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In vitro chelating, cytotoxicity, and blood compatibility of degradable poly(ethylene glycol)-based macromolecular iron chelators

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ABSTRACT

Desferrioxamine (DFO) is used to treat an excess accumulation of iron in the body and is currently the most commonly used iron chelator for the treatment of 'iron overload' disorder. However, the disadvantages of DFO surround its high toxicity and very short plasma half-life. Here, the detailed *in vitro* evaluation of a novel class of high molecular weight iron chelators based on DFO and polyethylene glycol methacrylate is reported. Reversible addition fragment chain transfer (RAFT) copolymerization afforded polymer conjugates (P-DFO) with well-controlled molecular weight (27–127 kDa) and substitution of DFO (5–26 units per chain) along the copolymer. Human umbilical vein endothelial cell (HUVEC) based cell viability assays showed that the cytotoxicity of P-DFO decreased more than 100-fold at identical concentrations of DFO. The hemocompatibilities of various P-DFO samples were determined by measuring prothrombin time (PT), activated partial thromboplastin time (APTT), thrombelastograph parameters (TEG), complement activation, platelet activation, and red blood cell aggregation. Furthermore, the iron binding properties and chelating efficiency of P-DFO were compared to DFO by measuring the spectral properties upon binding to iron(III), while the prevention of iron(III) mediated oxidation of hemoglobin was also determined. Degradation of the P-DFO conjugates via cleavable ester linkages between the polymer backbone and the PEG side chains was evaluated using gel permeation chromatography (GPC) and NMR. Since the chelating ability of DFO remains intact after conjugation to the copolymer backbone, these macromolecular, blood compatible and degradable conjugates are promising candidates as long circulating, non-toxic iron chelators.

1. Introduction

Under normal physiological conditions, iron metabolism and bioavailability are tightly controlled through the dietary absorption, trafficking, and recycling of iron within the body. Importantly, humans do not have an iron excretion pathway and therefore excess iron is usually stored primarily in the liver, spleen, and bone marrow in the form of ferritin molecules [1]. However, under certain pathological conditions, an excess accumulation of free iron in the body occurs as a consequence of enhanced dietary uptake (hemochromatosis), medical treatment (chronic blood transfusions), destabilized hemoglobin (sickle cell disease), reduced hemoglobin (thalassemia), or as a result of conditions such as cardiomyopathies, hepatic fibrosis and diabetes mellitus [2–5]. 'Iron overload' leads to the presence of bio-reactive iron and the iron-driven free radical oxidation of lipids, proteins, carbohydrates and nucleic acids [6].

To treat iron overload conditions such as transfusion-associated 'secondary iron overload', iron chelation therapy is often employed [3]. Currently, the most commonly used drug for iron chelation therapy is desferrioxamine (DFO): a bacterial siderophore isolated from *Streptomyces pilosus* [7–10]. Interest in DFO and several other iron chelators are also linked to their anti-proliferative activity against aggressive tumors in clinical trials for the treatment of neuroblastoma and leukemia [11–14]. Although DFO may be clinically effective, it is beset by many disadvantages, the most significant of which are its high toxicity and very short plasma half-life (~5.5 min) [15]. Other problems include the high cost of the drug [16] and the high hydrophilicity of the ligand resulting in it being poorly absorbed through the gastrointestinal tract [17]. Hence, more than 30 years of research on alternative low molecular weight [9,18] and, to a lesser extent, high molecular weight chelators have

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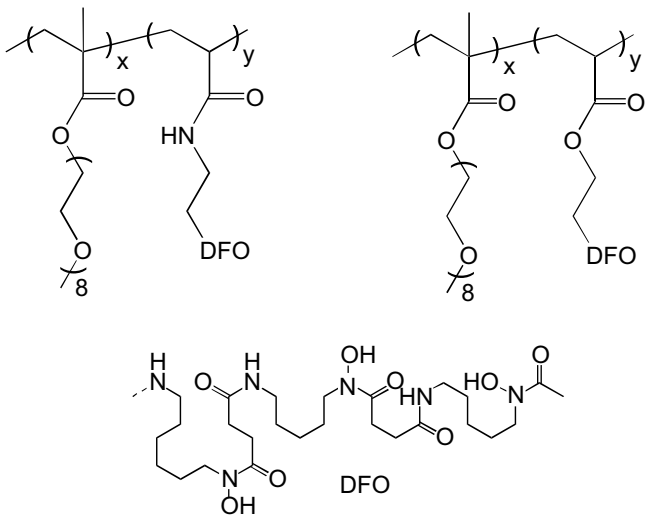
been conducted. While low molecular weight chelators demonstrate some toxicity at near therapeutic dosing, the rather limited work concerning high molecular weight chelators is very promising [15,19,20].

Recent advances in the field of polymer therapeutics have focused on improving the therapeutic index of drugs through the development of novel, poly(ethylene glycol) (PEG) based drug-polymer conjugates [21–23]. In most cases, the drug is attached to the polymer via a degradable linkage, such as a hydrolysable ester bond, to ensure release of the drug at a predetermined site of action. Once the payload has been delivered, it is essential that the polymer is then cleared from circulation [21]. In contrast, there are few examples of polymer-drug conjugates (macromolecular therapeutics) whereby the drug remains with the polymer support while the therapeutic dose is being delivered [24–26]. One such example involved the conjugation of DFO to a dextran or starch polymer [15,27,28]. In this case, DFO chelates iron while attached to a polymeric support and demonstrates a reduced toxicity and increased plasma residence time relative to monomeric DFO [27,28].

Controlled polymerization methods such as reversible addition fragment chain transfer (RAFT) [29–33] and atom transfer radical polymerization (ATRP) [34] developed over the last decade afforded the synthesis of polymer conjugates with predetermined molecular weight, composition, biostability, and immunological and pharmacological properties. In this paper, we report the *in vitro* evaluation of a novel class of macromolecular iron chelators (Scheme 1) synthesized by RAFT method [35]. DFO was conjugated to a PEG-containing copolymer in order to develop a highly blood compatible and long circulating macromolecular chelator which can bind iron in the body and be excreted through the kidney [35]. The presence of PEG and a resulting increase in the Stokes radius of the bound DFO might improve vascular retention, making it more suitable for systemic vascular applications [25]. DFO is conjugated to the polymer support through stable amide linkages, while PEG chains are conjugated via degradable ester bonds. Therefore, DFO should remain conjugated to the polymer at all times *in vivo*, while chelating excess iron. Furthermore, cleavage of the PEG side chains (M_n 400) via hydrolysable ester linkages may cause the copolymer chelator to decrease in size and be cleared through the kidney at faster rates. As part of ongoing studies, the *in vitro* properties of the P-DFO copolymers are described here. In particular, the iron binding performance, blood compatibility, cytotoxicity, and degradation of novel P-DFO conjugates are discussed. Although we report the use of DFO as the iron chelating molecule, the modular design of the polymer will be make it an effective platform for the conjugation of other clinically relevant chelators, organic molecules, peptides, or proteins for potential systemic vascular applications.



Scheme 1. P-DFO copolymer structures.

2. Materials and methods

2.1. Materials

For *in vitro* testing, P-DFO solutions of required concentrations were freshly made in 150 mM aqueous NaCl solution and filtered through 0.2 μm filters before use. Ammonium iron (III) sulphate dodecahydrate, Ultra minimum 99% $NH_4Fe(SO_4)_2 \cdot 12H_2O$, desferrioxamine mesylate (DFO), L-Glutathione (GSH) reduced 99% and Drabkin's reagent were purchased from Aldrich. PD-10 desalting column was obtained from Amersham Bioscience Piscataway, NJ, USA.

Fe(III) complexed P-DFO (P-DFO–Fe) samples were prepared and tested using similar procedures described for P-DFO. Briefly, nitrilotriacetic acid (NTA) solution (0.1 M) was added to ferric chloride in 1:1 molar ratios. P-DFO samples were dissolved in Fe-NTA solution and stirred for several hours. The solutions were neutralized, and then dialyzed against distilled water for 24 h. Water was replaced frequently, and the dialyzed solutions were finally lyophilized to recover the P-DFO–Fe product. PT and APTT reagents were purchased from Dade Behring. Human umbilical vein endothelial cells (Huvec's) were obtained from Lorus Pharmaceuticals (Allendale, NJ); EGM-2 BulletKit (CC-3162) media were purchased from Lonza, Inc.

2.2. Blood and plasma

Blood and plasma were obtained from consenting informed healthy volunteer donors at Centre for Blood Research, University of British Columbia, for *in vitro* iron chelation and blood compatibility measurements according to accepted and previously reported methods (see supporting information for full details) [36,37].

2.3. Apparatus

A Thermo Spectronic, Helios Alpha UV–Vis Spectrophotometer was used to determine hemoglobin concentrations. Prothrombin time (PT) and activated partial thromboplastin time (APTT) were determined by a coagulation analyzer using mechanical end point determination (ST4, Diagnostica Stago). TEG® hemostasis system 5000 series (TEG® analyzer and TEG® analytical software, Haemoscope Corporation, IL) was used to determine the effect of polymer solution on the efficiency of coagulation or clot formation of the blood. Optical microscopy was performed on Zeiss Axioskop 2plus microscope and images were captured with microscope-mounted black and white CCD camera (Qimaging Retiga 1300; exposure times less than 500 ms). Platelet activation analysis was performed on a BD Biosciences FACS Canto II flow cytometer.

Cell viability was determined using an MTS assay kit (CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI); absorbance measurements were recorded on a Labsystems Multiskan Ascent photometric plate reader using Ascent Software version 2.4.2 (Labsystems, Helsinki, Finland). Absorbance spectra of Fe-bound P-DFO samples were analyzed using a Thermo Spectronic UV–Vis spectrometer. Dialysis was carried out using a Spectra/Por dialysis membrane (MWCO 1000). The molecular weights of the polymers were determined by gel permeation chromatography (GPC) using a DAWN-EOS multi-angle laser light scattering (MALLS) (Wyatt Technology, Inc.) and Optilab RI detectors in aqueous 0.1 N $NaNO_3$ solution; the details have been described previously [38]. A d*n*/d*c* value of 0.132 was used for the calculation of the molecular weight of the copolymers in 0.1 N $NaNO_3$ solution. ^{1}H NMR spectra were recorded on a Bruker Avance 300 MHz NMR spectrometer; d_6-DMSO and D_2O (Cambridge Isotope Laboratories) were used as solvents, with the relevant solvent peak as reference.

2.4. Polymer synthesis

Polymer-DFO conjugates (P-DFO) were synthesized according to our published procedures [35]. Briefly, polyethylene glycol (M_n 400) methyl ether methacrylate (PEGMA) was copolymerized with one of two dioxolane based monomers in dimethylformamide at 70 °C under inert atmosphere: (2,2-dimethyl-1,3-dioxolane)methyl acrylate (DDMA) and (2,2-dimethyl-1,3-dioxolane) methyl acrylamide (DDMAA) using a charge transfer agent such as S,S′-bis(α,α′-dimethyl-α″-acetic acid)trithiocarbonate and the initiator 4,4′-azobis(4-cyanovaleric acid). The dioxolane functional groups of the resulting copolymers were deprotected using glacial acetic acid. Subsequent oxidation of the resulting diols was performed with periodic acid to form aldehyde moieties. Finally, the copolymers were covalently conjugated to the amine groups present in DFO to form a Schiff base and later reduced with $NaCNBH_3$.