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In vivo circulation, clearance, and biodistribution of polyglycerol grafted functional red blood cells

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ABSTRACT

The *in vivo* circulation of hyperbranched polyglycerol (HPG) grafted red blood cells (RBCs) was investigated in mice. The number of HPG molecules grafted per RBC was measured using tritium labeled HPGs (^{3}H-HPG) of different molecular weights; the values ranged from 1×10^5 to 2×10^6 molecules per RBC. HPG-grafted RBCs were characterized *in vitro* by measuring the electrophoretic mobility, complement mediated lysis, and osmotic fragility. Our results show that RBCs grafted with 1.5×10^5 HPG molecules per RBC having molecular weights 20 and 60 kDa have similar characteristics as that of control RBCs. The *in vivo* circulation of HPG-grafted RBCs was measured by a tail vain injection of ^{3}H-HPG60K-RBC in mice. The radioactivity of isolated RBCs, whole blood, plasma, different organs, urine and feces was evaluated at different time intervals. The portion of ^{3}H-HPG60K-RBC that survived the first day in mice (52%) remained in circulation for 50 days. Minimal accumulation radioactivity in organs other than liver and spleen was observed suggesting the normal clearance mechanism of modified RBCs. Animals gained normal weights and no abnormalities observed in necropsy analysis. The stability of the ester-amide linker between the RBC and HPG was evaluated by comparing the clearance rate of ^{3}H-HPG60K-RBC and PKH-26 lipid fluorescent membrane marker labeled HPG60K-RBCs. HPG modified RBCs combine the many advantages of a dendritic polymer and RBCs, and hold great promise in systemic drug delivery and other applications of functional RBC.

1. Introduction

Cell surface modification using hydrophilic polymers has a great potential in tissue engineering, stem cell research and embryology [1]. Functional cells have been investigated to control the host immune response in cell and organ transplantation, to investigate cell–cell interaction and division, and to construct systemic and targeted delivery vehicles of therapeutics to treat diseases such as cancer, vascular occlusion, and HIV [2]. Pancreatic islet surface modification has been investigated to eliminate immunogenicity by grafting or coating with PEG polymers [3–6]. In a recent study, Islets grafted with PEG have remained functional *in vivo* for 1 year when accompanied with the administration of low doses of cyclosporine, an immuno-suppressant [5]. To investigate cell–cell interaction and differentiation, others have modified cell surfaces by introducing lipid/PEG/oligoDNA or lipid/PEG/biotin on cell surfaces [1]. Cell–cell interaction was found to play a significant role in the stem cell differentiation and embryonic development [7,8].

RBC modification is another active area of research with the aims of developing universal blood donor cells and systemic and targeted delivery vehicles for therapeutics [2]. Cell surface grafting of polymers has been extensively investigated to protect major and minor RBC surface antigens [9,10]. Techniques that are used to modify the surface of RBCs with hydrophilic polymers include covalent conjugation [10], adsorption [11], and electrostatic interaction [12]. In the case of RBC modification, covalent conjugation is advantageous in terms of resisting degradation and transportation stresses during the 120 day life span of human RBCs, during which RBCs travel ~250 km [2]. These methods have resulted in various levels of antigen protection. *In vivo* studies using PEG-grafted RBCs

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revealed that PEG-modified RBCs have normal circulation below certain grafting concentrations [9,10]. However, no information is currently available on the number of PEG molecules grafted or the distribution or clearance of PEG-modified RBCs.

The very long life span (120 days in humans), participation in biodistribution of molecules, the presence of a protective glycocalyx layer, and the natural mechanism of clearance from the body have made RBCs an interesting vehicle for drug delivery [2]. Surface modified RBCs have been used for the delivery of tissue plasminogen activator [13] and urokinase plasminogen activator [14]. Hypertonic encapsulation of drugs such as adenosine deaminase [15], thymidine phosphorylase [16], and others in RBCs have reached clinical trials.

All these reports show the potential of cell surface modification to manipulate interactions of cells with proteins, other cells or to be used in drug delivery. Also, they substantiate the need for the development of biologically friendly functional cell surface modification methods to achieve this goal. Towards this, we have recently reported a method for the surface conjugation of functional dendritic polymers on RBC surfaces to camouflage cell surface antigens and to realize the full therapeutic potential of surface modified RBCs [17,18]. Hyperbranched polyglycerol (HPG) grafted RBCs showed excellent *in vitro* properties [18] and the compact and functional nature of HPG has many advantages over linear polymers.

Currently there is limited knowledge available on the amount of polymer grafted (PEG or HPG) on the surface of RBCs other than the solution concentrations used for the modification. Also, no data is available on the clearance of modified RBC from circulation and the biodistribution of polymer modified RBCs. Splenic macrophages are the main homeostatic regulators of senescent red blood cells [19]. The liver and the rest of reticuendothelial system (RES) participate to a lesser extent in the removal of senescent red blood cells [20,21]. As cells age, they undergo physiological changes that trigger the *in vivo* death signaling pathways, the exact mechanism of which remains not yet completely determined [22]. In the case of modified red blood cells, high levels of modification with streptavidin/biotin (260,000 molecules per RBC) have led to a rapid complement mediated lysis in the circulation. With lower levels of modification with streptavidin/biotin (90,000 molecules per RBC), RBCs were not cleared as rapidly, being removed by both complement mediated lysis and opsonization of RBCs and their uptake by RES macrophages of the spleen and liver [23]. Red blood cells grafted with 13,400 and 27,000 molecules of biotin per cell yielded functional RBCs that have a life span of 50 days similar to control RBCs [24]. Whereas grafting of 126,000 biotin molecules per RBC has caused a moderate decrease in the circulation of functional RBCs [24].

In this manuscript, we report a method for measuring the number of surface grafted HPG molecules per RBC using tritium labeled HPG (^{3}H-HPG) and the influence of grafting on both *in vitro* properties and *in vivo* circulation. *In vitro* characteristics of HPG modified human RBCs were investigated by measuring electrophoretic mobility, complement mediated lysis, and osmotic fragility. The *in vivo* circulation and clearance of ^{3}H-HPG-grafted RBCs were investigated by injecting a single dose of ^{3}H-HPG grafted RBCs (mouse RBC) in mice, and measuring the radioactivity of isolated RBCs from mice at different time intervals as well as the radioactivity in plasma, whole blood, and organs that are responsible for the removal of senescent RBCs. The clearance of ^{3}H-HPG from the system was investigated by isolating a group of animals in a metabolic cage and measuring the radioactivity of urine and feces, and other organs in which polymer accumulation is likely. The stability of the HPG-RBC linker was investigated by comparing the circulation of ^{3}H-HPG-grafted RBCs to the circulation of membrane labeled HPG-grafted RBCs.

2. Materials and methods

Chemicals and solvents used in polymer synthesis and modification including glycidol, potassium methylate, 1,4- dioxane, succinic anhydride, 4- dimethylaminopyridine, N,N′-diisopropylcarbodiimide and N-hydroxysuccinimide (NHS), pyridine, dimethyl formamide, methanol, and acetone were purchased from Sigma Aldrich (ON, Canada). Trimethylolpropane (TMP) was obtained from Fluka (ON, Canada). All the reagents were used without purification unless mentioned. Glycidol was distilled and dried prior to use. Amberlite IR120 H-ion-exchange resin was purchased from Sigma Aldrich. Cellulose ester dialysis membranes were obtained from Spectra/Por Biotech (CA, USA). Slide-A-Lyzer Dialysis Cassettes were purchased from Thermo Scientific (IL, USA). Hydrogen peroxide (30%) was purchased from Fisher Scientific (NY, USA). Tissue solubilizer protosol was purchased from New England Nuclear, and Atomlight scintillation cocktail was purchased from Perkin Elmer (MA, USA). PKH-26 Fluorescent cell lipid membrane marker was purchased from Sigma Aldrich (ON, Canada).

The molecular weights of polymers were determined by gel permeation chromatography (GPC) using DAWN-EOS multi-angle laser light scattering and Optilab refractive index detectors (Wyatt Technology Inc., USA) in aqueous 0.1N $NaNO_3$ solution. A value of 0.120 for dn/dc for HPG was used for molecular weight determination [25]. ^{1}H NMR analysis was performed on a 300 MHz Bruker Avance using 20 mg/mL deuterated solutions of polymer at room temperature.

2.1. HPG synthesis and functionalization

HPG was synthesized by ring opening multi-branching anionic polymerization of glycidol [25,26]. The obtained polymer was precipitated in acetone and was dialyzed against water for 3–4 days (3.5–5 kDa MWCO cellulose membrane) with twice daily changes in water. The polymer was recovered by lyophilization. The purified polymer (M_n ~45, 000 g/mol and M_w/M_n - 1.6) was fractionally precipitated from methanol into acetone to obtain different molecular weight HPGs (Table 1). Fractionated HPGs were dialyzed to remove the residual solvents and lyophilized.

2.2. Synthesis of succinimidyl succinate functionalized HPGs

The measured hydrodynamic size of the HPG was used for the estimation of the effective molecular surface area as HPGs have a globular structure [25]. Accordingly, different molecular weights HPG were functionalized to have one succinimildyl succinate (SS) binding groups on every 20 nm^2 of the surface area (Table 1). Polymers were acid-functionalized initially by succinic anhydride according to a procedure modified from Rossi et al. [18] and Ye et al. [27]. For a typical reaction, lyophilized HPG60K (0.5 g, 0.0083 mmol) was placed in a round bottom flask and dried overnight under vacuum at 90 °C. Dried HPG was dissolved in dry pyridine (3 mL) and a catalytic amount of dimethylaminopyridine (one drop of 5 mg/mL solution in pyridine) was added. To this mixture, succinic anhydride (0.0067 g, 0.0664 mmol) dissolved in 0.5 mL pyridine was added drop wise over 10 min. The mixture was stirred overnight at room temperature under argon atmosphere. The polymer solution was precipitated in 40 mL of cold acetone in a 50 mL centrifuge tube, and centrifuged using a Beckman J2-MC centrifuge at 27,000 × g for 15 min. The solvent was decanted, and the residual acetone was removed by flushing with argon. To activate the carboxyl groups with SS groups, the acid-functionalized polymer was dissolved in 3 mL of anhydrous DMF. N-hydroxysuccinimide (0.0077 g, 0.0664 mmol) and N,N′-diisopropylcarbodiimide (0.0084 g, 0.0664 mmol) were added to the polymer solution and the mixture was stirred overnight at room temperature under argon. Succinimidyl succinate functionalized HPG was purified by precipitation in acetone as before. The purity of synthesized polymers and the degree of acid and SS functionalization were determined by NMR analysis.

^{1}H NMR of HPG: (300 MHZ, d-D2O, ppm) δ: 3.25–4.0 (m, HPG backbone $-CH_2O-$, -–CHO–).

^{1}H NMR of carboxylated HPG: (300 MHZ, d-MeOD, ppm) δ: 3.40–4.30 (m, HPG backbone $-CH_2O-$, –CHO–), 2.64 (s, $-CO_2CH_2CH_2CO_2-$).

^{1}H NMR of SS-HPG: (300 MHZ, d-MeOD, ppm) δ: 3.40–4.30 (m, HPG backbone –CH2O–, –CHO–), 2.87 (s, $COCH_2CH_2CO$, ppm), 2.8 and 3 (s, $CO_2CH_2CH_2CO_2$, ppm).

Table 1
The physical properties of HPG polymers. HPGs are spherical molecules and they were designed to have a reactive succinimidyl succinate (SS) binding group for every 20 nm^2 of the surface area.

Polymers	M_n (KDa)[a]	M_w/M_n[a]	R_h (nm)[b]	SA (nm^2)	OH groups	SS groups[c]
HPG20K	19.8	1.09	2.6	82.4	267	4
HPG60K	60	1.07	3.7	169.3	810	8
HPG 98K	98	1.09	4.3	232.40	1323	11

SA: surface area.
[a] Determined by GPC.
[b] Determined by PFG-NMR.
[c] Determined by ^{1}H NMR.

2.3. Radio-labeling of HPGs (^{3}H-HPGs)

A typical example is given. Lyophilized HPG60K (900 mg) was placed in a 25 mL round bottom flask and dried in an oil bath heated to 90–95 °C overnight in a vacuum line. The dried polymer was dissolved in 4.5 mL of N-methylpyrolidone, and sodium hydride (27 mg) was added to deprotonate approximately 10% of the hydroxyl groups; the mixture was stirred for 2 h. Tritium labeled methyl iodide (C^3H_3I) (0.0044 g, 0.031 mmol, 6.82×10^9 DPM) diluted in DMSO (3 mL), was added to methylate less than 1% of hydroxyl groups of HPG. The reaction was continued at room temperature for 24 h. After methylation, water (4.5 mL) was added and the mixture was placed in a 2000 MWCO dialysis cassette using a syringe. The polymer was dialyzed for 3 days to remove unreacted reagents. The radioactivity of the polymer solution and the volume was measured. The solution was lyophilized and dried. The specific activity is determined from these values (Table 2). Approximately 6% of the HPG was lost during the reaction and dialysis steps. Similar procedure was used for HPG20K and HPG98K. The acid functionalization and NHS activation of ^{3}H-HPGs were performed similar to cold HPGs given in section 2.2. Approximately 20–25% of polymers were lost during these steps.

2.4. Determination of hydrodynamic radius using the pulsed-field gradient NMR (PFG-NMR)

PFG-NMR experiments were performed using a Bruker Avance 400-MHZ spectrometer at 298 K, using D_2O as a solvent. T_1 spin/lattice relaxation, P_1 (90° pulse), δ (the width of a bipolar pair), and Δ (the diffusion time) were optimized, and the data was acquired using Ledbpgp2s, a pulse program developed by Bruker. The gradient strength (G) was varied from 1 to 32 G/cm in 16 steps. Each of the free induction decays was averaged over 8 scans, using 4 dummy scans. The diffusion coefficient was extracted using XWINNMR-3 software, by performing a non-linear square curve fitting to the relationship between echo attenuation and pulse field gradient, given by the following equation [28,29]:

$$\ln\left(\frac{A}{A_0}\right) = -D\left[\gamma^2 G^2 \delta^2 \left(\Delta - \frac{\delta}{3}\right)\right]$$

The hydrodynamic radius was calculated using the Stock's equation. For HPG, attenuation of ether backbone signals, located between 3.3 and 3.95 ppm were used to estimate the diffusion coefficient.

2.5. HPG grafting to red blood cells

Whole blood was collected from consented healthy human donors into citrate vacutainer tubes and centrifuged at 1000 × g for 4 min. The protocol was approved by University of British Columbia Clinical ethical committee. The supernatant containing plasma along with the buffy coat (intermediate layer containing white blood cells and platelets) were removed using a Pasteur pipette. RBCs were washed three times with saline and suspended in PBS buffer (pH 8). Immediately after precipitation (see Section 2.2), 100 mg of HPG60K was dissolved in 120 μL of PBS buffer. In a typical reaction to obtain 1 mM of HPG60K graft concentration, 88 μL of HPG60K solution was added to the RBC suspension to obtain a final volume of 650 μL and 20% hematocrit. The suspension was vortexed gently and placed on an orbital shaker at room temperature for 1 h. Cells were washed twice with PBS buffer and once with saline, and suspended in saline to obtain 20% hematocrit. HPG-grafted RBCs were characterized by measuring electrophoretic mobility, complement mediated lysis, and osmotic fragility.

2.6. In vitro characterization of HPG-grafted RBCs

2.6.1. Electrophoretic mobility

Electrophoretic mobility experiments were performed using the Rank Brothers Mark I Electrophoresis apparatus (Rank Brothers, Bottisham, UK). A drop of 20% hematocrit RBC suspension was diluted in 10 mL saline (0.15 M, PH 7.4) and the velocity of individual cells located in the stationary region was measured at a constant applied voltage [18]. Electrophoretic mobility was calculated using the following equation:

$$EM = \frac{\upsilon \times L}{V}$$

Where, υ is the velocity of individual cells, V is the applied voltage (40.5 V), and L is the effective electrical length of the chamber. At least 10 individual cells were selected randomly and their velocity was measured, results were presented as the mean ± standard deviation.

2.6.2. Complement mediated lysis

Fresh serum was prepared by collecting the whole blood from healthy human volunteers in serum tubes and allowing the blood to clot at room temperature for 30 min. The tube was then centrifuged at 2000 × g for 2 min and the serum was collected. To prepare the heat inactivated serum, the serum was placed in a water bath at 60 °C for 30 min. Complement mediated lysis was evaluated by exposing 20% hematocrit HPG modified RBCs in 50% fresh autologous serum for 1 h at 37 °C. To quantify the amount of cells lysed as a result of exposure to autologous or heat inactivated serum, the cell suspension and supernatant were treated separately with Drabkin's reagent and the amount of hemoglobin was quantified. Briefly, 5.9 μL of 20% hematocrit cell mixture was placed in a 96 well plate and 294 μL of Drabkin's reagent was added, and mixed. To determine the amount of hemoglobin in the supernatant, the cell suspension was centrifuged at 13000 × g for 1 min and 50 μL of the supernatant was placed in each well of a 96 well plate, and 250 μL of Drabkin's reagent was added and mixed. The absorbance of the hemoglobin cyanoderivative was determined in triplicate at 540 nm using SPECTRA MAX 190 plate reader. The percent of lysed cells was determined as the ratio of the hemoglobin concentration in the supernatant to the total hemoglobin concentration in cell suspension.

2.6.3. Osmotic fragility

The osmotic fragility of HPG-grafted RBCs was measured immediately after polymer grafting and after one day following a procedure reported previously [18].

2.7. Quantification of HPG grafting on red blood cells

2.7.1. ^{3}H-HPG grafting to red blood cells

A similar procedure described in Section 2.5 was used for grating ^{3}H-HPGs to red blood cells. ^{3}H-HPG20K, ^{3}H-HPG60K and ^{3}H-HPG98K were used at different concentrations.

2.7.2. Red blood cell digestion protocol for radioactivity measurements

^{3}H-HPG-modifed RBCs were digested to determine the activity associated with the RBCs. A modified version of the reported procedure [11]. Protosol (0.5 mL) and absolute ethanol (0.5 mL) were added to 10–100 mg of packed RBCs in eppendrof tube. The mixture was heated in a water bath at 60 °C for 1 h. The vials were cooled on ice and 0.5 mL of 30% of hydrogen peroxide was added to bleach the green color formed during the previous step. After 10–15 min, vials were placed at 60 °C for additional 60 min. Five hundred microliters of 0.5 M HCl, and 15 mL of scintillation fluid was added, and the activity was measured using Packard TRI-CARB 2100-TR liquid scintillation analyzer.

2.7.3. Determination of number of HPG molecules grafted onto RBC surface

The number of HPG molecules covalently grafted to RBC was quantified by comparing the activity of ^{3}H-HPG-grafted RBCs to the specific activity of HPG polymers (Table 2). The loss in HPG activity due to color quenching was ignored in the analysis. It was found that 80 μL of packed red blood cells incorporates less than 2% error in HPG activity evaluation due to color quenching (Fig. 1S). Furthermore, considering the narrow polydispersity index of prepared HPG polymers, it was assumed that the molecular weight and specific activity did not change during the synthesis and purification steps.

2.8. In vivo studies of HPG-grafted RBCs

^{3}H-HPG60K grafted mouse red blood cells were used for the studies. Study protocols were approved by University of British Columbia animal ethics committee.

2.8.1. Study protocol

Balb/c strain mice in the weight range 20–26 g were used in this study. On 0th day of the study, eighteen mice were terminated by CO_2 asphyxiation. Upon last breath, mice were removed from the inhalation chamber and approximately 500–700 μL of blood was collected from each mouse by cardiac puncture in heparin

Table 2
Specific activity and percent of HPGs covalently linked to RBCs at different graft polymer solutions concentrations. HPG polymers with different molecular weights were tritium labeled by partial methylation (~0.5%) of hydroxyl groups that were reacted with ^{3}H-methyl iodide.

Polymers	Specific activity	Percent polymer grafted to H-RBCs [%]			
	DPM/g	0.5 mM	1.0 mM	1.5 mM	3 mM
HPG20K	1.18×10^9	0.54 ± 0.05	0.55 ± 0.05	0.48 ± 0.12	0.52 ± 0.05
HPG60K	2.33×10^9	0.51 ± 0.05	0.42 ± 0.04	0.45 ± 0.05	N/A
HPG98K	1.16×10^9	0.35 ± 0.02	0.54 ± 0.07	0.65 ± 0.06	1.16 ± 0.13
HPG60K[a]	1.82×10^9	0.39 ± 0.01	N/A	N/A	N/A

[a] Mouse RBCs, used for in vivo study.

anticoagulant solution (20 U/mL) and the blood was pooled. Mouse whole blood (9 mL) was spun for 6 min at 1000 × g, and the RBCs were separated. RBCs were washed 3 times with saline and were suspended in a 50 mL Falcon tube in PBS buffer at pH 8. Tritium labeled NHS ester activated ^{3}H-HPG60K was dissolved in PBS buffer to obtain a grafting concentration of 0.5 mM, and was added to RBC suspension (20% hematocrit). To insure sterility, the experiment was conducted in a biological safety cabinet using sterile materials and containers. After the addition of HPG, the Falcon tube was placed on an orbital shaker, and the experiment was conducted at room temperature for 1 h ^{3}H-HPG-grafted RBCs were washed with saline three times to remove unreacted polymer. The radioactivity of the wash buffer was measured to insure the removal of unreacted polymer from RBC suspension. After the last wash ^{3}H-HPG60K grafted mouse RBCs were suspended in PBS buffer (pH = 7.4) to obtain 50% hematocrit and injected in the tail vein of mice at a dose of 200 μL per 20 g of mouse.

2.8.2. Data collection

On days 1, 2, 3, 5, 10, 20, 30, and 50, three mice from each group were terminated and blood was collected via cardiac puncture. The radioactivity in whole blood, plasma and washed RBCs were measured. Upon termination, the liver, spleen, lung, kidney and heart were removed, weighed and processed for scintillation counting to evaluate the accumulation of HPG. Liver was homogenized to obtain 20% homogenate in water using a Polytron tissue homogenizer. Aliquots of 200 μL of homogenate were transferred for scintillation counting. Other organs were digested and analyzed using a protocol similar to the RBC digestion protocol described earlier.

The group of animals that were sacrificed on day 10 were initially placed in a metabolic cage. The urine and feces of these animals were collected and pooled on days 1, 2, 3, (4 and 5), and on days 6 through 10 to measure the radioactivity excreted through urine and feces. From 500 μL of whole blood collected from each mouse, 100 μL was digested, and was used to measure the activity in whole blood. The remaining blood was centrifuged to separate RBCs from plasma. The radioactivity of plasma was evaluated by measuring the activity in 50 μL in triplicates. The RBCs were washed three times with 5 mL saline and divided into 3 parts, digested using a similar protocol described in section 2.7 and the radioactivity was measured.

2.8.3. Labeling RBCs with PKH-26 lipid marker

Washed packed mouse RBCs (1 mL) were suspended in Diluent C (5 mL), a component of the PKH-26 kit. PKH-26 (1 mM, 25 μL) was diluted in Diluent C (5 mL). Diluted PKH-26 was added to the cell suspension, while mixing gently in a vortex. The labeling reaction was performed on an orbital shaker at room temperature in dark for 4 min. To terminate the labeling reaction, saline solution (11 mL) supplemented with 1% BSA was added and mixed gently. The mixture was spun for 6 min at 1100 × g, cells were removed and washed three times with saline. PKH-26 labeled RBCs were divided into two parts, the first part was used as a control, and the second part was treated with 0.5 mM of succinimidyl succinate functionalized HPG60K in the presence of 7.5 mM of HPG33K neutral additive polymer that enhances the binding efficiency of HPG to RBC [17]. HPG-grafted PKH-26 labeled RBCs were washed 3 times with saline to remove non-grafted HPGs. On days 1, 2, 3, 7, 9, 14, 16, 21, 23, 28, 30, 35, 37 and 44, mice were weighed and 15 μL of blood from each mice in each group was collected in heparin anticoagulant solution (2 U/mL) by saphenous vein collection. Collected blood was diluted in 0.5 mL saline and analyzed using flow cytometry. Ten thousand events were acquired on the RBC gate, the percentage of green events and mean green intensity measured.

2.9. Statistics

All the data are presented as mean ± standard deviation unless otherwise mentioned. Unless mentioned, one-way ANOVA test was performed with Tuckey analysis using Origin 7.0. Paired comparisons were considered significant if $p < 0.05$.

3. Results

3.1. HPG grafting to RBCs

HPGs with three different molecular weights were synthesized and covalently linked to primary amines on lysine residues of proteins on the surface of RBCs using an ester-amide linker (Fig. 1). The polydispersities of the HPGs used were less than 1.1 (Table 1). The hydrodynamic sizes of the HPG20K and HPG60K as determined by PFG-NMR were less that of the reported thickness of the glycocalyx of RBCs (7.5–7.8 nm) [30,31](Table 1). Since HPGs are reported to have a globular structure in aqueous solution [25], the following criterion was used for the modification of hydroxyl groups with succinimidyl succinate (SS) groups to generate comparable surface binding activities. All three HPG molecules were functionalized so that on average each SS group is present per ~20 nm^2 of the surface of HPG. Based on this, we synthesized HPG20K, HPG60K, and HPG 98 K with 4, 8 and 11 SS groups respectively (Table 1). The numbers of SS groups was determined from the intensity of the SS methylene signal at 2.41 ppm relative to the intensity of methylene and methine signals of the ether backbone of HPG at 3.25–4.1 ppm from ^{1}H NMR analysis [18]. The variation in the target number of carboxyl units was less than 10%. The number of hydroxyl groups in each polymer was assumed to be equal to the degree of polymerization, calculated by dividing the number average molecular weight obtained from GPC with the molecular weight of glycidol (Table 1) [25,26]. Only a small fraction of hydroxyl groups was functionalized with SS groups; 1.5% in HPG20K and less than 1% in HPG60K and HPG98K.

3.2. Determination of the number of HPG molecules attached to RBC

The number of HPG molecules covalently grafted to human RBC was quantified from the specific activity of tritium labeled HPGs and the activity of ^{3}H-HPG grafted RBCs. To avoid donor variability, a single male donor was used for this experiment. The effects of molecular weights of the HPGs and polymer concentration on the number of HPG molecules grafted were investigated. Results are given in Fig. 2. The concentration given was for the polymer present in the grafting solution. The average numbers of covalently bound HPGs to RBC with grafting concentration were linear in the range of 0.5–3 mM for HPG20K, and 0.5–1.5 mM for HPG60K. In the case of HPG98K, it was linear between 0.5 and 1.5 mM, but increased significantly at grafting concentration of 3 mM (Fig. 2).

The average number of HPGs grafted on RBCs was increased for the highest molecular weight at relatively high grafting concentrations. When RBCs were treated with 3 mM solution of HPG20K, $(9.53 \pm 0.93) \times 10^5$ molecules were attached per RBC, compared to $(2.15 \pm 0.25) \times 10^6$ for HPG98K (Fig. 2). Below 1.5 mM grafting concentration, there was not much dependence of the molecular weights of the HPG on the number molecules grafted. These results were quite different from the solid surface grafting of linear polymers where the number of molecules grafted decreased almost exponentially with molecular weight of the chains [32] and is opposite to trend obtained in the adsorption of alkylated HPGs on RBCs [11]. The increase in the number of molecules grafted with increasing concentration may be due to enhanced cell surface grafting in presence of concentrated polymer solutions reported recently [17]. The amount of polymer present at 3.0 mM concentration for 98 K is more than 4 times compared to 20 K, so the grafting enhancement is more for 98 K HPG.

The percent of HPG molecules covalently linked to RBCs with respect to the total amount added was determined from the ratio of the activity of ^{3}H-HPG grafted RBCs to the activity of the polymer added to react with RBCs (Table 2). The activity of added polymer was calculated from the amount of added polymer and its specific activity. The percent of grafted polymer on RBC was very low around 0.5% in the linear grafting region, and it was 1.2% at grafting concentration of 3 mM in the case of HPG98K (Table 2).

3.3. In vitro characterization of HPG-grafted RBC

In order to determine the potential for *in vivo* survival of HPG-grafted RBCs, modified cells with different HPG molecular weights and graft concentrations were characterized by measuring the electrophoretic mobility, complement mediated lysis, and osmotic fragility.

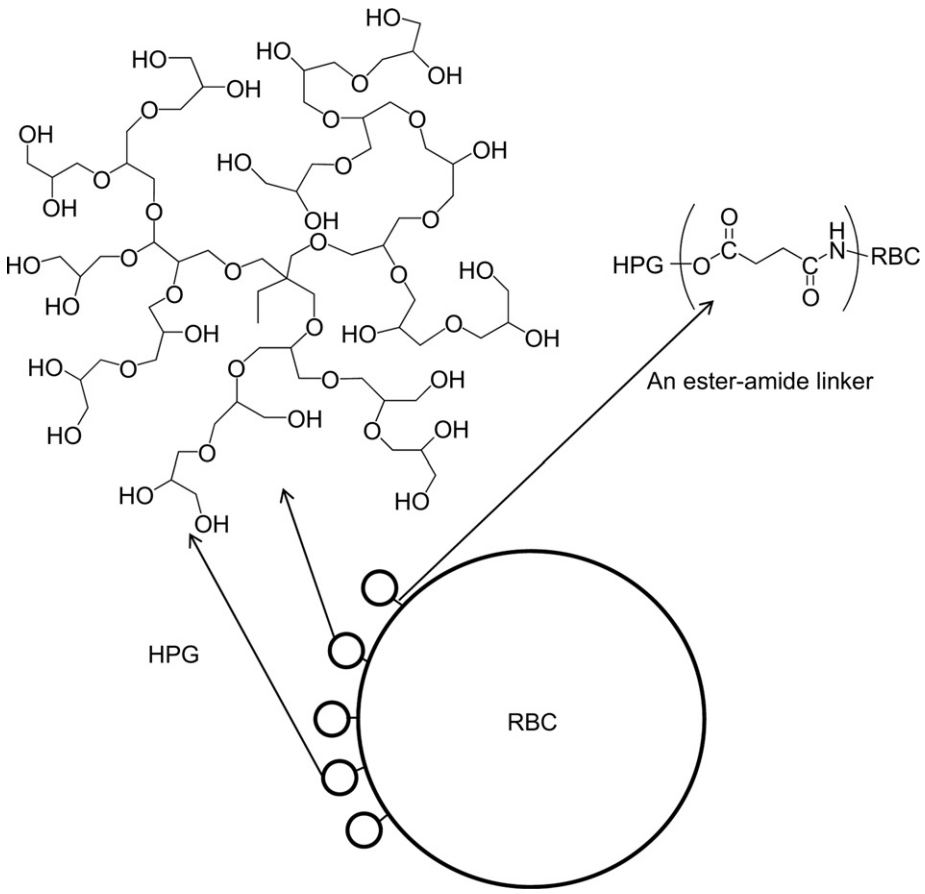

Fig. 1. Red blood cell surface modification by covalent attachment of globular hyperbranched polyglycerol polymers to surface proteins using an ester-amide linker.

3.3.1. Electrophoretic mobility (EPM)

Electrophoretic mobility of RBCs is governed by the surface charge density due to presence of negatively charged sialic acid on the surface, and by the hydrodynamic flow in the region of the double layer [31,33]. Surface charge density is a significant indicator of proper function of RBCs [34]. For example, RBCs that are deficient in glycophorin A and B that provide rich sialic acid structures to the RBC membrane are subject to agglutination and short circulation [35]. Results from EPM experiments indicated that HPG-grafted RBCs have reduced electrophoretic mobility (Fig. 3). The reduction in EPM with concentration appeared exponential, faster

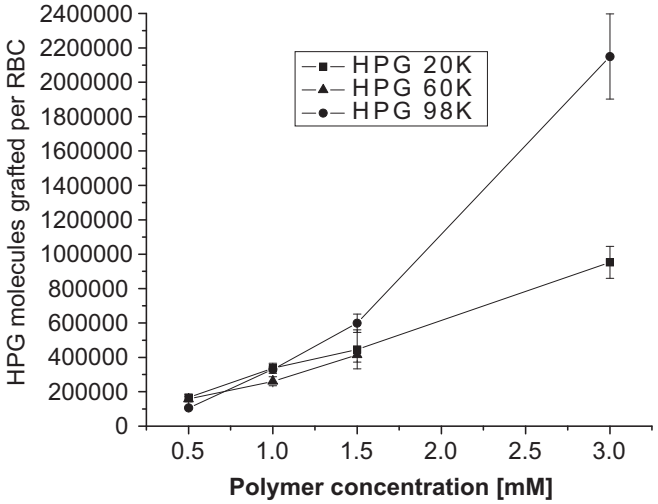

Fig. 2. Effect of molecular weight and polymer concentration on the number of HPG molecules covalently linked to RBC at different polymer concentrations and molecular weights.

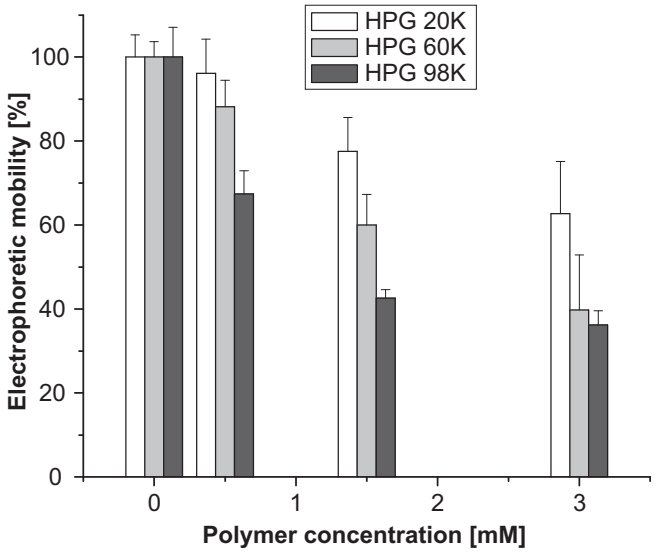

Fig. 3. Effect of molecular weight and polymer concentration on the electrophoretic mobility of HPG-grafted RBCs at different graft polymer solution concentrations and molecular weights.

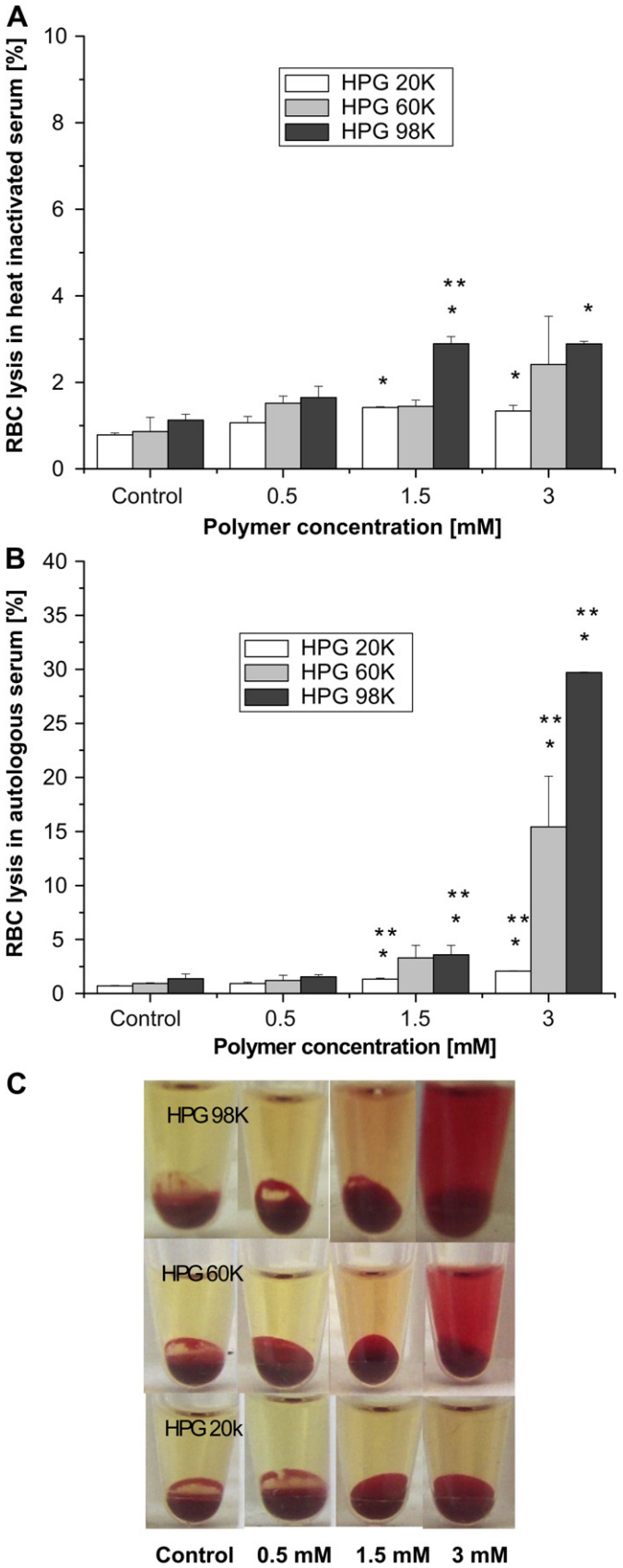

Fig. 4. A) Effect of molecular weight and polymer concentration on the lysis of HPG-grafted RBCs in 50% heat inactivated serum, treated for 1 h at room temperature. B) Effect of molecular weight and polymer concentration on the lysis of HPG-grafted RBCs in 50% fresh autologous serum, treated for 1 h at room temperature. C) Images of HPG grafted RBCs in 50% heat fresh autologous serum treated for 1 h at room temperature. * indicates to samples that are statistically different from the control, ** indicates to samples that are statistically different from the previous concentration of the same molecular weight.

initially, and it became slower with increase in number of HPG-grafted molecules. The grafting of high molecular weight HPG decreased EPM more than the low molecular weight HPGs even though the numbers of molecules grafted per RBCs were similar (Figs. 2 and 3). This data suggest that drag exerted by high molecular weight HPG is higher than low molecular weight HPG as expected.

3.3.2. Complement mediated lysis of HPG-grafted RBCs

Introducing new materials to cells and biological surfaces renders them foreign and subject to immune system mediated lysis and clearance [23]. To investigate whether HPG-grafted RBCs are subject to complement mediated lysis, cells were treated with human autologous serum and heat inactivated serum and percent of lysed RBCs were determined.

RBCs grafted with HPG20K and HPG60K underwent no or very little lysis in heat inactivated serum at grafting concentrations of 0.5, 1.5, and 3 mM, where up to 800,000 HPG molecules were attached. RBCs grafted with larger HPG98K lysed at slightly greater percent, $2.9 \pm 0.17\%$ at grafting concentration of 1.5 and $2.9 \pm 0.06\%$ at grafting concentration 3 mM, compared to $1.1 \pm 0.13\%$ of control RBCs.

In autologous human serum, a different lysis behavior was observed. In the case of HPG20K, lysis in autologous serum was similar to lysis in heat inactivated serum, where no or very little lysis was observed in all three polymer grafting concentrations (0.5, 1.5, and 3) mM, where up to $(9.53 \pm 0.93) \times 10^5$ molecules were grafted. Similarly to HPG20K, HPG60K and HPG98K lysed to at most a modest amount more than the control, when the grafting concentration was 0.5 and 1.5 mM. At grafting concentration of 3 mM, however, HPG60K and HPG98K-grafted RBCs underwent significant lysis, possibly the result of complement mediated lysis; $15.5 \pm 4.7\%$ of HPG60K grafted RBC, and $29.7 \pm 0.05\%$ of HPG98K-grafted RBCs were lysed (Fig. 4B). The supernatant of the grafted RBCs treated with serum was red at 3 mM graft concentration for both HPG60K and HPG98K (Fig. 4C).

Our group has reported that RBCs functionalized with HPG26K (4.8 mM) undergo only 0.5% lysis in autologous serum [18]. Similar results were found in the current study with the smallest HPG molecule; red blood cells grafted with $(9.53 \pm 0.93) \times 10^5$ molecules of HPG20K underwent 2.1% lysis when treated with autologous serum. However, unlike HPG20K, red blood cells functionalized with larger HPG molecules were susceptible to significant complement mediated lysis when they were grafted with similar numbers of HPG molecules (Fig. 4B), suggesting an importance of the molecular size.

3.3.3. Osmotic fragility of HPG-grafted RBCs

The osmotic fragility experiment indicates whether mechanical properties and deformability of RBC membrane has been compromised as a result of HPG grafting. In this experiment modified RBCs are exposed to deformation stress by treating with different concentrations of NaCl, and quantifying percent of lysed cells. Osmotic fragility experiments indicated that molecular size and number of HPG molecules grafted to RBC play significant role in modifying the membrane mechanical properties of RBCs. For example grafting of 6.4×10^{-6} ng of HPG20K per RBC (1.67×10^5 molecules) and 13×10^{-6} ng of HPG60K per RBC (1.57×10^5 molecules) did not alter RBC membrane properties whereas

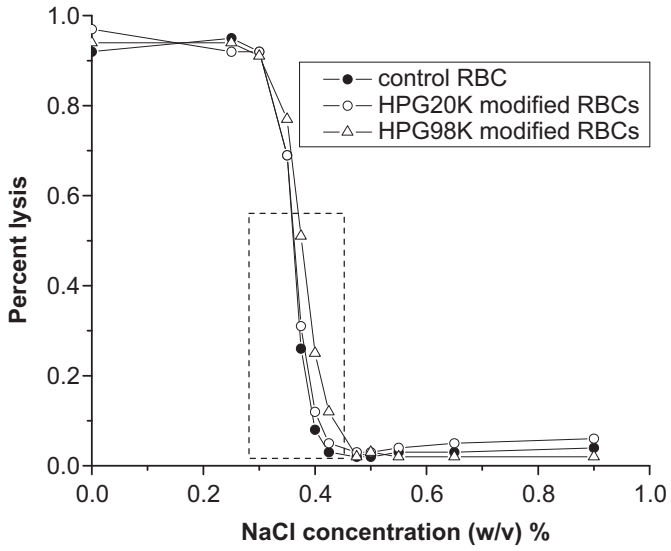

Fig. 5. Impact of molecular weight of HPG on osmotic fragility of RBCs. HPGs were grafted at ~150,000 molecules per RBC. The dashed rectangle indicates to the NaCl concentration region, where a HGP98K grafted RBCs were subject to significant lysis.

grafting of 17.3 $\times$ 10^{-6} ng of HPG98K per RBC (1.06 $\times$ 10^5 molecules) altered RBC membrane properties (Fig. 5 and Table 1S), suggesting that the weight of the molecules grafted is altering membrane stability. However, data from heavily grafted low molecular weight HPG (12.9 $\times$ 10^{-6} ng) of HPG20K (3.37 $\times$ 10^5 molecules) and high molecular weight HPG60K (13 $\times$ 10^{-6} ng grafted; 1.57 $\times$ 10^5 molecules) suggest that over grafting is more damaging than the weight of the HPG grafted (Table 1S). The weight of HPGs grafted to RBC is presented in Table 2S.

4. *In vivo* circulation and clearance of ^{3}H-HPG-grafted erythrocytes

After the *in vitro* analysis, HPG60K at 0.5 mM grafting concentration was selected for the *in vivo* survival studies. The radioactivity of mouse RBCs grafted at 0.5 mM grafting concentration of ^{3}H-HPG60K was slightly lower than that of the human RBCs grafted at similar polymer concentration, indicating that human RBC were slightly more functionalized (Table 2). Injected ^{3}H-HPG60K-RBCs were diluted around 10 times when injected at a dose of 200 μl/20 g. A significant amount of ^{3}H-HPG60K-RBC was removed from circulation at day 1 (48.6 ± 4.5% of injected dose). The remaining ^{3}H-HPG-RBC circulated for more than 50 days similar to the life span of normal mouse RBCs (50 days) [10]. At day 50, 6.1 ± 1.9% of ^{3}H-HPG-RBCs were still present in circulation. The initial fast clearance is most probably due to the removal of senescent RBCs and cells that were damaged cells during the modification. Modified cells that remained in circulation after day 1 showed no appreciable lysis or stability issues and behaved similarly to unmodified RBCs. The presence of radioactivity associated with the isolated washed RBCs following the i.v. injection of ^{3}H-HPG60K at different post-injection days (up to 50 days) indicated (Fig. 6) that ^{3}H-HPG grafted to RBCs are stable. We have previously shown that unmodified HPGs have a minimal absorption on the surface of human RBCs and the current data support the stability of the covalent conjugation [11]. The amount of radioactivity detected in whole blood and in plasma is presented in Fig. 7. Values are presented as μg of ^{3}H-HPG60K per RBC, and as a percent of the injected dose, calculated assuming 2.5 g of blood is present in mice [36]. The percent of radioactivity detected in whole blood was slightly greater than the percent activity detected in RBCs (Figs. 6 and 7). The difference is due to the presence of radioactivity in the plasma (Fig. 7). To investigate whether the degradation of the ester-amide linker and the shedding of ^{3}H-HPGs have contributed to radioactivity of plasma, the circulation of ^{3}H-HPG60K-RBC was compared to the circulation of HPG grafted to PKH-26 membrane labeled RBCs.

4.1. The stability of ester-amide linker in circulation

The *in vivo* circulation of membrane labeled HPG-grafted RBCs showed a similar profile to RBCs carrying radiolabeled HPGs (Fig. 8). After an initial rapid clearance of some of the HPG-grafted cells (~45%), the remaining modified RBCs circulated for 50 days in a pseudo linear fashion similar to normal mice RBCs [9]. The results suggest that HPGs and RBCs from HPG-RBCs in this cohort were cleared from circulation together and there was no independent clearance of each fraction. The results also indicate that the ester-amide linker between HPG and RBC is stable in circulation and initial rapid decrease and subsequent slow clearance of ^{3}H-

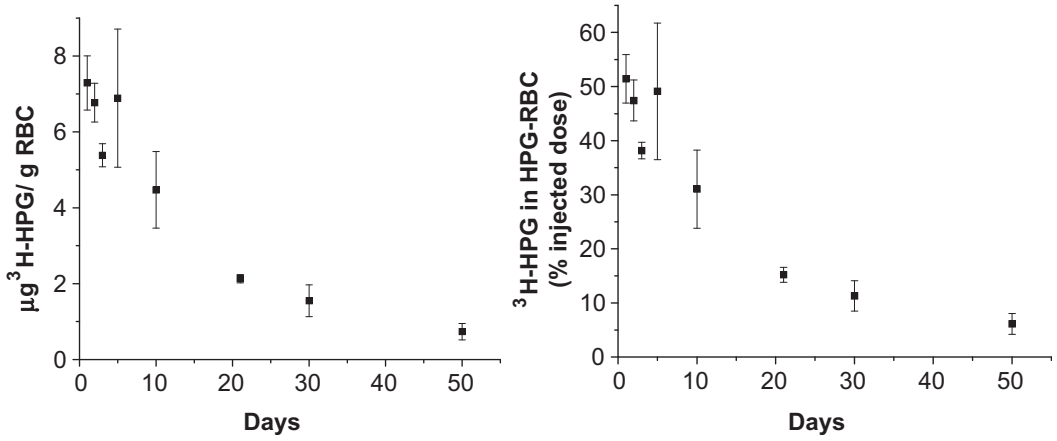

Fig. 6. The amount of ^{3}H-HPG present on washed RBCs with respect to time and corresponding percent of injected dose. RBCs were functionalized by treating with 0.5 mM of HPG60K solution for 1 h at room temperature, and injected in the tail vein of mice at a dose of 200 μl per 200 g animal. RBCs were digested prior radioactivity counting. The data represent the average from three animals and error bars represent the standard deviation around the mean. The specific activity of ^{3}H-HPG60K is 1.82 $\times$ 10^9 DPM/g. Percent of injected dose calculations assumed mice with ~2.5 g of blood.

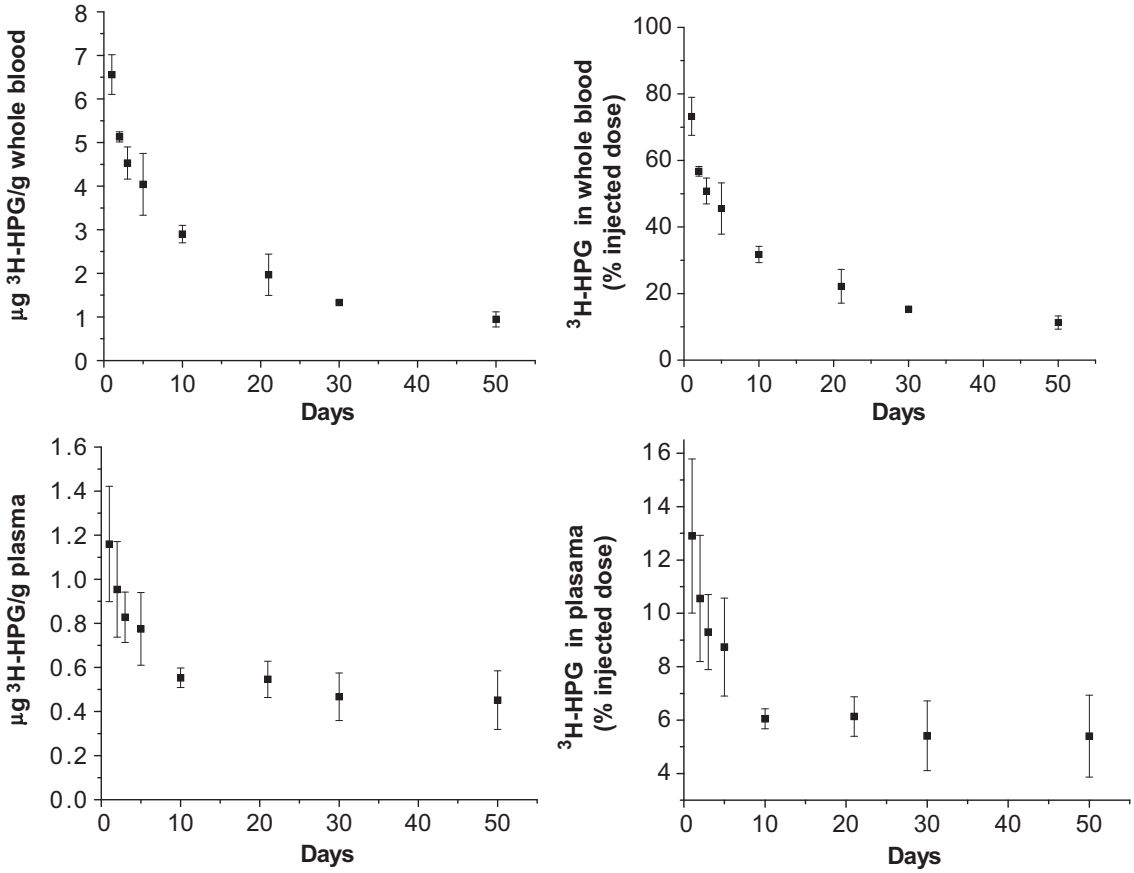

Fig. 7. The amount of ^{3}H-HPG in blood and plasma with respect to time and corresponding percent of injected dose. RBCs were functionalized by treating with 0.5 mM of HPG60K solution for 1 h at room temperature, and injected in the tail vein of mice at a dose of 200 μl per 200 g animal. Blood were digested prior radioactivity counting. The blood and plasma data represent the average of three animals and error bars represent the standard deviation around the mean. The specific activity of ^{3}H-HPG60K is 1.82×10^{9} DPM/g. Percent of injected dose calculations assumed mice with ~2.5 g of blood.

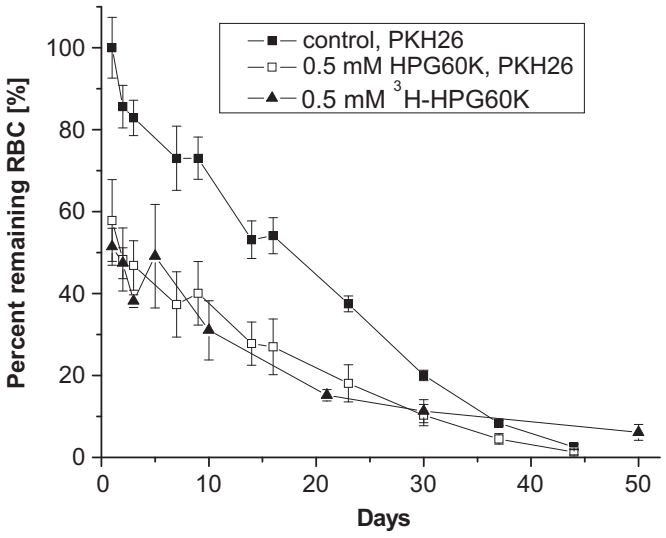

Fig. 8. Stability of HPG polymer on the surface of RBC evaluated by comparing the clearance of ^{3}H-HPG-RBC to the clearance of PKH-26 fluorescent membrane labeled and HPG-grafted RBCs. Similar clearance profiles indicate that ester-amide linker is stable and HPG remain on the surface of RBCs during 50 days of circulation. PKH-26 labeled RBCs were derivatized by treating with 0.5 mM of HPG60K solution in the presence of 7.5 mM of HPG33K additive polymer for 1 h at room temperature, and injected in the tail vein of mice at a dose of 200 μl per 200 g animal. The data of PKH-26 labeled RBCs represent the average of four animals and error bars represent the standard deviation around the mean.

HPG60K-RBC (Fig. 6) is due only to natural clearance of RBCs and not due to the cleavage of HPGs from RBCs.

4.2. Biodistribution

Upon termination, the spleen, liver, lungs, kidneys, and heart from mice were removed, weighed, and digested for radioactivity measurements. The data was not corrected for the residual plasma and red blood cells present in these organs.

The highest radioactivity was detected in the spleen and liver, the organs responsible for the removal of senescent blood cells. The amounts of radioactivity detected in the spleen and liver, calculated as a percent of the injected dose at various time points, are presented in Fig. 9; 18.2 ± 1.4% of the injected dose was detected in the spleen and 10.8 ± 1.9% of injected dose was detected in the liver at day 1. The activity detected in the liver decreased and remained constant after day 10, being 5.5 ± 0.7% of injected dose at day 50. The activity detected in the spleen was at the highest at day 3 (21% ± 1.9 of injected dose) and decreased with time, being 5.3 ± 0.5% of injected dose at day 50 (Fig. 9).

The activity detected in other organs was significantly lower than the activity detected in the spleen and liver. The activity in all tested organs decreased with respect to time, the activity in the lungs and kidney was around 3% of the injected dose at day 50, and it was 1% of the injected dose in the heart (Fig. 7). The amount of ^{3}H-HPG detected in kidneys was around 5% initially, and it was 3 ± 0.3% at day 50 of the study. The comparable hydrodynamic size

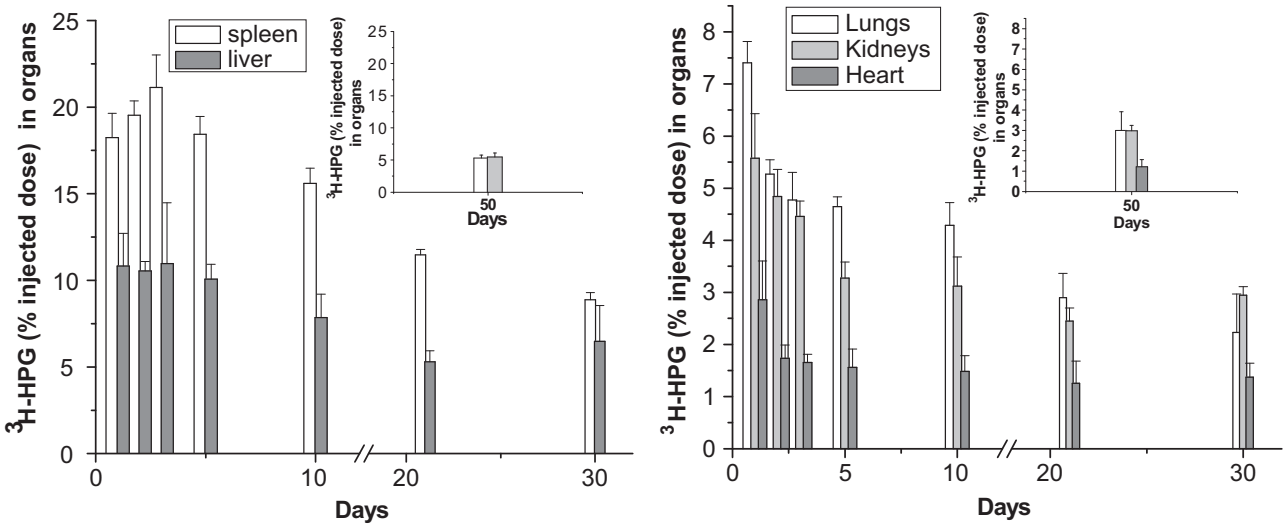

Fig. 9. Percent of ^{3}H-HPG injected dose detected in the spleen, liver, lungs, kidneys and heart at different time points. Organs were digested prior radioactivity evaluation. The data represent the average of three animals and error bars represent the standard deviation around the mean.

of HPG60K (3.7 nm) to the effective glomerular filter pore size (3.7–3.8 nm) [37,38], likely prevented the accumulation of HPGs in the kidneys. The stability of ^{3}H-HPG on the surface of RBCs (Fig. 8), the low activity in plasma (Fig. 7), and high activity detected in RBC removing organs (Fig. 9) suggest the normal clearance of the HPG-grafted RBCs. The removal of ^{3}H-HPG60K through urine and feces was investigated by locating a group of animals in a metabolic cage, and analyzing the radioactivity of urine and feces collected at various time points (Table 3). An equal amount of activity was removed from urine and feces at day 1 (~3% of the injected dose). During the first 10 days, 2 times more activity was removed through feces (25.3% of the injected dose) compared to the urine (12% of the injected dose).

A normal weight gain was observed in recipient mice during the study (Fig. 1S, supporting information). No abnormalities were observed in the necropsy analysis of thymus, spleen, and different nodes such as the pancreatic, renal brachial and others, when mice were terminated on day 50.

5. Discussion

The number of HPG molecules covalently grafted to RBCs ranged from 1×10^5 to 2×10^6 molecules/RBC (Fig. 2). Regardless of the molecular weight of grafted HPGs, a comparable number of molecules were attached to RBCs at low grafting concentrations (Fig. 2). This indicates that HPGs with different hydrodynamic sizes have comparable binding activity when the numbers of surface binding units were normalized with respect to the surface area. The number of polymer molecules grafted to RBCs in this study was comparable to the number of biotin molecules covalently grafted per RBC (1.26×10^5) [24] or to the number of streptavidin/biotin molecules grafted per RBC (5×10^5) [23].

Being hydrophilic, only a small fraction of HPG molecules were covalently linked to the surface of RBCs in spite of its large solution concentration. The percent grafted was generally around 0.5%, and it was 1.2% of total amount added in the case of viscous, high molecular weight HPG98K (Table 2). A similar phenomenon of enhanced grafting in the presence of viscous polymer solution was reported by Rossi et al. [17].

The *in vitro* HPG-RBC characterization experiments indicated that the size and the number of grafted molecules are important parameters to be considered when designing long circulating HPG-RBC functional RBCs. HPGs with smaller hydrodynamic size cause less alteration to RBC membrane mechanical properties, and trigger minimum complement activation. The smaller HPG molecules have a hydrodynamic size less than the thickness of the glycocalyx (~7.5–7.8 nm [30,31]), and are possibly grafted within the hairy glycocalyx, thus reducing the interaction of HPGs with biological surfaces. Importantly smaller HPGs have significantly lower content of hydroxyl groups per molecule which could play a significant role in reducing complement activation (Table 1). It was shown that polymers such as dextran and PVA with large number of hydroxyl groups activate the complement system when bound to surfaces [39]. HPG20K triggered minimal complement activation at grafting concentration of 3 mM, despite introducing mechanical changes to the cell membrane (Table 2S). HPG60K, on the other hand, caused significant complement mediated lysis at 3 mM as well as causing comparable mechanical changes to that of HPG20K (Table 1S). Thus, the greater number of hydroxyl groups in HPG60K and HPG98K and the large hydrodynamic size may have played a role in complement activation. *In vivo*, however, the survival of polymer grafted RBCs is contingent on the availability of self-proteins on the surface of RBCs [40]. We are currently investigating this aspect to understand the complement mediated lysis of HPG-RBCs.

RBCs that were presumably damaged during the HPG modification step were removed from circulation by day 1. This is based on the fact that PKH labeled control RBCs had a difference clearance profile than HPG-grafted RBCs (Fig. 8). The low activity detected in the plasma (18% of the activity of blood) at day 1 suggests that ^{3}H-

Table 3
Amount of ^{3}H-HPG (% injected dose) released through urine and feces of animals located in a metabolic cage.

Days	HPG in urine [%]	HPG in feces [%]
1	3.12	3.2
2	0.81	2.7
3	0.45	2.3
5	0.29	5.2
10	7.3	11.9

Error bars are not provided since urine and feces of three animals located together in a metabolic cage were collected and analysed.

HPG-RBCs were removed through nonlytic fixation of complement factors and consequent removal by splenic and hepatic macrophages as reported in the case of RBCs modified with biotin and streptavidin by Muzykantov et al. [23]. This is also supported by the high initial activity observed in the spleen and liver. The presence of activity in the plasma could not be due to ^{3}H-HPG being shed off the surface, rather it may be due to a mild complement mediated lysis or to the remaining radioactive metabolites of the liver, spleen and other organs returning to the circulation. Further investigation is needed to confirm these arguments. The amide-ester linker between the RBC and HPG remained protected from enzymatic degradation as evident from the presence of HPG-RBC in circulation for 50 days as well almost identical clearance of membrane labeled and HPG labeled HPG-grafted RBCs (Fig. 8). This is supported by the *in vivo* circulation reported for PEGylated polyester core dendrimers; the polyester core underwent no hydrolytic or enzymatic degradation being in circulation for longer than 2 days [41].

RBCs at grafting levels of 1.0×10^5 HPG molecules per RBC have a great potential in vascular drug delivery applications [2]. HPG-RBCs with a remarkable long circulation in mice have a great potential in targeted drug delivery and modified cell applications. Future studies will concentrate on eliminating the initial rapid clearance of HPG-RBC, and investigating the circulation of proteins conjugated to HPG-RBC.

6. Conclusions

The number of covalently grafted HPG molecules to RBCs ranged from 1×10^5 to 2×10^6 molecules. Being hydrophilic, only a small portion of HPG (0.5–1.2%) used were linked to RBCs. The size and number of HPG-grafted molecules are parameters that need to be considered in the design of long circulating HPG-RBC functional RBCs. *In vitro* characterization studies indicated that the grafting of $\sim 1.5 \times 10^5$ of HPG20K and HPG60K molecules per RBC yields functional RBCs that have similar complement mediated lysis, osmotic fragility, and electrophoretic mobility to that of control RBCs. The ester-amide linker between HPG and RBC was stable *in vivo*, and it did not undergo hydrolysis. *In vivo* circulation experiments indicated that a large fraction (>50%) of HPG-grafted RBCs are functional, and remained in circulation for more than 50 days. The presence of low radioactivity in the plasma on day 1 indicates that ^{3}H-HPG-RBC did not undergo immediate complement mediated lysis in circulation. The initial high radioactivity in spleen and liver indicates that ^{3}H-HPG60K-RBCs are being removed by the RES macrophages of the spleen and liver, similar to the normal RBC clearance mechanism. Minimal radioactivity was accumulated in organs other than liver and spleen. Animals gained weight normally and no abnormalities were observed in necropsy. HPG functionalized RBCs have great potential in the systemic delivery of therapeutics, biomedical applications of functional cells and in blood transfusion.

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Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2011.12.053.

References

[1] Teramura Y, Iwata H. Cell surface modification with polymers for biomedical studies. Soft Matter 2010;6:1081–91.
[2] Muzykantov VR. Drug delivery by red blood cells: vascular carriers designed by mother nature. Expert Opin Drug Deliv 2010;7:403–27.
[3] Hume PS, Bowman CN, Anseth KS. Functionalized PEG hydrogels through reactive dip-coating for the formation of immunoactive barriers. Biomaterials 2011;32:6204–12.
[4] Kizilel S, Scavone A, Liu XA, Nothias JM, Ostrega D, Witkowski P, et al. Encapsulation of pancreatic islets within nano-thin functional polyethylene glycol coatings for enhanced insulin secretion. Tissue Eng Part A 2010;16:2217–28.
[5] Lee DY, Nam JH, Byun Y. Functional and histological evaluation of transplanted pancreatic islets immunoprotected by PEGylation and cyclosporine for 1 year. Biomaterials 2007;28:1957–66.
[6] Salvay DM, Rives CB, Zhang XM, Chen F, Kaufman DB, Lowe WL, et al. Extracellular matrix protein-coated scaffolds promote the reversal of diabetes after extrahepatic islet transplantation. Transplantation 2008;85:1456–64.
[7] Beier JP, Bitto FF, Lange C, Klumpp D, Arkudas A, Bleiziffer O, et al. Myogenic differentiation of mesenchymal stem cells co-cultured with primary myoblasts. Cell Biol Int 2011;35:397–406.
[8] Talavera-Adame D, Wu G, He Y, Ng TT, Gupta A, Kurtovic S, et al. Endothelial cells in co-culture enhance embryonic stem cell differentiation to pancreatic progenitors and insulin-producing cells through BMP signaling. Stem Cell Rev 2011;7:532–43.
[9] Murad KT, Mahany KL, Brugnara C, Kuypers FA, Eaton JW, Scott MD. Structural and functional consequences of antigenic modulation of red blood cells with methoxypoly(ethylene glycol). Blood 1999;93:2121–7.
[10] Bradley AJ, Murad KL, Regan KL, Scott MD. Biophysical consequences of linker chemistry and polymer size on stealth erythrocytes: size does matter. Biochim Biophys Acta 2002;1561:147–58.
[11] Liu ZH, Janzen J, Brooks DE. Adsorption of amphiphilic hyperbranched polyglycerol derivatives onto human red blood cells. Biomaterials 2010;31:3364–73.
[12] Mansouri S, Merhi Y, Winnik FM, Tabrizian M. Investigation of layer-by-layer assembly of polyelectrolytes on fully functional human red blood cells in suspension for attenuated immune response. Biomacromolecules 2011;12:585–92.
[13] Murciano JC, Medinilla S, Eslin D, Atochina E, Cines DB, Muzykantov VR. Prophylactic fibrinolysis through selective dissolution of nascent clots by tPA-carrying erythrocytes. Nat Biotechnol 2003;21:891–6.
[14] Murciano JC, Higazi AAR, Cines DB, Muzykantov VR. Soluble urokinase receptor conjugated to carrier red blood cells binds latent pro-urokinase and alters its functional profile. J Control Release 2009;139:190–6.
[15] Bax BE, Bain MD, Fairbanks LD, Webster ADB, Ind PW, Hershfield MS, et al. A 9-yr evaluation of carrier erythrocyte encapsulated adenosine deaminase (ADA) therapy in a patient with adult-type ADA deficiency. Eur J Haematol 2007;79:338–48.
[16] Moran NF, Bain MD, Muqit MMK, Bax BE. Carrier erythrocyte entrapped thymidine phosphorylas etherapy for MNGIE. Neurology 2008;71:686–8.
[17] Rossi NAA, Constantinescu I, Brooks DE, Scott MD, Kizhakkedathu JN. Enhanced cell surface polymer grafting in concentrated and nonreactive aqueous polymer solutions. J Am Chem Soc 2010;132:3423–30.
[18] Rossi NAA, Constantinescu I, Kainthan RK, Brooks DE, Scott MD, Kizhakkedathu JN. Red blood cell membrane grafting of multi-functional hyperbranched polyglycerols. Biomaterials 2010;31:4167–78.
[19] Bennett GD, Kay MMB. Homeostatic removal of senescent murine erythrocytes by splenic macrophages. Exp Hematol 1981;9:297–307.
[20] Bowring CS, Ferrant AE, Glass HI, Lewis SM, Szur L. Quantitative measurement of splenic and hepatic red-cell destruction. Br J Haematol 1975;31:467–77.
[21] Ferrant A, Cauwe F, Michaux JL, Beckers C, Verwilghen R, Sokal G. Assessment of the sites of red-cell destruction using quantitative measurements of splenic and hepatic red-cell destruction. Br J Haematol 1982;50:591–8.
[22] Antonelou MH, Kriebardis AG, Papassideri IS. Aging and death signalling in mature red cells: from basic science to transfusion practice. Blood Transfus 2010;8:S39–47.
[23] Muzykantov VR, Murciano JC, Taylor RP, Atochina EN, Herraez A. Regulation of the complement-mediated elimination of red blood cells modified with biotin and streptavidin. Anal Biochem 1996;241:109–19.

[24] Suzuki T, Dale GL. Biotinylated erythrocytes - in vivo survival and in vitro recovery. Blood 1987;70:791–5.
[25] Kainthan RK, Muliawan EB, Hatzikiriakos SG, Brooks DE. Synthesis, characterization, and viscoelastic properties of high molecular weight hyperbranched polyglycerols. Macromolecules 2006;39:7708–17.
[26] Sunder A, Hanselmann R, Frey H, Mulhaupt R. Controlled synthesis of hyperbranched polyglycerols by ring-opening multibranching polymerization. Macromolecules 1999;32:4240–6.
[27] Ye L, Letchford K, Heller M, Liggins R, Guan D, Kizhakkedathu JN, et al. Synthesis and characterization of carboxylic acid conjugated, hydrophobically derivatized, hyperbranched polyglycerols as nanoparticulate drug carriers for cisplatin. Biomacromolecules 2011;12:145–55.
[28] Price WS. Pulsed-field gradient nuclear magnetic resonance as a tool for studying translational diffusion .1. basic theory. Concepts Magn Reson 1997;9:299–336.
[29] Zhang Y, Amsden BG. Application of an obstruction-scaling model to diffusion of vitamin B-12 and proteins in semidilute alginate solutions. Macromolecules 2006;39:1073–8.
[30] Hochmuth RM, Evans EA, Wiles HC, McCown JT. Mechanical measurement of red-cell membrane thickness. Science 1983;220:101–2.
[31] Levine S, Levine M, Sharp KA, Brooks DE. Theory of the electrokinetic behavior of human-erythrocytes. Biophys J 1983;42:127–35.
[32] Sofia SJ, Premnath V, Merrill EW. Poly(ethylene oxide) grafted to silicon surfaces: grafting density and protein adsorption. Macromolecules 1998;31:5059–70.
[33] Neu B, Armstrong JK, Fisher TC, BaumLer H, Meiselman HJ. Electrophoretic mobility of human red blood cells coated with poly(ethylene glycol). Biorheology 2001;38:389–403.
[34] Piagnerelli M, Boudjeltia KZ, Brohee D, Piro P, Carlier E, Vincent JL, et al. Alterations of red blood cell shape and sialic acid membrane content in septic patients. Crit Care Med 2003;31:2156–62.
[35] Daniels G. Human blood groups. Massachusetts, USA: Blackwell Science Ltd.; 1995.
[36] Fox ME, Szoka FC, Frechet JMJ. Soluble polymer carriers for the treatment of cancer: the importance of molecular architecture. Acc Chem Res 2009;42:1141–51.
[37] Oliver JD, Anderson S, Troy JL, Brenner BM, Deen WM. Determination of glomerular size-selectivity in the normal rat with ficoll. J Am Soc Nephrol 1992;3:214–28.
[38] Venkatachalam MA, Rennke HG. Structural and molecular-basis of glomerular-filtration. Circ Res 1978;43:337–47.
[39] Arima Y, Kawagoe M, Toda M, Iwata H. Complement activation by polymers carrying hydroxyl groups. Acs Appl Mater Interfaces 2009;1:2400–7.
[40] Bradley AJ, Scott MD. Immune complex binding by immunocamouflaged [poly(ethylene glycol)-grafted] erythrocytes. Am J Hematol 2007;82:970–5.
[41] Guillaudeu SJ, Fox ME, Haidar YM, Dy EE, Szoka FC, Frechet JMJ. PEGylated dendrimers with core functionality for biological applications. Bioconjug Chem 2008;19:461–9.