

Pefluorocarbon inhibition of bubble induced Ca^{2+} transients in an *in vitro* model of vascular gas embolism

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Abstract

Endothelial injury resulting from deleterious interaction of gas microbubbles occurs in many surgical procedures and other medical interventions. The symptoms of vascular air embolism (VAE), while serious, are often difficult to detect, and there are essentially no pharmaceutical preventative or post-event treatments currently available. Perfluorocarbons (PFCs), however, have shown particular promise as a therapeutic option in reducing endothelial injury both *in-* and *ex-vivo*. Recently, we demonstrated the effectiveness of Oxycyte, a third-generation PFC formulated in a phosphatidylcholine emulsion, using an *in vitro* model of VAE developed in our laboratory. This apparatus allows live cell imaging concurrent with precise manipulation of physiologically sized microbubbles so that they may be brought into individual contact with human umbilical vein endothelial cells dye-loaded with the Ca^{2+} sensitive Fluo-4. Herein, we expand use of this fluorescence microscopy-based cell culture model. Specifically, we examined the concentration dependence of Oxycyte in reducing both the amplitude and frequency of large intracellular Ca^{2+} currents that are both a hallmark of bubble contact and a quantifiable indication that abnormal intracellular signaling has been triggered. We measured dose dependence curves and fit the resultant data using a modified Black and Leff operational model of agonism. The half maximal inhibitory concentrations of Oxycyte for (i) inhibition of occurrence and (ii) amplitude reduction were $229 \pm 49 \mu\text{M}$ and $226 \pm 167 \mu\text{M}$, respectively. This investigation shows the preferential gas/liquid interface occupancy of the PFC component of Oxycyte over that of mechanosensing glycocalyx components and validates Oxycyte's specific surfactant mechanism of action. Further, no lethality was observed for any concentration of this bioinert PFC, as it acts as a competitive allosteric inhibitor of syndecan activation to ameliorate cell response to bubble contact.

Keywords: Surfactant, vascular air embolism, syndecan-4, HSPG, microbubble, perfluorocarbon

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Introduction

Vascular air embolism (VAE) can easily occur in many surgical procedures and medical interventions when endogenously administered gas becomes entrained in the circulation.¹ It also arises during decompression sickness – a well-known risk in deep-sea diving and aviation – when sudden barometric change promotes the dissolution of supersaturated dissolved gas as bubbles into the blood stream.² Treatment is limited to hyperbaric oxygen therapy, which is logistically difficult and seldom used. Advances in monitoring have improved the detection of VAE occurrence, but prophylaxis remains vigilance in operating procedures.^{3,4}

The dearth of therapeutic options arises in part because the pathophysiology of VAE is poorly understood. It is unclear how physical and chemical responses of the endothelium to bubble contact gives rise to the clinical

presentation of the wide range of cardiovascular, pulmonary, and neurologic sequelae observed.³ Blood and endothelial surface layer (ESL) proteins have been shown to partition into and subsequently unfold upon the hydrophobic gas/liquid interface presented by a bubble. Our working hypothesis is that such interfacial interactions trigger events that lead to endothelial cell (EC) injury, dysfunction, and death.⁵ Surfactants preferentially partition onto the gas/liquid interface to compete with vascular macromolecules for interfacial occupancy. This competition should ameliorate endothelial-bubble interactions.⁶ Extensive work has shown that exogenous surfactants improve the dynamics of bubble clearance, reduce adherence, and ease endothelial damage *in vitro*, *ex vivo* and *in vivo*.^{7–11}

So that we could identify the specific glycocalyx components involved in the mechanotransduction of bubble contact and better understand the molecular mechanisms of

surfactant action, we developed an in vitro live-cell imaging model of VAE. The platform, described in earlier work and illustrated in Figure 1, enables generation of physiological-sized air microbubbles (50–150 μm), which may be individually manipulated into contact with single human umbilical vein endothelial cells (HUVECs).^{12–14} By recording cellular responses with phase contrast and epifluorescence microscopy concurrent with bubble contact, we have established that such contact elicits large intracellular Ca^{2+} transients associated with lethality.¹² This transient response requires an initial influx of external Ca^{2+} through a stretch-activated channel in the transient receptor potential vanilloid (TRPV) family, which in turn triggers release of Ca^{2+} from intracellular stores via the IP_3 pathway to produce the full signal.^{13,14} TRPV is gated open by the

heparan sulfate proteoglycan (HSPG), syndecan, that acts as a mechanosensor. Oxycyte, a third-generation perfluorocarbon/phosphatidylcholine (PFC/PC) emulsion intercedes syndecan activation by coating the gas/liquid interface to outcompete triggering HS side-chain interactions.¹³

Intracellular responses to bubble contact are not limited to Ca^{2+} transients. Contact also elicits mitochondrial membrane depolarization via a parallel, calcium-independent, $\text{PKC}\alpha$ -dependent pathway.¹⁵ The common upstream source of both pathways – initiation of syndecan signaling through HS interfacial adherence – is indicated by the elimination of mitochondrial depolarization in the presence of surfactant. Figure 2 illustrates the current understanding on early cell response to bubble contact.

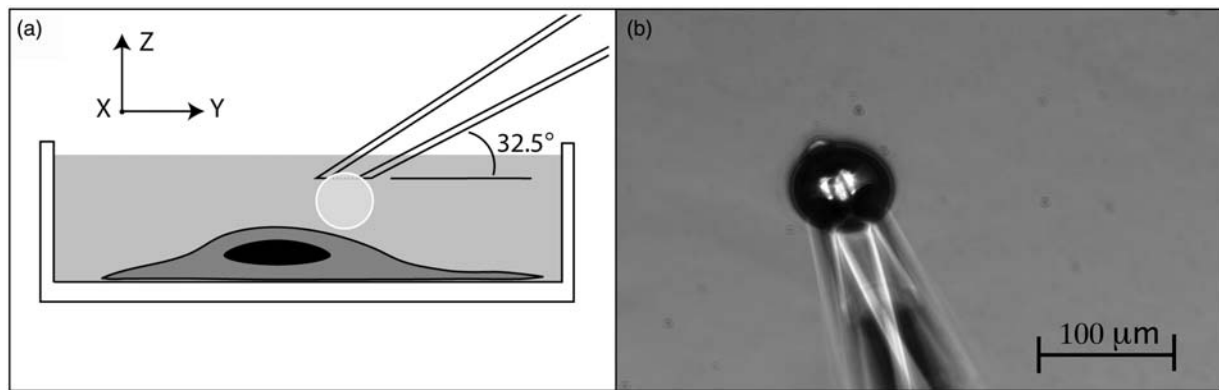


Figure 1 (a) Schematic of bubble probing apparatus (described in Methods) to bring bubble, on the end of a pulled and ground glass capillary tip, in contact with human umbilical vein endothelial cell (HUVEC) for real-time live-cell imaging using phase contrast and fluorescence epifluorescence microscopy. (b) Phase contrast image of 72 μm bubble on tip used to generate and manipulate bubbles

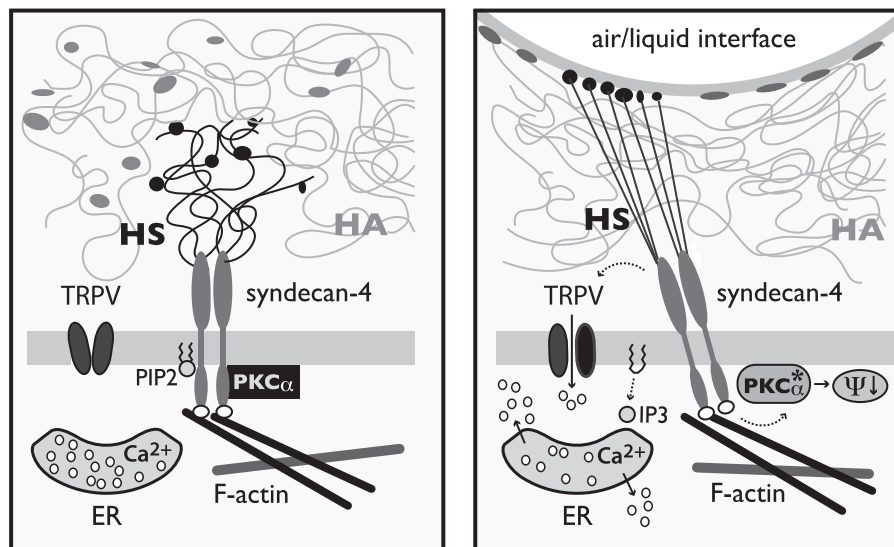


Figure 2 Graphical depiction of early cell response to bubble contact. (Left panel) Important glycocalyx, membrane, and intracellular components in the absence of bubble. Hyaluronic acid (HA) is represented as light gray curved lines with non-specifically bound ESL proteins such as albumin shown as light grey ovals. Syndecan-4 is shown with extracellular HS syndecan side chains depicted as black curved lines with bound protein ligands as black circles on its extracellular ectodomain, PIP2 associated with its membrane spanning region, and $\text{PKC}\alpha$ and actin binding proteins associated with its cytoplasmic V-domain. (Right panel) HS side chains are pulled into the air/liquid interface triggering two parallel intracellular signaling responses. (1) the IP_3 -dependent Ca^{2+} transient initiated by an external influx of Ca^{2+} through a TRPV family ion channel and (2) $\text{PKC}\alpha$ -dependent lowering of the mitochondrial membrane potential, $\Delta\psi$

Herein, we use the Ca^{2+} transient signal as an accurate and sensitive means to quantify the dose response of Oxycyte and further validate its surfactant mechanism of action. We adapt Black and Leff's operational model for agonism to this bubble-cell system to reproduce measured concentration dependence of Oxycyte to ameliorate cell response to bubble contact.¹⁶ As a vehicle control we also examine the inhibitory properties of PC, the emulsifying ingredient in Oxycyte's formulation.

Methods

Cell culture and dye loading

Primary HUVECs were purchased from Lifeline Cell Technologies (Walkersville, MD), cultured according to provider's specifications, and prepared for experiments as described previously.⁷ Briefly, cells were plated sparsely on 35-mm Mattek glass-bottomed Petri dishes coated with fibronectin. About 48 h after plating, cells were loaded with 1 μM of the calcium sensitive dye Fluo-4 and placed in a measurement solution of HBSS (no phenol-red, no bicarbonate, Invitrogen) supplemented with 1% FBS (Sigma), 2 mM GlutaMax (Invitrogen), and 0.01% Heparin Sodium (Fisher Scientific). Ethidium-Bromide dimer (500 nM, final concentration) was added before all experiments to monitor for membrane integrity loss or cell death.

Microscopy and microbubble manipulation

All experiments were performed on an inverted Olympus IX70 microscope as described previously.⁷ Image time courses were obtained with an IPLab script: typically one frame per second for 300 sec in three parallel time courses for each experiment: phase contrast, green fluorescence, and red fluorescence emission. Air bubbles between 50 and 150 μm in diameter were produced and micromanipulated into contact with the cells until a rapid and strong increase in Fluo-4 fluorescence intensity was observed. A cell was considered non-respondent if no Ca^{2+} transient was observed within 30 sec of bubble contact.

Surfactant preparation

Oxycyte, a PFC suspended in egg-phosphatidylcholine (egg-PC) micelles (Oxygen Biotherapeutics) was diluted to 30% in measurement solution, sonicated briefly, and syringe filtered (0.45 μm) to remove any large aggregates. The suspension was further diluted directly in the cell plates and allowed to sit for at least 5 min before measurement. Perfluorocarbon concentration was estimated for Oxycyte by lyophilizing and weighing remaining solid from presumed w/v percentage and assuming 70% PFC in emulsion preparation (M.W. 500.8). Dosing curves were started at 10% v/v to mimic animal studies, which use 10% assumed blood volume intravenous injection.⁷ Egg PC alone was tested as a vehicle control. A 100 μM ($>100\times$ critical micelle concentration) suspension of egg-PC (Avanti Polar Lipids, Birmingham, AL) was made in measurement solution and diluted to the appropriate final concentrations directly into the cell plates. Cell sensitivity to chemical activation of Ca^{2+} response was tested at the

highest concentrations of Oxycyte (10% v/v, 120 mM PFC) by direct addition of ATP to the cell plate (final concentration 10 μM).

Data analysis

Fluorescence intensity time courses from the microscopy images acquired were measured for each time point using ImageJ and then exported to Excel for further calculations. Fluorescence traces normalized to fold over baseline (after background correction) were calculated as described previously.¹³ A minimum of nine cells ($n=9$) were tested from at least three separate plates for each solution condition. Controls on cell plates were done both before and after test plates were run, giving a total of 145 control trials. Normalized fluorescence value after bubble contact is the parameter used for further statistical analyses. Data are presented either as mean $\pm$ standard deviation or as percent inhibitions. Percent inhibition was calculated in two ways. (1) Unity minus fraction of non-responding cells and (2) Unity minus fraction of mean amplitude of responding cell with respect to those of control cells. Graphing and curve fit analysis was performed with GraphPad Prism version 5.0 c for Mac OS X (GraphPad Software, San Diego, CA, USA). Statistical comparisons were made using Student's *t*-test, with $p < 0.05$ considered to be significant.

To formulate the relationship of inhibitor concentration to the amplitude to the reduction of amplitude and occurrence of bubble-induced transients, we adapt the operational model of agonism developed by Black and Leff.¹⁶ In this model, *E* is posited to be a rectangular hyperbolic function of the concentration of receptor with bound agonist, [AR]. Therefore, the governing equation is

$$E = E_m \frac{[AR]}{K_E + [AR]}$$

in which *E* = effect at a given level of [AR], *E_m* = maximal effect, and *K_E* = value of [AR] that elicits the half-maximal effect.

Syndecan-4 is defined as the "receptor" for our system. Mechanotransduction of bubble presence into cell response occurs upon the adherence of syndecan HS side-chains onto the gas/liquid interface. Our measurable effect, the Ca^{2+} transient amplitude, is dependent on the concentration of syndecan-4 with its HS side-chains so engaged, or 'activated receptor,' [AR].

Formation of [AR] does not occur in a typical agonist equilibrium binding reaction. Rather, it occurs upon irreversible adherence of syndecan-4 HS bound ligand to the bubble surface. Thus [AR] is dependent on available interfacial surface area or, equivalently, the number of available dynamic adherence sites. Bubble surface occupancy by surfactant reduces the number of adherence sites, thus lowering [AR] as described by mass action interfacial partitioning of the surfactant. We model the reduction in [AR] also with a rectangular hyperbola. The half maximal inhibitory concentration, *IC*₅₀, is defined as the concentration of surfactant to reduce [AR] and thus the Ca^{2+} transient amplitude or frequency of occurrence by one half.

Results

Normal HUVEC response to bubble contact

Figure 3 illustrates a typical intracellular Ca^{2+} response to bubble contact (solid line). Under control conditions a transient signal with mean amplitude 6.4 ± 0.4 times over baseline Ca^{2+} levels was observed on average 14 sec after the air/liquid interface of the bubble was brought into proximity with the cell surface. These results are consistent with our prior work.^{13–15} Over the control trials conducted (i.e., no surfactant present), bubble contact resulted in transient increases of intracellular Ca^{2+} levels in >98% of experiments.

Oxycyte inhibits bubble-induced calcium transients in a dose-dependent fashion

Figure 3 also shows a typical normalized trace of the Ca^{2+} response of a cell in the presence of a 10% Oxycyte PFC/PC suspension (120 mM final PFC concentration, dotted line). Under these conditions, a dramatic reduction of the occurrence of induced Ca^{2+} influx was observed. When surfactant was present, no transient Ca^{2+} response was observed following bubble contact in over three quarters of the trials (77 out of 100). Under control conditions of no surfactant being present, less than 2% of the cells failed to show a transient Ca^{2+} response after contact. The reduction in triggering frequency was accompanied by a nearly two-fold reduction in mean normalized amplitude (3.3 ± 1.01) of Ca^{2+} transients in those cells that did show a transient response. This was smaller than the mean amplitude observed in the control system (6.4 ± 0.4 , $p < 0.0001$).

Graphs displaying the concentration dependence of Oxycyte inhibition appear in Figure 4. The top graph shows percent inhibition calculated with respect to the fraction of cells that respond to bubble contact. These are raw counts of fixed events having a binary outcome: cells either respond to bubble contact with a Ca^{2+} transient or they remain quiescent. Therefore, no error bars can be displayed for the occurrence data; however, the upper and lower 95%

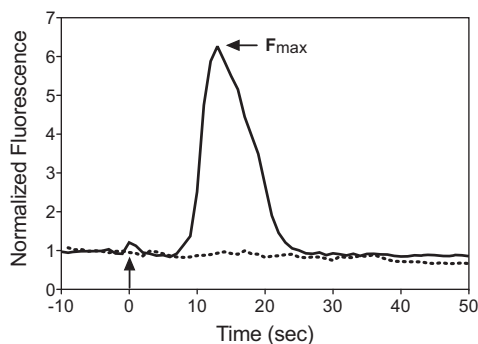


Figure 3 Normalized Fluo-4 fluorescence time courses for control human umbilical vein endothelial cell (HUVEC) contacted with a bubble (solid line) and cell contacted by a bubble in the presence of 10% Oxycyte (dotted line). Bubble contact occurrence is indicated with arrow at $t = 0$. The amplitude of the Ca^{2+} transient is defined as the maximum normalized fluorescence signal (F_{max}) after contact and is also indicated

confidence intervals for the nonlinear fit are included. The lower graph illustrates the concentration dependence for the same cell plates as fraction of the mean Ca^{2+} transient amplitude of responding cells with respect to that of control cells. Error bars for the standard deviations in these continuous data, as well as upper and lower 95% confidence intervals for the nonlinear fit, are included. The best fit analyses of inhibition of occurrence and decrease in signal amplitude yield IC_{50} values of $229 \pm 49 \mu\text{M}$ and $226 \pm 167 \mu\text{M}$, respectively, with IC_{50} defined as the concentration of surfactant reducing normalized Ca^{2+} transient amplitude or frequency of occurrence by 50%.

ATP was added after the experiments to a final concentration of $10 \mu\text{M}$ to cells with the highest Oxycyte concentration (10% v/v). Normal chemo-induced Ca^{2+} signaling pathways remained viable with no inhibition in the number of cells that produce ATP-induced Ca^{2+} transients and with no significant inhibition of transient signal amplitude. Normalized signal was 8.1 ± 0.6 ($n = 6$) for control cells and 7.0 ± 3.0 ($n = 6$) for cells in 10% v/v Oxycyte ($p = 0.4$). No cell death or loss of membrane integrity, monitored in all experiments with 500 nM Ethidium Bromide dimer, was seen for any concentration of Oxycyte.

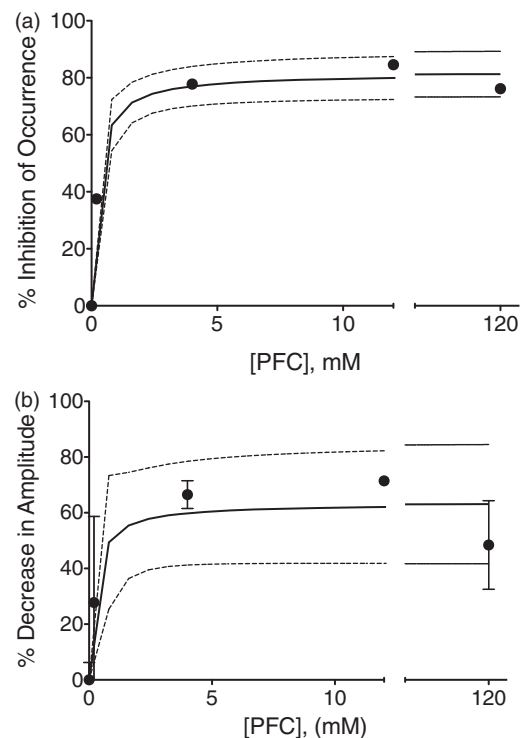


Figure 4 Inhibition of (a) Ca^{2+} transient occurrence and (b) maximum transient amplitude in the presence of Oxycyte graphed as a function of (PFC). No error bars are present in the upper graph since occurrence is calculated from counting binary events over total instances. Error bars in the lower graph represent standard deviations from the mean. The error bars for the 12 mM data point ($71.4 \pm 0.8\%$) is smaller than can be seen in the graphic. Solid lines in both graphs represent best-fit curves to inhibition of occurrence and amplitude reduction data using a one-component hyperbolic function (described in Methods) to yield IC_{50} values of $229 \pm 49 \mu\text{M}$ and $226 \pm 167 \mu\text{M}$, respectively. Dashed lines represent upper and lower 95% confidence intervals

Vehicle control experiments

The dose response of a PC suspension alone was tested to explore the possible contribution from the PC present in the Oxycyte suspension formulation. These data are presented in Figure 5. Inhibition of bubble induced Ca^{2+} transients is shown as bar graphs. The inhibition curve is biphasic with a maximum effect occurring near the critical micelle concentration (CMC) of egg-PC (50 nM). This maximum effect is well below (three orders of magnitude) the concentration of egg-PC in the lowest active Oxycyte suspension. Negligible egg-PC inhibition (2%) is seen at the micromolar concentrations that are present in active Oxycyte. Also shown in Figure 5, in black circles, are the amplitudes of occurring bubble-induced Ca^{2+} transients as a function of [egg-PC]. There is on average (with large standard deviations) a 50% reduction in Ca^{2+} transient size at every concentration of egg-PC tested (10 nM–1 μM range).

Discussion

Work from our laboratory has offered important new insights into the poorly understood pathophysiology of endothelial damage arising from VAE.^{6–12} Most recently we have identified the mechanosensing trigger and two of the resulting intracellular signaling pathways that occur in early EC response to microbubble contact.^{13–15} This response is characterized by (1) a strong IP3-dependent intracellular Ca^{2+} transient with concurrent rise in mitochondrial calcium and (2) a parallel Ca^{2+} -independent, PKC α -dependent loss of mitochondrial membrane potential. Both pathways are induced upon imposition of adhesion forces to the apical glycocalyx when syndecan HS side chains are drawn to the hydrophobic gas/liquid interface of the microbubble. The resulting tensile force activates a TRPV ion channel, and this (external Ca^{2+} dependent) influx in turn initiates the IP3-dependent Ca^{2+} release from the ER. In addition, bubble perturbation of syndecan-4 activates PKC α , causing mitochondrial membrane depolarization. The two known intracellular signaling response pathways to bubble contact are graphically summarized in Figure 2.

Ample evidence argues that these observed bubble-induced responses would be deleterious *in vivo*. It is not

possible, however, given the experimental constraints of our live cell imaging model system to demonstrate explicitly that these bubble-induced pathways are generative mechanisms for ultimate EC dysfunction and death. Specifically, our studies have both temporal and spatial limitations. The fluorescence microscopy technique employed does not allow cells to be studied for longer than a few hours. The study of individual cells on sparsely populated plates not only precludes the use of traditional population-based molecular biology techniques but also lacks cell-cell interactions that are likely to be present with confluent ECs *in vivo*. Additionally, *in vivo* VAE bubbles in blood flow pass across the surface of the ECs, providing a shear stimulus in the ESL interaction that we cannot reproduce completely in this experimental model. Even so, what we have shown explicitly is the molecular specificity of how bubble contact is mechanotransduced, and which particular intracellular pathways contribute to the earliest endothelial response resulting from that contact. More importantly, our work has established that surfactant presence prevents triggering of these potentially harmful signaling responses by rendering the gas/liquid interface virtually undetectable to the EC glycocalyx mechanosensory apparatus.

Now, we have continued our investigations, using the strong intracellular Ca^{2+} transient response as a metric, to quantitate Oxycyte inhibition of cell response to bubble contact. Inhibition is well described by a rectangular hyperbolic curve with an IC_{50} of $\sim 230 \mu\text{M}$. At this concentration, preferential partitioning of the PFC component of Oxycyte to the gas/liquid interface effectively reduces by half the number of (non-specific) available adherence sites for the HS syndecan side chains. This reduction lowers both the population fraction of the HSPG syndecan that can be (and the probability it will be) activated. In this way, Oxycyte acts in some way as an allosteric inhibitor for initiation of Ca^{2+} influx (and presumably PKC α activation) upon bubble contact.

These results concur well with earlier work from our laboratory, which through quantitative imaging techniques measured a lower surface concentration of protein adsorbed to the gas/liquid interface in the presence of surfactant.^{6,25} Thus, as surface occupancy of surfactant decreases the interfacial area available for protein adsorption to the gas/liquid interface, it diminishes interactions of specific cell surface layer constituents with the bubble interface. Both the number of syndecan HS side-chains available to adhere and the probability that they will do so are reduced by a mechanism that is well described by interfacial partitioning of Oxycyte.

We have shown further that the mechanism of action by which Oxycyte inhibits EC mechanoactivation is predominately through its PFC component. PFC is essentially bioinert, thus it is highly specific to the gas/liquid interface and has little or no interaction with the rest of the EC system. The results with egg-PC micelles emphasize the importance of that specificity in achieving relevant concentrations of surfactant for inhibition of EC response to bubble contact. Similarly to the PFC in Oxycyte, PC monomers also partition to the gas/liquid interface and should reduce

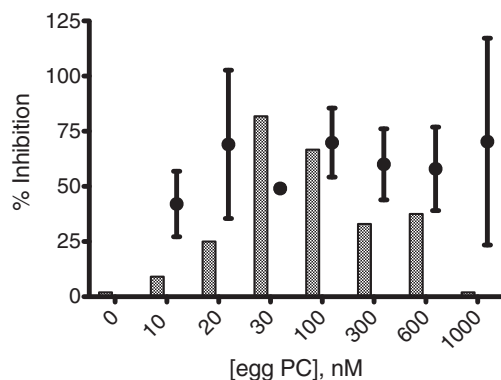


Figure 5 Chart indicating percent inhibition of Ca^{2+} transient occurrence (bar graph) and signal reduction (black circles) by egg phosphatidylcholine (PC)

triggering of cell response. However, unlike the PFC in Oxycyte, lipid monomers prefer to self-associate when above their CMC and show little adherence to the gas/liquid interface.

Further key advantages of a biologically inert surfactant agent in the pharmacological interruption and prevention of vascular bubble injury are also revealed in our studies. As Oxycyte specifically targets the bubble surface, much lower doses of this PFC emulsion may be used than what has been estimated from earlier *in vivo* and *ex vivo* studies using different agents.^{7,17} Even at the highest Oxycyte concentration we see no lethality or interference in normal EC function. Finally, ATP-initiated Ca^{2+} response is not affected, and we expect little to no perturbation by the PFC surfactant agent to the dynamic regulatory and mechanotransduction functions of the extended ESL. Thus, by all measures, benign nature of the Oxycyte emulsion is reassuring as it indicates that transient local high concentrations of Oxycyte that may be reached in therapeutic application will not be harmful.

PFCs have been evaluated for decades to treat VAE.¹⁸ The primary mechanism of action of PFCs proposed by most researchers in this field is related to the ultra-high solubility of gases in PFC emulsions (100,000 times greater than in plasma).^{19,20} The efficiency of PFC as vascular gas transporters has shown effectiveness in reducing bubble volume and increasing clearance in animal models.^{17,19,21–24} This current work demonstrates an alternate, complementary mechanism for the PFC to reduce endothelial injury from microbubble presence in the vasculature by interruption, through surfactant action, of the mechanotransductional trigger that initiates EC response to gas emboli contact. Oxycyte eliminates development of triggering adhesion forces between the bubble and the cell glycocalyx by coating the gas/liquid interface with bioinert PFC. With preferential surface occupancy, the PFC emulsion competitively inhibits the binding of syndecan heparan sulfate side chains, which would normally be pulled onto the physio-chemically active bubble surface.

In conclusion, Oxycyte inhibits syndecan potentiation and resultant EC response to bubble contact – lowering of mitochondrial membrane potential and triggering of spurious intracellular Ca^{2+} concentration spikes. This happens through Oxycyte's elimination of tensile forces imposed on HS glycocalyx components by the gas/liquid interface. Using the frequency and amplitude of bubble induced Ca^{2+} transients as a measure of Oxycyte inhibitory activity, we have in this work validated a surfactant mechanism of action for the PFC component of the Oxycyte formulation. This action is well modeled by the saturable mass action reduction of both the number of and probability of HS adherence sites on the bubble by the PFC component of Oxycyte with an IC_{50} near 230 μM . While gas bubble entry into the vasculature is often not preventable in clinical care or decompressive events, it is evident from these findings that the pre-existing presence of PFC in the circulation should serve to avert ignition of the mechanotransduction signaling cascade which would otherwise result from bubble interaction with the EC ESL.

Important future work should include increasing the biological complexity of the model system used for study. This could be achieved via the use of excised, perfused microvessels with transient passage of injected bubbles or possibly *in vivo* using a microcirculatory bed for intravital microscopy. We also suggest modification of the culture apparatus used on the microscope to enable control of the external gaseous environment of the cells so that links between Oxycyte's effectiveness for gas transport as well as its interfacial properties can be investigated. Also, adaptation of the bubble producing apparatus to allow flow and multi-cell studies would enable more detailed cell-biology investigations on intracellular signaling under general embolic stress.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; ALK, BP, and DME designed the experiments, ALK and BP conducted the experiments and performed analysis. ALK, JK, and DME interpreted studies and wrote the manuscript.

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