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Surfactants Attenuate Gas Embolism–induced Thrombin Production

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Background: **There are no pharmacologic strategies to prevent embolism bubble–induced blood clot formation. The authors conducted experiments to measure thrombin production in sheared whole blood in the presence and absence of bubbles and three surface-active compounds.**

Methods: **Blood samples were obtained from six volunteers seven times. The thrombin-specific substrate Boc-VPR-MCA was added to citrated blood diluted with HEPES-buffered saline. Experimental groups were as follows: sparging (air microbubble embolization) with surfactant present; sparging alone; surfactant alone; and neither surfactant nor sparging. The surfactants were Dow Corning Antifoam 1510US, Perftoran, and Pluronic F-127. Blood was sheared by a cone-plate viscometer at 100 and 500 s^{-1} for 5, 10, and 20 min at 37°C, pipetted into excess stop buffer, and evaluated fluorimetrically. Mean values of fluorescence intensity ± SDs for each group were compared using ANOVA. Differences were considered significant at $P < 0.05$ using the Bonferroni correction.**

Results: **For fixed shear rate, thrombin production increased 2.3- to 5.7-fold ($P < 0.05$) as shear duration lengthened. For fixed shear duration, thrombin production increased 1.9- to 3.9-fold ($P < 0.05$) with increasing shear rate. For fixed shear rate and duration, sparging increased thrombin production 2.1- to 3.7-fold ($P < 0.05$). Surfactant addition without sparging did not change thrombin production ($P > 0.05$). Surfactants attenuated thrombin production in sparged samples 31.8–70.9% ($P < 0.05$).**

Conclusions: **Thrombin production is shear rate and duration-dependent. Sparging increases thrombin production. Surfactants added before sparging attenuate thrombin production. Surfactants may have a clinical application to attenuate gas embolism–induced clotting.**

INTRAVASCULAR gas bubbles provide an interface for the adsorption of macromolecules present in blood. Gas–liquid surface adsorption of proteins is known to provoke molecular conformational changes such as unfolding. Large-scale changes in protein shape potentially expose regions of a protein that can signal the immune system to respond or trigger activation of biochemical pathways involving regulation of vessel tone or initiation of blood coagulation.[1–4] Activation of the coagulation process can precipitate vessel occlusion and reduce or eradicate distal tissue perfusion. By initiating thrombotic pathways, intravascular gas bubbles can lead to the development of pathophysiology, severely affecting the patient.[5] Occluding brain or heart blood flow even briefly may cause transient or permanent cerebral or cardiac injury. Development of a pharmacologic strategy to minimize the extent of blood flow obstruction resulting from clot formation initiated by blood exposure to bubbles may help to protect against the development of end organ injury.

Currently, hyperbaric oxygen therapy, if available and if used, is the standard treatment for gas embolism. Initiation of emergency hyperbaric treatment is usually delayed by several hours from the occurrence of an inciting gas embolic event or after clinical assessment, because of the absence of specific diagnostic criteria for gas embolism and the relative difficulty in accessing hyperbaric chambers. Hyperbaric therapy does not prevent the onset of pathophysiologic processes such as blood coagulation stimulated by embolism bubbles. New strategies to treat gas embolism will depend on knowledge of the molecular mechanisms by which tissue responses to bubble exposure are initiated. Clinical application of preventive therapies based on the molecular basis of disease and applied preemptively in the population at risk for gas embolism could potentially reduce morbidity, lower mortality, and decrease care-associated costs.

We hypothesized that adsorption of blood-borne macromolecules to the surface of an embolism bubble provides a biochemical signal to activate blood clotting. We further hypothesized that surfactants, by preferentially populating gas–liquid interfaces and limiting the interfacial area available for occupancy by those moieties stimulating clot formation, reduce activation of blood clotting stimulated by blood exposure to bubbles. Our main objective was to quantify the relative contributions of shear rate, shear duration, and exposure to gas embolism bubbles to the production of thrombin, an end product of activation of blood clotting. We also sought to quantify the extent to which three novel surface active compounds, added to blood samples before gas embolization, attenuate the clotting response under conditions of imposed shearing. Therefore, we measured thrombin production in human whole blood samples subjected to venous and arterial levels of shear stress over various durations, with and without the addition of gas embolism bubbles and with and without the addition of a surfactant. Experiments were also conducted to quantify the specific contributions of each of the surfactants and

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of platelet-mediated thrombin production to the total amount of thrombin measured.

Materials and Methods

The Institutional Review Board of the University of Pennsylvania approved all experimental procedures used in this study.

Whole Blood Sample Preparation

Whole blood samples were obtained from six healthy volunteers on each of seven occasions. Samples were immediately citrated (9 parts blood to 1 part citrate) and equal-volume diluted into calcium-free HEPES-buffered saline. The factor IIa (thrombin)–specific fluorogenic substrate N-tert-butoxycarbonyl (Boc)-VPR peptide substrate-methylcoumarin (MCA) (boc-VPR-MCA) (Peninsula Laboratories, San Carlos, CA) was added to a 50-μM final concentration. This fluorogenic substrate is specifically cleaved by thrombin, and the cleavage product fluoresces with intensity several orders of magnitude greater than the uncleaved substrate. Blood samples were assigned to four groups: sparging (controlled delivery of air microbubbles for gas embolization) in the presence of one of three surfactants; sparging in the absence of surfactant; no sparging but one of three surfactants present; and no surfactant and no sparging. In a preliminary experiment, recalcification of citrated whole blood with $CaCl_2$ to a final calcium concentration of 2 mM did not neutralize the excess citrate and led to zero thrombin production. Recalcification to 5- and 10-mM final concentrations resulted in identical thrombin production curves for the conversion of Boc-VPR-MCA. For these experiments, blood samples were recalcified to 10 mM to ensure neutralization of the excess citrate for each subject studied. This is a lower final calcium concentration than has previously been used in molecular studies of clotting function.[6,7] A study surfactant, if called for, was added. The three surfactants are described in the next section. Samples were allowed to sit for 1 min or were subjected to controlled sparging for 1 min. Sparging was performed by air delivery at 600 μl/min through a 34-gauge nonmetallic syringe needle (MicroFil; World Precision Instruments, Sarasota, FL) using a Harvard 22 syringe pump (Harvard Apparatus, Holliston, MA). Samples were then placed onto siliconized (AquaSil; Hampton Research, Laguna Niguel, CA) glass coverslips mounted on the shear instrumentation.

Samples were sheared at 37°C using a Rheomat RM265 cone-plate viscometer (Rheometric Scientific, Piscataway, NJ). Shear exposure was performed using two shear rates, 100 and 500 s^{-1}, for three different durations, 5, 10, and 20 min. Immediately after the prescribed level and duration of shear exposure, the specimen was pipetted into excess stop buffer (50 mM EDTA), placed in a quartz cuvette, and then evaluated with a RF-5301PC spectrophotofluorimeter (Shimadzu Scientific Instruments, Columbia, MD) coupled to a personal computer. For this and all subsequent fluorimetry measurements, the excitation wavelength, λ_{EM}, was set to 355 nm, and the fluorescence intensity was measured over the range of emission wavelengths (440–610 nm). Data from each experiment were digitized by the fluorimeter and stored for off-line analysis. Peak fluorescence intensity occurred at an emission wavelength of 460 nm. Baseline fluorimetry measurements of EDTA, each of the surfactants in HEPES-buffered saline, and the fluorogenic substrate in deionized water were also acquired for offset of the experimental data.

Surfactants

We used three prototypical surface-active agents: Pluronic F-127 (PF-127; Molecular Probes, Eugene, OR); Dow Corning Antifoam 1510US (DCA; Dow Corning, Midland, MI); and Perftoran (OJSC SPC Perftoran, Moscow, Russia). The concentrations used were 0.1% (vol/vol) for PF-127, 1.5% (vol/vol) for DCA, and 10% (vol/vol) for Perftoran. The surfactant concentrations chosen reduce the surface tension of blood halfway between its native surface tension ($\approx$ 52 mN/m) and the surface tension of the neat (or pure) surfactant.[8,9]

Assaying Thrombin (Factor IIa) Activity by Fluorimetry

To demonstrate that thrombin activity can be measured by fluorimetric analysis, Boc-VPR-MCA at a 0.1-mM final concentration was added to thrombin (final concentration, 0.01 U/ml) in calcium-free HEPES buffer. The sample was analyzed fluorimetrically in a quartz cuvette.

To demonstrate that thrombin production can be measured in whole blood under conditions of blood activation, recalcified whole blood with Boc-VPR-MCA added at a 0.1-mM final concentration was gently vortexed for 5 s, placed in a quartz cuvette, and inserted into the fluorimeter. Either the sample was allowed to sit or histamine (H 7125; Sigma–Aldrich Chemicals, St. Louis, MO) at the final concentration of 10 μM was added to the reaction mixture to activate blood. Under both conditions (n = 3 subjects each), fluorescence intensity was continuously measured for 30 min.

Surfactant Thrombogenicity and Effects on Platelet-mediated Thrombin Production

To determine if the surfactants are thrombogenic and to elucidate their effects on platelet activation, Boc-VPR-MCA (final concentration, 0.1 mM) was added to platelet-rich plasma. Platelet-rich plasma (n = 3 subjects) was prepared by centrifuging citrated whole blood at 100*g* for 15 min. The samples were recalcified and gently vortexed without surfactant or in the presence of PF-127, DCA, or Perftoran at the above-listed concentra-

tions. The samples were then placed in a quartz cuvette. Fluorescence intensity, expressed as the percentage of baseline, was measured over time for quiescent conditions and with the addition of 1 μM adenosine diphosphate (ADP [M-152]; Sigma–Aldrich Chemicals) after 1 min to elicit platelet activation.

Assessment of Contact Activation

To assess the contribution of contact activation to thrombin production in the sparging and surfactant experiments, we conducted an additional brief experiment. We added Boc-VPR-MCA (final concentration, 0.1 mM) to recalcified, citrated whole blood samples from three donors that were equal-volume diluted in HEPES-buffered saline. Samples were placed either onto untreated glass coverslips or onto siliconized (Aqua-Sil–treated) glass coverslips. Samples were sheared at 500 s^{-1} for 10 min at 37°C. Samples were pipetted into excess stop buffer (50 mM EDTA), placed in a quartz cuvette, and evaluated fluorimetrically.

Data Analysis and Statistics

For each experiment, the fluorescence intensity value used for data analysis was the peak value measured at λ_{EM} = 460 nm and recorded in the computer files. For each set of experimental conditions for the whole blood study (*i.e.*, sparging, surfactant, shear level, and exposure time), the mean values ± SDs of the measured fluorescence intensity data were calculated for each treatment group. The resultant values were normalized to the values obtained for the baseline experiment consisting of 5 min of shearing at 100 s^{-1} without sparging and without any surfactant present. Data were expressed as relative fluorescence intensity as a percentage of this baseline value. Results between different experimental conditions were compared using ANOVA. Differences between groups were considered significant at $P < 0.05$ using the Bonferroni correction. For each of the other experiments (*e.g.*, assaying thrombin activity, surfactant thrombogenicity, and contact activation), the data were normalized to the peak fluorescence intensity occurring at the initial time point with an emission wavelength of 460 nm.

Results

Assaying Thrombin (Factor IIa) Activity by Fluorimetry

For a thrombin sample, the fluorescence intensity measured over the emission wavelength range of 440–610 nm using an excitation wavelength set at 355 nm is shown in figure 1A. Peak fluorescence intensity was found to occur at an emission wavelength of 460 nm, as shown by the vertical dashed line in figure 1A. Figure 1B shows results of the fluorescence intensity measured

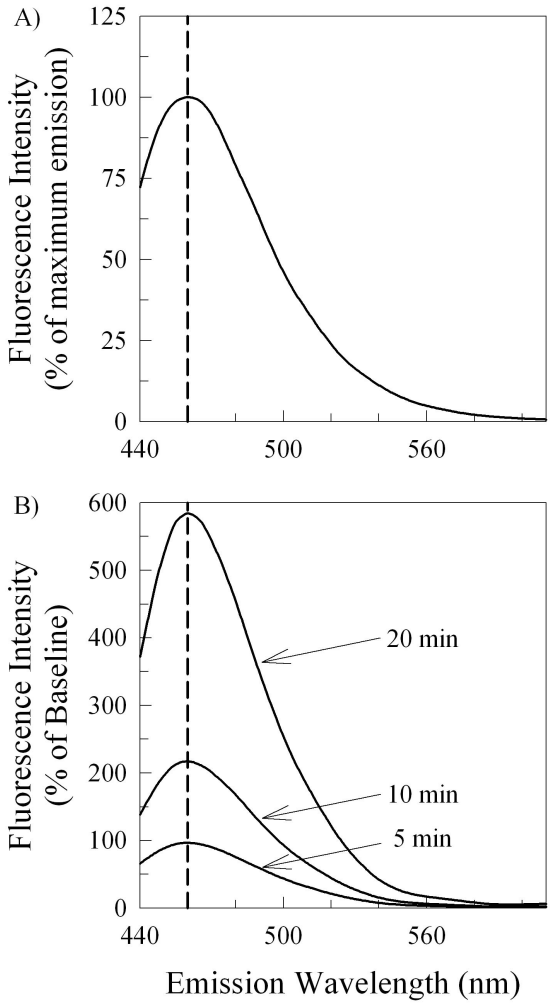

Fig. 1. (*A*) Fluorescence emission spectrum of Boc-VPR-MCA in the presence of thrombin. (*B*) Fluorescence emission spectrum of Boc-VPR-MCA in a sample of whole blood sheared at 100 s^{-1} for various durations. Excitation wavelength was 355 nm in all cases.

over the same full emission wavelength range using 355 nm for whole blood samples from a single individual. Separate curves are shown for measurements made after 5, 10, or 20 min of shear exposure at a shear rate of 100 s^{-1} from a no-surfactant and no-sparging case. Peak emission, indicated by the vertical dashed line, occurred at an emission wavelength of 460 nm. Noting that the curves preserve the characteristic response elicited in figure 1A, these data show that thrombin production can be assessed in whole blood samples prepared as described in Materials and Methods. For this representative trio of experiments shown in figure 1B, 10 min of shearing increased thrombin production by 112% over the value obtained after 5 min of shearing (baseline). With 20 min of shearing, thrombin production increased 481% above baseline.

The time-dependent enhancement of thrombin production presented in figure 1B also appears in the data

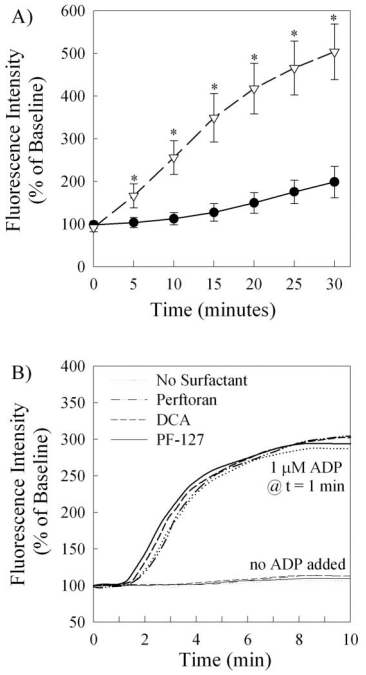

Fig. 2. (*A*) Continuous measurement of thrombin production induced by addition of histamine to whole blood. ● = whole blood; ∇ = whole blood plus histamine. * $P < 0.05$ compared with the whole blood group (n = 3 per group). (*B*) Continuous measurement of platelet activation–mediated thrombin production induced by addition of adenosine diphosphate (ADP) in the presence of the three study surfactants. DCA = Dow Corning Antifoam 1510US; PF-127 = Pluronic F-127.

obtained using quiescent whole blood samples with and without exposure to histamine, as is depicted in figure 2A. Only means ± SDs of the peak fluorescence intensity data (emission wavelength, 460 nm) for each group at each time point are shown. The level of blood activation increased slightly more than 2-fold in the untreated quiescent sample after 30 min. In the histamine-treated sample, thrombin production increased more than 5-fold from the baseline value after 30 min. After the initial time point, significantly more thrombin was formed in the histamine-activated samples ($P < 0.004$ in all cases).

Background Fluorescence

The separate emission spectra obtained for the three study surfactants at their respective study concentrations in HEPES-buffered saline showed that no appreciable fluorescence was emitted in the range of 400–500 nm, indicating that the surfactants did not interfere with the wavelengths used to assess the thrombin-specific substrate. The EDTA and the fluorogenic substrate in deionized water also showed minimal fluorescence (< 0.1% of intensity measured in whole blood after 5 min of shearing at 100 s^{-1}). Taken together, the measurements presented in figures 1 and 2 illustrate our ability to use Boc-VPR-MCA as a fluorogenic reporter of thrombin activity in whole blood under varying activation conditions.

Surfactant Thrombogenicity and Effects on Platelet-mediated Thrombin Production

The curves presented in figure 2B show the general effects of each of the surfactants on thrombin production and their specific effects on thrombin production concomitant with ADP-induced activation of platelets. Each curve is the mean for three samples. The 95% confidence intervals were narrow and are not shown, so that curves for each surfactant condition can be distinguished. Under quiescent conditions without ADP added, there was only 10–15% enhancement of fluorescence after 10 min compared with the initial time point. No statistical differences were identified between the various surfactants and no surfactant ($P > 0.88$ for all cases). The addition of ADP led to an approximately 3-fold increase in fluorescence by 10 min. This was significant ($P < 0.009$) for all matched surfactant conditions in comparison with the ADP-free conditions, indicating a platelet activation–mediated increase in thrombin production. The presence of the surfactants had no effect on this response to ADP. No statistical differences were identified between the various surfactants and no surfactant ($P > 0.76$ for all cases).

Assessment of Contact Activation

Fluorescence intensity was 6.1% greater on untreated slides than on siliconized slides (normalized to results for 5-min shear at 100 s^{-1}; relative fluorescence intensity, 877 ± 171 *vs.* 827 ± 164). This was not statistically significant ($P = 0.68$). Coupled with the results for quiescent whole blood without histamine (fig. 2A) and quiescent whole blood without ADP (fig. 2B), this indicates that blood sparging and blood shearing play a major role in clot activation and that contact activation induced by the coverslip plays a minor role in the overall activation measured.

Sparging, Shearing, and Surfactant Effects in Whole Blood

The results of the experiments to determine the effects of shearing and sparging of whole blood in the presence or absence of the three study surfactants are shown in figure 3. Each panel depicts a separate condition of shear duration at a specific shear rate. All data in figure 3 were normalized to the mean peak fluorescence intensity measured after 5 min of shearing at 100 s^{-1} for the group that was neither sparged nor had any surfactant added. This value appears as the *left-most bar* in figure 3A.

Increasing either the shear duration or the shear rate, with all other factors in the experiment unchanged, enhances thrombin production. For instance, with unsparged samples sheared at 100 s^{-1} and no surfactant, increasing the shear duration from 5 to 10 min (fig. 3B) increased the thrombin production 2.3-fold ($P = 0.0061$). For fixed shear duration, the shear rate effect can be determined by comparing the results in figure 3,

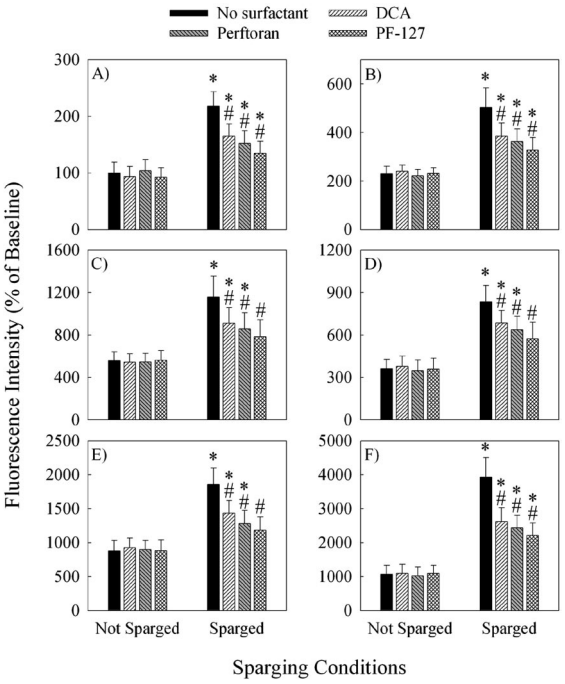

Fig. 3. Relative fluorescence intensity of sparged and unsparged blood samples with no surfactant, Dow Corning Antifoam 1510US (DCA), Perftoran, or Pluronic F-127 (PF-127) added (n = 6 per group). Shear conditions were as follows: (*A*) 100 s^{-1} for 5 min; (*B*) 100 s^{-1} for 10 min; (*C*) 100 s^{-1} for 20 min; (*D*) 500 s^{-1} for 5 min; (*E*) 500 s^{-1} for 10 min; and (*F*) 500 s^{-1} for 20 min. Baseline fluorescence intensity was the value measured without surfactant for samples sheared at 100 s^{-1} for 5 min but not sparged. * $P < 0.05$ compared with the not sparged group; # $P < 0.05$ compared with the sparged and no surfactant group.

A–C, with those in figure 3, D–F. As an example, without sparging or adding surfactant, increasing the shear rate from 100 to 500 s^{-1} increased thrombin production 3.8-fold at 10 min of shearing ($P = 0.0081$).

Sparging markedly increased thrombin generation in each set of experiments without surfactant present. When comparing values for sparged and not sparged conditions (no surfactant) within any part of figure 3, it is evident that at low shear, the increase due to sparging was 2.2-fold at both 5 and 10 min of shear exposure ($P < 0.0069$ for each case) and 2.1-fold at 20 min of shearing ($P < 0.0084$). At high shear, sparging increased thrombin production 2.3-fold at 5 min of shearing, 2.1-fold at 10 min of shearing, and 3.7-fold at 20 min of shearing ($P < 0.009$ for all cases).

In the absence of sparging, none of the surfactants had a significant effect on thrombin production at any combination of shear rate and shear duration. Compared with the levels of fluorescence intensity measured without surfactant present, the relative range of fluorescence intensity levels measured was 94–105% with DCA present ($P > 0.86$ for all cases), 96–104% with DCA present ($P > 0.89$ for all cases), and 92–102% with DCA present ($P > 0.78$ for all cases).

The surfactants significantly reduced thrombin generation in sparged and sheared blood under all experimental conditions. The data presented for sparged blood samples in each part of figure 3 show that the fluorescence intensity was always maximal if surfactant was not added. Thrombin production was always appreciably attenuated in the sparged samples with surfactants present as compared with sparging alone ($P < 0.028$ all cases). Among the three surfactant groups at any combination of shear duration and shear rate, there were no differences as to the overall level of thrombin production with sparging ($P > 0.26$ for all cases). In three cases, thrombin production in the presence of PF-127 was no different with or without sparging ($P < 0.041$ for each case). For all other experimental combinations, thrombin generation was significantly higher with sparging than without exposing blood samples to bubbles ($P > 0.066$ for all cases).

Table 1. Percentage of Surfactant-induced Attenuation of Gas Embolism–caused Thrombin Production

Shear Rate, s^{-1}	Time, min	Surfactant, %		
		DCA	Perftoran	PF-127
100	5	44.8	55.7	70.9
100	10	43.1	51.8	64.3
100	20	41.1	50.2	62.8
500	5	31.8	41.8	55.2
500	10	43.2	59.0	69.1
500	20	45.8	52.3	60.2

DCA = Dow Corning Antifoam 1510US; PF-127 = Pluronic F-127.

Another way to assess the effect of each surfactant is to calculate the degree to which the additional thrombin production provoked by sparging is attenuated at each shear level–shear rate combination by the presence of the study compounds. The percent attenuation of additional thrombin production is given by:

$$\% \text{ Attenuation} = \frac{\text{FI(Sparged, No Surfactant)} - \text{FI(Sparged, Surfactant)}}{\text{FI(Sparged, No Surfactant)} - \text{FI(Not Sparged, No Surfactant)}} \times 100 \quad (1)$$

In this expression, the values of FI (sparging condition, surfactant condition) are the fluorescence intensities measured under each combination of sparging and surfactant at a fixed shear rate and shear duration. The values (range, 31.8–70.9%) are listed in table 1.

Discussion

One approach to developing new therapy for vascular gas embolism is first to identify the molecular basis of events leading to initiation of injury and then "target" them with a therapeutic intervention aimed at preventing onset of the undesired pathophysiologic effect. We hypothesized that adsorption of blood-borne macromolecules to the bubble surface provides an important biochemical signal that accelerates clotting. Proteins can

adsorb to gas–liquid interfaces through ordinary hydrophobic interactions,[10,11] and intravascular bubbles provide surface area for adsorption of circulating molecules. Details of plasma protein structure, function, size, concentration, and diffusivity are necessary to fully understand surface adsorption, denaturation, and conformational change. Studies of plasma protein adsorption onto solid–liquid interfaces[12] and of adsorption out of aqueous media onto hydrophobic liquid drops[13] have been conducted. To our knowledge, similar studies for plasma protein adsorption to gas–liquid interfaces have not been performed; therefore, few of the molecular events associated with resultant biofunctional phenomena such as activation of clotting mechanisms have been clearly identified. Protein layers adsorbing to the bubble interface do, for instance, have mechanical implications for adhesion interactions between the bubble surface and the vasculature[14–16] and may slow gas reabsorption from bubbles.[17]

In this study, we used a fluorescence technique to assess thrombin production in sheared whole blood. As shown in figure 1, the peak fluorescence intensity for the fluorophore used in whole blood samples occurred at the same emission wavelength corresponding to the maximum emission measured with thrombin, as has been detailed previously.[18] Given that we added an excess of the uncleaved fluorophore to the samples, the measured intensity was proportional to the thrombin concentration in all clotting samples, so that relative effects of different experimental conditions could easily be determined. Based on this, we showed that the addition of gas bubbles to whole blood samples subjected to shearing markedly increased clot activation. The magnitude of the effect of embolism bubble exposure is to increase thrombin levels 2.1- to 3.9-fold, depending on the shearing conditions. These results, shown in figure 3, also indicate that thrombin levels increase with higher shear exposure (500 *vs.* 100 s^{-1}) and prolongation of shearing. This is likely due to enhanced mixing of the samples that occurs in longer experiments and with faster rotational speeds of the viscometer. In those cases involving sparging, the further increase in thrombin production results from both interface-induced clotting and enhanced mixing, bringing critical clotting elements into contact with each other. In the histamine and ADP experiments (fig. 2), there was no shear-induced mixing. Thrombin formation proceeded rapidly in those cases in which a specific activator (histamine or ADP) was added. In the cases in which no activator was added and there was no sample stirring during the measurement period, thrombin formation proceeded slowly.

The effects of the surfactants to attenuate thrombin formation in the presence of bubbles could have several causes, including direct inhibition of thrombosis, interference with the fluorescence measurement method, or inhibition of bubble-induced signaling activating clot formation. Many different classes of compounds (solvent, colloidal, and polymeric) are known to be surfactants. They include, but are not limited to, the three classes of compounds we included for study: perfluorocarbons (Perftoran), polydimethylsiloxanes (DCA), and nonionic polyols (PF-127). Although these represent three distinct chemical families (halogenated solvents, antifoaming agents, and long-chain polymers), they share certain important features. They are well characterized for physical characteristics (*e.g.*, aqueous solubility, vapor pressure, boiling point, and viscosity). They are stable, chemically inert, and essentially nontoxic. Their physical properties exhibit relatively small variation in temperature (important to consider, for instance, in application to cardiopulmonary bypass). There is increasing literature regarding their biocompatibility and effects on inflammation,[19–21] as well as their specific interactions with blood components[22,23] and in altering clearance of embolism bubbles from the circulation.[8,9] Specifically, DCA is a nonionic, silicone emulsion–type surfactant. Perftoran is a proprietary 10 vol% perfluorocarbon emulsion containing perfluorodecalin ($C_{10}F_{18}$; molecular weight = 462 daltons) and perfluoromethylcyclohexylpiperiden ($C_{12}F_{23}N$; molecular weight = 595 daltons). We previously showed that DCA[8] and Perftoran[9] enhance the rate of arteriolar embolism bubble clearance *in vivo*. The third surfactant used, PF-127, is a polyethylene oxide–polypropylene oxide–polyethylene oxide block copolymer with a single hydrophobic polypropylene chain and two hydrophilic polyethylene oxide chains. PF-127 forms gels and therefore has pharmaceutical applications as a delivery vehicle for modulating drug release.

Figure 2B shows that none of the surfactants directly stimulated or inhibited thrombin production resulting from either contact activation or platelet activation caused by addition of ADP. This is consistent with the findings that none of the surfactants enhanced or depressed shear-induced thrombin production in the absence of sparging, illustrated in each panel of figure 3. Thus, the surfactants are biologically inert as direct inhibitors or activators of thrombin formation, yet lower thrombin concentrations resulted in the sparging experiments with the surfactant present. One mechanism for this is adsorption of the relative small surfactant molecules to the bubble interface. Surfactant occupancy of the interface can reduce or prevent population of the interface by other, larger plasma-borne molecules having the potential to initiate clotting. Experimental and theoretical studies of adsorption of mixed systems of proteins (*e.g.*, human and bovine serum albumin, β-lactoglobulin, and β-casein) and surfactants have shown that the complex process involves surfactant–protein interactions that alter protein structure, modify surface coverage, and change mechanical properties of the interface.[24–27] A nonionic surfactant, for instance, can nearly completely occupy the surface and prevent almost all protein molecules from adsorbing.[25] In the extreme case, Mackie *et*

al.[28] showed that proteins, which have already occupied the interface, can actually be displaced by a surfactant. In the "orogenic" mechanism responsible, the surfactant adsorbs into small spatial defects in the protein layer along the surface. The surfactant region increases in size, compressing the protein region until the surface pressure is sufficiently high to displace protein from the interface. In any case, our results are consistent with the concept that the surfactant coverage of a bubble's surface prevents protein adsorption, unfolding, and exposure of regions of the protein that signal the initiation of clotting.

Other mechanisms in addition to the formation of procoagulant multiprotein complexes on the bubble surface could also explain the molecular mechanisms involved in activation of gas embolism–induced thrombin formation. Bubble-induced stimulation of pathways leading to initiation of cell-based coagulation mechanisms and, ultimately, leading to thrombin production could also occur. For example, an increase in expression of tissue factor and subsequent platelet activation resulting from cellular injury caused by exposure of vascular endothelium to bubbles or activation of platelets directly contacting the bubble surface could provide a sufficiently large primary surface for promulgation of thrombin generation. In all of these potential mechanisms, it is possible that the presence of a surfactant adsorbed to the bubble surface could protect against thrombin formation by effectively shielding blood from exposure to a surface that elicits activation of a thrombogenic response.

Many studies of intravascular gas bubble reabsorption and growth[16,29–33] demonstrated that small bubbles ($<$ 100 nl) can persist for minutes to hours, essentially continuously providing a gas–liquid interface. In the current study, we demonstrated that sparging (or gas embolization of) blood samples, in addition to shearing them, markedly increases thrombin production. The conclusion that exposure of blood to an air interface provokes blood clot formation is not novel, but attempting to render the gas–blood interface biologically inert as a stimulant to clotting by adding a surfactant to blood, as we did, has not previously been reported. Previously reported *in vivo* studies showing that exogenous surfactants can accelerate the rate of bubble clearance from vessels after embolization[8,9] are complemented by our findings that three chemically different surfactants reduce the level of thrombin production stimulated by *in vitro* gas embolization.

In conclusion, we have demonstrated that the shear rate–dependent and shear duration–dependent production of thrombin, as measured fluorimetrically, is greatly enhanced by gas embolization of blood samples. The presence of any of the three surfactants studied in samples before gas embolization resulted in significant diminution of thrombin produced. This demonstration that the thrombin formation stimulated by gas emboli can be attenuated by surfactants opens the possibility that therapy based on competition with plasma proteins for bubble interfacial occupancy is a rational approach for the future study of the pathophysiology and treatment of gas embolism–related injury. Experiments should be designed to determine which potential molecular mechanisms are involved, including identification of the specific role of the bubble surface in increasing thrombin production by activation of platelets and stimulation of expression of tissue factor. Knowledge of these plus characterization of any identifiable procoagulant macromolecular complexes formed on an embolism bubble surface will provide therapeutic targets for future drug design.

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