences compared to the control. However, in experiments performed with thrombocytopenic blood, MLV-FBN decreased the CT with respect to MLV (153.33 ± 2.82 versus 128.00 ± 4.62;  $P < 0.05$ ), whereas no differences were found between MLV-FBN and control (Figure 1).

As expected, the results using whole blood in the EXTEM test showed a reduction in clot formation and clot firmness. This test reflected the role of platelets and coagulation processes in hemostasis. In contrast, results obtained with the FIBTEM tests, which eliminated platelet function, showed significant differences in thrombocytopenic blood.

### Platelet aggregation studies

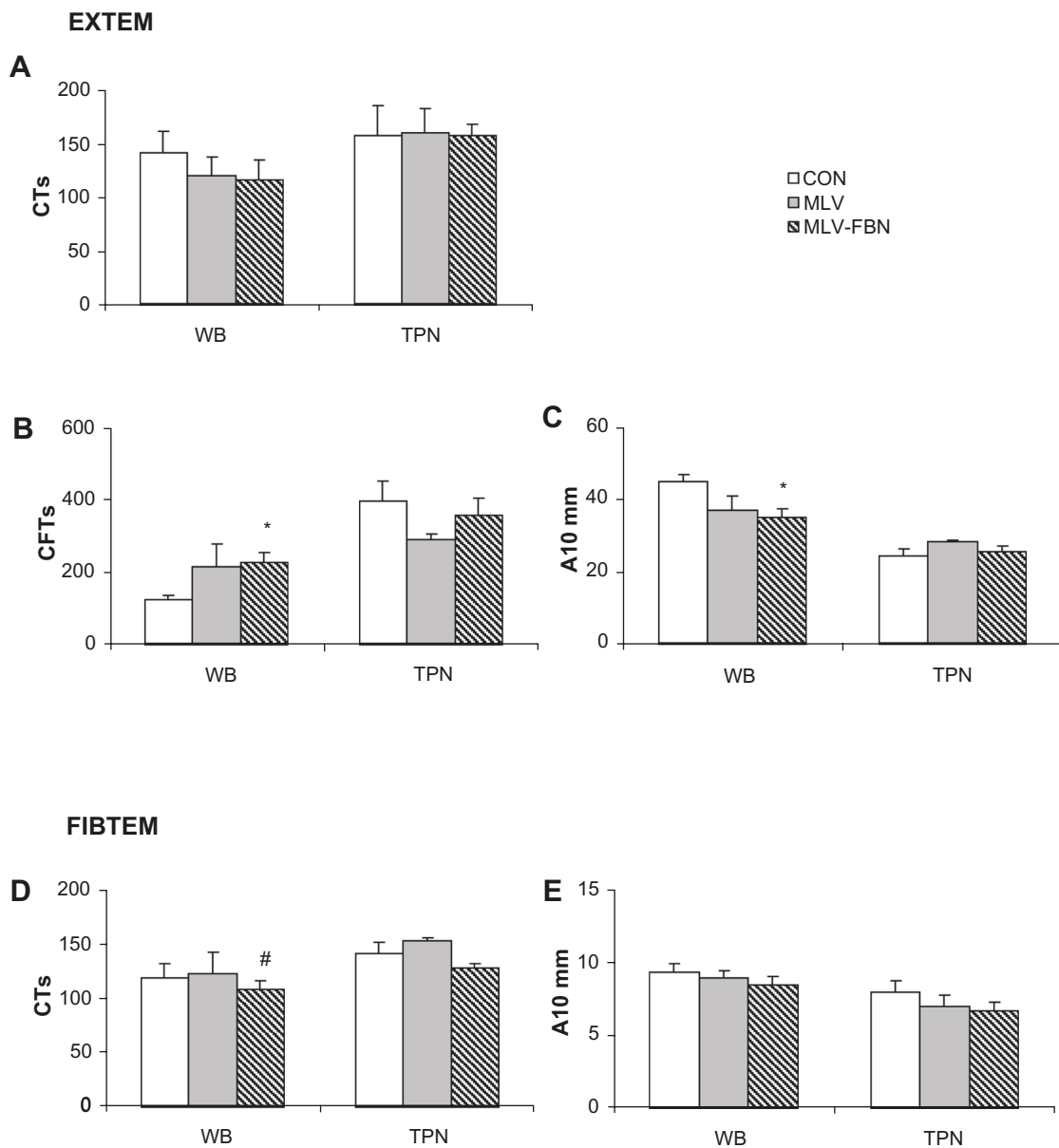
MLV modified aggregation profiles only when arachidonic acid was used as an agonist (50.9% ± 7.9% versus 88.0% ± 2.1% in control;  $P < 0.01$ ). Interestingly, MLV-FBN also inhibited aggregation induced by arachidonic acid (52.1% ± 8.1% versus 88.0% ± 2.1% in control;  $P < 0.01$ ) and ristocetin (16.7 ± 3.2 versus 68.3% ± 5.8%;  $P < 0.01$ ). Aggregations induced by ADP and collagen were not modified by either MLV or MLV-FBN. However, we observed a reduction in the maximal aggregation induced by ADP in the case of MLV-FBN, but this was not statistically significant. These results are summarized in Figure 2.

### PFA-100® studies

The addition of liposomes to whole blood did not statistically modify closure times in the PFA-100® device. COL-EPI cartridges showed values of 171 ± 48.1 s for MLV, 194 ± 27.3 s for MLV-FBN, and 184 ± 27.4 s for control. Results using COL-ADP cartridges were 113.5 s ± 21.6 s for MLV, 124.3 ± 20.5 s for MLV-FBN, and 95 ± 16.9 s for control.

### Perfusion experiments

Perfusions with whole blood resulted in a platelet coverage surface of 22.01% ± 2.05%. Addition of MLV and MLV-FBN to whole blood samples decreased the coverage surface of platelets (13.25% ± 2.4% and 9.85% ± 2.41%, respectively, versus 22.01% ± 2.05%;  $P < 0.01$ ). A more detailed analysis of platelet interaction also showed a decrease in the presence



**Figure 1** Thromboelastometric results of EXTEM- (A–C) and FIBTEM (D and E) derived parameters (CT and CFT expressed in s, and A10 in mm). Experiments were performed in whole blood (WB) and thrombocytopenic blood (TPN) following in vitro addition of MLV and MLV-FBN.

**Notes:** Data presented as mean  $\pm$  SEM,  $n = 6$ . \*MLV-FBN versus control  $P < 0.01$ ; #MLV-FBN versus MLV liposomes  $P < 0.01$ .

**Abbreviations:** CT, clotting time; CFT, clot formation time; CON, control; MLV, raw multilamellar liposomes; MLV-FBN, fibrinogen-coated liposomes; SEM, standard error of the mean.

of large aggregates measured as % T ( $8.44\% \pm 2.33\%$  and  $3.36\% \pm 1.01\%$ , respectively, versus  $14.64\% \pm 1.89\%$  in control experiments;  $P < 0.01$ ). Moreover, a significant decrease in fibrin deposition was observed when liposomes were present in perfusates (MLV  $8.12\% \pm 4.73\%$  and MLV-FBN  $0.84\% \pm 0.52\%$  versus control  $36.48\% \pm 6.76\%$ ;  $P < 0.01$ ). Representative microscopic fields are shown in Figure 3.

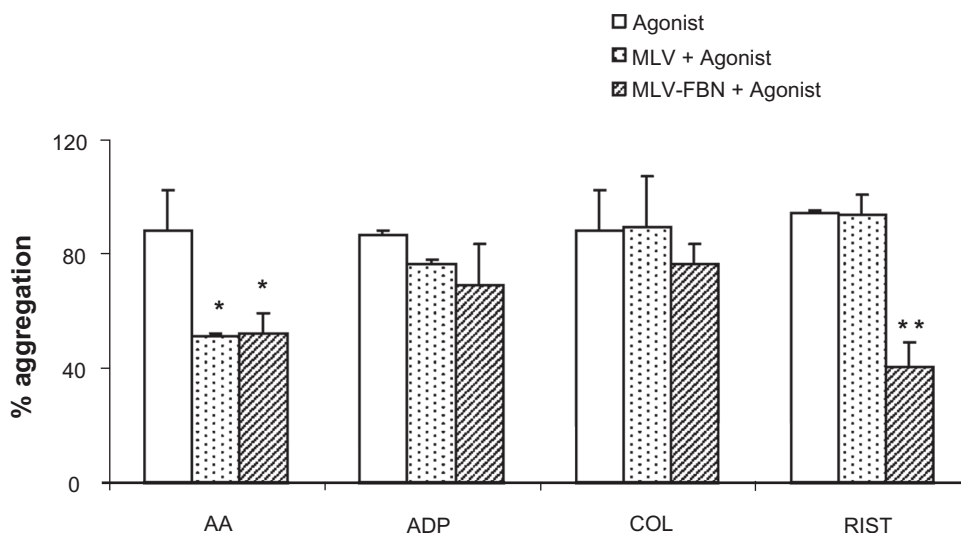
Experiments performed under conditions of moderate thrombocytopenia revealed that the presence of liposomes resulted in a significant reduction ( $P < 0.05$ ) of the percentage of covered surface by platelets (MLV  $7.30\% \pm 1.17\%$  and

MLV-FBN  $6.99\% \pm 0.94\%$  versus  $12.50\% \pm 1.31\%$  in thrombocytopenic blood;  $P < 0.05$ ). MLV did not modify fibrin deposition on the subendothelium ( $34.58\% \pm 5.21\%$ ), but there was a significant decrease in fibrin deposition after the addition of MLV-FBN to thrombocytopenic blood ( $14.47\% \pm 6.34\%$ ;  $P < 0.05$ ). These results are summarized in Figure 4.

## Discussion

The present study focused on the preparation of FBN-coated liposomes in order to improve hemostatic mechanisms, such as platelet reactivity and coagulation mechanisms.





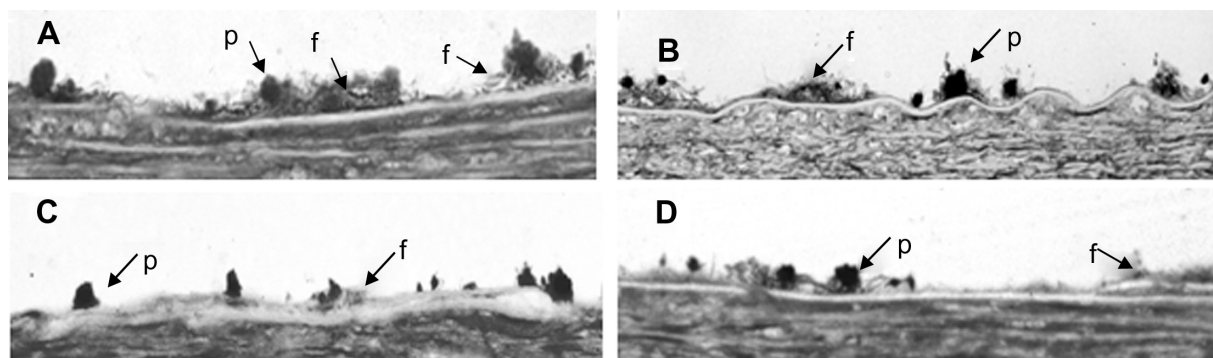
**Figure 2** Bar diagram summarizing changes in platelet aggregation induced by different agonists (AA, ADP, COL, and RIST) in samples treated with MLV or MLV-FBN. Results are expressed as mean  $\pm$  SEM;  $n = 6$ ; \* $P < 0.05$ ; \*\* $P < 0.01$ .

**Abbreviations:** AA, arachidonic acid; ADP, adenosine diphosphate; COL, collagen; RIST, ristocetin; MLV, raw multilamellar liposomes; MLV-FBN, fibrinogen-coated liposomes; SEM, standard error of the mean.

In recent years, several groups have explored the use of modified liposomes and microcapsules as coagulants to improve hemostasis.<sup>5-9,31,32</sup> Our group demonstrated that raw preparations of phospholipids were able to activate the coagulation system and promote fibrin formation on damaged vascular surfaces under thrombopenic conditions.<sup>3</sup> A reduction of platelet counts is quite common in oncohematological patients who exhibit hemorrhagic episodes that sometimes may require platelet transfusion. Originally, the present study was designed to evaluate the possible effect of FBN-containing liposomes as a prohemostatic strategy in different conditions, such as normal platelet counts and thrombocytopenic conditions, in order to mimic patients potentially requiring platelet transfusion.

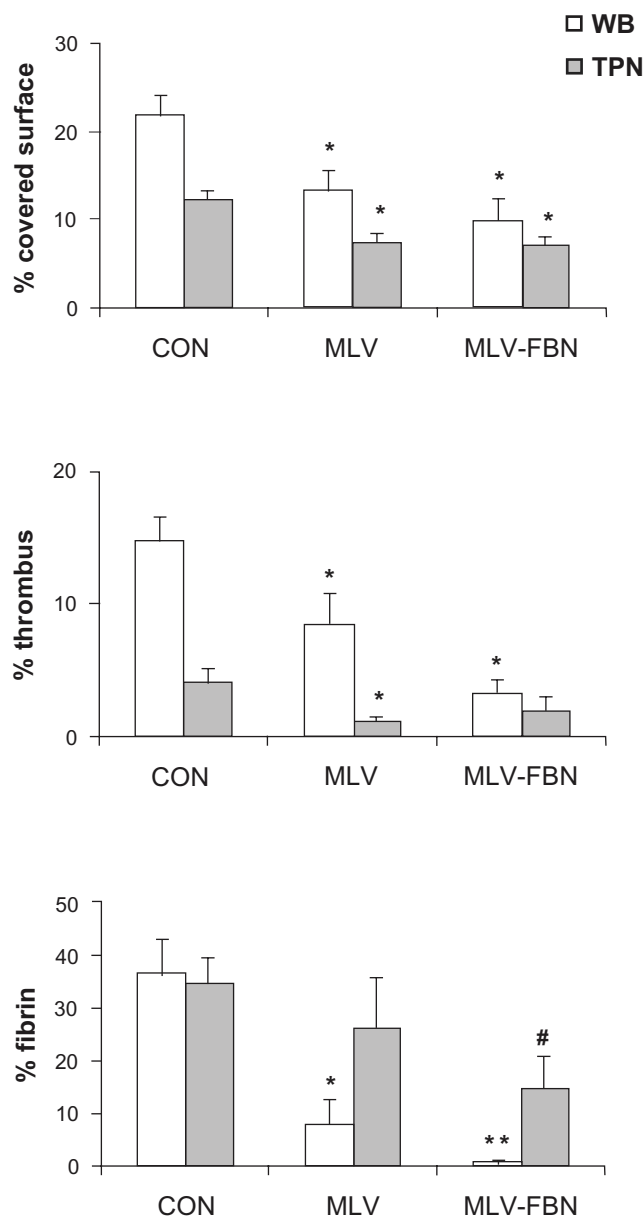
Under our experimental conditions, FBN-coated liposomes modified blood coagulation parameters, as indicated

by the thromboelastometric and fibrin deposition results. In the FIBTEM thromboelastometric studies, in which platelets were inhibited, MLV-FBN reduced the CT in thrombocytopenic blood, indicating a positive effect on the coagulation mechanism. Interestingly, studies using EXTEM tests on whole blood, when both platelets and the coagulation mechanism are active, have demonstrated a partial inhibition of overall hemostatic parameters that includes a reduction in CFT and clot firmness. Moreover, our results of the experiments under moderate flow conditions indicate that MLV-FBN induced lower clotting activity, as demonstrated by a decrease in fibrin deposition on the subendothelium. Furthermore, MLV-FBN acted on primary hemostasis, as demonstrated by decreased platelet interactions in the perfusion experiments performed at a moderate shear rate. However, no differences were observed at a high shear rate,



**Figure 3** Light micrographs representative of changes induced by MLV-FBN in platelet interaction (p) and fibrin deposition (f) on the subendothelium observed in perfusions using whole blood (WB) and thrombocytopenic blood (TPN). (A) WB control; (B) TPN control; (C) WB + MLV-FBN; (D) TPN + MLV-FBN.

**Abbreviations:** MLV, raw multilamellar liposomes; MLV-FBN, fibrinogen-coated liposomes.



**Figure 4** Bar diagram summarizing changes in platelet interaction in samples treated with MLV or MLV-FBN.

**Notes:** Results are expressed as % covered surface by platelets, % of large aggregates (thrombus), and % of fibrin deposition on the subendothelium. Mean  $\pm$  SEM; n = 6. Comparisons with respect to control: \* $P < 0.01$ ; \*\* $P < 0.005$ ; comparison of MLV-FBN versus MLV – raw liposomes \* $P < 0.01$ .

**Abbreviations:** CON, control; MLV, raw multilamellar liposomes; MLV-FBN, fibrinogen-coated liposomes; SEM, standard error of the mean.

as shown by the closure time obtained with the PFA-100®. In fact, it is well established that the shear rate has a role in platelet adhesion and fibrin formation. Under high shear rates, platelet interactions predominate over fibrin formation, whereas under moderate and low shear rates, coagulation mechanisms prevail over platelet interactions.<sup>33–35</sup> We also observed an inhibition in the aggregation profiles induced by ristocetin and arachidonic acid. In the presence of liposomes, this reduction could be explained by a direct effect

of MLV-FBN on the GpIb $\alpha$  complex<sup>32</sup> in the ristocetin assay and by the phospholipidic composition of the liposomes. Indeed, it has been shown conclusively that the net charge of liposomes and the firmness of the membranes determine the effect on hemostasis.<sup>4,32,36</sup>

Although the antihemostatic activity observed in our experiments with FBN-coated liposomes cannot be ascribed to the  $\gamma'$  chain of FBN, our results (reduced thrombin generation, inhibition of ristocetin-induced aggregation, decrease in fibrin density and inhibition of thrombus formation) show behavior similar to that described for the  $\gamma'$  chain.<sup>11,37</sup> This could be explained by a global negative charge in our liposomes<sup>17</sup> and the use of thiolated FBN during the binding procedure, which could induce residue sulfation and generate conformational changes in the molecule similar to those observed in the  $\gamma'$  chain. Interestingly, the effect of MLV-FBN suggests that with a high number of platelets, FBN acts mainly in primary hemostasis, whereas its participation in coagulation is moderate.

Inconsistent effects of FBN have been described to date, probably because FBN can undergo several posttranslational modifications, such as glycosylation, glycation, nitration, oxidation, sulfation, phosphorylation and proteolytic degradation, and genetic differences among individuals.<sup>32</sup> In fact, contradictory results in studies on FBN binding to liposomes have been published. Whereas some studies show that conventional approaches to binding proteins to liposomes do not work effectively with FBN to provide a final product with hemostatic properties,<sup>38</sup> others demonstrate that the binding to liposomes of the dodecapeptide H12, corresponding to a fibrinogen  $\gamma$ -chain carboxy-terminal sequence, yields a product with demonstrated hemostatic activity.<sup>31</sup>

The biological function of FBN can be modified by changes in protein folding.<sup>39</sup> It is accepted that structural features of fibrinogen are related to multiple biological functions, including binding to thrombin, fibrinolysis, regulation of FXIII activity, and interactions with blood cells, such as platelets and leukocytes. Residues A414 to L427 of the  $\gamma'$  chain have been reported to play a central role in thrombin binding because of their overall negative charge, which disrupts the platelet binding domain in the  $\gamma$ A chain and introduces a binding site for zymogen factor XIII and thrombin.<sup>10–13</sup> Moreover, the sulfation of residues Y418 and Y422 further enhances thrombin binding by increasing the net charge and inhibiting thrombin activity.<sup>32,40</sup> On the other hand,  $\gamma$ XL sites in FBN are also involved in the binding to FXIII and to the platelet integrin GPIIb/IIIa. It is not surprising that when chemical modification occurs,

the liposomalization of FBN gives liposomes the ability to bind to the GPIIb/IIIa of platelets, thus competitively inhibiting the formation of platelet aggregates. According to Sivaraman and Latour,<sup>39</sup> the conformation of FBN is the critical determinant of platelet adhesion. Adsorption-induced unfolding can expose two distinctly different types of platelet-binding sites in FBN: one induces platelet adhesion alone, and the other induces both platelet adhesion and activation.<sup>11,29,41</sup>

The present study, in contrast to its initial purpose, offers the possibility of developing new clinical strategies for regulating arterial or venous thrombosis based on the use of liposomes. Our data suggest that the antihemostatic effect observed on liposomes is probably related to the conformational structure adopted by the FBN after binding to the liposome. These changes seem to play an important role not only in blood clot formation and stabilization, but also in primary hemostasis. Future investigations will need to address how the binding procedure may interfere with hemostasis.

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## Disclosure

The authors report no conflicts of interest in this work.

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