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The influence of poly-*N*-[(2,2-dimethyl-1,3-dioxolane)methyl]acrylamide on fibrin polymerization, cross-linking and clot structure

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ABSTRACT

Poly-*N*-[(2,2-dimethyl-1,3-dioxolane)methyl]acrylamide (PDMDOMA) is a neutral synthetic water-soluble polymer. In this report, we evaluated the influence of PDMDOMA on blood hemostasis by studying the fibrin polymerization process, the three-dimensional clot structure, and the mechanical properties and fibrinolysis. PDMDOMA altered the normal fibrin polymerization by changing the rate of protofibril aggregation and resulting in a 5-fold increase in the overall turbidity. Fibrin clots formed in presence of PDMDOMA exhibited thinner fibers with less branching which resulted in a more porous and heterogeneous clot structure in scanning electron micrographs. The overall strength and rigidity of the whole blood clot also decreased up to 10-fold. When a combination of plasminogen and tissue-plasminogen activators were included in clotting reactions, fibrin clots formed in the presence of PDMDOMA exhibited highly shortened clot lysis times and was supported by the enhanced clot lysis measured by thromboelastography in whole blood. Further evidence of the altered clot structure and clot cross-linking was obtained from the significant decrease in D-dimer levels measured from degraded plasma clot. Thus, PDMDOMA may play an important role as an antithrombotic agent useful in prophylactic treatments for thrombosis by modulating fibrin clot structure to enhance fibrinolysis.

1. Introduction

Antithrombotic agents serve the purpose of treating and preventing thrombus formation in diverse clinical conditions such as atrial fibrillation [1] and venous thromboembolism [2] or in various types of invasive surgeries. Inhibition of thrombosis can be achieved by three different approaches: inhibit the coagulation cascade, increase endogenous fibrinolytic activity, and alter platelet activation and aggregation [3–5]. Classic examples of therapeutic agents that utilize these approaches include heparin and warfarin (indirect inhibitors of thrombin), hirudin [6] and argatroban [7] (direct inhibitors of thrombin), low molecular weight heparins and fondaparinux [8] (indirect inhibitors of coagulation factor Xa), tissue-plasminogen activator and streptokinase [9] (fibrinolytic agents) and ASA and clopidogrel [4] (antiplatelet agents). Despite their success in preventing thrombosis, current therapies have limitations ranging from bleeding complications to allergic responses [10–12]. Thus, the development of new and improved antithrombotic agents is very important.

Another target for antithrombotic agents in the blood coagulation cascade is the fibrin clot, the final product of the coagulation. Upon cleavage of fibrinogen by thrombin, fibrin monomers polymerize to give a fibrin clot with great diversity in structural properties [13]. The polymerization process first involves fibrin monomers interacting with each other in a half-staggering, end-to-end fashion to form double-stranded protofibril. The protofibrils then aggregate in a lateral manner to form fibers and finally fibers branching in various directions to produce the overall structural network of a fibrin clot [14]. With the three-dimensional gel mesh, the fibrin clot is then stabilized by formation of intermolecular N^{ϵ}-(γ-glutaml) lysine covalent cross-links between adjacent or neighboring fibrin molecules catalyzed by coagulation factor XIIIa [15]. The overall stability of a fibrin clot is therefore dependent on both the three-dimensional network branching between fibrin fibers and the cross-linking of gamma chains between fibrin monomers [16,17]. An alteration to the branching network or the stabilizing cross-links in the fibrin clot would render the clot less rigid and less elastic, and more prone to fibrinolysis by plasmin [18,19]. Thus, designing an agent that specifically affects the normal fibrin clot polymerization and the cross-linking process offers a possible target in the attenuation of thrombosis [20].

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A few inhibitors acting against fibrin clot cross-linking have been reported. Compounds derived from natural sources, Tridegin (leech) [21], cysteine proteinase inhibitor (potatoes) [22] and alutacenoic acids (fungi) [23], and synthetic compounds such as, 2-[(2-oxopropyl)thio] imidazolium [24,25], 1,2,4-thiadiazole [26], and tyrosine melanin [27] have demonstrated ability in modulating cross-links within fibrin clot and enhancing fibrinolysis. Scheiner et al. [28] have also reported a monoclonal antibody inhibiting the clot cross-linking and enhancing fibrinolysis by targeting the C-terminus of the γ chain of fibrinogen. However, there is limited knowledge on agents that change the fibrin polymerization process.

In current work, we report the influences of neutral synthetic polymer PDMDOMA on overall fibrin clot structure and clot properties. The changes in clot characteristics are remarkably different from other synthetic water-soluble polymers studied.

2. Experimental

2.1. Materials

2.1.1. Chemical

Poly-*N*-[(2,2-dimethyl-1,3-dioxolane)methyl]acrylamide (PDMDOMA) and poly (*N*,*N*-dimethylacrylamide) (PDMA) were synthesized by following our previous report [29].Two different molecular weights of PDMDOMA were studied, high molecular weight-PDMDOMA (HMW-PDMDOMA) and low molecular weight-PDMDOMA (LMW-PDMDOMA). The polymers were purified by dialysis against water after incubation with EDTA solution (0.1 M) to remove any adsorbed copper species during the synthesis. The molecular weights of the polymers were determined by gel permeation chromatography (GPC) system coupled with a multi angle light scattering detector (Wyatt Technology Corp., CA) [29]. Partially de-protected, PDMDOMA-diol (PDMDOMA-co-PDHPA) and 100% dioxolane de-protected PDMDOMA-diol (PDHPA, poly-*N*-[(2,3-dihydroxypropyl)acrylamide]) were prepared by the controlled acidic cleavage of dioxolane groups in the PDMDOMA polymer. Poly (ethylene glycol) (PEG) of molecular weight 35,000 Da was purchased from Fluka (Oakville, ON). The properties and structures of all the polymers used in the study are shown on Table 1. 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid sodium salt (HEPES), $CaCl_2$ (99.99%) and $NaCl_2$ (99%) were purchased from Aldrich (Oakville, ON) of the highest purity. Distilled water used in all experiments was further purified using a Mili-Q Plus water purification system (Millipore Corp., Bedford, MA).

2.1.2. Biological

Blood from healthy donors was collected into 3.8% sodium citrated tube with a blood/anticoagulant ratio of 9:1 at Centre for Blood Research, University of British Columbia after obtaining consents. Platelet-poor plasma (PPP) was prepared by centrifuging whole blood samples at 1200 × *g* for 20 min in an Allegra X-22R Centrifuge (Beckman Coulter, Canada). Human plasma proteins such as fibrinogen (91% of clottable protein, F3879), plasminogen (≥2 units/mg protein), tissue-plasminogen activator (≥500,000 IU/mg) (tPa), and thrombin (≥2000 NIH units/mg protein) were all purchased from Sigma–Aldrich (Oakville, ON) and used as received unless specified. Human plasma factor XIII (FXIII) and activated plasma factor XIII (FXIIIa) were purchased from Haematologic Technologies Inc. (VT, USA). All of the lyophilized plasma proteins were made into solution by HEPES-saline buffer (10 mM HEPES, 150 mM NaCl, pH 7.4). The reagents were kept at −80 °C until use. Reagents for conventional coagulation assays APTT and PT, actin FSL and recombinant thromboplastin, were purchased from Dade Behring (Marburg, Germany).

Table 1
Characteristics of the polymer samples studied.

Polymer	Mn (Mw/Mn)
HMW-PDMDOMA	45,000 (1.51)
LMW-PDMDOMA	6300 (1.14)
PDMDOMA-co-PDHPA	42,000 (1.51)
PDHPA	34,800 (1.51)
PEG	35,000 (1.22)
PDMA	6400 (1.74)

Mn, number average molecular weight; Mw/Mn, polydispersity; HMW, high molecular weight; LMW, low molecular weight.

2.2. Methods

2.2.1. Coagulation assays: prothrombin time (PT) and activated partial thromboplastin time (APTT)

Sodium citrate anticoagulated PPP is used for PT and APTT analysis. The effect of polymer in HEPES-saline on the coagulation cascade was examined by mixing PPP with the polymer solution (9:1 v/v; 1 mg/mL final concentration) at 37 °C. Control experiments were performed with identical volumes of HEPES-saline solution to PPP. Each experiment was repeated in triplicates on a STart®4 coagulometer (Diagnostica Stago, France). Detailed experimental procedure was as described by our group previously [30].

2.2.2. Fibrin polymerization assay

Human fibrinogen (180 μl; 3 mg/mL) was first pre-incubated with either 20 μL of HEPES buffer or HEPES-buffered polymer solutions (1 mg/mL, final concentration) at 37 °C for 10 min in a 96-well microtitre plate [31]. Fibrin polymerization was initiated by the addition of 20 μL of 3 NIHU/mL human thrombin, 20 μL of 25 μg/mL activated plasma factor XIII (FXIIIa), and 20 μL of 40 mM $CaCl_2$ (3 mM final concentration) to each well. The optical density (OD) values were monitored at 405 nm using a SpectraMax plate reader (Molecular Devices, USA) every minute. Final turbidities of fibrin clots were quantified after the clots had reached plateau OD value after 1 h. Each measurement was carried out in triplicates. Rate of lateral aggregation of protofibril was measured by the change in OD values from the baseline value to the highest OD value obtained over the period of time for this change in OD values. A similar fibrin polymerization assay was carried out using fibrinogen dialyzed overnight against HEPES buffer to monitor the effect of high citrate from the commercially available fibrinogen.

2.2.3. Scanning electron microscopy (SEM) analysis of clot structure

For SEM analysis, fibrin clot was prepared from 360 μL of pure fibrinogen (3 mg/mL), and 40 μL of 2 NIHU/mL thrombin, 20 μL of 30 μg/mL FXIIIa, 20 μL of 40 mM $CaCl_2$ (1.67 mM final concentration) in presence or absence of the polymer samples. The mixtures were thoroughly premixed and were incubated for 1 h at 37 °C to allow clotting to proceed. Clots were first washed 3 times with 50 mM phosphate buffer (pH 7.4) to remove excess salt and fixed with 2% glutaraldehyde. We followed a method reported by Weisel et al. [32] with a slight modification for the preparation of the clots for SEM imaging. Clots were frozen and fractured for 1 h, and critical point dried with CO_2 in a DCP-1 critical point drying apparatus (Denton Vacuum Co., USA). The specimens were mounted and sputter-coated with gold-palladium, and then examined with a Hitachi S-4700 field emission scanning electron microscope (Hitachi High-Technologies Inc., Canada). The electron micrographs were taken at magnifications between 5k and 10k. From the images, the size of each of the fibrin fiber strands was determined using Image J (NIH, USA), by drawing a line to bisect foreground fibers to obtain a pixel value and relating to pixel value obtained for the scale bar on the image. Fifteen different fibrin strands on each image were analyzed. A minimum of two images were analyzed for each sample. The diameter of individual fibrin strands on the images was reported as an average for all fiber strands measured.

2.2.4. Thromboelastography analysis of clot formation

Thromboelastograph (TEG) Hemostasis System 5000 series (Haemoscope Corporation, USA) was used to examine the viscoelastic properties of whole blood clots. Citrated human whole blood was first incubated with HEPES-saline buffered polymer samples at ratio 9:1 v/v. Two separate analyses were performed: (i) effect of different polymers (1 mg/mL final concentration) and (ii) effect of concentration of PDMDOMA (0.125 mg/mL, 0.25 mg/mL, and 1 mg/mL final concentration). Whole blood added with buffer in the absence of polymer was used as control. Aliquots of reaction mixtures (340 μL) were added to TEG cup and the clotting was initiated by the addition of 20 μL of 0.2 M $CaCl_2$ (10 mM final concentration). The TEG analysis was allowed to proceed to a 2 h preset completion time or until the analysis was prematurely terminated by TEG system itself. The overall coagulation profile was recorded by TEG system. The time for initial fibrin formation (R-time), overall clot strength (maximum amplitude (MA)), clot elasticity (G′) and percent of lysis at 30 min (%LY30) after the clot reaches maximum strength were measured and analyzed. Both MA and G` recorded are direct representation of the dynamic properties between fibrin and platelet bonding via GP IIb/IIIa. The %LY30 after reaching maximum amplitude is indicative of fibrinolysis and degradation of fibrin clot [33,34].

2.2.5. Fibrinolysis assay

Purified human fibrinogen (108 μL, 3 mg/mL) was first pre-incubated with either 12 μL of HEPES buffer or HEPES-buffered polymer solutions (1 mg/mL final concentration) at 37 °C for 10 min. A total of 5 μL of 0.77 μg/mL tPA and 5 μL of 300 μg/mL plasminogen were added to each well before the addition of 12 μL of 3 NIHU/mL human thrombin, and 12 μL of 40 mM $CaCl_2$ (3 mM final concentration) to initiate fibrin polymerization. The optical density (OD) values were monitored at 405 nm using a SpectraMax plate reader (Molecular Devices, USA). The overall turbidities of fibrin clots were monitored overnight. Each measurement was carried out in triplicates. The turbidity data were normalized to maximum turbidity due to