Surface Modification of Red Blood Cells Using Novel Plasma Membrane Anchors

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Statement of Purpose: Red Blood Cells (RBCs) comprise approximately 45% of circulating blood volume and have the ability to reach almost every organ. Moreover, RBCs are most robust circulating blood cells and have a flexible plasma membrane. These characteristics have made them suitable candidates for drug delivery systems. Resealed erythrocytes and surface modified erythrocytes are the two main approaches to utilize RBCs for drug delivery systems. Surface modification of RBCs can be achieved by lipid based plasma membrane anchors; however, the effect of lipid chain size on the localization efficiency of membrane anchors has not been well documented.

We hypothesize that lipid chain length of a phospholipid-like anchoring molecule will significantly affect interaction with RBC membrane. We utilized custom designed fluorescently labeled lipids containing 0, 6, and 16 carbon chain lengths and compared their distribution on RBCs (cytoplasm vs. membrane) using confocal imaging. We also studied biocompatibility of the most efficient membrane anchor using hemolytic index.

Methods: Each membrane anchor designed for the study had a varying length of lipid (alkyl) chain attached to a multifunctional core. Fluorescein isothiocynate (FITC) was attached to the core for fluorescence imaging studies. Figure 1 shows a schematic representation of the three different compounds synthesized for the study.

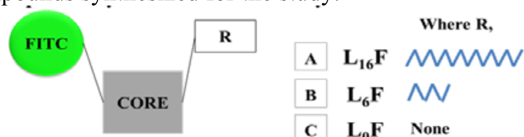

Figure 1 Schematic representation of the compounds synthesized. FITC is linked to Lipid chain anchor via a core. A) $L_{16}F$ - 16 C chain compound B) L_6F - 6 C chain compound C) L_0F - compound without chain (only core and FITC).

Freshly isolated human RBCs at 10% hematocrit (Ht) in Phosphate Buffered Saline (PBS) were used for localization studies. 20µg of each compound ($L_{16}F$, L_6F, L_0F) was added to 200µl RBCs in respective centrifuge tubes. Cells with only PBS and plain FITC served as two controls. Samples were allowed to incubate for one hour at 37 ºC. After incubation, cells were washed thrice with 3 ml PBS by centrifugation (5 min at 250*g and 4 ºC). Fluorescence microscopy (Zeiss LSM 510 META) was done to compare whether compounds were localized on the surface or internalized by RBCs.

Hemolytic index based on hemoglobin (Hb) release was used to see if the 16C chain anchor (mw 658.88 g/mol) was causing any membrane damage. 200 µl of RBCs was incubated with varying amounts (50 to 2000 µg) of 16C chain anchor. After incubation cell pellet was removed by centrifugation and supernatant was used for absorbance measurement (BioMate 3S) at 576 nm (λ_{max} for Hb). PBS and DI water were used as negative and positive controls respectively.

Results: Figure 2 shows a comparison of center 2D plane of RBCs incubated with different compounds. Each group shows a phase contrast image with the corresponding fluorescence image. As anticipated RBCs incubated with FITC showed fluorescence throughout the cell which confirmed internalization. Distribution profile of anchors found to be affected by the size of the lipid chain. Molecule with no lipid chain (L_0F) showed minimal membrane attachment and internalization. L_6F showed weak internalization and no clear membrane anchoring. $L_{16}F$ showed plasma membrane localization evident by the "fluorescence ring" appearance in the image. Figure 3 A) & B) show multiple $L_{16}F$ loaded RBCs in a single field.

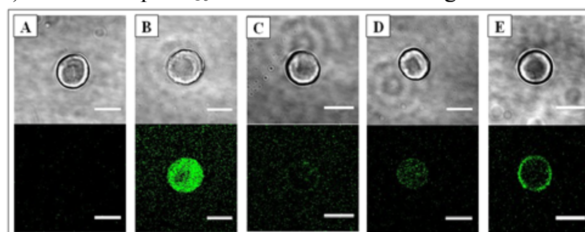

Figure 2 Comparison of distribution profiles of anchors with 0, 6, 16 C lipid chain and FITC. A) PBS control with no fluorescence, B) FITC with distribution throughout cells, C) L_0F found to be faintly localized on the membrane, D) L_6F was internalized weakly throughout the cells, and E) $L_{16}F$ was localized specifically on the membrane of the cells. [Scale bar 7 µm]

Figure 3 C) shows that hemolysis of RBCs is not linearly dependent on the concentration and hemolysis increases suddenly by raising compound amount beyond 500 µg.

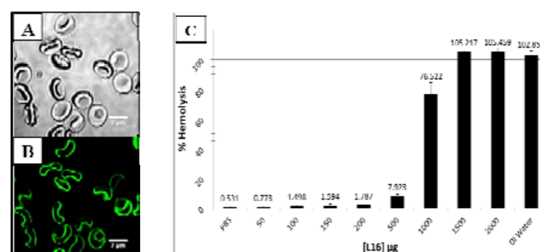

Figure 3 A) & B) Phase contrast and corresponding fluorescence image showing RBCs with $L_{16}F$ localized on their membrane. C) % hemolysis vs. amount of L_{16}. PBS and DI water were used as references, 0 and 100% hemolysis respectively.

Conclusions: The core used for the study was large and hydrophilic enough to prevent uptake by RBCs i.e. (L_0). Addition of lipid chain increased the interaction with cells; however, compared to 16C lipid the 6C lipid interacted weakly and got internalized. Presumably the balance between hydrophilicity of the core and lipophilicty of the lipid favored initial interaction with the membrane and then trans-membrane passage. This intriguing nonlinear effect of lipid chain size on membrane anchors demands further studies for the understanding of the exact mechanism of molecular interaction. In hemolysis study, sudden increase in hemolysis instead of gradual increase prompts us to speculate that the cell damaging effect observed beyond 500 µg can be due to hypertonicity and not a characteristic of membrane anchor. Thus, L_{16} can potentially be used as a biocompatible membrane anchor for RBCs by attaching drug of choice to the core.

Reference: Biagiotti S, Iubmb Life. 2011;63.

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