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Separation and purification of methoxypoly(ethylene glycol) grafted red blood cells via two-phase partitioning

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Abstract

Alloimmunization to donor blood group antigens remains a significant problem in transfusion medicine. To attenuate the risk of alloimmunization, we have pioneered the membrane grafting of methoxypoly(ethylene glycol) (mPEG) to produce immunocamouflaged red blood cells (RBC). Grafting of the mPEG was accomplished using cyanuric chloride activated mPEG (CmPEG; $M_r = 5000$), benzotriazole carbonate methoxyPEG (BTCmPEG; $M_r = 5000$ or 20,000); or *N*-hydroxysuccinimidyl ester of mPEG propionic acid (SPAmPEG; $M_r = 2000$, 5000, or 20,000). Because of the heterogeneity of grafting, a crucial tool in developing the stealth RBC is an ability to purify the modified RBC from unmodified (immunogenic) donor cells. As demonstrated, a (5, 4) dextran:PEG aqueous two-phase polymer partitioning system cleanly separated the immunologically silent mPEG-grafted human RBC from control or lightly modified cells. Cell mixing experiments employing varying ratios of mPEG-modified and control RBC confirmed the purification efficacy of the phase partitioning system. Proportional changes in PEG-rich phase partitioning were achieved by increasing either the quantity of surface mPEG or the mPEG molecular weight. The biological viability of purified mPEG-RBC (BTCmPEG; $M_r = 20{,}000$) was demonstrated by their normal in vivo survival at immunoprotective grafting concentrations ($\leq$2 mmol/L). The effective immunocamouflaging of RBC antigens coupled with efficient purification of the immunocamouflaged population provides encouragement for the further development of the stealth erythrocyte.

Keywords: Purification; Aqueous two-phase systems; Partitioning; Methoxypoly(ethylene glycol)

1. Introduction

The transfusion of red blood cells (RBC) remains the most common, and best tolerated, form of tissue transplantation. Surprisingly, while ABO/RhD matched transfusions are generally considered as safe, a significant incidence of mild to moderate morbidity occurs due to immunologic recognition of the non-ABO blood antigens and the resultant alloimmunization of the transfused individual [1,2]. This immunologic risk is further exacerbated in patients receiving chronic transfusion therapy and may lead to significant treatment concerns. Currently, no satisfactory solutions exist to prevent either the initial alloimmunization or the subsequent transfusion reactions upon re-exposure to antigen positive RBC.

To this end, we and others have investigated the covalent grafting of methoxypoly(ethylene glycol) (mPEG) to the red cell membrane [3–18]. As a consequence of the grafted mPEG, RBC antigens are physically and biochemically masked from the recipients immune system. Subsequent to this biophysical immunocamouflage, the inherent antigenicity and immunogenicity of the cell is attenuated thereby reducing the risk and incidence of alloimmunization and transfusion reactions. The effect of membrane grafted mPEG is readily observed using the highly immunogenic RhD antigen (Fig. 1A). As shown when RhD^+ human RBC treated with anti-RhD typing antibodies these cells readily agglutinate in response to the antibody. However, with increasing amounts of membrane grafted mPEG, the RhD^+ cells fail to agglutinate and test as RhD negative.

However, RBC derivatized via the bolus addition of activated mPEG exhibit significant heterogeneity in the amount of grafted mPEG per cell with some cells potentially retaining significant immunogenicity (Fig. 1B). Thus, to clinically utilize these stealth RBC, it has been necessary to develop a technique by which the immunocamouflaged RBC can be separated from the immunogenic cells within a given population. To achieve this aim we have utilized an aqueous two-phase (dextran:PEG) partitioning system [9,12,19]. The

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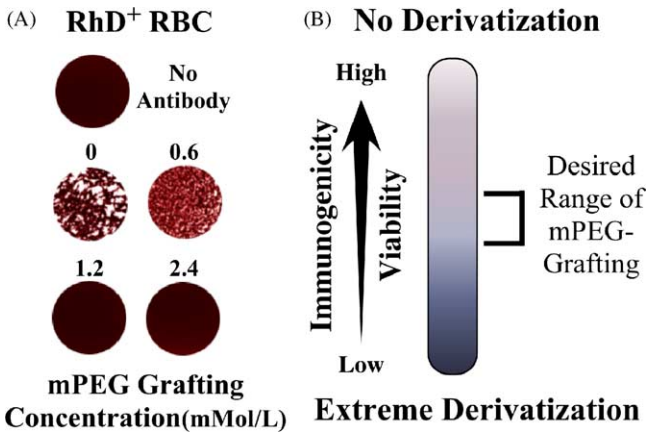

Fig. 1. Membrane grafted mPEG is efficient at camouflaging highly immunogenic RBC antigens (panel A); however, this immunocamouflage is heterogeneous in nature (panel B).

partitioning of pegylated cells via the two-phase system is governed by the ratio of dextran:PEG, the density of grafted PEG and the size (number of ethoxy units) bound to the RBC. Hence, partitioning allows for the efficient separation and purification of the modified RBC from the unmodified, or under-modified, highly immunogenic donor cells [12]. Importantly, the described partitioning system has no deleterious affects on the viability of pegylated RBC as demonstrated by in vivo murine studies.

2. Methods

2.1. Blood collection

Following informed consent, whole blood was drawn from normal healthy human volunteers into heparin Vacutainer tubes. Murine RBC were collected in heparinized syringes from Balb/c mice via cardiac bleed. Following collection, all erythrocytes were washed three times with isotonic saline.

2.2. PEG derivatives and cellular grafting

Methoxypoly(ethylene glycol)-benzotriazolyl carbonate (BTCmPEG; $M_r = 5$ and 20 k) and *N*-hydroxysucciminidyl ester of mPEG propionic acid (SPAmPEG; $M_r = 2$, 5 and 20 k) were obtained from Shearwater Polymers Inc. (Huntsville, AL). SPAmPEG-5 k was purchased from Fluka Chemie (Sigma–Aldrich, St. Louis, MO). Cyanuric chloride activated mPEG (CmPEG; $M_r = 5$ k) was purchased from Sigma. Derivatives were used without further purification.

Erythrocytes were modified as washed cells at a 12% hematocrit as previously described [5,7,11,12]. Stock solutions of PEG-derivatives were made in 50 mmol/L dibasic potassium phosphate, 105 mmol/L NaCl, pH 8.0 (PEG buffer). Final mPEG concentrations ranged from 0 to 5 mmol/L. Derivatization reactions were carried out at room temperature for either 30 min or 1 h. Modified RBC were then washed three times with saline. The control group consisted of RBC incubated in mPEG-free PEG-buffer (0 mmol/L mPEG-RBC).

2.3. Partitioning in aqueous two-phase polymer systems

Partitioning of control and mPEG-modified RBC was carried out in an aqueous two-phase system consisting of PEG8000 (Sigma) and dextran T500 (molecular weight ∼ 500,000; Pharmacia). PEG and dextran stock solution concentrations were determined by refractive index measurements. Phase systems were made up in 50 ml polypropylene tubes and allowed to equilibrate over night at room temperature. The upper (PEG-rich) phase was drawn off into a separate tube and the lower (dextran-rich) phase was obtained by puncturing the tube to collect the solution. Phase systems were used within 3 days. The two-phase system denoted (5, 4) was prepared as described previously [12,20] and consisted of 5% (w/w) dextran, 4% (w/w) PEG, 0.15 mol/L NaCl, 6.84 mmol/L Na_2HPO_4, and 3.16 mmol/L NaH_2PO_4 (pH 7.4). Similarly, (5, 3.5) and (5, 5) systems consisted of 5% (w/w) dextran and either 3.5 or 5% PEG, respectively.

Control and mPEG-RBC were labeled with ^{51}Cr to allow for quantitation. The partitioning experiment was performed in duplicate and consisted of the addition of 12 μl (load) of ^{51}Cr-RBC ± mPEG (3.7×10^8/ml) to 0.75 ml each of the upper and lower phases. Tubes were inverted 20 times and the phases were allowed to separate at room temperature for 20 min. Samples (350 μl) from both phases were removed for gamma counting. To determine the total ^{51}Cr-RBC ± mPEG loaded, at least three separate 12 μl samples of the ^{51}Cr-RBC ± mPEG were counted and the average total loaded counts per minute (cpm) was utilized. Results were expressed as the percentage of ^{51}Cr-RBC ± mPEG in the PEG-rich upper phase and were calculated as: $(\text{cpm}_{\text{upper phase}}/\text{cpm}_{\text{total loaded}}) \times 100$.

2.4. Cell mixing studies

To test the ability to separate mPEG-modified human RBC from unmodified human RBC, mixtures of these cells were loaded into the (5, 4) aqueous two-phase system described above. ^{51}Cr-labelled mPEG-RBC (2.5 mmol/L mPEG for 5 k or 0.9 mmol/L for mPEG 20 k) were mixed with ^{51}Cr-labelled control cells at the indicated percentages.

2.5. In vivo survival of mPEG-modified RBC

RBC survival studies were performed in Balb/c mice. RBC were collected from donor mice via cardiac bleed and derivatized with BTCmPEG (5 or 20 k) as previously described [5,7]. The concentration of mPEG used for the in vivo studies ranged from 0 to 2 mmol/L. Murine RBC were labeled using the fluorescent, membrane anchored marker PKH-26 (Sigma). Survival of fluorescently labeled

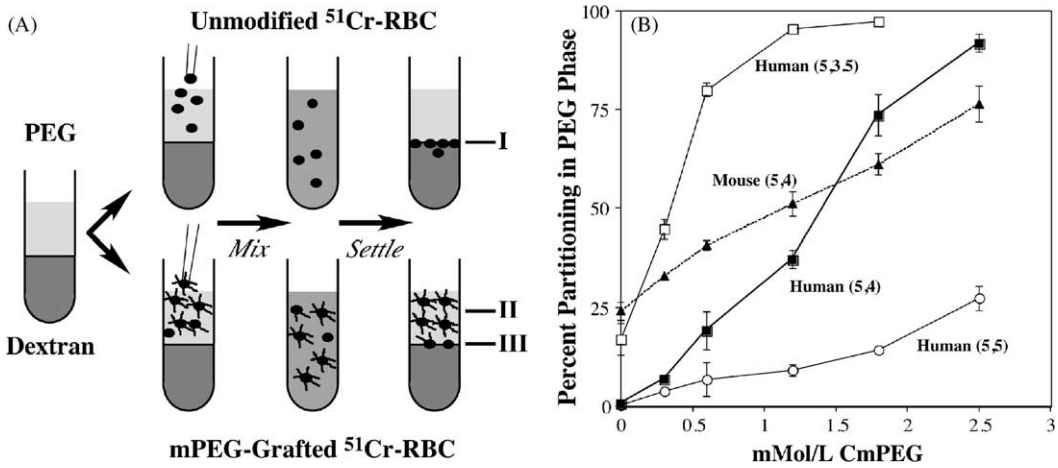

Fig. 2. Separation of mPEG-modified erythrocytes from unmodified cells is readily accomplished via two-phase partitioning systems utilizing dextran and PEG.

mPEG-treated and control RBC was monitored by analyzing the percent of fluorescently labeled RBC using flow cytometry. A minimum of five mice were used for each mPEG concentration tested [12].

3. Results

Partitioning in an aqueous two-phase polymer system has been used previously to monitor the consequences of PEG-derivatization of proteins and the incorporation of PEG into liposomes and to study erythrocyte surface properties [21–25]. Based on the successful use of this technique for these purposes, we adapted the partitioning method for use in purifying pegylated RBC. Partitioning in an aqueous two-phase polymer system containing dextran and PEG (the two-phases form due to the immiscibility of the two polymers) exploits the affinity of PEG-RBC for the PEG-rich upper phase. Fig. 2A is a schematic that shows an ideal partitioning system where unmodified control cells partition at the interface and in the lower, dextran-rich phase (I). Pegylated RBC partition into the upper PEG-rich phase (II). In contrast, RBC having low levels of grafted mPEG or that remain unmodified localize to the interface or are in the lower dextran phase (III).

Studies using different dextran:PEG systems, (5, 3.5), (5, 4), and (5, 5) systems, were conducted to find the most appropriate system for separating pegylated and control human RBC (Fig. 2B). The (5, 4) (dextran:PEG) system was found to be ideal for assessing and comparing changes in RBC membrane derivatization. In this two-phase system, less than 1% of unmodified RBC partition to the upper PEG-rich phase while ∼65% remain at the dextran:PEG interface and 34% are in the dextran-rich phase. The transition of RBC from the interface/dextran layer to the PEG-rich upper phase was a function of mPEG-derivative concentration, with almost complete PEG-phase partitioning of cells that had been derivatized with 2.5 mmol/L CmPEG (Figs. 2B and 3 insert). In comparison, more of the lightly derivatized and even 17% of control cells partitioned to the PEG-rich phase in the (5, 3.5) system. The opposite trend was achieved in the (5, 5) system. Here, very few (27%) of the highly modified (2.5 mmol/L CmPEG) cells partitioned into the PEG-rich phase. As the proportion of PEG 8000 in the dextran:PEG phase system was increased, the amount of surface modification required for the PEG-RBC to partition into the upper PEG-rich phase became greater.

Since the animal model used to determine in vivo survival is the Balb/c mouse, PEG-modified and control mouse RBC were also assessed in the (5, 4) partitioning system (Fig. 2B). As with human RBC, PEG-phase partitioning increased proportionally with the amount of CmPEG added to the RBC. The underlying differences in surface

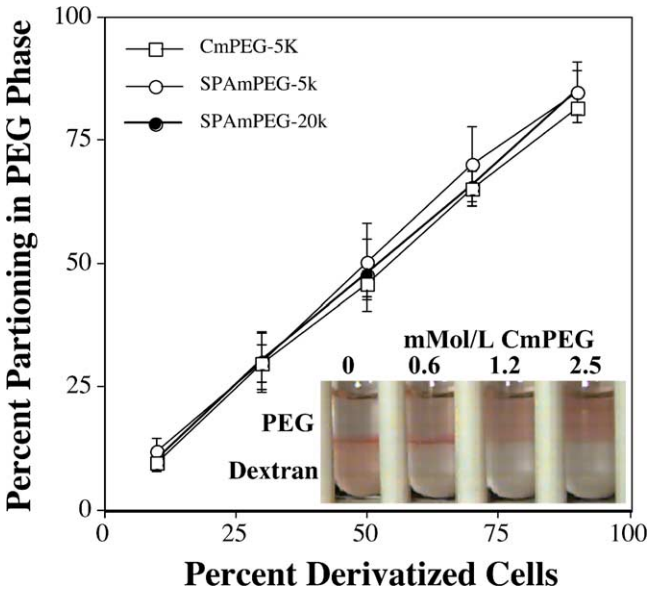

Fig. 3. mPEG-modified human erythrocytes are readily separated from unmodified cells via two-phase partitioning.

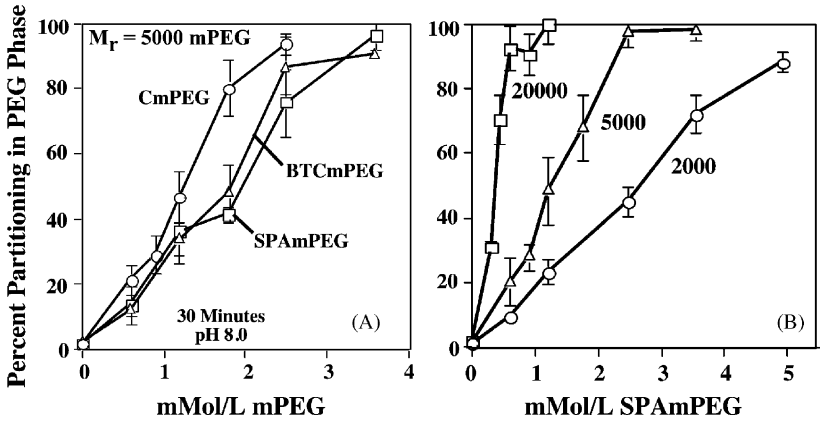

Fig. 4. Aqueous two-phase partitioning is used to monitor the effect of different linker chemistries (panel A) and polymer size (panel B) on surface properties of mPEG-modified human RBC.

characteristics between human and mouse RBC resulted in differences in partitioning patterns. For instance, the slope of the mouse RBC partitioning curve was decreased compared to that of human RBC.

A key clinical requirement for the use of pegylated RBC in the prevention of alloimmunization will be the necessity of removing unmodified or insufficiently modified RBC. As demonstrated in Fig. 3, the (5, 4) two-phase partitioning system is well suited for this task as mixtures of mPEG-modified RBC and unmodified RBC were successfully separated. The relationship between the proportion of highly PEG-modified RBC in the mixture and the percent partitioning in the PEG-rich phase was linear and 1:1, showing that virtually all of the modified cells were in the upper phase. In terms of actual retrieval, approximately 90% of highly mPEG-derivatized RBC (e.g. derivatized with 2.5 mmol/L CmPEG) were recovered from the PEG-rich layer by removing the top phase, diluting this phase in saline, then concentrating the RBC by low speed centrifugation.

The two-phase partitioning system was also employed to assess the overall membrane derivatization achieved using three different chemical linker agents. Each of these linker moieties (cyanuric chloride, BTC and SPA) were efficient at derivitizing RBC membranes with 5 k mPEG (Fig. 4A). As we have previously shown, the grafting reaction rate of the CmPEG was greater than that achieved with either BTC or SPAmPEG. Given a 1-h reaction time, BTC and SPAmPEG reached the same level of derivatization as the CmPEG after 30 min [12].

The molecular weight of the mPEG-derivative also had an enormous effect on RBC surface properties as illustrated by partitioning (Fig. 4B). To achieve ∼50% partitioning into the PEG-rich phase, approximately 2.5 times more SPAmPEG-5 k derivative or 5.5 times more SPAmPEG-2 k was required compared to the SPAmPEG-20 k. Similarly, to achieve over 80% partitioning into the PEG phase, 3.5 times more SPAmPEG-5 k or 8.5 times more SPAmPEG-2 k was required compared to the SPAmPEG-20 k. Similar results have previously been shown for BTCmPEG of varying molecular weights [12].

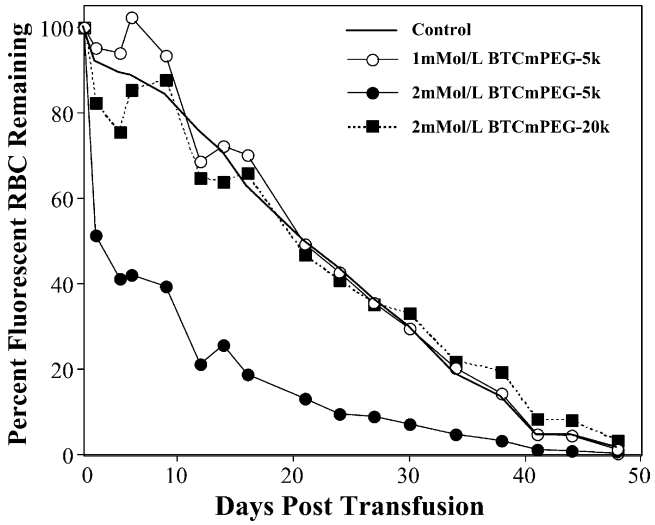

Fig. 5. In vivo survival of mouse mPEG-modified RBC is normal at immunoprotective levels of derivatization (1–2 mmol/L) with BTCmPEG-20 k.

Finally, in order to show biological efficacy, in vivo survival studies were conducted in a mouse model. These in vivo survival studies also demonstrated a molecular weight effect in that BTCmPEG-20 k was vastly superior to the lower molecular weight BTCmPEG-5 k derivative (Fig. 5). At a concentration of 2 mmol/L BTCmPEG-5 k, in vivo survival of murine RBC was significantly shortened. However, the use of a longer polymer, the BTCmPEG-20 k, resulted in normal survival kinetics (i.e. ∼50 days) at derivatization concentrations of at least 2 mmol/L.

4. Discussion

The RBC is a highly immunogenic cell. As a consequence, blood group typing is done to the most immunogenic

sites, the ABO/RhD antigens, prior to all transfusions. However, even after these precautions, alloimmunization to non-ABO/RhD blood group antigens remain a common consequence of blood transfusions, especially in the chronically transfused patient. To prevent this alloimmunization we have pioneered the immunocamouflage of RBC antigens via the grafting of methoxypoly(ethylene glycol) to the RBC membrane. As previously demonstrated, these immunocamouflaged RBC exhibit significantly reduced antigenicity and immunogenicity as evidenced by the attenuation of antigen detection via typing antibodies and by a decrease in antibody formation in response to allogeneic and xenogeneic transfusion in murine models [4–7,9].

However, the grafting of mPEG to the RBC membrane is a stochastic process yielding a heterogeneous population of cells (Fig. 1). Within a RBC population subjected to mPEG derivatization, a significant minority of cells are either unmodified or only lightly modified thus retaining significant immunogenic potential. To overcome this inherent problem, it has been necessary to develop a means by which these immunogenic cells can be removed. Importantly, the methodology used must not impair normal RBC structure, function, or viability. While an antibody affinity column chromatography method could be used to remove those cells exhibiting residual antigenicity, this process is expensive and not suitable for large scale blood banking. As a consequence, we have investigated the use of aqueous two-phase partitioning using a dextran:PEG system.

As shown in Fig. 2, partitioning of the mPEG-grafted cells can be manipulated based on the partitioning formulation used as well as the amount of membrane grafted mPEG. Based on the near linear separation (as a function of the amount and size of the grafted mPEG) noted with the (5, 4) system, this scheme was chosen for use with human RBC. Using this system we have demonstrated the efficient separation of pegylated RBC from unmodified cells when these populations are experimentally mixed (Fig. 3). As demonstrated, unmodified cells remain at the interface or within the dextran layer thereby assuring that only those cells with sufficient covalently bound mPEG are found within the PEG-rich layer (Fig. 3 insert). In contrast, while murine RBC exhibit increasing PEG-phase partitioning with increasing mPEG grafting in the (5, 4) system, a significant percentage (∼25%) of the unmodified murine cells naturally partition to the PEG-rich layer (Fig. 2B). Hence it is clear that the partitioning system must be tuned for each species.

Importantly, these data also demonstrate that a two step partitioning system can be used to purify cells with a relatively narrow range of derivatization. An initial purification step using the (5, 4) system removes cells that are unmodified or under modified. The population of the cells in the PEG-rich phase would then be washed and subjected to a second partitioning step using a (5, 5) or (5, 6) system in which only heavily derivatized enter the PEG-rich phase. These overly derivatized cells, which would exhibit impaired in vivo survival (Fig. 5), are discarded while the mPEG-RBC at the interface are collected for use. Importantly, the two-phase partitioning system is highly scalable for use in a blood banking environment and also very suitable for mechanization/automation following simple modification of existing blood processing instrumentation.

5. Conclusions

Ongoing research suggests that the immunocamouflage of donor RBC may prove to be a very powerful tool in preventing alloimmunization and in the treatment of patients already alloimmunized. However, key to using these cells is an ability to separate appropriately modified RBC from those devoid of or lacking sufficient mPEG grafting. As demonstrated in the current study, efficient separation and purification of the derivatized RBC from poorly or unmodified cells is readily achieved using a two-phase (dextran:PEG) partitioning system. In this simple purification technique, derivatized RBC are added to an immiscible dextran:PEG two-phase system, rapidly mixed and allowed to separate. The partitioning of pegylated cells via the two-phase system is governed by the ratio of dextran:PEG, the density of grafted mPEG and the size (number of ethoxy units) of the polymer grafted to the RBC. Importantly, in vivo murine studies demonstrate that neither the pegylation process or the partitioning procedure adversely affect RBC survival.

Acknowledgements

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