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Preparation and characterization of anionic oligopeptide-modified tissue plasminogen activator for triggered delivery: An approach for localized thrombolysis

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ABSTRACT

Purpose: The study sought to synthesize anionic peptide-conjugated tissue plasminogen activator (tPA) for its targeted/triggered delivery, where tPA's activity would be masked in the circulation and regenerated at the thrombus site by a commonly used anticoagulant, heparin, to minimize tPA associated bleeding complications.
Methods: tPA was conjugated to Polyglutamate, and the activity of oligoanion-modified tPA was tested by fibrinolytic assay. Separately human serum albumin (HSA) was conjugated to protamine and the formation of its electrostatic complex with anionic peptide was monitored by Förster Resonance Energy Transfer (FRET). The masking of tPA-activity via steric hindrance created by albumin, and subsequent regeneration with *therapeutic dose* of heparin was tested by enzymatic assay. Stability of 'camouflaged-tPA' was determined in human plasma. Using fluorescence microscope, binding of camouflaged-tPA with activated platelets was monitored. Heparin modulated clot-lysis was evaluated in human blood clot.
Results: The anionic tPA retained ~97% activity of the unmodified-tPA. FRET experiments confirmed the electrostatic interaction between polyglutamate and protamine which was subsequently reversed by heparin. Complexation with HSA-protamine masked ~60% of tPA activity which was fully regenerated by heparin. The complex retained its prodrug character in human plasma after incubation at 37 °C. Fluorescence microscopic study confirmed binding of the construct with activated platelets. In lysing human clot, the camouflage could mask tPA-activity until it was triggered at a heparin level of 0.4 U/mL.
Conclusion: Oligoanion-modified tPA could be used for targeted/triggered delivery where its enzymatic activity could be masked by HSA-protamine conjugate and successfully regenerated by *therapeutic dose* of heparin.

Introduction

Tissue plasminogen activator (tPA) is the most commonly used enzyme drug in the treatment of acute thrombotic disorders [1,2]. It catalytically converts plasminogen, to plasmin [3,4], which degrades the fibrin mesh and thus exerts thrombolytic action. Like other enzyme drugs, tPA is unable to distinguish thrombus-associated plasminogen from the circulating ones, and hence produces systemic plasmin that creates a 'lytic state' by excessively degrading key clotting factors such as fibrinogen, factor V and factor VIII, leading to fatal bleeding complication, the major side-effect of tPA therapy [5] that affects a significant number of patients treated with this thrombolytic agent [6,7]. Although tPA is considered a fibrin-specific agent, as its activity can be stimulated by 2–3 orders of magnitude in the presence of fibrin [8], targeted thombolysis is compromised because of two major events: i) the short half-life of tPA (~2–5 minutes) that requires administration of a high dose of the drug which offsets the potential benefit of fibrin-specific increment in catalytic action [8–10]; ii) systemic level of soluble fibrin monomer increases significantly during thrombotic event, resulting in systemic stimulation of tPA-activity in the circulation[11,12]. Thus the inherent fibrin-specificity alone could not prevent tPA from developing systemic bleeding complication, hence leading to the need of an effective delivery strategy.

Although the use of enzymes as therapeutic agents has gained a considerable attention due to their high substrate specific action, significant off-target effects are not uncommon when the substrates are ubiquitous throughout the body [13,14]. An effective way to overcome

Abbreviations: tPA, Tissue Plasminogen Activator; HSA, Human Serum Albumin; FRET, Förster Resonance Energy Transfer; ADEPT, Antibody Directed Enzyme-linked Targeted Prodrug; PEG, PolyEthylene Glocol; PA, Plasminogen Activator; LMWH, Low Molecular Weight Heparin; HSA-Pro, Human Serum Albumin Protamine Conjugate; tPA-Glu, Polyglutamate conjugated tPA; DTT, Dithiothreitol; SPDP, N-Succinimidyl1, 3-(2-pyridyldithio) Propionate; PDPH, SPDP Hydrazide; FPLC, Fast protein liquid chromatography; Glu_7-Cys, Polyglutamate Containing Free Cysteine; FITC, Fluorescein Isothiocyanate; Rho B, Rhodamine B Isothiocyanate; ANOVA, Analysis of Variance.

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this shortcoming could be targeted delivery [8,15]. Especially, a pre-targeting strategy resembling ADEPT (Antibody Directed Enzyme-linked Targeted Prodrug) approach could offer localized activation of drug molecules [16–18].

A number of strategies have already been attempted to offer local thrombolytic action of tPA: PEGylation [19], the use of anti-fibrin antibody [20–22], delivery systems, i.e., nanoparticles, liposomes, polymeric hydrogels [23–25] that successfully offered an increased circulation half-life of tPA. However, the success of these approaches may be limited by compromised activity [7] or immunogenic responses [26]. Thrombus-specific delivery systems were also attempted using RGD-peptide to target the integrin GPIIb/IIIa expressed on activated platelets [27–30]. However, without modulating the active site of tPA, this approach does not prevent systemic activation of plasminogen. Therefore a delivery strategy is warranted to prevent tPA from systemic action while preferably retaining its catalytic activity at the desired site.

To this end, we propose a heparin-triggered delivery system for tPA by introducing electronegative functionality in the drug, wherein its activity in the systemic circulation will be masked by forming electrostatic complex with a positively charged camouflaging moiety, albumin-protamine conjugate (HSA-Pro). Upon target accumulation with the help of a targeting moiety (the peptide sequence of the D-domain of fibrinogen that binds with the GPIIb/IIIa on the activated platelet) [31], its action would be regenerated by administering a triggering agent e.g. heparin (Fig. 1). We have previously tested the proof-of-principle of this strategy [32] by chemically modifying tPA with low molecular weight heparin (LMWH) that can act as a docking site for positively charged HSA-Pro. Since LMWH itself is a therapeutically active molecule, we sought to use a relatively inert negatively charged compound to synthesize oligoanion-modified tPA to avoid any potential side-effects. In the current work, we evaluated the feasibility of using 7-mers of glutamate oligopeptide as a negatively charged

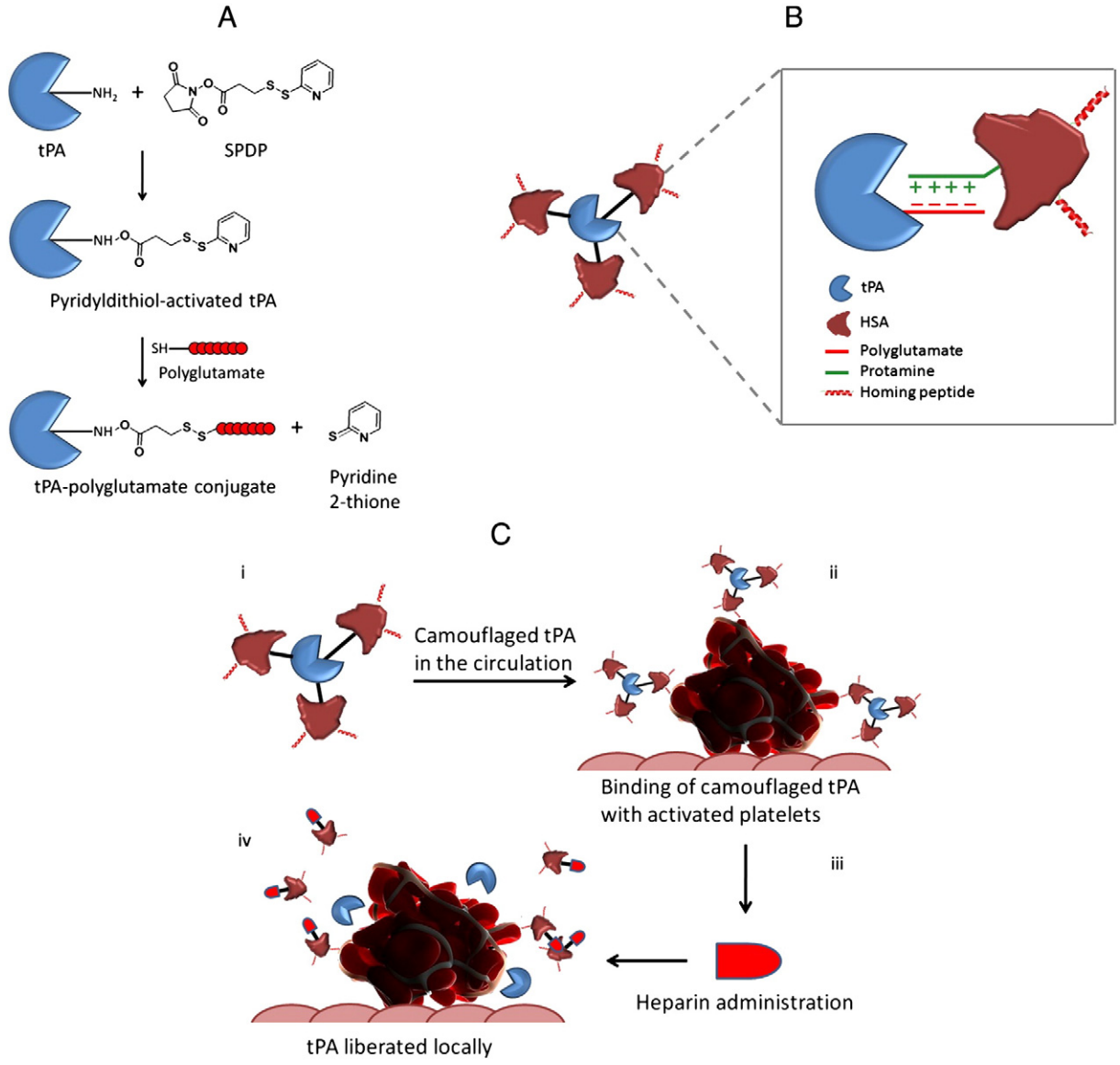

Fig. 1. (A) Schematic diagram of synthesis of tPA-polyglutamate conjugate; (B) The construct of camouflaged tPA consisting of targeting peptide-HSA-protamine and tPA-polyglutamate (tPA-Glu). The camouflaged-tPA is formed by the electrostatic interaction between Polyglutamate and protamine; (C) The schema of the activity of camouflaged tPA in systemic circulation: (i) the albumin bound with tPA will provide steric hindrance to tPA-binding macromolecules in plasma, (ii) accumulation of the complex on the surface of the activated platelets associated with the thrombus via targeting peptide-GP IIb/IIIa binding, (iii) administration of heparin after accumulation of the complex at thrombus site, (iv) triggered release of tPA for local plasminogen activation and fibrinolysis.