Engineering Erythrocytes to Improve Hemostatic Properties of Platelets

Pandya SG[1,2], Evani SJ[1,2], Nair PM[1,2], Ramasubramanian AK[1], Agrawal CM[1].
[1]University of Texas at San Antonio, [2]University of Texas Health Science Center at San Antonio.

Statement of Purpose: Trauma induced coagulopathy results in hemorrhage due to low coagulation and high fibrinolysis. RGD functionalized polymeric and liposomal particles have been shown to enhance platelet aggregation and improve hemostasis during coagulopathy. RBCs have also been explored to carry RGD moieties cross-linked on their surface. However, covalent modification with chemicals results in membrane damage and leads to potential clearance. We hypothesized that RBC surface can be easily modified using biomimetic phospholipid anchors with RGD units and these RBCs can take active part in platelet aggregation to aid in hemostasis.

Here, we have studied the effect of lipid and PEG chain size on the loading efficiency of the anchors on RBCs. Moreover, lipid-cRGD (cyclo-Arg-Gly-Asp) modified RBCs were studied for their platelet binding potential.

Methods: Dual chain pegylated phosphoethanolamine (PE) derivatives were used for anchoring efficiency analysis. As shown in Figure 1A, three different DRPE-PEG2000-Fluorescein i.e. 10, 16, 18 C and three different DSPE-PEGx-Fluorescein i.e. 2000, 3400, 5000 (Nanocs Inc®) were used for anchoring efficiency analysis. Figure 1B shows fabrication scheme for DSPE-PEG2000-cRGDfC using maleimide derivative (conjugation confirmed using UV and Mass Spectroscopy).

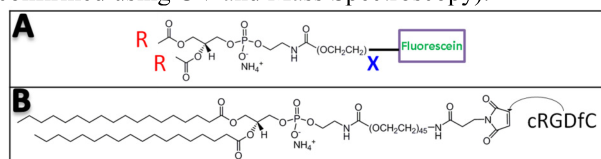

Figure 1 A) Schematic representation of different pegylated phosphoethanolamine anchors where R and X represents variation in lipid and PEG chain sizes. B) Fabrication scheme for lipid-cRGD using DSPE-PEG2000-Maleimide.

RBCs (40% Ht) were incubated with ~300 µM lipid (in PBS) for 10 min followed by 3x wash. Confocal microscopy was used to study distribution of lipid on RBCs and flow cytometry was used to quantify the anchoring efficiency of each lipid. Optimal lipid-PEG combination was chosen for further studies. Platelet to lipid-cRGD binding was confirmed by platelet aggregometry where high concentration of cRGD competitively reduced platelet-fibrinogen binding. To study binding between the cRGD modified RBCs and activated platelets, 10 µL PRP (Platelet Rich Plasma) was incubated with $\sim10^6$ RBCs with or without pre-activation with ADP. Dual color flowcytomtery was employed where RBCs were labeled with Calcein AM (FL-1 Green) and platelet activation was quantified using Phycoeryhrin Mouse Anti-Human CD62P (FL-2 Red). Presence of both FL-1 and FL-2 positive events in RBC gate was considered RBC-Platelet binding.

Results: Figure 2A-F and G-H shows effect of lipid and PEG chain size on anchoring efficiency of different PEs. 10C chain led to membrane damage on RBCs (B) which can be attributed to non-native nature of the lipid. Increasing lipid chain increased binding and increasing PEG size reduced binding. Presumably at high PEG chain, hydrophilic interactions between molecules and media prevented stable anchoring.

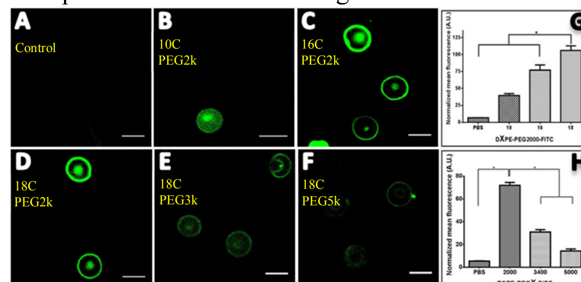

Figure 2 A-F) Representative confocal fluorescence micrographs showing distribution profiles of different lipid-PEG-Fluorescein anchors on RBCs [Scale bar 7 µm]. G-H) Flowcytometry showed statistically significant higher binding for DSPE-PEG2000 compared to all other pegylated lipids (*p<.001).

Based on these results, DSPE-PEG2000-Maleimide was used to fabricate DSPE-PEG2000-cRGD. As shown in figure 3A, Calcein AM levels on cRGD modified RBCs showed a concentration independent drop in fluorescence intensity which can be attributed to binding of hydrophobic Calcein AM and excess lipid in media during incubation minimizing Calcein AM levels (RBC viability was confirmed in a separate study). Figure 3B shows presence of platelet specific events on gated RBCs to identify competitive RBC platelet binding in presence of all plasma components. Control RBCs did not bind to platelets even after activation of ADP. A small population of cRGD modified RBCs bound to platelets without ADP activation which can be attributed to a small stored platelet population which gets activated with time without any agonist. However, addition of ADP resulted in a marked increase in RBC population positive for platelet dye indicating agonist dependent RBC platelet binding.

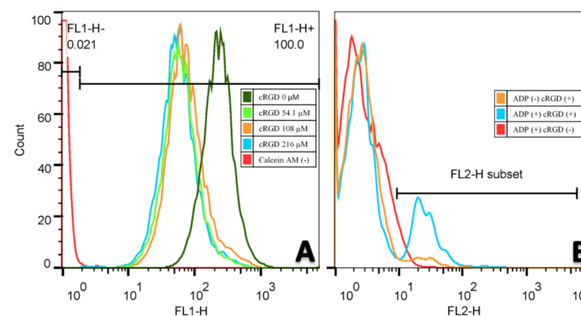

Figure 3 Flowcytometric evaluation of A) Calcein AM fluorescence levels between control and RBCs modified with varying concentration of lipid-cRGD. B) Levels of FL2 (platelets) positive RBCs when incubated with PRP in presence and absence of ADP activation.

Conclusions: RBCs can be non-covalently functionalized using semisynthetic membrane components. cRGD modified RBCs can potentially be transfused in trauma induced coagulopathy to enhance hemostatic property of platelets. However, further analysis is needed to evaluate the effect of such modification on RBC functions and time dependent stability of the surface ligands.

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