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Platelet microparticle-inspired clot-responsive nanomedicine for targeted fibrinolysis

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ABSTRACT

Intravascular administration of plasminogen activators is a clinically important thrombolytic strategy to treat occlusive vascular conditions. A major issue with this strategy is the systemic off-target drug action, which affects hemostatic capabilities and causes substantial hemorrhagic risks. This issue can be potentially resolved by designing technologies that allow thrombus-targeted delivery and site-specific action of thrombolytic drugs. To this end, leveraging a liposomal platform, we have developed platelet microparticle (PMP)-inspired nanovesicles (PMINs), that can protect encapsulated thrombolytic drugs in circulation to prevent off-target uptake and action, anchor actively onto thrombus via PMP-relevant molecular mechanisms and allow drug release via thrombus-relevant enzymatic trigger. Specifically, the PMINs can anchor onto thrombus via heteromultivalent ligand-mediated binding to active platelet integrin GPIIb-IIIa and P-selectin, and release the thrombolytic payload due to vesicle destabilization triggered by clot-relevant enzyme phospholipase-A_2. Here we report on the evaluation of clot-targeting efficacy, lipase-triggered drug release and resultant thrombolytic capability of the PMINs in vitro, and subsequently demonstrate that intravenous delivery of thrombolytic-loaded PMINs can render targeted fibrinolysis without affecting systemic hemostasis, in vivo, in a carotid artery thrombosis model in mice. Our studies establish significant promise of the PMIN technology for safe and site-targeted nanomedicine therapies in the vascular compartment.

1. Introduction

Vascular pathologies like myocardial infarction, stroke and peripheral arterial disease are major causes of morbidities and mortalities on a global scale [1]. A common clinical presentation in such disease pathologies is the formation of occlusive clots (thrombi) in blood vessels, that restrict blood flow to critical organs [2]. Therefore, rapid removal of occlusive thrombi to restore blood flow is a critical component of treating these conditions. One established clinical strategy for clot removal is the intravascular administration of thrombolytic (fibrinolytic) drugs [3]. These drugs, e.g. streptokinase (SK), urokinase-type plasminogen activators (uPA) and tissue plasminogen activators (tPA), act by facilitating conversion of plasminogen to plasmin, which in turn can break down the fibrin in the clot. While this fibrinolytic action is essential for therapeutic activity at the clot site, systemic off-target action of these drugs to convert circulating plasminogen to plasmin is harmful because this circulating plasmin can then break down circulating fibrinogen (systemic *fibrinogenolysis*), which affects normal hemostatic capabilities and leads to hemorrhagic side-effects [4,5]. According to the American Academy of Emergency Medicine (AAEM) and National Institute of Neurological Disorders and Stroke (NINDS), about 6% of stroke patients undergoing tPA-based thrombolytic therapy suffer from intracranial hemorrhage with about 45% fatality risk [6]. Another issue with the direct intravascular administration of thrombolytic agents is their rapid deactivation by plasma components (e.g. by plasminogen activator inhibitors) and resultant short circulation life, that in turn reduces their availability at the clot site [7]. All these issues can be potentially mitigated by designing technologies that can (i) encapsulate and protect the drug in

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circulation, (ii) anchor actively onto clot site under hemodynamic flow environment and (iii) allow triggered release of the drug specifically at the clot site to minimize off-target effects.

In consideration of these design criteria, we have developed a vascular nanomedicine technology inspired by platelet-derived microparticles (PMPs). PMPs, originally reported by Wolf in the 1960s as 'platelet dust', are membrane fragments shed from activated platelets [8]. These PMPs are characteristically known to be lipid bilayer vesicles 100nm-1μ in diameter, with a high surface-presentation of pro-coagulant anionic phosphatidylserine (PS) lipid, active integrin GPIIb-IIIa, P-selectin, GPIb-type receptors, thrombospondin, C-X-C type chemokine receptors and thrombin receptors [9,10]. Fig. 1A shows representative cartoon of a PMP, with (A1) showing fluorescence microscopy image of red fluorescent active platelets (shown with blue arrows) shedding PMPs (shown with yellow arrows) and (A2) showing representative scanning electron microscopy (SEM) images of the same at high resolution, demonstrating that the PMPs are sub-micron size vesicular structures. The PS-rich surface of PMPs facilitate intrinsic coagulation mechanisms, while the presence of active integrin GPIIb-IIIa and P-selectin moieties facilitate binding interactions with fibrinogen (Fg), fibrin, stimulated GPIIb-IIIa motifs on active platelets via fibrinogen-mediated interactions and P-selectin Glycoprotein Ligand-1 (PSGL-1) on leukocytes and active platelets (schematic shown in Supplementary Figure S1). Also, PMPs secrete several chemokines, cytokines, pro-thrombotic and pro-inflammatory molecules in the clot milieu [9–12]. Therefore, PMPs essentially represent (i) lipid vesicle nano-containers loaded with bioactive molecules, that can (ii) actively bind to thrombus-associated cellular phenotypes (e.g. platelets and leukocytes) via heterotypic ligand-receptor interactions, and (iii) secrete their payload locally to influence the thrombus pathology. Drawing inspiration from these structural and mechanistic aspects of PMPs (but not their pro-thrombotic functional aspect), we have chosen to construct (i) liposomal nanovesicles with non-coagulant lipid membrane, that can (ii) undergo active anchorage to platelet-rich thrombi via PMP-inspired heteromultivalent ligand-receptor interactions, and (iii) upon active anchorage to thrombus, can release encapsulated thrombolytic drug to render site-specific thrombolytic action. We rationalized that these PMP-inspired nanovesicles (PMIN) will enhance therapeutic availability at the clot site, while protecting the encapsulated drug from plasma and minimizing systemic off-target side effects.

For the PMIN design (Fig. 1B), the clot-specific active anchorage was rendered by heteromultivalently surface-decorating glycerophospholipid-based liposomal vesicles with peptide ligands that can specifically bind to stimulated integrin GPIIb/IIIa and P-selectin on activated platelets. Activated platelets are an ideal cellular target for PMIN binding to thrombi, since aggregation of activated platelets and platelet-mediated promotion of coagulation mechanisms are hallmark events in thrombosis [13]. Specifically, the fibrinogen-derived peptide sequence GSSSG***RGD***SPA was used for active GPIIb-IIIa-binding and the sequence DA***EWVDV***S was used for P-selectin binding [14,15]. For thrombolytic drug encapsulation, we selected streptokinase (SK) as a model fibrinolytic drug, especially since its direct systemic use is known to cause significant off-target hemorrhagic side-effects (and therefore will be a good control to compare). For clot-relevant stimulus, we selected secreted phospholipase A_2 ($sPLA_2$, group II) as a candidate, since it is reported to be produced from activated platelets and inflammatory cells in athero-thrombotic milieu and can cleave the sn-2 ester bonds in glycerophospholipids, thereby destabilizing lipid vesicles for payload release [16–18]. In fact, PLA_2 action can result in release of arachidonic acid from PMPs to augment transactivation and aggregation of platelets in thrombosis [9]. Building on these design components, our central hypothesis was that intravenously administered SK-loaded PMINs can actively anchor onto thrombi (Fig. 1C, C1), where thrombus-associated $sPLA_2$ activity can destabilize the vesicles to render clot site-selective SK release for targeted fibrinolytic action (Fig. 1C, C2 and C3).

2. Materials and methods

2.1. Materials

Phosphate Buffered Saline (PBS), 3.8% w/v sodium citrate, Bovine Serum Albumin (BSA), chloroform, methanol, ethanol, AlexaFluor 488-conjugated fibrinogen (AF488-Fg) and 1,2-Bis-BODIPY®FL-C_{11}-*sn*-Glycero-3-Phosphocholine (bis-BODIPY®FL C_{11}-PC) were obtained from Thermo Fisher Scientific (Pittsburgh, PA, USA). Cholesterol, secreted phospholipase A2 (sPLA2), calcium chloride and collagen were obtained from Sigma Aldrich (St. Louis, MO, USA). Adenosine Diphosphate (ADP) was purchased from Bio/Data Corporation (Horsham, PA, USA). Cysteine-terminated peptides *CGSSSGRGDSPA* and *CDAEWVDVS* were custom-synthesized and purchased from Genscript (Piscataway, NJ, USA). Distearyl phosphatidyl choline (DSPC), maleimide-terminated polyethylene glycol-conjugated distearyl phosphatidyl ethanolamine (DSPE-PEG_{2000}-Mal) and Rhodamine-B-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (DHPE-RhB) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Polycarbonate membrane filters with 200 nm pores for vesicle extrusion were obtained from Whatman (Kent, UK). For streptokinase analysis the chromogenic assay Chromogemix S-2251 was purchased from Diapharma (West Cjester, OH, USA). The Parallel Plate Flow Chamber (PPFC) system was purchased from Glycotech (Gaithersburg, MD, USA). Aggregometry studies were done on a ChronoLog aggregometer. All inverted fluorescence microscope studies were carried out using a Zeiss Axio Observer D1 microscope fitted with a CCD camera. For in vivo studies on mice, anesthesia agents ketamine was obtained from Fort Dodge Animal Health, IA, USA and xylazine was obtained from Hospira, IL, USA. Rhodamine 6G for in vivo mouse platelet labeling and ferric chloride ($FeCl_3$) for carotid artery thrombus induction were obtained from Sigma. All intravital microscopy studies were carried out using a Leica DMLFS fluorescent microscope with a Gibraltar Platform (EXFO, Quebec, Canada).

2.2. Preparation of heteromultivalently ligand-decorated PMINs

The peptides *CGSSSGRGDSPA* and *CDAEWVDVS* were conjugated via thioether reaction to DSPE-PEG_{2000}-Mal through the cysteine termini to form DSPE-PEG_{2000}-*GSSSGRGDSPA* and DSPE-PEG_{2000}-*DAEWVDVS* conjugates. The peptides and resulting DSPE-PEG-peptide conjugates were characterized by mass spectrometry. For PPFC-based in vitro platelet-rich clot-targeting studies DSPC, DhPE-RhB, cholesterol and DSPE-PEG-peptide conjugates were dissolved in 1:1 chloroform: methanol, and this lipid mixture was subjected to standard reverse phase evaporation and lipid hydration technique followed by extrusion through 200 nm pore size polycarbonate membrane in a pneumatically controlled lipid extruder (Northern Lipids). Resultant vesicles were characterized for their size distribution using dynamic light scattering (DLS) and cryo transmission electron microscopy (cryoTEM) and the size was found to be approximately 150–170 nm in diameter. For the various vesicle formation, DSPC content was always maintained at 50 mol% and DHPE-RhB content was always maintained at 1 mol% of total lipid. For homomultivalently decorated vesicles (single peptide decoration), the corresponding DSPE-PEG-peptide content was maintained at 2.5, 5, or 10 mol % of total lipid, while for heteromultivalently decorated vesicles (bearing both peptides) the total