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Enhanced Cell Surface Polymer Grafting in Concentrated and Nonreactive Aqueous Polymer Solutions

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Abstract: Macromolecular cell surface modification techniques have shown tremendous utility in various biomedical applications. However, a major drawback concerns inefficient cell surface modification caused by the poor association of hydrophilic macromolecules with cell surfaces. Here, a novel, highly efficient, and universal strategy in which nonreactive "additive" macromolecules are used to modulate the grafting efficiency of cell surface reactive, hydrophilic macromolecules is described. Unprecedented enhanced cell surface modifications by up to 10-fold were observed when various concentrations of a suitable "additive" polymer was present with a constant and low concentration of a "reactive" macromolecule. The importance of this increased efficiency and the possible mechanisms involved are discussed. The cell compatible technique is demonstrated in the case of four different cell types—red blood cells (RBC), leukocytes, platelets, and Jurkat cells. A practical application of grafting macromolecules to cell surfaces in concentrated polymer solutions is demonstrated by the enhanced camouflage of RBC surface antigens for the development of RhD null RBC. In principle, the technique can be adapted to various macromolecular systems and cell types, with significant potential for biomedical applications such as live cell based technologies.

Introduction

The conjugation of macromolecules to cell surfaces[1,2] has been increasingly explored as an efficient method for improving in vivo compatibilities, minimizing immune responses, and reducing enzymatic degradation.[3] For example, the development of a universal donor red blood cell (RBC) has been investigated by covalently attaching poly(ethylene glycol) (PEG) chains to the membranes of RBC to "camouflage" surface antigens.[1] Similarly, the grafting of PEG chains to virus particles has decreased immunogenicity and specific interactions with cell surfaces.[2] Several other emerging technologies, such as the development of macromolecular tools for studying the cell−cell interactions that govern biological processes, have recently been investigated.[4] In addition, it has been shown that cell adhesion and patterning can be studied using cell surfaces conjugated with biological macromolecules such as DNA.[5]

Although such cell surface modification techniques have shown tremendous utility in various biomedical applications, the inefficiency with which the hydrophilic macromolecules associate with cell surfaces (due to repulsion) is a major drawback.[6] This inefficient derivatization is costly, since a large stoichiometric excess of expensive functionalized macromolecules is required, while the use of excess cell interactive macromolecules can cause unwanted side effects and toxicity.[7]

To overcome such difficulties and substantially enhance efficiency, we present a novel and universal cell surface

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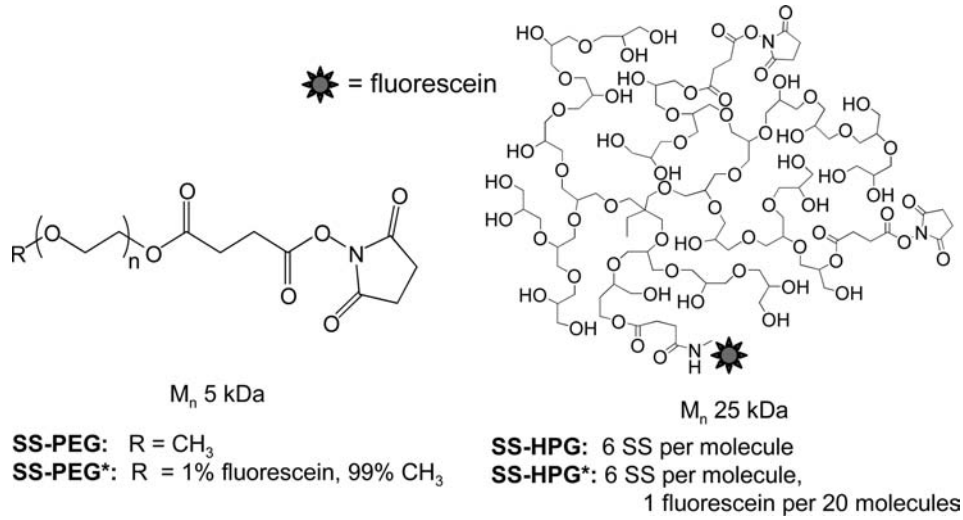
Scheme 1. Unlabeled and Fluorescein-Labeled, Amine Reactive polymers: (Left) Succinimidyl Succinate Functionalized Poly(ethylene glycol) (SS-PEG, SS-PEG*); (Right) Succinimidyl Succinate Functionalized Hyperbranched Polyglycerol (SS-HPG, SS-HPG*)



modification technique that utilizes cell-compatible, nonreactive "additive" polymers such as dextran or hyperbranched polyglycerol (HPG) in combination with cell surface reactive polymers to significantly improve cell surface modification. Primary amine reactive succinimidyl succinate functionalized PEG (SS-PEG, M_n 5 kDa) and HPG (SS-HPG, M_n 25 kDa) were employed as model cell surface reactive polymers to study cell surface modification. Fluorescently labeled cell surface reactive polymers were used to measure relative concentrations of grafted polymer on the surfaces of the cells upon grafting in concentrated additive polymer solutions. The effects of enhanced polymer grafting were investigated using four different cell types—RBCs, peripheral blood mononuclear cells (leukocytes), platelets, and Jurkat (human tumor T lymphocyte) cells (Jurkat cells)—to highlight that this technique has significant potential for use in various live cell based technologies. Enhanced polymer grafting was also measured as a function of additive concentration, molecular weight, and structure. The applicability of this new technique is demonstrated by enhanced camouflage of Rhesus (RhD) antigens on the RBC surface. The process highlights a macromolecular mechanism and technique that can, in principle, be adapted to other macromolecular systems and cell types.

Results and Discussion

Red Blood Cell Surface Grafting. The cell surface reactive polymers described are either hyperbranched (SS-HPG)[8] or linear (SS-PEG)[9] and contain succinimidyl succinate functional groups which can react with primary amines of lysine residues present on the surface of the cells to form covalent amide linkages (Scheme 1; also, see the Supporting Information for synthetic protocols). Although the grafting of PEG to cell surfaces has been previously reported,[1,2] the grafting of SS-HPG represents the first example of a multifunctional, hyperbranched polymer being covalently bound to a cell surface. Commercially available, non fluorescently labeled, cyanuric chloride functionalized PEG (CM-PEG, M_n 5 kDa) was also used in certain cases.

Initially, conventional grafting of the reactive polymers to RBC surfaces for 1 h under physiological conditions was performed (Figure 1A). Incubation times of 1 h were determined to be optimal (as highlighted in Figure 1S in the Supporting

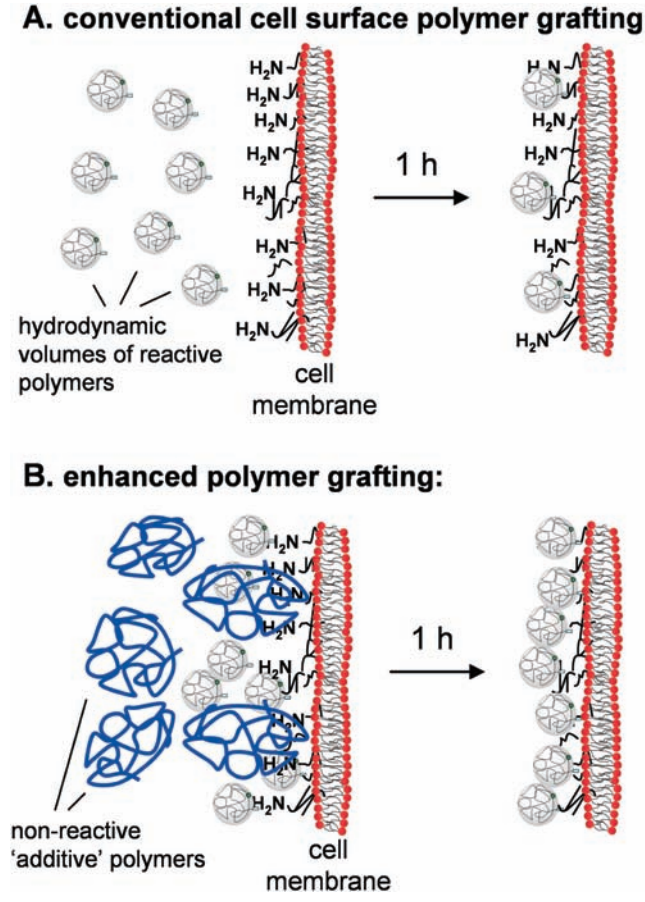


Figure 1. Enhanced cell surface polymer grafting in the presence of a nonreactive polymer additive: (A) representation of cell surface modification using conventional method using primary amine reactive polymers; (B) cell compatible surface grafting process in the presence of a nonreactive polymer additive (e.g., dextran or HPG).

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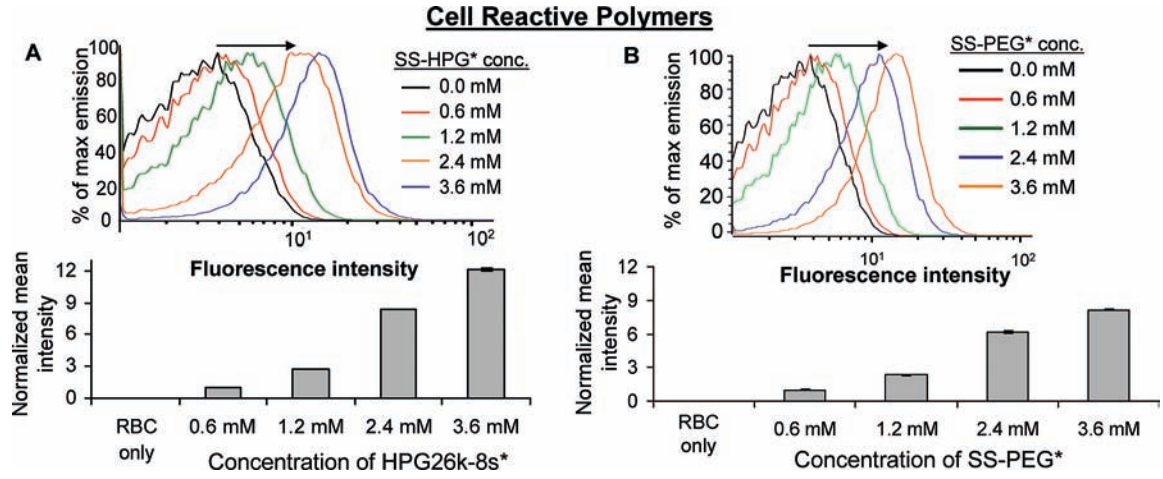

Figure 2. Cell surface reactive polymers: extent of RBC modification after 1 h grafting under physiological conditions with fluorescein-labeled polymers (A) SS-HPG* (26 kDa) and (B) SS-PEG* (5 kDa), as measured by flow cytometry; mean cell fluorescence intensity of 50 000 cells. Insets: overlays of flow cytometry plots (percentage of maximum emission vs fluorescence intensity). Normalized mean fluorescence intensity values were obtained by subtracting the fluorescence intensity of the unmodified RBC and setting the resulting fluorescence of cells grafted with the lowest reactive polymer concentration (0.6 mM) to 1 (arbitrary units). Error bars represent standard deviations (SD) of three independent measurements; values given are "mean ± SD".

Information) in all cases. In this study, it was possible to determine the relative concentrations of reactive polymer on the cell surface using flow cytometry. This was achieved by incorporating a fluorescein marker (5-(aminoacetamido)fluorescein) onto a small percentage (approximately 1% of SS-PEG and 5% of SS-HPG) of the reactive polymers. These fluorescently labeled polymer mixtures are denoted as SS-PEG* and SS-HPG* to distinguish them from non fluorescently labeled polymers (i.e., SS-PEG and SS-HPG).

The results of SS-PEG* and SS-HPG* grafting onto the RBC in the absence of a nonreactive polymer additive (conventional grafting) is shown in Figure 2. As expected, an increase in the concentration of the reactive polymer led to an increase in cell surface modification. For example, an increase in the grafting concentration of SS-PEG* from 0.6 to 3.6 mM enhanced the amount of PEG attached to the cell surface by ~11-fold (assuming the fluorescence intensity is linearly related to the amount of polymer attached). Similarly, an increase in the grafting concentration of SS-HPG* from 0.6 to 3.6 mM resulted in an ~8-fold increase in cell surface fluorescence (Figure 2A). Higher grafting concentrations (in both conventional and additive enhanced grafting experiments) of reactive PEG were not investigated, since a significant amount of cell damage occurs above 2.4 mM grafting concentrations.[10]

Enhanced Cell Surface Polymer Grafting. Figure 1B describes the grafting of the same reactive polymers to RBC surfaces in the presence of two kinds of cell compatible and nonreactive macromolecular additives: dextran (10−70 kDa) and HPG (6−120 kDa). In Figure 3, cell surface modification performed in the presence of these additive polymers using a constant concentration of SS-PEG* or SS-HPG* (2.4 mM) is highlighted. In this case, the amount of reactive polymer attached to the cell surface increased severalfold as increasing amounts of additive polymer were used, even though the concentration of reactive polymer remained constant. Figure 3A highlights the enhanced cell surface reactivity of SS-HPG* at a constant grafting concentration of 2.4 mM in the presence of varying concentrations of "additive" dextran (40 kDa, 0−3 mM). At a dextran concentration of 3.0 mM, the amount of SS-HPG* attached to the RBC surface increased approximately 10-fold compared to when no dextran additive (0 mM) was used. Enhanced grafting was also observed at lower concentrations of SS-HPG* (such as 1.2 mM SS-HPG*; Figure 2S in the Supporting Information). Similarly, an increase in the concentration of nonreactive dextran additive also resulted in increased cell surface modification with SS-PEG* (Figure 3B). Note that the normalized mean fluorescence intensity values were obtained in all cases by subtracting the fluorescence intensity of the unmodified RBC and setting the resulting fluorescence of cells grafted with reactive polymer in the absence of polymer additives (0 mM) to 1 (arbitrary units). For clarity, a normalization procedure was performed, since the background fluorescence intensity (noise) can vary depending on the source (donor) of the RBCs.

A similar enhancement in cell surface modification was also observed for another type of additive polymer, HPG (M_n 35 kDa), when used in conjunction with SS-HPG* or SS-PEG* (Figure 3C,D). Enhanced modification was observed, although to a lesser extent (up to ~2.5 fold) than for the systems involving dextran. A third nonreactive polymer additive—PEG (M_n 6 and 35 kDa)—was also investigated. However, upon addition of PEG, no significant increase in cell surface derivatization by SS-HPG* or SS-PEG* was observed, suggesting that the choice of additive macromolecule is crucial (Figure 4 and Figure 3S in the Supporting Information). It was found that the addition of PEG caused significant aggregation and lysis of the RBCs (Figure 4S in the Supporting Information). The remaining cells which had not been lysed or irreversibly aggregated showed no signs of improved polymer grafting; for example, when SS-PEG* was grafted to cells in the presence of nonreactive PEG additive (6 kDa), the mean cell fluorescence intensity did not increase and there was no noticeable decrease in electrophoretic mobilities (parts A and B of Figure 4, respectively). In fact, the extent of grafting decreased with increasing PEG additive concentration, probably as a result of poor mixing due to heavily aggregated cells.

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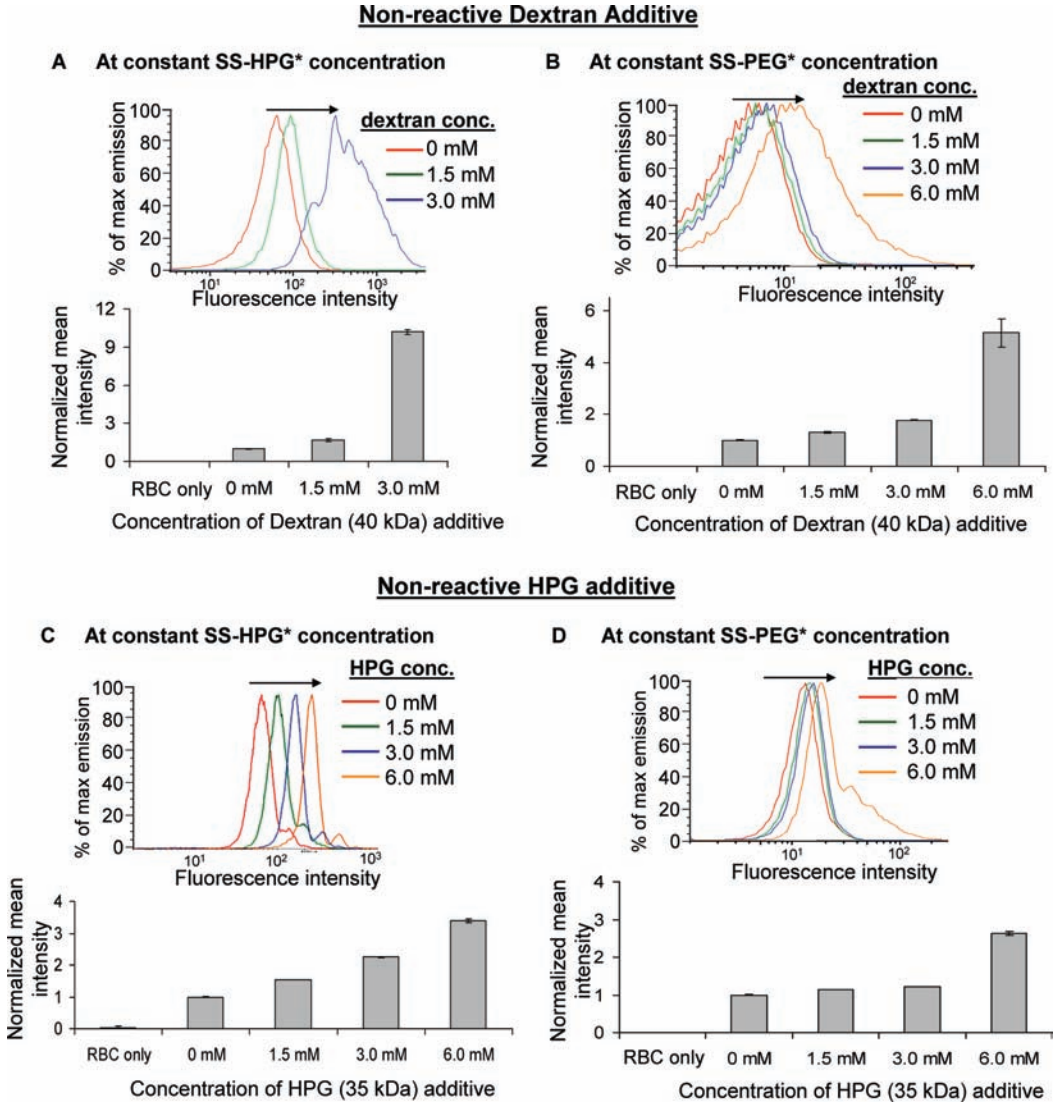

Figure 3. Effect of nonreactive, macromolecular additives: extent of RBC modification after 1 h grafting with fluorescein-labeled, surface reactive polymers under physiological conditions. Effect of concentration of nonreactive additive dextran T40 with (A) 2.4 mM reactive SS-HPG* and (B) 2.4 mM reactive SS-PEG*. Effect of concentration of nonreactive HPG (35 kDa) additive with (C) 2.4 mM reactive SS-HPG* and (D) 2.4 mM reactive SS-PEG*; mean cell fluorescence intensity of 50 000 cells. Overlays of flow cytometry plots are shown in each case. Normalized mean fluorescence intensity values were obtained by subtracting the fluorescence intensity of the unmodified RBC and setting the resulting fluorescence of cells grafted with reactive polymer in the absence of polymer additives (0 mM) to 1 (arbitrary units). Error bars represent standard deviations (SD) of three independent measurements; values given are "mean ± SD".

A second, independent method for analyzing cell-surface polymer grafting is particle electrophoresis.[11] RBC motion due to the applied electric field was observed using an optical microscope focused on the stationary phase of a glass capillary. Any changes in the electrophoretic mobility of the RBC can be correlated with the extent of polymer attachment on the surface of the cell. Hence, enhanced polymer grafting was also proven using particle electrophoresis: a significant decrease in the electrophoretic mobility of the RBC was observed upon grafting with a constant concentration of SS-HPG (2.4 mM) and increasing amounts of nonreactive HPG (35 kDa) additive (Figure 5). This indicates a higher amount of polymer is being covalently bound to the cell surface. Control cells, which were only incubated with nonreactive polymer additive (4.8 mM HPG), did not show any change in electrophoretic mobility, confirming that the effect is not due to polymer adsorption.

Enhanced cell surface polymer modification can be explained by the synergetic effects of enhanced macromolecular transport[12] and increased concentration of reactive polymer molecules near the cell surface due to improved penetration of polymer into the glycocalyx of the cell membrane.[13] In the first instance, the diffusion of one type of macromolecule in solution can be enhanced by the presence of a suitable second macromolecule in higher concentration.[12] In describing the transport process of macromolecules in multicomponent systems, Laurent et al. demonstrated that the diffusion of polyvinylpyrrolidone was significantly enhanced in the presence of concentrated aqueous solutions of various molecular weight dextrans. From the results presented here, the rapid diffusion of reactive polymer (SS-

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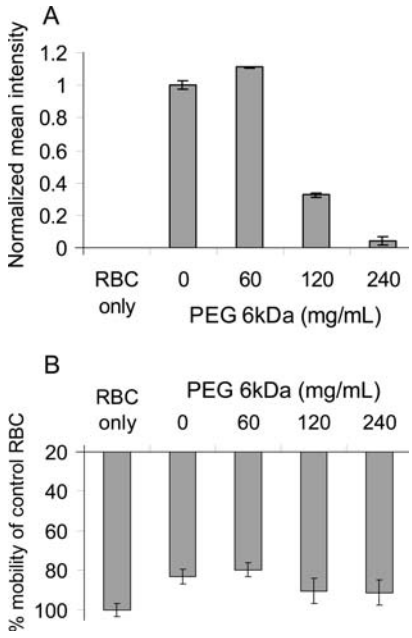

Figure 4. Effect of nonreactive PEG (6 kDa) additive as measured by (A) mean fluorescence intensity and (B) electrophoretic mobility after 1 h grafting with fluorescein-labeled, surface reactive SS-PEG* (0.6 mM, 5 kDa) under physiological conditions. Mean cell fluorescence intensity of 50 000 cells; mobility is measured as a percentage of the mobility of the unmodified, control RBC ("RBC only"), which is set as 100%. Error bars represent standard deviations (SD) of mobility of several RBCs; values given are "mean ± SD".

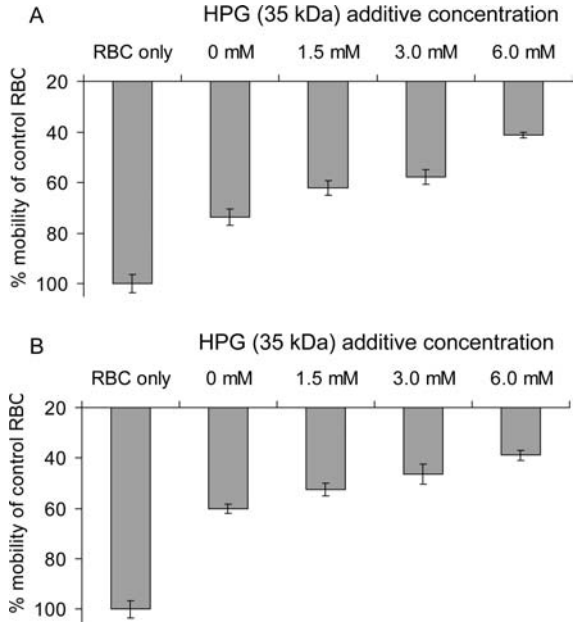

Figure 5. Effect of additive polymer (HPG, 35 kDa) on the electrophoretic mobility of RBC derivatized with cell surface reactive (A) SS-HPG* (2.4 mM) and (B) SS-PEG* (2.4 mM) for 1 h at room temperature in PBS (pH 8, 0.15 N NaCl). Mobility is measured as a percentage of the mobility of the unmodified, control RBC ("RBC only"), which is set as 100%. Error bars represent standard deviations (SD) of mobility of several RBCs; values given are "mean ± SD".

HPG or SS-PEG) to the cell surface could generate an apparent increase in concentration near the cell surface, resulting in enhanced surface modification. Since the SS-PEG or SS-HPG is covalently grafted to the cell surface during the process, it would then stay attached even after the additive HPG or dextran was washed away.

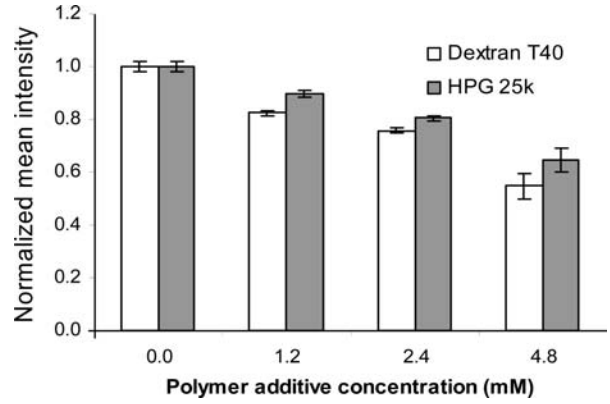

Figure 6. Small-molecule coupling: effect of nonreactive additive polymer concentration (dextran (40 kDa) or HPG (25 kDa)) on the mean fluorescence intensity of RBC derivatized for 1 h at room temperature with 20 μM cell surface reactive fluorescein-5-EX, succinimidyl ester in PBS (pH 8, 0.15 N NaCl). Normalized mean fluorescence intensity values were obtained by subtracting the fluorescence intensity of the unmodified RBC and setting the resulting fluorescence of cells grafted with fluorescein-5-ex in the absence of polymer additives (0.0) to 1 (arbitrary units). Error bars represent standard deviations (SD) of mobility of several RBCs; values given are "mean ± SD'".

This explanation for enhanced grafting was further investigated by testing whether similar additive effects were observed for systems in which nonmacromolecular additives or reagents were used. To demonstrate that enhanced grafting is exclusive to macromolecules, the extent of cell surface grafting with an amine reactive, fluorescent small molecule—fluorescein-5-EX, succinimidyl ester (Invitrogen)—in the presence of nonreactive polymer was investigated. In this case, the nonreactive additive polymer did not increase the grafting efficiency of the molecule; on the contrary, the amount of fluorescent molecules coupled to the RBC surface actually decreased (Figure 6). Further investigations also revealed that alternative low-molecular-weight additives such as dextrose, NaCl, and mannitol did not enhance cell surface modification of SS-HPG* or SS-PEG* (Figure 3S in the Supporting Information).

Since low molecular weight additives did not enhance polymer grafting, the molecular weight effect of nonreactive macromolecules was examined with respect to enhanced polymer grafting. During grafting with SS-PEG or SS-HPG, it was found that the addition of low molecular weight dextran (10 kDa) did not enhance cell surface grafting as much as higher molecular weight dextran (40 and 70 kDa) at similar concentrations (80 mg/mL). This is highlighted by the electrophoretic mobility measurements (Figure 7), and by the flow cytometry data shown in Figure 8. According to the flow data, an increase in fluorescence by approximately 30−40% was observed when higher molecular weight dextran was used. Similarly, low molecular weight HPG (6 kDa) additive was less effective than higher molecular weight HPG (33 kDa and 164 kDa) (Figure 7B).

Since the rapid diffusion of macromolecules in concentrated systems is shown to decrease above certain concentrations of polymer,[14] an alternative and complementary mechanism must also exist. In the presence of concentrated polymers (e.g., aqueous solutions of dextran), the aggregation of cells and the

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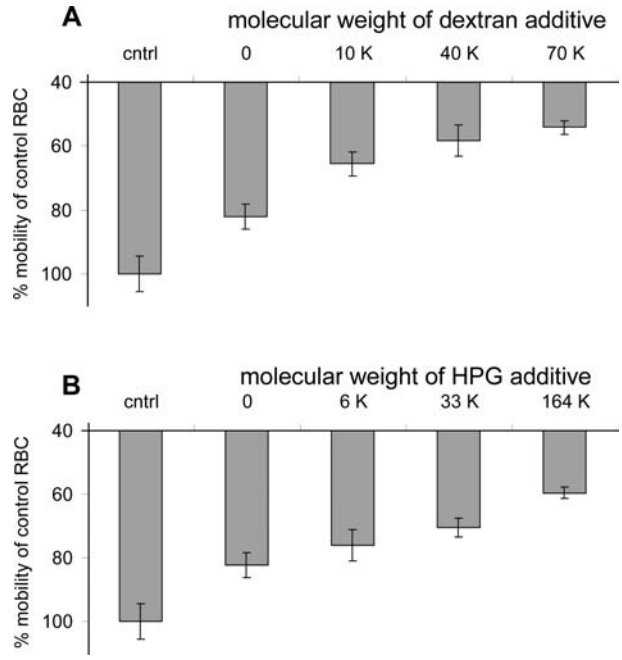

Figure 7. Effect of nonreactive polymer molecular weight on the extent of grafting with surface reactive SS-PEG* (0.4 mM) under physiological conditions for 1 h: (A) grafting of SS-PEG* in the presence of dextran additives (80 mg/mL concentration; 10, 40, and 70 kDa); (B) grafting of SS-PEG* in the presence of HPG additives (80 mg/mL concentration; 6, 33, and 164 kDa). "0" denotes grafting in the absence of any additive. Mobility is measured as a percentage of the mobility of the control RBC (0 mM SS-HPG), which is set as 100%.

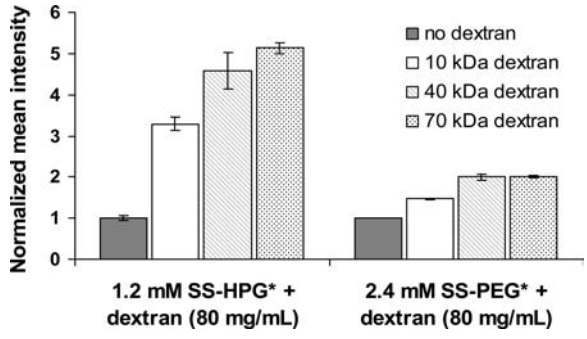

Figure 8. Effect of nonreactive polymer molecular weight on the extent of grafting with surface reactive SS-HPG* (1.2 mM) and SS-PEG* (2.4 mM) under physiological conditions for 1 h in the presence of dextran additives (80 mg/mL concentration; 10, 40, and 70 kDa). Normalized mean fluorescence intensity values were obtained by subtracting the fluorescence intensity of the unmodified RBC and setting the resulting fluorescence of cells grafted with reactive polymer in the absence of dextran additives ("no dextran") to 1 (arbitrary units). Error bars represent standard deviations (SD) of three independent measurements; values given are "mean ± SD".

formation of a depletion layer between the bulk polymer and the glycocalyx has been shown to occur.[13] Upon addition of low molecular weight PEG, this cell aggregation process in concentrated dextran solutions is reversed as the depletion layer becomes enriched with PEG. The initial formation of the depletion layer" between the glycocalyx and the bulk polymer is also dependent on the interaction of polymers (i.e., type) with the glycocalyx, as emphasized by previous reports that dextran forms larger depletion zones than does PEG.[13] This interaction is facilitated by the soft hairy nature of the glycocalyx, which already contains a layer of macromolecules that can be penetrated by the free polymer in solution.[13] This is highlighted by the fact that the same effect is not observed for the hard and smooth surfaces of plastics and metals.[15] From the point of view of cell surface grafting, the presence of a large amount of additive polymer (dextran and HPG) resulted in a reversible cell aggregation that enhanced the concentration of reactive polymers near the cell surface (in the present case PEG and HPG) and therefore the reactivity with cell surface amine groups.

As demonstrated by Neu and Meiselman, the concentrating effect of the polymer in the depletion layer also depends on the type of both the bulk polymer and the added small molecular weight polymers.[14] PEG has shown to have the lowest affinity for and penetration of the glycocalyx; this can also clarify the difference in the enhancement by different polymers (e.g. dextran (10-fold) compared to PEG (no enhancement)). PEG was found not to be suitable, since it caused irreversible aggregation of the cells at similarly high (additive) concentrations. Furthermore, the molecular weight dependence of "additive" dextran during the grafting process can be explained by the increased size of the depletion layer, which leads a higher amount of reactive polymer near the glycocalyx.[15]

The concentrating effect of polymer in the depletion layer may also be affected by the polymer−polymer repulsion, which could increase the concentration of reactive polymer near the glycocalyx.[13] It is known that dextran and PEG form two-phase systems in concentrated aqueous solutions.[16] Similarly, it was found that HPG and dextran also formed two-phase systems (unpublished results), and as a result the increased polymer grafting in presence of dextran can thus partially be also explained by PEG/HPG-dextran exclusion. However, the grafting enhancement observed in HPG additive solutions cannot be explained by exclusion. It is likely that a combination of several factors, including rapid diffusion, concentrating effects due to a depletion layer near the glycocalyx, and polymer−polymer repulsion/exclusion are contributing to the observed enhanced polymer grafting on cell surfaces in concentrated polymer solutions.[12−15]

Application to Other Cells. Macromolecular conjugation to cell surfaces is of particular interest to a wide range of biomedical applications.[1−5] In order to determine the universality of the enhanced grafting process, the apparent increase in grafting efficiency of both SS-HPG* and SS-PEG* caused by nonreactive macromolecular additives (dextran 40k or HPG 35k) was tested for three other types of cells: peripheral blood mononuclear cells (leukocytes, Figure 9A), platelets (Figure 9B), and Jurkat cells (Figure 9C). Using the same flow cytometry techniques discussed previously, similar enhancements in the cell surface modification were observed. As demonstrated for the RBC surface, the results shown here suggest that the technique is applicable to many different cell types, both nucleated and non-nucleated (see Figures 5S−7S in the Supporting Information for detailed analysis).

Cell Compatibility of the Process. Furthermore, the viability of the different cells (RBC, leukocytes, platelets, and Jurkat cells) were measured in the presence of increasing concentrations of different polymer additives (Figures 8S−14S in the

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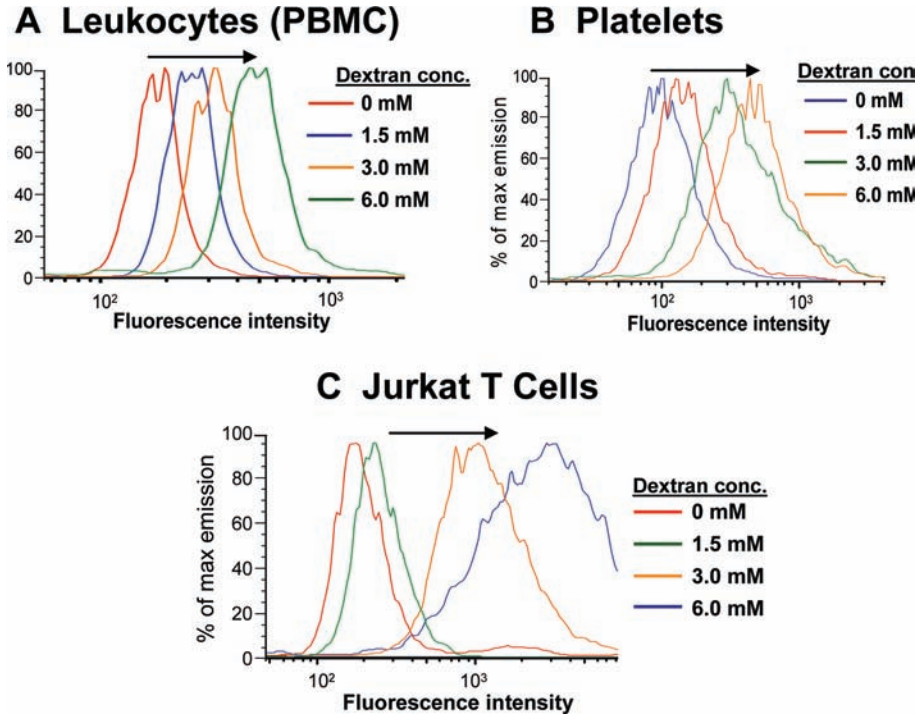

Figure 9. Universality of the *enhanced cell surface modification* process: extent of (A) leukocyte (peripheral blood mononuclear cell), (B) platelet, and (C) Jurkat cell modification at a constant concentration (2.4 mM) of SS-PEG* in the presence of various concentrations of nonreactive dextran (40 kDa) additive as measured by flow cytometry.

Supporting Information) in the absence of cell reactive polymer. Little or no effect was observed in terms of the size, shape, and number of healthy cells after incubating with the polymers for 1 h, even at relatively high concentrations of >3 mM (for dextran (M_n 40 kDa), 3 mM equates to 120 mg/mL). For example, Figure 8S shows the optical microscopy images of the RBCs after incubation with either HPG or dextran, with no sign of the cells undergoing crenation. These observations are not altogether surprising, since dextran (40 kDa) has been used as an FDA-approved plasma expander for many years and is often administered intravenously as a 10% solution (final concentration in plasma approximately 2.5%).[17]

A Practical Application. The covalent attachment of polymers to the membranes of RBC in order to camouflage surface antigens has been investigated extensively.[1] To highlight the potential applications and potential usefulness of the enhanced grafting technique, the extent of RBC surface antigen protection is shown as a practical example. The masking of Rhesus (RhD) antigens on the RBC surface was measured using a highly reactive fluorescently labeled monoclonal anti-D antibody (FITC-Anti-D, Alba Bioscience (Edinburgh, U.K.)). A decrease in the detection of fluorescence caused by the presence of FITC-Anti-D bound on the cell surface is correlated to the extent of RhD camouflage and the efficiency of polymer cell surface attachment.[18] Figure 10 compares the extent of RhD masking using conventional PEG grafting (2.4 mM) and PEG grafting (2.4 mM) in various concentrations of nonreactive additive polymer. As controls, RhD+ RBC incubated with FITC-Anti-D are set at 100% fluorescence, whereas 0% fluorescence is

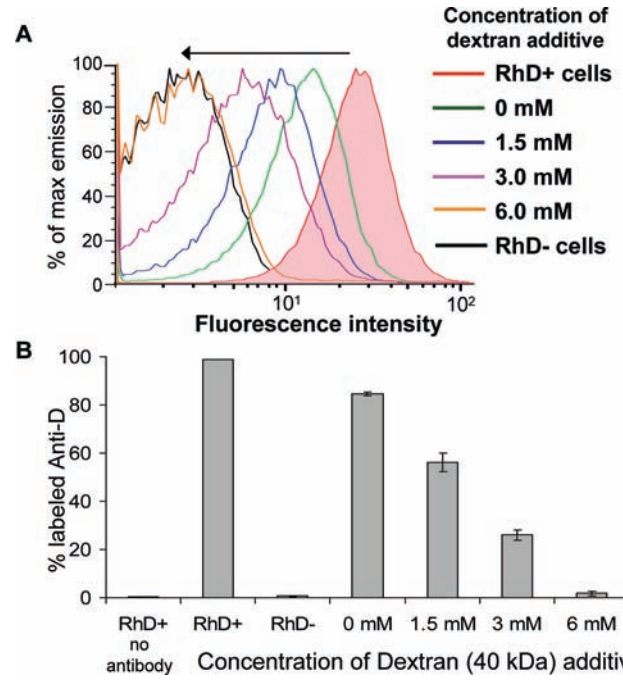

Figure 10. Comparison of conventional and enhanced polymer grafting on the extent of RhD antigen protection on the RBC surface, using a constant CM-PEG (2.4 mM, 5 kDa) grafting concentration: (A) overlays of the extent of camouflage; (B) percentage of cells labeled by fluorescently labeled FITC-Anti-D antibody as measured by flow cytometry (50 000 cells). RhD+ RBC without FITC-Anti-D (RhD+, no antibody), and RhD+ RBC (RhD+) and RhD− RBC (RhD−) with FITC-Anti-D are shown as controls. Error bars represent standard deviations (SD) of three independent measurements; values given are "mean ± SD".

observed for RhD− (O−) blood cells. In the case of conventional PEG grafting, only 15% of the RhD antigens were protected. However, by increasing the concentration nonreactive

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dextran additive, the percentage of protected antigens increased to more than 98%, indicating that effectively RhD null RBC were created by this technique. The result is further proof that higher amounts of reactive polymer are grafted to the cell surface, facilitating the physical camouflage of antigens. As was shown for SS-HPG* earlier, the increase in the amount of CM-PEG grafted to the surface was also independently confirmed using electrophoresis. Eliminating the possibility that additive polymer might simply be absorbed onto either the RBC surface or the polymer-derivatized RBC surface, antigen camouflage was not enhanced by simply mixing the cells with the additive polymer either (a) before or (b) after surface grafting with a reactive polymer (Figure 15S in the Supporting Information).

Conclusion

The mechanism of enhanced cell surface polymer grafting can be explained in part by a combination experimental result published on diffusion macromolecules in concentration polymer solutions, the demonstrated mechanism of reversible cell aggregation and polymer−polymer exclusion.[12−15] Given the nature of this phenomenon that has led to enhanced cell surface modification by up to 10-fold at a low and constant polymer reagent concentration, we have termed the technique *Diffusion Modulated Macromolecular Cell Derivatization (DMMCD)*. This technique can be applied to different macromolecular combinations, structures, and molecular weights and to various cell types (both nucleated and non-nucleated). The method can potentially be adapted to other macromolecular cell surface interacting agents. Importantly, the technique significantly enhances cell surface modification and decreases the polymeric reagent concentration required for cell surface engineering, thereby minimizing toxicity and cost.

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Supporting Information Available: Text, tables, and figures giving detailed experimental protocols, reaction schemes, additional results, and analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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