Interactions of Fluorescein Isothiocyanate-labeled Poloxamer P188 with Cultured Cells

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Abstract — **Poloxamer 188 (P188) is a tri-block copolymer with well-established membrane sealing properties. Langmuir trough experiments using lipid monolayers have suggested that P188 interacts only with damaged membranes and that it is capable of being "squeezed out" upon the restoration of membrane integrity. Here, we investigate the interactions of P188 with cultured cells by producing a fluorescent version of P188 in which the molecule is tagged with fluorescein isothiocyanate (FITC). Uninjured cells were incubated in the presence of the fluorescent poloxamer, which was then found inside the cells. This indicates that cells are capable of internalizing the molecule such that the mechanisms by which the poloxamer is retained or released by the cell may be more complex than previously thought.**

I. Background

Poloxamer 188 (P188) is a member of the poloxamer family which consists of a variety of tri-block copolymers made of polyethylene glycol-polypropylene glycol-polyethylene glycol polymers of various ratios and sizes (Figure 1) [1] .

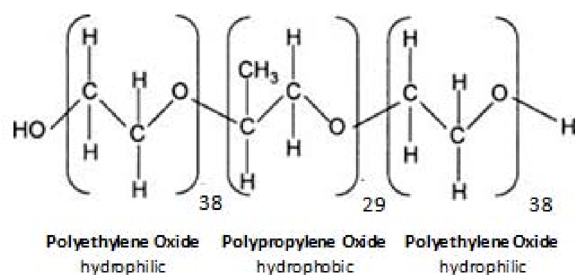

Figure 1. Chemical structure of Poloxamer 188

P188 has a variety of industrial and clinical uses. It has been used as a food additive, a stool softener, a topical wound cleanser, and as a treatment to decrease discomfort in patients with sickle cell[2]. More recently, the interactions of P188 with cell membranes has become of great interest. It has membrane sealing properties when used in concentrations less than its critical micelle concentration. These membrane sealing properties have great potential as therapeutic tools in radiation injury[3], thermal burns[4], frostbite, electrical shock[5], and traumatic brain injury[2, 6, 7].

The exact mechanism by which P188 seals permeabilized membranes has yet to be elucidated. An understanding of this mechanism is crucial to the use of P188 as a therapeutic agent. Many researchers have attempted to investigate poloxamer-membrane interactions by using lipid membranes; either monolayer, bilayer[8], or vesicles[9]. Results from Langmuir trough monolayer experiments have implied that P188 only inserts into damaged portions of membranes, and that it is "squeezed out" if the membrane is able to regain its integrity[10]. This supposition was of great significance as it allayed fears that therapeutic use of P188 might interfere with normal, healthy membranes. However, it is important to remember that monolayers are not perfectly representative of cell membranes which consist of lipid bilayers along with a variety of proteins.

In order to be able to confidently believe that P188 does not interfere with normal cell membranes, more representative experiments were needed. To achieve this, we fluorescently tagged P188 to allow for direct visualization of the fluorescent molecule with live cells.

II. Materials and Methods

P188 (Sigma) was fluorescently tagged by adding an amine group at the end of each of the PEO legs that was then able to react with the isothiocyanate group of fluorescein isothiocyanate (FITC). The amine modification was performed by dissolving P188 in dimethylformamide (DMF) and then activating the solution with 1,1'-carbonyldiimidazole(CDI) while stirring at room temperature for three hours. An excess of ethylenediamine(EDA) was added to the solution and modification was allowed to proceed for 48 hours while stirring at room temperature[11]. This solution was then dialyzed extensively to result in a purified P188 solution with functional NH_2 groups which was then lyophilized before being fluorescently labeled. The labeling procedure involved dissolving the P188-NH_2 powder in 0.1M sodium bicarbonate solution and then stirring overnight with FITC dissolved in DMSO. Gel permeation chromatography using Sephadex G-25 beads was used to ensure that FITC was attached to the poloxamer, as well as serving to separate the labeled P188 from the free dye. The final solution was then lyophilized and stored at -20°C before use.

Bovine aortic endothelial cells were used as a model cell due to their ease of use and availability. Cells were grown to confluence on glass coverslips and plated with DMEM media with 10%FBS and antibiotics.

The cells were treated with either 100 μM FP188, 5 μM FITC, or 100 μM P188 + 5 μM FITC. A control treated with the cell culture media. Gel permeation chromatography revealed 5 μM to be the highest estimate of free FITC in the FP188 solution, so that concentration of FITC was used as a comparison to FP188. A mixture of unlabeled P188 and free FITC was used in order to see if P188 had a permeabilizing effect on the cell membrane that allowed free FITC to pass through, as has been shown for other members of the poloxamer family.

The cells were washed twice with PBS before the addition of the assigned treatment dissolved in DMEM. They were

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incubated for two hours at 37°C and 5% CO_2 before being fixed with 1% paraformaldehyde for twenty minutes. The coverslips were rinsed and glued onto coverslips along with ProLong® Gold antifade reagent with DAPI (Invitrogen). The cells were then imaged using confocal microscopy.

III. Results and Discussion

Confocal microscopy revealed the presence of FP188 inside the cells (Figure 2). While 5 μM FITC was also able to pass through the cell membrane, it is unlikely that the fluorescence seen in the FP188 treated cells is free FITC due to the difference in fluorescence pattern. The FITC treated cells show diffuse fluorescence through the entire cytoplasm while the FP188 treated cells show a punctate pattern of fluorescence indicative of an endocytotic vesicular entry process. Further evidence that the fluorescence seen in the FP188 cells is due to the entry of FP188 and not of free FITC is presented in the cells treated with both 5 μM FITC and 100 μM unlabeled P188, which presented less fluorescence than cells that were treated with FITC alone.

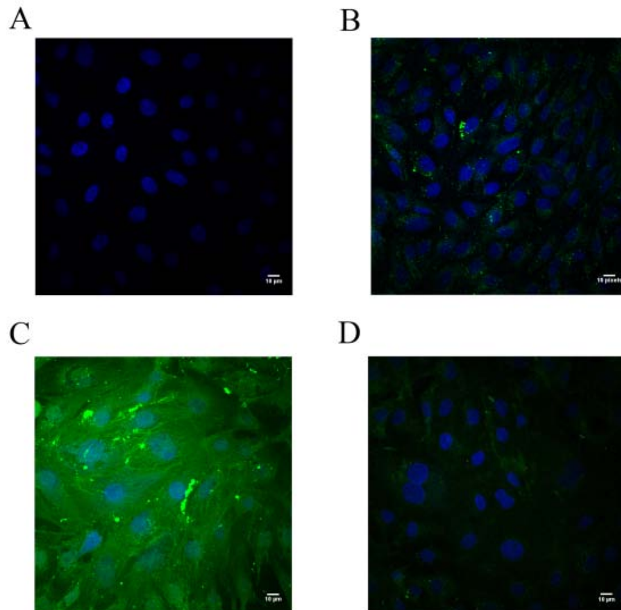

Figure 2. Z-stack projection of confocal images. Blue is the DAPI stain indicating the cell nucleus and green is FITC. A) Untreated cells B) Cells treated with 100 μM FP188 C) Cells treated with 5 μM FITC D) Cells treated with 5 μM FITC and 100 μM P188

These results show that cells are capable of internalizing FP188. This calls into question the manner in which P188 interacts with injured cell membranes and to what extent it will be cleared from the cell after being used as a treatment to restore cell membrane integrity.

These results are only applicable to use of P188 insofar as FP188 is representative of P188. The attachment of the FITC molecule to P188 changes the structure and may therefore affect its functionality in unknown ways. While the size of the FITC molecule (MW = 389.39) is very small compared to P188 (MW = 8400), it is much more hydrophobic than the polyethylene leg it is attached to.

There are various ways in which the interaction of P188 and cell membranes can be further examined. The use of different, perhaps more hydrophilic, fluorophores may provide insight to how the FITC fluorophore may change the interaction of P188 with cell membranes.

While the ability of P188 to seal damaged membranes has been well established, the mechanism of this action is still poorly understood. The use of a fluorescent P188 has great potential for furthering the understanding of this mechanism and helping to unleash the true therapeutic potential of P188.

IV. Acknowledgements

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