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Surfactant Copolymer Annealing of Chemically Permeabilized Cell Membranes

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Abstract

Structural breakdown of the cell membrane is a primary mediator in trauma induced tissue necrosis. When membrane disruption exceeds intrinsic membrane sealing processes, biocompatible multi-block amphiphilic copolymer surfactants such as Poloxamer 188 (P188) have been found to be effective in catalyze or augment sealing. Although in living cells copolymer induced sealing of membrane defects has been detected by changes in membrane transport properties, it has not been directly imaged. In this project we used Atomic force microscopy (AFM) to directly image saponin permeabilized and poloxamer sealed plasma membranes of monolayer cultured MDCK and 3T3 fibroblasts. AFM image analysis resulted in the density and diameter ranges for membrane indentations per $5 \times 5 \mu\text{m}$ area. For control, saponin lysed, and P188 treatment of saponin lysed membranes, the supra-threshold indentation density was 3.6 ± 2.8 , 13.8 ± 6.7 , and $4.9 \pm 3.3/\text{cell}$, respectively. These results indicated that P188 catalyzed reduction in size of AFM indentations which correlated with increase cell survival. This evidence confirm that biocompatible surfactant P188 augment natural cell membrane sealing capability when intrinsic processes are incapable alone.

II. INTRODUCTION

The development of biomimetic materials to aid tissue repair and regeneration is a developing and promising aspect of biomedical research. Because traumatic injury remains the primary cause of disability and death of humans under 40 years old, development of treatment options is extremely important [1,2]. Physical trauma disrupts tissue structure,

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which then leads to recovery responses at the cellular, tissue and organismal levels. The ultimate outcome is governed by the balance between injury and repair.

Healing subsequent to cell injury involves molecular regeneration or repair. Because the cell membrane lipid bilayer is a supramolecular assembly of amphiphilic phospholipids stabilized by entropic forces, the energy required to disrupt it is less than that required to disrupt hydrogen or covalent bonds stabilizing proteins and other macromolecules. [3] Consequently, plasma membrane disruption is a common immediate consequence of tissue trauma [4,5]. The intrinsic highly evolved and regulated membrane repair (i.e. membrane sealing) capacity of cells is a particularly important survival capability that is critically important for cell survival on this planet.

Body trauma can cause tissue necrosis mediated by disrupting cell membranes beyond the capability of cell membrane repair (ie sealing) processes. In this condition, trauma therapies are necessary to augment membrane sealing and enhance cell survival. Amphiphilic triblock copolymer surfactants such as Poloxamer 188 (P188) are known to catalyze the sealing of disrupted cell membranes [6]. P188 consists of a central polyoxypropylene block flanked by two polyoxyethylene blocks. At sub-CMC concentrations, it has been shown to seal damaged membranes of many cell types, including skeletal myocytes disrupted by electroporation, ionizing irradiation or hyperthermia [7–9], mechanically damaged neurons [10], and mutant cells with defective membrane sealing proteins [11,12]. Poloxamer 188 can be used to seal cell membranes both *in-vivo* and cell culture [11,13].

The mechanism of copolymer surfactant sealing has been studied extensively in pure bilayer lipid membrane models [6,14]. These studies suggest that the hydrophobic domain of P188 targets delivery of the copolymer to sites of membrane disruption then absorbs in the membrane defect. This concentrates the copolymer leading to corralling of the edge lipids. The hydrophilic component alters the local water structure, altering local thermodynamics such that sealing of the membrane defect is catalyzed. Finally, as membrane sealing occurs the copolymer is squeezed out [14,15], as illustrated in Figure 1.

In addition to physical trauma, cell membrane disruption is commonly mediated by altering the chemical structure of membrane fatty acids or detergent extraction of membrane lipids. Undulations and membrane attached vesicles function as lipid bilayer reservoir to maintain membrane integrity and control cell plasma membrane tension [16]. In this capacity, the membrane reservoir buffers acute changes of cell membrane tension induced by environmental factors, and plays a role in the cell membrane morphology and function. The buffering effect of the membrane reservoir can be measured by straining the membrane using a laser optical tweezers (LOT) to pull a membrane attached dielectric bead under quasistatic strain-rate conditions while recording the force required. The maximum length that the membrane can be strained before the bead breaks away is the tether length which depends on the mechanical properties of the membrane and the surrounding water structure.

The purpose of this study is to test the hypothesis that P188 acts to catalyze sealing of mammalian cell membrane defects created by extraction of membrane lipids into micelles. The defects can be created by using various detergents including saponin. Saponins

preferentially on the plasma membrane because of their high binding affinity to cholesterol [17]. In low concentrations, saponin might integrate transiently into the cell membrane to create small spontaneously sealing defects that transiently increase the permeability of the cell membrane [18]. In higher concentrations, larger stable structural defects are produced, resulting in cell death [13,19,20]. Saponin's specific ability to extract lipids in cell membranes has contributed to its wide use in cell research [18,21].

To directly observe the defects and sealing, we choose to use tapping mode Atomic force microscopy (AFM) that allows recording of the cell surface topography at a spatial resolution down to nanometer range [22,23], in physiologically relevant conditions [24]. Under cell culture conditions, mammalian cells are typically soft which makes precise structural interpretation of the images challenging. For this investigation we used Madin-Darby Canine Kidney (MDCK) epithelial cell line and an NIH 3T3 fibroblast cell line. We use AFM and LOT to demonstrate P188's ability to reverse saponin-induced membrane defects. The membrane damage and repair was correlated with cell survival assays.

III. MATERIALS AND METHODS

Materials

Research-quality Poloxamer 188 was purchased from Maroon Biotech Corp (Chicago, Illinois). Polyethylene Glycol (molecular weight 8,000 g/mol) and octane were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture

Madin-Darby canine kidney cells (MDCK) were obtained from the Karl Matlin lab, Department of Surgery at the University of Chicago. NIH 3T3 fibroblast cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA). Both cell lines were monolayer cultured in Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine, 10% fetal bovine serum and 1% penicillin streptomycin (GIBCO, Carlsbad, CA on tissue culture plastic). The cells were maintained in standard cell culture incubator at 37°C with 5% CO₂ in an air filter clean room.

Plasma Membrane Permeabilization

The cells were harvested with trypsin/EDTA, and plated on collagen Type I coated cover slides purchased from BD Biosciences (Franklin Lakes, NJ). Collagen coated slides were used in order to minimize the number of cells lost to detachment. When cells were in sub-confluent condition, they were treated with 0.01% saponin for 15 minutes, washed with PBS, and incubated with 1mg/ml P188 for 30 minutes. The growth medium was washed away by PBS 3 times. 0.01% saponin was added to the Petri dish to cover the slides. After 10 minutes, the saponin was washed away by PBS 2–3 times. The cells were recovered by incubating for 30 minutes in a solution of 10 mg/mL of either P188 (experimental group) or PEG (control) in DMEM.

Optical Viability Imaging Assay

After recovery, the cells were labeled with LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells (Invitrogen, Carlsbad, CA). Calcium Am (final concentration 1 μ M) was used to label the live cells, and ethidium homodimer-1 (final concentration 1 μ M) was used to label the cell membrane permeated cells. A cover slide was mounted on the glass slide for observation. An Olympus IX81 DSU confocal microscope was used to image the cells. Slidebook software V4.2 (Intelligent Imaging Innovations Inc. Denver, CO) was used to collect and analyze images.

Cell Surface Topology by Atom force microscopy

Following the various cell treatment protocols used, cells on coverslips were fixed with 2% glutaraldehyde for 30 min, then 4 % Osmium tetroxide solution for 30 minutes. The membrane surface topography was recorded using tapping-mode atomic force microscope (AFM) (Novascan Technologies, Ames, IA) mounted on an inverted microscope (Nikon, Tokyo, Japan).

Scanning was carried out in water using a triangular contact-mode AFM probe with soft silicone nitride cantilevers (Veeco Co., Santa Barbara, CA) with a spring constant of 0.06 N/m. This resulted in a force of 0.1–0.5 nN applied to the surface. The membrane surface was scanned at 1.5 Hz line speed and high Proportional/Integral/Derivative (PID) feedback gain. The scan area was 5 μ m $\times$ 5 μ m and was positioned on the non-attached cell surface away from both the lateral edges and the nucleus.

Membrane Tension Assay by Laser optical tweezers

Fluorescent 0.5 μ m diameter polystyrene beads (FluoSpheres, 515 nm emission, Molecular Probes, Eugene, OR) were used as the observation target. CD 29 antibody was conjugated to the beads using the protocol provided by the manufacturer. MDCK for study were grown on coverslips in a standard cell culture dish. After 1–2 days incubation, cells were sub-confluent. The coverslips were removed for study. Some were treated with 0.01% saponin for 10 minutes, washed three times in a 3% BSA PBS solution for 15–20 minutes. Then 100 μ l of antibody-conjugated fluorescent polystyrene beads were added to the solution for 15 minutes at room temperature. The coverslip was washed by PBS twice and mounted on a microscope slide. The laser optical tweezers system consisted of a Nd/YAG laser beam (1064 nm, 5W maximum output power) focused with an oil-immersion microscope objective (100x/NA 1.4) and translated in the specimen plane at a constant speed of 1 μ m/s. Fluorescent images of trapped beads were recorded with a CCD camera at a 10 Hz frame rate. The beads were held to the cell surface for 4 s before pulling at constant speed, as in past work by Raucher [16] and Dai [25]. The tether growth was observed as the membrane tether was pulled, until the bead escaped from the trap and quickly returned to the original position.

Cell Viability by Flow Cytometry

The MDCK cells were incubated to confluent condition in 10% serum medium in a cell culture flask. Trypsin/EDTA was used to dissociate the MDCK cells from the culture flask. Growth medium with serum was added to the flask and the cell suspension was transferred

to a sterile centrifuge tube and centrifuged. After centrifugation, as much of the supernatant was removed as possible. The cells were re-suspended in medium without serum to a cell density of $5 \times 10^5/\text{ml}$. The suspended cells were treated with 0.005% saponin for 5 minutes. The tube was centrifuged again and the supernatant was removed. The cells were re-suspended into 2–20mg/ml solution of P188 or polyethylene glycol (PEG) in DMEM for 30 minutes. Calcein AM solution and ethidium homodimer-1 stock [LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells (Invitrogen, Carlsbad, CA)] were added to each tube for 20 minutes to stain the cells. A BD LSRII Flow Cytometer was used in this experiment. FACS-DiVa Software (Version 6.1) was used for data collection, and Flowjo software (Version 8.8.4) was used for data analysis.

Only cells labelled only by calcein (green fluorescence) were considered viable and to have intact plasmalemma membranes. Cells labelled with Ethidium Homodimer, regardless of whether they also contained calcein, were considered non-viable. This meant that cells transiently permeabilized by experimental manipulation and still viable would be considered non-viable.

Data Analysis

Statistical comparisons were made between the uninjured control and saponin-permeabilized cells, the injured and post-permeabilized polymer treated cells, and the uninjured control and polymer treated cells. In all three pairs, the Welch's two-sample t-test was used to evaluate differences in the mean density, mean diameter, and mean depth of topological features. Between the uninjured and injured cells, the null hypothesis was that the saponin injury did not increase the number density, mean diameter, or mean depth of the defects. Between the injured and treated cells, the null hypothesis was that the P188 treatment did not reduce the number density, mean diameter, or mean depth of the defects. Between the uninjured and treated cells, the null hypothesis was that the two populations had the same number density, mean diameter, and mean depth of defects.

IV. RESULTS

The population doubling time of our MDCK and Swiss 3T3 fibroblast cells was approximately 20 hours. Under cell culture conditions, they remained viable and proliferative over the six population doubling levels used in this investigation. In order to avoid to losing the cell because of detachment, the cells were cultured in the collagen coated glass cover slides.

Optical Viability Imaging

Nearly all cells survived the transplantation on the 22×22 poly-D-lysine-coated coverslips (Figure 2A). MDCK cells were permeabilized by saponin as indicated by the increase in number of EH labelled cell (Figure 3B). Cells treated with P188 alone were similar to control cells in viability (Figure 2C). P188 sealed cells after saponin treatment as indicated by a qualitative increase in viable cells. In contrast, PEG treatment of saponin permeabilized cells did not restore cell viability (Figure 2D).

The NIH 3T3 fibroblast cell line manifested similar results to MDCK cells. Comparison of fluorescent micrographs in Figures 3A and 3B reveal that most of the 3T3 fibroblasts did not survive the saponin injury protocol. In addition, the 3T3 fibroblasts were much less adherent to collagen coated cover slides than healthy controls. P188 treated cells showed both good attachment and high viability (Figure 3C). PEG treatment restored neither viability nor attachment (Figure 3D). This result indicates that P188 repairs the cell membrane defects and helps cell survival.

Analysis AFM Cell Surface Topology

AFM imaging of cell surfaces in physiological aqueous media are soft enough to substantially reduce resolution of the AFM imaging. Figure 4 contains four representative images of more than 26 cells from each arm of the study. Figure 4A illustrates a typical AFM topographical image of normal MDCK cell in culture and reflects the baseline degree of roughness we observed. In these images, brighter area indicates positive displacement of the AFM cantilever. Typical cantilever displacements were no more than 200 nm, comparable to that seen in past AFM imaging of wet cells. [24]. Cell surface topographic variations observed for control viable cells were considered to be free of structural defects and thus were used to define the normal cantilever displacement amplitude and width ranges for cells anchored to collagen coated coverslips. AFM tip indentations with magnitudes greater than two standard deviations above the control range were classified as “supra-threshold” and considered to be caused by structural defects in the cell membrane.

Figure 4B reveals the characteristic AFM image obtained for normal MDCK cells in which the media was supplemented with 1 mM P188. Treatment with P188 alone did not alter substantially the topographical features in the membrane surface (Figure 4B). After injury with 0.01% saponin for 15 minutes, AFM images reflected substantial changes. The number density of supra-threshold displacements became significantly larger, indicating that the cell membrane became more fragile. We expect the larger displacements and widths reflected actual membrane defects. (Figure 4C). Cells permeabilized by saponin, then treated with P188 exhibited less membrane roughness which likely represents consequences of membrane sealing. (Figure 4D).

Graphical plots obtained by analyzing the AFM data over 26 MDCK cells in each treatment group, roughness increased from 3.6 ± 2.8 to 13.8 ± 6.7 ($p < 0.001$) per cell in a $5\mu\text{m} \times 5\mu\text{m}$ area (Figure 5). For the saponin permeabilized cells permeabilized by saponin and then treated with P188, the membrane surface roughness was more normal than saponin treatment group alone. The roughness decreased from 13.8 ± 6.7 to 4.9 ± 3.3 ($p < 0.001$), comparable to uninjured cells (Figure 5A). The average cantilever displacement wide diameter did not significantly increase (442 ± 279 nm to 486 ± 229 nm; $p = 0.08$) after saponin injury. P188 treatment decreased the defect diameter to 389 ± 233 nm ($p < 0.001$), comparable to uninjured cells. The distribution of the number of AFM amplitudes among the treatments are shown in Figure 5B and the spectrum of displacement widths are shown in Figure 5C.

As Figures 4 and 5 reveal visually, P188 and PEG treatment of non-permeabilized control MDCK cells alone did alter the cell membrane topography to small but reproducible degree. Additional studies are being done to better understand this effect.

Laser optical tweezers and cell tether length

Changes in mechanical quasistatic stiffness was recorded to correlated to the AFM images. In each group, more than 30 tethers from 30 different cells were pulled by laser optical tweezers. In the control group, the average tether length was $18.4 \pm 2.0 \mu\text{m}$. P188 did not change the tether length (Figure 6). Saponin injury significantly decreased the average cell tether length to $7.5 \pm 2.0 \mu\text{m}$ ($p < 0.5$), indicated that the modulus was increasing. As a result, the tether lengths were shorter in permeabilized membranes. Culturing the cells with PEG did not change this decrease ($6.9 \pm 1.6 \mu\text{m}$). P188 partially restored the tether length to $13.7 \pm 3.7 \mu\text{m}$. This result suggests that P188 aids sealing for membrane defects and reduces membrane water content.

Quantitative Cell Survival

The viability of saponin-treated MDCK cells was about 70% in P188 culture, but only about 55% in PEG culture ($p = 2 \times 10^{-5}$, Figure 7). To determine if P188 membrane sealing was cell type dependent, NIH 3T3 fibroblast cells were permeabilized then P188 sealed under the same conditions as MDCK cells. With P188 culture, the fibroblast viability was about 45%, but with PEG culture, the viability was only about 25%. The viability of saponin permeabilized-P188 treated MDCK cells viability was not P188 dose dependent in the P188 concentration range of 0.2mM to 2mM in DMEM culture media (Figure 7A). Interestingly, the addition of octane did interfere with the therapeutic efficacy of P188, lowering cell viability from 70% to 46% ($p = 5.2 \times 10^{-8}$, Figure 7B). Poloxamer 188 had no beneficial membrane sealing effect on octane pretreated cells.

V. DISCUSSION

Although, Atomic force microscopy has been used to demonstrate surfactant sealing of supported pure lipid bilayer membranes, this is the first reported investigation of cell membrane typography of disrupted cell membranes sealed with surfactant copolymers. [23]. These studies demonstrate recovery toward normal cell membrane structure of P188 treated saponin-permeabilized cell membranes. Saponin was equally effective in permeabilizing 3T3 fibroblast and MDCK cells.

The addition of saponin to the cells created a significant structural alteration in plasma membrane that corresponded to a significant reduction in cell viability. The rather specific targeting of saponins and its permeabilizing action on cell membranes is well documented, thought to be mediated by the interaction between saponins and the cholesterol molecules in the cell membrane [17,21]. Scanning electron microscopy of saponin-treated cells has revealed membrane defects that range in size from 100 nm to 1 μm , consistent with the size of the membrane defects found in our study [26]. Many other methods of inducing injury to the cellular membrane have been used, including electroporation [27,28], ionizing

irradiation [8], and hyperthermia [29,30], but saponin-induced membrane permeabilization is unique in that it extracts the lipids from the membrane bilayer.

In this study, the addition of P188 to injured cells catalyzes sealing of saponin defects a rescue effect, decreasing the number and the diameter of membrane defects, and restoring viability of injured cells. We were able to directly visualize by AFM the structural restoration P188 provided to disrupted membranes. The ability of P188 to restore the integrity of cellular membranes has been demonstrated in a variety of settings, including thermal injury [29,30], ionizing radiation [8,31], traumatic brain injury [32], neuronal injury [33], and mechanical damage [34]. The membrane-sealing effects of P188 are largely attributed to its amphiphilic nature. P188 has been observed to selectively insert into injured portions of the membrane where local lipid packing density is reduced and then is “squeezed out” once normal surface pressure is restored [15]. This suggests that the copolymer surfactant co-localizes to exposed hydrophobic regions in a disrupted plasma membrane (Figure 1), and the hydrophobic core of the P188 allows for the selective insertion of the molecule into the disrupted membrane.

This proposed mode of surfactant sealing is supported by our studies indicating that n-octane prevented membrane-sealing effects of P188. Pretreatment of saponin-lysed cells with 20 mM n-octane reduced the cytoprotective effects of P188 on saponin permeabilized 3T3 fibroblasts. The most plausible explanation is that n-Octane absorbs into the exposed hydrophobic regions at the edge of bilayer lipid membrane defects, thus blocking the target sites that P188 can potentially repair. Cell death thus ensues since octane does not exert the membrane annealing effects that P188 does. Ongoing studies focused on understanding the effects of octane on cell membrane defects would shed light on the extent of interaction between the hydrophobic moieties: whether the interaction involves only the disrupted parts of the membrane or the entire bilayer, altering certain membrane properties.

This ability to target defects in the membrane distinguishes P188 from PEG. PEG has been shown to have protective effects against myocardial reperfusion injury [35] and to hasten recovery from spinal cord injury in dogs [33] through a mechanisms that may mediated by effects on mitochondria. However, the concentrations of PEG used in this study are substantially lower to be equivalent in molar concentration with P188. On the other hand, P188 is capable of catalyzing the sealing of disrupted cell membranes at sub-CMC millimolar concentrations. Such low concentrations of P188 have been found to be well tolerated in humans.[36]. The safety of P188 for short-term use in humans has been demonstrated for the treatment of vaso-occlusive crises in sickle cell patients [36]. The time it takes for damaged membranes to be sealed is short with the beneficial effect of structural recovery taking place within half an hour of adding P188. This suggests the delivery of P188 in boluses by intravenous administration may be effective with few adverse effects.

Conclusion

This study used AFM to directly image the effects of a widely used blood compatible amphiphilic copolymer P188 on the surface topography of normal and detergent permeabilized cell membranes. The images documented recovery of more normal membrane architecture that correlated with increased cell survival.

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Lay Summary and Future Studies

The fundamental purpose of this study was to compare the topography of living mammalian cell membranes before and after detergent disruption using direct atomic force microscope (AFM) measurements membrane surface topography. Because increased roughness was observed, as reflected in greater depth and diameter of AFM tip indentations, the effect of Poloxamer 188 (P188, ~9kDa) on the membrane were investigated. P188 is a copolymer surfactant that is known to seal disrupted pure lipid bilayer membranes. We observed that P188 reduced the roughness disrupted cell membranes consistent with defect sealing. The pure hydrophilic (10 kDa) neutral polyethylene glycol did not induce sealing.

Future investigation will focus on optimizing the molecular design of block copolymer surfactants to maximize their membrane sealing efficacy. The optimal structure or membrane sealing is likely to be cell phenotype specific.

Figure 1.
A conceptual illustration of copolymer surfactant absorption onto sites of lipid bilayer structural defects mediated by hydrophobic effects.

Figure 2.

Fluorescent photomicrographs (100x) of monolayer MDCK cells incubated in calcein AM and Ethidium Homodimer vital dyes. (A) The normal MDCK cells (no saponin exposure) just calcein labelled indicating viability above 99%. (B) 30 minutes after 0.01% saponin exposure, many cells manifested Ethidium homo-dimer labelling, indicating loss of plasma membrane integrity and some have detached from the coverslip. (C) Photograph of MDCK cells after both saponin disruption and 1 mM P188 treatment that reveals increased cell viability over saponin treatment alone. (D) Photograph of MDCK cells after both saponin disruption and PEG(10kDa) treatment that reveals similar percentage of calcein labelled cells as pure saponin treatment alone.

Figure 3.

The fluorescent photomicrographs (100x) of NIH 3T3 fibroblasts 20 minutes after labelled with Calcein-AM/EtHD after exposure to various experimental conditions. (A) Normal fibroblasts without saponin exposure manifested > 90% viability. (B) Fibroblasts after 0.01% saponin disruption of the plasma membrane lipid bilayer, viability was substantially reduced. (C) After culturing with P188, the fibroblast cell viability was almost entirely restored. (D) Polyethylene glycol (PEG) treatment also enhanced cell survival but was not as effective as P188.

Figure 4.

AFM image of an MDCK cell membrane in a $5\ \mu\text{m} \times 5\ \mu\text{m}$ area. Darker colors indicate deeper surface features. (A) A normal cell. There are a few pores in the surface of the cell membrane. (B) A normal cell with P188. P188 alone did not change the cell membrane topography. (C) A cell with 0.01% saponin after 15 minutes. The number of the peaks and indentations on the cell membrane surface increased significantly. (D) A cell injured with saponin and treated with P188. The cell membrane became less rough again.

Figure 5(a):

Figure 5(b):

Figure 5(c):

Figure 5.

(A) Distribution of the number of defects from 26 MDCK cells. The defect number significantly increased from 3.6 ± 2.8 to 13.8 ± 6.7 after saponin injury, but decreased to 4.9 ± 3.3 after treatment with P188. Each point represents one cell. (B) Distribution of the number of supra-threshold indentations from 26 MDCK cells. (C) Distribution of the diameters of supra-threshold indentations (26 MDCK cells).

Figure 6.

P188 and PEG have no effect on a healthy MDCK cell membrane reservoir. The average cell tether length is around 18 μm . After saponin injury, the tether length significantly decreased from $18.4 \pm 2.0 \mu\text{m}$ to $7.5 \pm 2.0 \mu\text{m}$. Culturing with dextran did not affect the cell tether length ($6.9 \pm 1.6 \mu\text{m}$, $p > 0.05$), but culturing with P188, significantly increased the cell tether length to $13.7 \pm 3.7 \mu\text{m}$. *: p-value is smaller than 0.05.

Figure 7.

Quantitative comparison of the viability results by flow cytometry. (A) After saponin injury, 0.2 mM and 2.0 mM P188 were equally effective in restoring viability ($p>0.05$). Viability with P188 treatment was about 70%, but only about 55% with PEG treatment ($p<0.05$). (B) 20 mM n-Octane pretreatment blocks the effect of the P188 and PEG on cell viability.