Biomaterials 35 (2014) 2518–2528

Contents lists available at ScienceDirect

Biomaterials

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

Abnormal blood clot formation induced by temperature responsive polymers by altered fibrin polymerization and platelet binding

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ARTICLE INFO

Article history:
Received 28 September 2013
Accepted 8 December 2013
Available online 31 December 2013

Keywords:
Thermoresponsive polymers
Blood clotting
Fibrin polymerization
Platelet binding
Blood coagulation
Fibrin clot

ABSTRACT

Thermoresponsive polymers (TRPs) have been extensively investigated as smart devices, drug delivery systems and protein conjugates due to their unique phase transition properties. Here, we report the unusual influence of TRPs in blood clotting and the mechanism by which TRPs change the three dimensional organization of blood clot structure. Ten different TRPs with lower critical solution temperatures ranged from 26 to 80 °C are studied. TRPs altered the fibrin polymerization by increasing the rate of protofibril aggregation, decreased the fibrin fiber diameter and changed the platelet integration within the clot. The mechanical properties of the clot decreased considerably in presence of TRPs due to the poor platelet binding. The poor integration of platelets within the clot is not due to the inhibition of platelet activation by TRPs but may due to the unusual organization of fibrin structure. The plasma phase of the blood coagulation is not affected in presence of TRPs. We anticipate that our results will have significant implications on the use of TRPs in applications where blood contact is essential. These observations may also open up new avenues, for example, in the design of new generation antithrombotics.

1. Introduction

Synthetic polymers are being extensively researched in the development of drug delivery vehicles, smart materials for diagnosis, and imaging agents [1–8]. Among the polymeric systems, thermoresponsive polymers (TRPs) have been studied intensively for various biotechnology applications including the development of smart drug delivery systems, smart protein and peptide conjugates, in tissue engineering scaffolds [9–19]. TRPs have a unique property; they undergo coil-to-globule transition above certain temperatures in aqueous solution and is referred as lower critical solution temperature (LCST) [15,18,19]. The phase transition of TRPs in aqueous solution with increase in temperature is due to the changes in hydrogen bonding and water structure around the polymer chains [15,18,19]. The LSCT of the polymers can be finely tuned by the composition of polymer or by changing the chemical nature of the polymer [15]. Most of the water soluble LCST polymers are amphiphilic in nature and it has been shown that the hydrophobic character of the polymer increases above their LCST [16].

Blood coagulation involves a tightly regulated enzymatic cascade consists of several proteases resulting in the formation of insoluble fibrin clot from soluble fibrinogen which is cross-linked by factor XIIIa as a final product [20–22]. The polymerization of the fibrin monomers is triggered after its cleavage from fibrinogen, which generate fibrin clot with great structural diversity. Platelets and red blood cells will get incorporated into the clot to provide strength of the clot. This process is required to arrest the bleeding resulting from an injury.

Fibrin polymerization is a very complex process that starts with spontaneous assembly of fibrin monomers into double-stranded protofibril. Protofibrils then assembles to fibers and finally fibers branching in various directions to form the complex network of fibrin clot [23,24]. The three dimensional structure of the clot is then stabilized by the formation of cross-links between fibrin molecules by the coagulation factor XIIIa [25]. The overall stability of the clot depend on the formation of three dimensional fibrin clot structure, and any alteration in the structure (such as branching or fiber diameter) will result in the instability of the clot, and subsequently render the clot less rigid and elastic, which will result in bleeding at the site of injury [26–30]. Changes in clot structure are

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http://dx.doi.org/10.1016/j.biomaterials.2013.12.003

also shown to influence the wound healing process due to the alteration of clot degradation, cell adhesion and proliferation [31]. It has been reported that local pH, ionic strength, concentrations of calcium, polyphosphate, fibrinogen, fibrin(ogen) binding peptides and varying proteins and thrombin concentration can influence the structure of the fibrin clot by affecting the fiber thickness, branching and network density [29,30,32,33]. Alteration to the fibrin network structure is indicated as the advancement and outcomes of diseases such as haemophilia, venous thromboembolism, ischemic stroke, myocardial infraction, Alzheimer's disease and other disorders [29,32].

On the other side of the spectrum, an agent that can specifically change the fibrin clot formation process and fibrin degradation process can offer a potential target in the treatment of thrombosis [34–42]. For instance, compounds derived from saliva of leech, alutacenoic acids from fungi, synthetic compounds such as 1,2,4-thiadiazole and 2-[(2-oxopropyl)thiol] imidazolium and monoclonal antibodies demonstrated their ability in changing clot cross-linking and enhancement in the endogenous fibrinolysis process [35–42]. In addition to these findings, synthetic polymers such as poly-N-[(2,2-dimethyl-1,3-dioxolane)methyl]acrylamide, poloxamers, and dextran have been reported with altering the clot stable structure [43–46]. In the case of poloxamers, the alteration of fibrin clot structure was useful for their clinical application in the prevention of surgical adhesion [44,45]. Poloxamers and dextrans were shown to increase the speed of fibrin assembly at relatively high concentrations (>5 mg/mL) [44–46]. Thus the design of the polymers which can alter the clot-structure could be beneficial.

In this manuscript, we describe the abnormal blood clot formation in presence of thermoresponsive polymers. We have screened over 10 different thermoresponsive polymers having different LCSTs (26 to >80 °C), which represent broader class of such polymers reported in literature [15,17], to determine their influence on blood clot formation using fibrin polymerization assay, various blood coagulation assays and scanning electron microscopy analysis.

2. Materials and methods

2.1. Materials

2-(2-Methoxyethoxy)ethyl methacrylate (MEO_2MA, 95%, Sigma–Aldrich), *N*-vinylcaprolactam (VCL, 98%, Sigma–Aldrich), N,N′-dimethylacrylamide (DMA,-Sigma–Aldrich), oligo(ethylene glycol) methyl ether methacrylate ($OEGMA_{8.5}$, $M_n \sim 475$ g/mol, Aldrich), 1,1,4,7,10,10-hexamethyl triethylene tetramine (HMTETA, Aldrich, 97%), tris[2-(dimethylamino)ethyl]amine (Me_6TREN, Aldrich), CuCl (Aldrich, 99+%), $CuCl_2$ (Aldrich, 99.99%), methyl 2-chloropropionate (MCP, Aldrich, 97%) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Aldrich) were used as supplied. Aqueous solution of poly(vinyl methyl ether) (PVME) (50% solution) and poly(ethylene glycol) (PEG) was purchased from Polysciences and Aldrich respectively. N-isopropylacrylamide (NIPAm, Aldrich, 97%) was purified by recrystallization in hexane and dried under vacuum at 25 °C. Water was purified using a Milli-Q Plus water purification system (Millipore Corp., Bedford, MA) and was used in all experiments. ^{1}H NMR spectroscopy was performed on a Bruker AV400 MHz NMR spectrometer.

Absolute molecular weights of the polymers were determined by gel permeation chromatography (GPC) on a Waters 2690 separation module fitted with a DAWN EOS multi-angle laser light scattering (MALLS) detector from Wyatt Technology Corp. with 18 detectors placed at different angles (laser wavelength $\lambda = 690$ nm) and a refractive index detector from Viscotek Corp. operated at $\lambda = 620$ nm. The detailed procedure was given in previous reports [47,48].

The LCST of the thermoresponsive polymers used in this study was measured with a Varian 4000 UV-Vis spectrometer equipped with multi-cell, thermoelectric temperature controller (±0.1 °C). Transmittance through aqueous solutions of polymer (5 mg/mL) at $\lambda = 500$ nm was recorded at every 0.5 °C interval. For each temperature, the solution was equilibrated for 10 min. The LCST was defined as the midpoint of the temperature-transmission curve.

2.2. Synthesis and characterization of thermoresponsive polymers

All the thermoresponsive polymers used in this study were synthesized via atom transfer radical polymerization (ATRP) [47]. All ATRP reactions were carried out at ambient temperature (22 °C) in a glove box filled with argon. Methyl 2-chloropropionate was used as ATRP initiator. The detailed polymerization conditions including solvents and ligands employed, polymerization temperature, and monomer conversion was given in Table 1. For a typical reaction for the synthesis of PNIPAm, a schlenk flask was loaded with CuCl (15 mg, 0.15 mM), Me_6TREN (69 mg, 0.30 mM), NIPAm (1.13 g, 10 mM) in of DMF (5 mL) as solvent. The flask was sealed with a rubber septum and cycled three times between argon and vacuum to remove oxygen. Methyl 2-chloropropionate (18.4 mg, 0.15 mM) was added into the vial using a syringe and the reaction was allowed to proceed at room temperature with stirring for 12 h. The degree of monomer conversion was estimated from ^{1}H NMR spectra at the end of polymerization. The reaction mixture was then dialyzed (MWCO 1000) against water for 3 days with three times daily changes in water, filtered and lyophilized. The polymer was further incubated with 0.1 M EDTA solution and dialyzed against 0.1 M EDTA for a day and then dialyzed against MilliQ water for three days with three times daily changes in water to remove any copper species present before the biological evaluation. The solution was filtered and lyophilized to recover the polymer. The resulting PNIPAm was characterized by GPC-MALLS and NMR spectroscopy. Other polymers were also prepared and characterized in a similar way and characterized (Table 1). All polymers were dissolved with HEPES-buffered saline (10 mM HEPES, 150 mM NaCl, pH 7.4) as a stock solution (10 mg/mL concentration) for their biological evaluation.

2.3. Biological materials

Whole blood from healthy consented donors was collected into either 3.8% sodium citrated blood collection tubes, with a blood to anticoagulant ratio of 9:1 or serum tube with no anticoagulant, at the Centre for Blood Research, University of British Columbia. The protocol was approved by the clinical ethic committee of the University of British Columbia. With an Allegra X-22R Centrifuge (Beckman Coulter, Canada), platelet-rich plasma (PRP) was obtained by centrifuging citrated whole blood tubes at 150 × *g* for 10 min and platelet-poor plasma (PPP) was collected by spinning the citrated whole blood at 1200 × *g* for 20 min. Human serum was collected, after allowing whole blood in serum tube to clot for 30 min at room temperature, by centrifugation at 1200 × *g* for 20 min. Using ADVIA® 120

Table 1
Polymerization conditions and molecular weight characteristics of thermoresponsive polymers.

Samples	Polymer abbreviation	Solvent and ligands	Polymerization time, and conversion %[a]	M_n[b] (g/mol)	M_w/M_n	LCST (°C)
PAM1	PNIPAm	DMF, Me_6TREN[c]	12 h, 65%	14,000	1.23	32
PAM2	PNIPAm-co-PDMA (a)	DMF, HMTETA[c]	12 h, 53%	22,500	1.28	39
PAM3	PNIPAm-co-PDMA (b)	DMF, HMTETA[c]	12 h, 47%	29,200	1.19	44
PAM4	PDMA	DMF, Me_6TREN[c]	18 h, 69%	18,000	1.22	>85
PAP1	$PMEO_2MA$	EtOH, bipyridine	4 h, 68%	21,000	1.46	26
PAP2	$PMEO_2MA$-co-POEGMA (a)	EtOH, bipyridine	4 h, 72%	36,900	1.23	39
PAP3	$PMEO_2MA$-co-POEGMA (b)	EtOH, bipyridine	4 h, 54%	49,200	1.18	45
PAP4	POEGMA	EtOH, bipyridine	4 h, 82%	19,000	1.45	>85
PVME[d]	/			41,000	1.17	33
PVCL	/	NMP	12 h, 68%	34,000	1.22	35
PEG[d]	/			35,000	1.05	/

[a] All polymerizations were carried out at ambient temperature. Methyl 2-chloropropionate was used as ATRP initiator.
[b] Molecular weight of PAM1 to PAM3, PAP1 to PAP3 was determined by GPC-MALLS in THF. Molecular weights of PAM4, PAP4 were determined by GPC-MALLS in 0.1 M $NaNO_3$ aqueous solution.
[c] HMTETA: 1, 1, 4, 7, 10, 10-hexamethyltriethylenetetramine; Me_6TREN:tris[2-(dimethylamino) ethyl] amine.
[d] PVME and PEG was purchased from Polysciences and Aldrich respectively.