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Reversible hemostatic properties of sulfabetaine/quaternary ammonium modified hyperbranched polyglycerol

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ABSTRACT

A library of hyperbranched polyglycerols (HPGs) functionalized with different mole fractions of zwitterionic sulfabetaine and cationic quaternary ammonium ligands was synthesized and characterized. A post-polymerization method was employed that utilized double bond moieties on the dendritic HPG for the coupling of thiol-terminated ligands via UV initiated thiol-ene "click" chemistry. The proportions of different ligands were precisely controlled by varying the ligand concentration during the irradiation process. The effect of the polymer library on hemostasis was investigated using whole human blood. It was found that polymer with ≥40% of alkenes converted to positive charges and the remainder to sulfabetaines caused hemagglutination at ≥1 mg/mL, without causing red blood cell lysis. The quaternary ammonium groups can interact with the negative charged sites on the membranes of erythrocytes, which provides the bioadhesion. The zwitterionic sulfabetaine evidently provides a hydration layer to partially mask the adverse effects that are likely to be caused by cationic moieties. The polymer was also found able to enhance platelet aggregation and activation in a concentration and positive charge density-dependent manner, which would contribute to initiating hemostasis. In a variety of other assays the material was found to be largely biocompatible. The polymer-induced hemostasis is obtained by a process independent of the normal blood clotting cascade but dependent on red blood cell agglutination, where the polymers promote hemostasis by linking erythrocytes together to form a lattice to entrap the cells.

1. Introduction

Enhancement of hemostasis at the site of a wound is a very attractive method to limit bleeding and to reduce the need for blood transfusion support. The ideal hemostatic agent would be easy to use, highly efficacious, nonantigenic, fully absorbable and inexpensive [1,2]. Another factor for consideration is to have the hemostat disassemble or "reversible" to avoid undesired ongoing clotting. Synthetic hemostatic agents such as cyanoacrylates and glutaraldehyde cross-linked albumin are reported to exhibit toxicity and potential mutagenicity [3]. The application of widely used zeolite-based QuickClot generates heat that can cause burn injuries [4]. Fibrin bandages overcome these problems, yet they still have some limitations such as high cost and scarcity of supply [5]. Thus despite much research in the field of hemostasis and a plethora of hemostatic agents, the need for an improved hemostatic material that can be easily and rapidly applied to severed vessels to reduce perioperative blood loss at all scales still exists.

Cationic polymers aggressively bind to a variety of biological cells because of their ability to interact strongly with negatively charged cell membranes; they bind more strongly than neutral and anionic substances due to the interaction between opposite charges [6]. Such ionic, multivalent interactions of cationic polymers with cell membranes can cause aggregation of cells in a concentration and surface charge density dependent manner [7], events which also offer other biomedical functions, i.e., hemostatic and antimicrobial [8]. The hemostatic potential of polycations, for example

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chitosan [9] and poly-L-lysine [10], has been investigated since the 1950s. Polycations have achieved levels of success in fields such as gene delivery [11], drug delivery [12] and tissue engineering [13,14], but they have also been faced with the challenge of biocompatibility. Their relatively high cytotoxicity often is caused by the disruption of the cell membrane [15,16], which has limited their therapeutic applications. Therefore, methods to achieve defined and adjustable polymer/biomembrane interactions to control the strength of cell adhesiveness and enhance biocompatibility are needed.

Zwitterionic materials with a balance of positively and negatively charged moieties within the same segment have been considered as "stealth" materials in the biomedical community due to their very weak interaction with bioentities, such as proteins and cells [17,18]. Almost all of the zwitterionic materials that have been reported are known to have excellent non-fouling properties, except methyl decorated choline phosphate, which displays a strong affinity for biological membranes [19–21]. The high degree of hydration of zwitterionic materials, which exceeds the hydration capacity of polyethylene glycol (PEG) [22], is thought to be responsible for the observed stealth effect of surfaces decorated with dipolar groups [23]. Several types of zwitterionic entities, including sulfobetaine [24,25], carboxybetaine [26,27] and phosphorylcholine [28,29], have been used as biocompatible coating materials for various nanoparticles, but to the best of our knowledge, sulfabetaine-modified surfaces have only been reported as lipid head groups [30], not associated with polymers. Unlike sulfobetaine, where the sulfur atom is directly linked to carbon characteristic of a sulfonate group, the carbon atom in sulfabetaine is linked to sulfur through an oxygen atom resembling a sulfate group. The presence of an additional heteroatom in the form of oxygen can introduce more hydrophilicity to the polymer, and also increase the size of the anionic moiety resulting in a lower charge density [31].

Hyperbranched polyglycerol (HPG) has a glycerol-based macromolecular architecture that is structurally related to PEG, but with a high degree of branching. Probably due to its globular nature, the hydration capacity of HPG is higher than that of PEG [32]. Moreover the terminal hydroxyl groups can be directly used as linker functionalities, allowing the coupling of multiple copies of bioactive molecules and chemical entities, enhancing the design potential for multifunctional nanostructures. Due to the low degree of molecular weight dispersity, good chemical stability, high hydrophilicity, inertness under biological conditions, and flexible design [33], HPG has emerged as a scaffold with a broad range of possible structural designs in biomedical applications, including drug conjugates [34,35], erythrocyte surface camouflage [32,36,37], antidotes for anticoagulation [38], and presentation of antibacterial peptides [39].

In this study we describe a novel class of multivalent water soluble HPG-based polymeric materials that combine several functions by incorporating multiple anti-fouling and/or adhesive bioactive agents. These bioactive agents, whether the zwitterionic moieties or the quaternary amine groups, are intended to function synergistically to form the basis of a biocompatible hemostatic wound dressing agent when coupled to a suitable flexible base material. We anticipate the resulting bioactive material to possess hemostatic functions with high efficacy since cationic moieties can strongly bind red blood cells and enhance the formation of a blood plug while both the zwitterionic ligands and HPG foundation can impart hemocompatibility to the structure to mask the cytotoxicity of polycations and also absorb wound fluids due to their strong hydration properties.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma–Aldrich (Oakville, ON) and all solvents were HPLC grade from Fisher Scientific (Ottawa, ON) and used without further purification unless otherwise stated. 1,1,1-Tris(hydroxymethyl)propane and potassium methylate solution (25 wt% in methanol) were purchased from Fluka (Oakville, ON). Dialysis membranes with different molecular weight cut-offs (Spectra/Por Biotech regenerated cellulose dialysis membranes) were obtained from Spectrum Laboratories, Inc. (Rancho Dominguez, CA). Glycidol (96%) was purified by distillation under reduced pressure and stored at 4 °C. Anti-CD62PE and goat anti-mouse PE antibodies were purchased from Immunotech Inc. (Vaudreuil-Dorion, QC). GVB2+ (0.1% gelatin, 5 mM Veronal, 145 mM NaCl, 0.025% NaN_3 with 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$, pH 7.3) and antibody-sensitized sheep erythrocytes were purchased from CompTech (Tyler, TX). Human umbilical vein endothelial cells (HUVECs) were obtained from Lorus Pharmaceuticals (Allendale, NJ).

2.2. Synthesis of sulfabetaine (SB) ligand

All air and moisture sensitive reactions were carried out in flame-dried glassware under an argon inert gas atmosphere. Bis(3-dimethylaminopropyl)disulfide was obtained from the corresponding HCl salt via extraction of an aqueous sodium hydroxide solution with diethylether, phase separation, drying over Na_2SO_4 and concentration under reduced pressure.

Bis(3-dimethylaminopropyl)disulfide (2.36 g, 0.01 mol, 1 equiv.) was dissolved in anhydrous acetone (15 ml) and cooled to 0 °C in an ice bath. 1,3,2-Dioxathiolane 2,2-dioxide (2.73 g, 0.22 mol, 2.2 equiv.) dissolved in anhydrous acetone, (20 mL) was added to the chilled bis(3-dimethylaminopropyl)disulfide solution under vigorous stirring in a dropwise manner via a syringe. The reaction was continued for 1 h at 0 °C then allowed to warm to room temperature overnight. A white precipitate was obtained during the reaction, which was filtered, washed thoroughly with acetone and dried under vacuum to obtain pure sulfabetaine disulfide as a colorless, very hygroscopic solid in 91% yield (4.4 g).

The corresponding sulfabetaine thiol (3.5 g, 80%) was obtained by reduction of the corresponding disulfide (2.20 g, 0.0045 mol, 1 eq) with triphenylphosphine (12 g, 0.045 mol, 10 eq) under emulsifying conditions in a solvent mixture of dichloromethane (DCM), trifluoroethanol (TFE) and water at room temperature for 48 h. The aqueous phase was separated and TFE was removed from it by rotatory evaporation. The remaining aqueous phase was freeze-dried to yield 3.5 g (80%) of the final thiol product as a white, hygroscopic solid, which was stored under an inert gas atmosphere.

^{1}H NMR (δ: ppm, 300 MHz, D_2O) 4.50 (t, 2H, $-CH_2SO_4$), 3.76 (t, 2H, $SO_4-CH_2CH_2N-$), 3.55 (t, 2H, $HSCH_2CH_2CH_2N-$), 3.20 (s, 6H, $-N(CH_3)_2$), 2.65 (t, 2H, $HSCH_2-$), 2.14 (m, 2H, $HSCH_2CH_2CH_2N$).

^{13}C NMR (δ: ppm, 75 MHz, D_2O) 64.1 ($-CH_2SO_4^-$), 62.5, 61.9 ($2\times CH_2N-$), 51.8 ($-N(CH_3)_2$), 26.2 ($HSCH_2-$), 20.5 ($HSCH_2CH_2CH_2-$).

2.3. Synthesis of quaternary ammonium (QA) ligand

Sodium thiosulfate pentahydrate (5.46 g, 0.022 mol, 1.1 equiv.) was dissolved in 20 mL deionized water and added into a flask with (3-bromopropyl)trimethylammonium bromide (5.20 g, 0.02 mol, 1 equiv.). The mixture was heated to 100 °C and kept under reflux and stirring for a total of 12 h. The resulting Bunte salt solution was then hydrolyzed without isolation to the corresponding thiol by adding concentrated H_2SO_4 to a final acid concentration of 1 M and