Biomaterials 35 (2014) 412–422

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Comparative efficacy of blood cell immunocamouflage by membrane grafting of methoxypoly(ethylene glycol) and polyethyloxazoline

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ARTICLE INFO

Article history:
Received 7 August 2013
Accepted 4 September 2013
Available online 25 September 2013

Keywords:
Immunocamouflage
Polyethyloxazoline
Methoxypoly(ethylene glycol)
Polymer
Red blood cell
Leukocyte

ABSTRACT

The grafting of low-immunogenic polymers to cells dramatically reduces antigenic recognition and immunogenicity of allogeneic donor cells consequent to steric and charge camouflage (*i.e.*, immunocamouflage). While methoxypoly(ethylene glycol) [mPEG] has historically been utilized for the immunocamouflage of cells, other low-immunogenic polymers such as polyethyloxazoline propionic acid (PEOZ) may also be capable of conferring immunoprotection. Moreover, PEOZ may have attributes that could have enhanced pharmacological and biological utility relative to mPEG. To evaluate the immunocamouflage efficacy of PEOZ relative to mPEG, human red blood cells (RBC) and leukocytes were modified with mPEG or PEOZ. The differential effects of mPEG and PEOZ was assessed via grafting efficacy, cell morphology and viability, immunocamouflage of surface antigens, and the prevention of *in vitro* immune recognition (RhD and HLA). Although membrane grafting of mPEG and PEOZ were similar, mPEG demonstrated superior immunocamouflage efficacy as measured by antibody binding and phagocytosis of opsonized RBC while PEOZ showed improved RBC morphology. While mPEG appears to be superior to PEOZ in the immunocamouflage of cells, PEOZ may still be a valuable addition to our repertoire of immunomodulatory polymers. Moreover, our results demonstrate the importance of indirect immunocamouflage of antigens found in membrane protein complexes.

1. Introduction

Covalent grafting of polyethylene glycol (PEG; PEGylation), and its derivatives such as methoxypoly(ethylene glycol) [mPEG], to biological substrates has been shown to prevent immunological recognition and prolong vascular retention [1–3]. Consequent to these effects, clinically approved PEGylated products (*e.g.*, polymer-modified proteins, microspheres, polypeptides, DNA and RNA) have been used, in some cases, for over 20 years [4–9]. More recently, PEGylation of intact, biologically viable cells, has demonstrated potential utility in transfusion and transplantation medicine where prevention of immune responses to allogeneic tissues is critical [10–18]. For example, PEGylation of human red blood cells (RBC) demonstrated the efficient immunocamouflage of non-ABO blood group antigens with no significant effects on cell structure or function at immunoprotective grafting concentrations. Moreover, polymer-modified murine RBC demonstrated normal *in vivo* survival [10,12,19]. The immunocamouflage imparted by the grafted polymer was not limited to RBC, as PEGylation of human or murine leukocytes prevented T lymphocyte dependent allorecognition both *in vitro* (human and mouse) and *in vivo* (mouse) [11,15,17,20]. Interestingly, PEGylation of mammalian host cells has also been shown to be a viable antiviral prophylactic strategy by preventing viral infections from a broad spectrum of enveloped and non-enveloped viruses *in vitro*. [21–23].

Despite the experimental and clinical success of PEGylation, some concerns exist as to the immunological recognition of PEG and the potential peroxidation of the PEG polymer itself leading to unwanted biological consequences. For example, a small subset of patients treated with PEGylated liposomal carriers demonstrated adverse hypersensitivity reactions [24], while other reports suggest that >25% of healthy blood donors had pre-existing antibodies to PEG using their methodology [25–28]. Moreover, accelerated blood clearance has been observed in a small percentage of patients chronically administered PEGylated products (*i.e.*, PEG-Asparaginase, PEG-liposomes) as part of their clinical treatment [27,29–31]. Despite these potential

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http://dx.doi.org/10.1016/j.biomaterials.2013.09.016

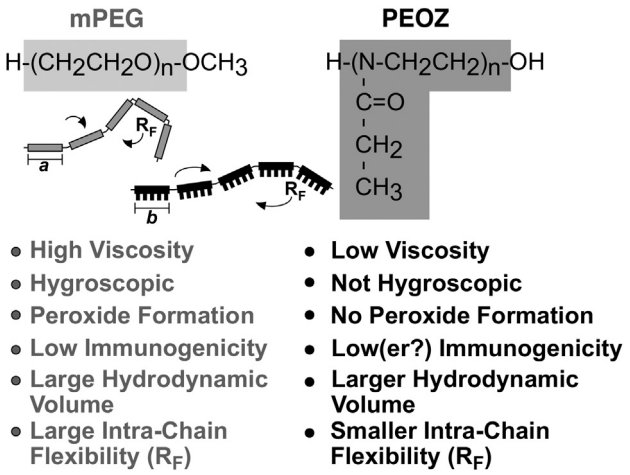

Fig. 1. Structure, chemical and biophysical characteristics of mPEG and PEOZ. The repeating structures of mPEG and PEOZ are denoted by the shaded areas. The (a) and (b) notations denote the independent rotational segments of mPEG and PEOZ (respectively) governing intra-chain mobility. The relative intra-chain mobility of the polymers, coupled with polymer size (e.g., 2 versus 20 kDa), underlies the radius of gyration of the grafted polymer. The relative radius of gyrations for mPEG and PEOZ is indicated by the arrows denoting the Flory radii (R_F; root mean square of end to end length of the polymer chain) of the polymers. As illustrated, the side-branches of PEOZ decrease intra-chain mobility producing a larger hydrodynamic volume relative to mPEG.

concerns, PEG remains the 'gold standard' for the immunocamouflage of biological components and allogeneic cells. However, a search for alternative polymers with improved pharmacological and biological properties relative to PEG may be beneficial in the development of new generations of immunocamouflaged proteins and cells.

Indeed, one of the first 'alternatives' to PEG was mPEG, as the substitution of the terminal hydroxyl (−OH) with a methyl ($-CH_3$) group yielded reduced biological reactivity. However, a wide variety of other non-PEG based experimental polymers such as hyperbranched polyglycerols (HPG) are being actively explored. For example, like mPEG, HPG has been used for the immunocamouflage of RBC blood groups [32–35]. Other polymers such as polyoxazolines may also be suitable for the induction of immunocamouflage. Polyoxazolines have historically been synthesized as food and cosmetic additives and have well-characterized toxicological profiles [36]. In contrast to PEG, polyoxazolines exhibit lower viscosity, increased stability and lowered degradation compared to current mPEG polymers [36]. However, limited or no information exists as to the *in vitro* and *in vivo* efficacy of polyoxazolines in inducing immunocamouflage of proteins, much less allogeneic cells [37].

In this study, we have examined the comparative efficacy of covalently grafted polyethyloxazoline (PEOZ) and mPEG on the direct and indirect immunocamouflage of human blood cells (RBC and leukocytes). The effects of polymer length and grafting density of PEOZ and mPEG on the immunocamouflage of blood group antigens (RhD and Kell) and RBC structure was assessed by the attenuation of antibody recognition and the prevention of phagocytic recognition. Moreover, the differential effects of PEOZ and mPEG grafting to leukocytes was evaluated by the immunocamouflage of cluster of differentiation (CD) markers, effect on cell viability and on the prevention of HLA-mediated allorecognition using mixed lymphocyte reactions (MLR).

2. Methods and materials

2.1. Cell collection and extraction

Whole blood was collected from healthy donors, following informed consent, into tubes containing sodium heparin. Peripheral blood mononuclear cells (PBMC) were isolated using histopaque (Sigma–Aldrich, St. Louis) according to manufacturer's protocol. For Packed Red Blood Cells (pRBC) collection, whole blood was washed 3× in PBS or saline and centrifuged for 5 min at 1000× *g*.

2.2. Polymer species and cell derivatization

Succinimidyl valerate activated mPEG (mPEG; 5, 20 and 30 kDa) was purchased from Laysan Bio Incorporated (Arab, AL, USA). N-hydoxysuccinimidyl ester of Polyethyloxazoline propionic acid (PEOZ; 20 and 30 kDa) was obtained from Serina Therapeutics Incorporated (Huntsville, AL, USA). The structures and characteristics of mPEG and PEOZ are shown in Fig. 1.

Isolated RBC and PBMC were resuspended to 12% hematocrit (5 g%Hb) and 4×10^6 cells/ml respectively in mPEG buffer (50 mM K_2HPO_4, 105 mM NaCl, pH 8.0) with desired amount of mPEG (5, 20, 30 kDa) or PEOZ (20, 30 kDa) to reach appropriate grafting concentration (0–4 mM). Cell suspensions (RBC or PBMC) were mixed by gentle inversion and reaction was performed for 1 h at room temperature as previously described [10–18]. RBC or PBMC were washed 3× with PBS or RPMI (Invitrogen, Carlsbad, CA, USA) respectively. PBMC were enumerated using a hemacytometer.

2.3. Microscopy

For morphology studies, freshly derivatized RBC (0–4 mM) were fixed in 4% methanol free formaldehyde and assessed using Zeiss Axioplan 2 (Micro-Optik, Deursen, Netherlands) or Olympus CK40 microscope (Olympus America Inc, Melville NY, USA). Transmitted light images were taken at 1000× magnification with Northern Ecilpse software or 200 and 400× magnification with Q-capture imaging software (v.2.8.1). For the mixed lymphocyte reaction (MLR) and viability studies, images were captured on Day 3, 7, 10 and 14 using Olympus CK40 microscope (Olympus America Inc, Melville NY, USA) fitted with Q-imaging camera (QICAMFAST Qimaging Corporation Surrey, BC, Canada). Transmitted light images were taken at 200 and 400× magnification with Q-capture imaging software (v.2.8.1).

2.4. Two-phase RBC partitioning and osmotic fragility

To quantitate the efficacy of polymer grafting, aqueous two-phase partitioning studies of control, mPEG or PEOZ-modified human RBC were performed as previously described [14,38]. In this system, polymer-modified RBC partition to the upper PEG layer, whereas unmodified or poorly modified cells partition to the lower Dextran layer or remain at the phase-interface. The two-phase system used consisted of 5% ww Dextran, 4% ww PEG8000, 0.15 mol/L NaCl and 6.84 mmol/L $NaHPO_4$. A 10 μl aliquot of the control RBC, mPEG-RBC or PEOZ-RBC suspension (5% Hb) was added to 0.5 ml of PEG rich phase, mixed then overlayed with the Dextran rich phase (0.5 ml) and mixed thoroughly by inversion. Phases were allowed to separate at room temperature for 20 min after which 250 μl was removed from the upper phase and washed 3× in PBS. Hemoglobin was assessed by Drabkin's assay [39]. The structural stability of the mPEG and PEOZ-modified RBC, relative to unmodified cells, was examined via osmotic fragility studies as previously described [10,12]. Samples were resuspended to 10% hematocrit and 100 μl of unmodified (0 mM) or polymer-modified RBC were added to microcentrifuge tubes containing 1 ml of the saline concentrations. Samples were mixed by inversion and processed immediately ($T = 0$) or incubated in a 37 °C water bath for 24, 48, and 68 h. Total and supernatant hemoglobin concentrations were determined at the indicated time points by Drabkin's reagent to determine percent lysis.

2.5. Monocyte monolayer assay (MMA)

The monocyte monolayer assay (MMA) was used to measure the effect of mPEG and PEOZ on immunologic recognition. The MMA measures FcγR-mediated phagocytosis *in vitro* using adherence-purified allogeneic monocytes isolated from PBMC [40–43]. RhD^+ or RhD^- RBC were incubated with OBS (negative control) or opsonized using a commercial anti-RhD antibody (Rho(D) Immune Globulin (Human) RhoGAM Ultra-Filtered PLUS; Ortho Clinical Diagnostics), washed and overlaid on the monocyte monolayer at 37 °C for 60 min. The number of adherent and phagocytosed control and opsonized RBC are enumerated per 100 monocytes (MI). Positive (*i.e.*, anti-D opsonized unmodified RhD^+ RBC) control values typically yields an MI of between 60 and 100. Using the MMA, MI values of ≤5–6 indicate that the donor cells can be given with minimal risk of an acute hemolytic reaction (though the MMA is less predictive of long-term circulation of donor RBC). Within the transfusion medicine community, the MMA is considered to be the best assay currently available for the *in vitro* evaluation of FcγR-mediated phagocytosis of antibody-coated human red cells, having more than 20 years of proven validity for the correlation of *in vitro* phagocytosis with *in vivo* clinical relevance. [44,45].

2.6. Surface marker analysis

Immunocamouflage of RBC RhD and Kell (k) blood group antigens and leukocyte Cluster of Differentiation (CD) surface marker analysis was determined via flow cytometry (FACSCalibur flow cytometer; BD Biosciences, San Jose, CA) following cell derivatization. For measuring the comparative efficacy of mPEG and PEOZ on the