Non-covalent Surface Modification of Erythrocytes: Study of Binding Strength and Effect on Membrane Integrity

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Statement of Purpose: Erythrocytes or Red Blood Cells (RBCs) owing to their quick circulation and high volume have been explored for drug delivery and diagnostics. Surface modification is a common approach to functionalize RBCs with appropriate ligands. For this purpose covalent chemical cross-linkers have been extensively used however lipid based plasma membrane anchors are less explored. We hypothesized that non-covalent phospholipid based anchors can be used to functionalize RBC surface with binding strength comparable to covalent modification and with minimal effect on membrane integrity. Here, we have compared erythrocyte functionalization using different reagents and evaluated the strength of the functionalization using ligand-receptor biding assay. Moreover, the effect of functionalization on the membrane integrity was also analyzed using different viability/cytotoxicity parameters.

Methods: Biotin-avidin chemistry was used as the model ligand-receptor system. Sulfo NHS-LC-Biotin® and NHS-PEG_4-Biotin (Thermo Scientific Inc.) were used for covalent surface biotinylation while pegylated phosphoethanolamine, DSPE-PEG2000-Biotin (Avanti Polar Lipids, Inc.) was used for non-covalent surface modification. To compare the strength of surface biotinylation, freshly isolated RBCs were biotinylated using different reagents for 1 hour at RT. After 3x washes with PBS, fixed number of modified cells were mixed with a solution of fluorescent Avidin Alexafluor® 488. After incubation for 10 minutes, samples were analyzed by fluorescence Confocal Laser Scanning Microscopy (CLSM). Level of aggregation and morphology of individual cells were compared between reagents. Effect of non-covalent surface modification on the membrane integrity was evaluated at different concentrations using combination of hemolysis index (Abs at 539 nm) and Calcein AM/Annexin-V apoptotic assay (flow cytometry). PBS and Triton X-100 (known hemolytic agent) were used as negative and positive controls respectively.

Results: Figure 1 shows CSLM images with aggregation profiles of biotinylated RBCs in presence of Avidin.

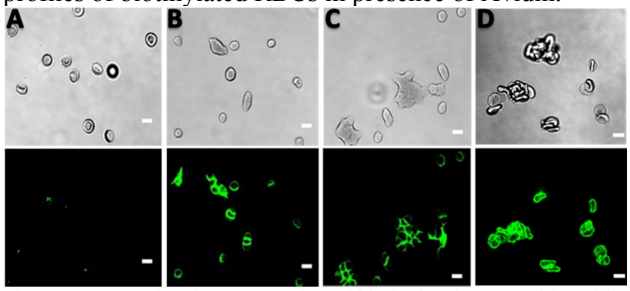

Figure 1 CLSM showing aggregation of biotinylated RBCs with Avidin Alexafluor® 488. A) Control, B-C) Covalent reagents Sulfo NHS-LC-B and NHS-PEG_4-B showed extensive aggregation with membrane fusion while D) DSPE-PEG2000-B showed aggregation of RBCs with clear cell shape and boundaries [Scale bar 7 µm].

Compared to the control, all reagents showed high level of aggregation. Morphologically, both covalent biotinylation reagents showed cell-cell fusion and apparent membrane damage with NHS-PEG_4-B showing higher aggregation, while DSPE-PEG2000-B showed aggregation with clear cell shape and boundaries. Presumably, short linker length of covalent reagents resulted in "agglutination" of RBCs with significant membrane fusion, while large PEG spacer in DSPE-PEG2000-B provided cell-cell aggregation without membrane fusion. For membrane integrity analysis, RBCs treated with different concentrations of lipid were analyzed for hemolysis and stained with Calcein AM/Annexin-V PE for viability analysis. Unlike Triton X-100, the Lipid was not found to damage RBC membrane (Figure 2B) or cause cell fragmentation (Figure 2A).

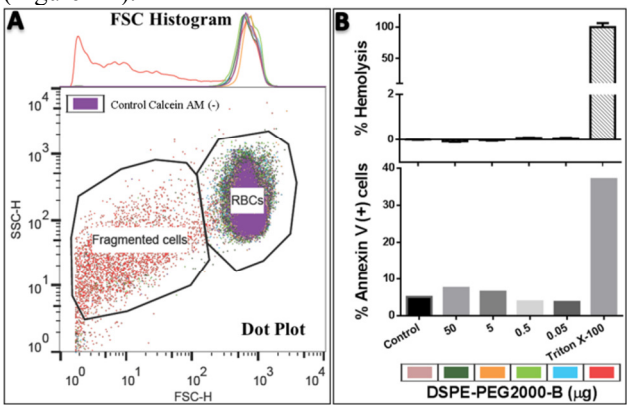

Figure 2 Effect of DSPE-PEG2000-B (0.05 to 50 µg) on RBC membrane compared to PBS and Triton X-100. A) Flow cytometry scatter profiles showed marked cell damage only in positive control. B) Lipid did not show significant hemolysis compared to PBS however; it showed minor concentration dependent increase in Annexin-V (+) cells.

Interestingly, hemolysis analysis showed minor drop in hemolysis at high lipid concentration compared to the PBS. However, Annexin-V analysis showed a concentration dependent increase in Phosphatidyl Serine (PS) exposure (apoptosis indicator). Decrease in hemolysis can be attributed to a "membrane stabilizing" effect of extra phospholipid molecules inserted in the membrane which reduced the damaging effect of centrifugation steps during the experiment (supported by high Calcein AM levels – data not shown). Increase in Annexin-V binding can be attributed to actual apoptotic potential (exposure of PS) or false positive staining (concentration dependent non-specific binding of Annexin-V to DSPE-PEG2000-B). A separate study of Annexin-V to lipid binding should be carried out to understand the exact molecular mechanism.

Conclusions: Pegylated DSPE can be used to functionalize RBCs with strength comparable to covalently linked surface ligands. It can potentially be used to modify RBCs without significantly altering or damaging their membrane integrity. This system should be further evaluated for its efficiency to modify other cell types and to attach macromolecular ligands such as antibodies on cells.