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Fibrinolytic PLGA nanoparticles for slow clot lysis within abdominal aortic aneurysms attenuate proteolytic loss of vascular elastic matrix

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Abstract

Abdominal aortic aneurysms (AAAs) involve chronic overexpression of proteases in the aortic wall that result in disruption of elastic fibers and consequent loss of vessel elasticity. Nearly 75% of AAAs contain flow-obstructing, fibrin-rich intraluminal thrombi (ILT), which act as a) a bioinert shield, protecting the underlying AAA wall from high hemodynamic stresses, and b) a reservoir of inflammatory cells and proteases that cause matrix breakdown. For these reasons, restoring flow through the aorta lumen and facilitating transmural diffusion of therapeutics from circulation to the AAA wall must be achieved by slow thrombolysis of the ILT to render it porous without rapid breakdown. Intravenously dosed tissue plasminogen activator (tPA) has been shown to rapidly lyse ILTs in acute stroke and myocardial infarctions. For future use in opening up AAA segments, in this study, we investigated the ability of tPA released from poly(lactic-co-glycolic acid) nanoparticles (PLGA NPs) to slowly lyse fibrin clots without inducing proteolytic injury and matrix synthesis-inhibitory effects on cultured rat aneurysmal smooth muscle cells (EaRASCs). Fibrin clot lysis time was greatly extended over that in presence of exogenous tPA. Surface functionalization of NPs with a cationic amphiphile allowed them to bind to anionic fibrin clot, release tPA at a slower rate and to lyse the clot as a front proceeding outwards unlike the more rapid and homogenous lysis that occurred due to anionic PLGA NPs. Elastic matrix content was decreased in EaRASC cultures exposed to byproducts of clot lysis with exogenous tPA, but not tPA-NPs, and was likely due to increased proteolytic activity (MMPs, plasmin) in EaRASC cultures exposed to exogenous tPA-lysed clots. Our results suggest that gradual ILT lysis via slow release of tPA from NPs will be likely beneficial over exogenous tPA delivery in preserving elastic matrix content and attenuating matrix lysis in the adjoining AAA wall, in vivo, while rendering the ILT porous to facilitate transmural delivery of endoluminally delivered AAA therapeutics.

Graphical abstract

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Keywords

Nanoparticles; Fibrinolysis; Elastic Matrix; Regenerative Matrix Repair; Aortic Aneurysms; Smooth Muscle Cells

1. Introduction

Abdominal aortic aneurysms (AAAs) represent a proteolytic disorder characterized by breakdown and loss of the elastic matrix in the aortic wall, which leads to its progressive weakening and subsequent rupture [1]. It is prevalent in between 1.0–9.0 % of men and 1.0–2.2 % of women in developed nations, and causes 1–3 % of all deaths among men between the ages of 65–85 in this demographic [2]. Extracellular matrix (ECM)-degradative enzymes called matrix metalloproteinases (MMPs), especially MMPs -2 and -9, are chronically overexpressed by inflammatory cells recruited to the AAA site and by activated smooth muscle cells (SMCs) within the AAA wall. These enzymes progressively breakdown the elastic matrix in the aortic wall to drive slow AAA growth [1]. Adult vascular cells [3], more so diseased aneurysmal cells, poorly auto-repair and/or regenerate elastic matrix by [4] and are impaired in their ability to recruit, crosslink and organize elastin precursors into a mature fiber-based matrix [5]. As a result, elastic matrix disruption within the AAA wall cannot be reversed. This prospect is also further diminished by the chronic proteolysis that occurs within AAAs, which prevents any significant net accumulation of intact new elastic matrix structures to overcome proteolytic disruption.

In developing treatment paradigms (e.g., via MMP inhibition and stimulation of de novo cellular elastic matrix assembly) for small, growing AAAs, we anticipate a need to deliver therapeutics to the AAA site in a targeted, localized and sustained manner, to avoid their systemic side effects. However, intraluminal thrombi (ILT), which tend to be present in ~75% of all AAAs [6] represent a barrier to the efficient delivery of AAA therapeutics via the endoluminal route [7]. The exact role of the ILT in AAA progression is still not clearly elucidated. On one hand, ILTs have been suggested to be critical pathophysiological determinants of AAA growth [8], based on a strong correlation shown between ILT size and AAA growth, and risk of rupture [9–11]. The thickness and composition of the ILT has been shown to correlate positively with the extent of matrix disruption within the AAA wall [12, 13], with the initially fibrin-rich clot becoming progressively thicker and platelet- and red blood cell-packed as the AAA grows [14]. As it grows, the ILT furthers proteolysis within the AAA wall, serving as a reservoir of inflammatory cells and proteases [7, 15–17]. Despite these adverse effects, the ILT also mechanically shields the underlying AAA wall from high

hemodynamic stresses against possible rupture [18, 19]. Based on these factors, we hypothesize that slow poration or lysis of the ILT within small AAAs will be important to a) reduce its contribution to proteolytic events that drive AAA growth, b) facilitate uptake of endoluminally delivered therapeutic agents to the AAA wall, and c) maintain the mechanical stabilizing effects of the ILT on the AAA wall. To enable this, new modalities to achieve slow, predictable lysis of ILTs within AAAs are necessary.

Thrombolysis following intravenous administration of tissue plasminogen activator (tPA) has been shown to rapidly restore blood flow in patients following thrombotic events such as stroke [20] and myocardial infarction (MI) [21]. tPA converts plasminogen in the bloodstream into its active plasmin form, which then circulates systemically and mediates thrombolysis. However, high systemic plasmin levels incite enhanced MMP expression [22] and elastic matrix degradation [23], both of which are undesirable from the standpoint of preventing AAA growth. Additionally, rapid clot lysis may be undesirable from the standpoints of a) sudden exposure of the underlying aortic wall to proteases and inflammatory cells present in the ILT, and b) loss of its (bio)mechanical shielding effect on the underlying wall. Therefore, there is a critical need for modalities to enable slow and controlled thrombolysis to modulate plasmin production, minimize systemic plasminemia, and prevent AAA rupture.

Previously, tPA release from liposomes [24, 25] and polymeric microcarriers [25–28] has been shown to cause rapid thrombolysis *in vitro* and *in vivo*, with enhanced thrombolytic efficacy over exogenously dosed tPA. This has been attributed to the ability of these carriers to infiltrate into the thrombus on account of their small size [26]. This mode of delivery was also shown to protect tPA from inactivation by components in the blood (e.g., tPA by PA-inhibitors (PAI)) and prevent depletion of circulating fibrinogen (critical to maintenance of hemostasis) and thus bleeding complications [29]. Based on these previous findings, we have sought to investigate if tPA-release from poly(lactic-co-glycolic acid) nanoparticles (PLGA NPs) can lyse fibrin-rich clots in a significantly more gradual manner than achievable by free tPA. We also sought to investigate the effects of anionic and cationic surface functionalization of the NPs on their fibrin clot binding abilities, and tPA release and clot lysis, and subsequent generation of fibrinolytic products on a) cell proliferation and migration, b) elastic matrix content, c) MMP-synthesis and enzyme activity, and d) plasmin activity in cultures of aneurysmal rat aortic SMCs (EaRASMCs). Our goal is to achieve gradual clot lysis with minimal tPA dosing, so as to limit plasmin production and activation, and thus prevent increases in local proteolytic activity for preserving existing elastic matrix structures. While complete clot lysis may not be necessary, we instead seek to increase the permeability of the clot (via controlled delivery of tPA), towards enabling the subsequent transmural transport of endoluminally infused matrix regenerative/anti-proteolytic AAA therapeutics (cell- or NP-based) to the AAA wall.

2. Materials and methods

2.1 Formulation of PLGA NPs

NPs were formulated from poly (*D*-lactic-co-glycolic acid) (PLGA; 50:50 lactide: glycolide; inherent viscosity 0.95–1.20 dL/g in hexafluoroisopropanol; Durect Corporation,

Birmingham, AL). Chloroform (Fisher Scientific, Fair Lawn, NJ) was used as the organic solvent for NP formulation with didodecyldimethylammonium bromide (DMAB; Sigma-Aldrich, St. Louis, MO) as the stabilizer, while dichloromethane (Sigma-Aldrich) was used as the solvent for NPs formulated using poly(vinyl alcohol) (PVA; Sigma-Aldrich) as the stabilizer.

A double emulsion-solvent evaporation technique was utilized to encapsulate tissue plasminogen activator (tPA, human; Calbiochem, San Diego, CA), as described for other therapeutic agents in recent studies by our group [30, 31]. Briefly, tPA (10 µg in 100 µL of nanopure water) was emulsified into the PLGA solution using a probe sonicator (Q500; QSonica LLC, Newtown, CT) to form a water-in-oil emulsion. This primary emulsion was then further emulsified into an aqueous solution of 0.25% w/v DMAB or PVA to form a water-in-oil-in-water emulsion. All sonication steps were carried out on ice for 1 min, at a 20% amplitude setting. The NP suspension thus formulated was stirred for 16 h at room temperature, and then desiccated for 1 h under vacuum, to remove traces of the organic solvent(s). NPs were recovered by ultracentrifugation at 35,000 rpm (Beckman L-80, Beckman Instruments, Inc., Palo Alto, CA). The NPs were further washed twice with nanopure water, sonicated and ultracentrifuged (30,000 rpm) to remove residual stabilizer and unencapsulated tPA, and then lyophilized for 48 h to obtain a dry powder.

2.2 Size and surface charge measurements on tPA-loaded PLGA NPs

The mean hydrodynamic diameters of the NP formulations were determined using a dynamic light scattering technique, and their mean zeta potential (i.e., surface charge) was quantified via a phase analysis light scattering technique using a commercial particle-sizing system (PSS/NICOMP 380/ZLS, Particle Sizing Systems, Santa Barbara, CA), as previously described by our group [30, 31].

2.3 Efficiency of tPA encapsulation and tPA release from NPs

In order to determine the encapsulation efficiency of tPA within the PLGA NPs, tPA was conjugated with Alexa Fluor® 633 (AF633; carboxylic acid, succinimidyl ester; Life Technologies, Carlsbad, CA). Briefly, a 0.1 mL aliquot of AF633 (containing 500 µg of dye) was added to 0.1 mL of tPA solution (containing 10 µg tPA) and allowed to undergo bioconjugation with gentle shaking for 1 h. Unconjugated AF633 was removed by dialysis (10 kDa molecular weight cutoff; Spectrum Laboratories, Rancho Dominguez, CA) against phosphate buffered saline (PBS; Sigma-Aldrich) for 24 h, with PBS being replaced every 8 h. AF633-conjugated tPA (f-tPA) was then encapsulated within PLGA at a loading of 10 µg, to generate NPs per methods in section 2.1. The supernatants from the washing/ultracentrifugation steps were pooled for each individual NP formulation. The unencapsulated f-tPA was quantified fluorometrically (excitation λ = 580 nm, emission λ = 650 nm) using a SpectraMax M2 plate reader (Molecular Devices, Inc., Sunnyvale, CA), based on a standard calibration curve generated with serial dilutions of a known concentration of f-tPA. The secondary excitation peak for AF633 at λ = 580 nm was used (instead of the primary peak at 633 nm), to avoid interference by the significant fluorescence due to PBS at 633 nm.

Release of f-tPA from the NPs was also assessed using fluorometry (excitation $\lambda = 580$ nm, emission $\lambda = 650$ nm), as described above. Briefly, 1.5 ml polypropylene microcentrifuge tubes ($n = 6$ per formulation) were filled with 1.0 mL of a 3.0 mg/mL suspension (in PBS; pH 7.4) of the DMAB- and PVA-functionalized fibrinolytic NPs (referred to as DMAB-FNPs and PVA-FNPs in the manuscript henceforth), and incubated at 37 °C on a shaker at 100 rpm. In initial tests, this NP concentration was found to release detectable f-tPA levels in the ng/mL range. Release was carried out over 48 h, with sampling of the f-tPA at specific time points (10, 20, 30, 45, 60 and 120 min over the first 2 h, followed by 24 h and 48 h). At each analysis time point, the samples were centrifuged (14,000 rpm, 5 min, 4 °C) in a microcentrifuge (Beckman Microfuge 16®, Beckman Coulter, Inc.), the supernatants were withdrawn to quantify the f-tPA content and the volume was replenished with fresh PBS. The f-tPA released was quantified fluorometrically, based on a standard calibration curve constructed using serial dilutions of a known concentration of f-tPA.

2.4 *In vitro* fibrinolysis by NPs

A preliminary fibrinolysis experiment was carried out with PVA- and DMAB-FNPs encapsulating 50 μ g tPA, to assess their ability to lyse fibrin clots, and compare the same with the effects of an exogenous tPA control (2.0 μ g/mL tPA solution). Based on the results from this initial clot lysis experiment (see Supplementary Figure S1) that showed clot lysis by the FNPs to be prolonged relative to clot lysis by exogenous tPA, in subsequent experiments, we sought to further prolong fibrinolysis by loading PVA- and DMAB-FNPs with only 10 μ g of tPA. To match the steady-state level of tPA released from the PVA-FNPs at ~48 h (see Figure 1), we also tested an equivalent dose (0.2 μ g/mL) of exogenous tPA.

As described previously [32], the fibrinolytic efficacy of the tPA-loaded FNPs was examined by monitoring the temporal decrease in absorbancies due to fibrin clots (relative to their initial absorbance prior to addition of FNPs) within a 96-well plate at $\lambda = 405$ nm. The fibrin clots were formed by mixing human fibrinogen (0.1 mL of 3.0 mg/mL solution; FIB 1; Enzyme Research Laboratories, South Bend, IN), human plasminogen (6 μ L of 1.0 mg/mL solution; Calbiochem), and human thrombin (0.1 mL of 1.0 U/mL solution; Calbiochem) in 40 mM Tris/HCl buffer (Sigma-Aldrich) containing 75 mM NaCl (Sigma-Aldrich). Clot formation was allowed to progress for 1 h at 24 °C. The absorbance ($\lambda = 405$ nm) of the clot was measured at 1 min intervals over this time, and was found to plateau thereafter. Following the formation of the clot, the FNPs (50 μ L) were added to the clot at concentrations of 0.1, 0.2 and 0.5 mg/mL, with similar volumes of tPA (0.2 μ g/mL concentration) and Tris/HCl buffer added instead as the exogenous and clot controls, respectively. The absorbances of the clot at different time points were determined by subtracting the absorbances of control wells containing only FNPs from that of wells containing the clot with FNPs as it lysed the clot.

2.5 Impact of surface charge of NPs on their clot binding affinity

PLGA NPs encapsulating AF633 were formulated using a protocol similar to that described earlier (in Sections 2.1 and 2.3) using PVA and DMAB as stabilizers. To compare their clot binding affinities, NPs (50 μ L) were added to already-formed fibrin clots (per method in Section 2.4) in a 96-well plate at three different NP concentrations (0.2, 0.5 and 1.0 mg/mL;

$n = 6$ per NP type) and incubated for 60 min. To assess non-specific binding of NPs to the wells (if any), empty wells were also incubated with NPs at the three concentrations mentioned. At the end of the incubation step, the supernatant solution was collected, all wells (fibrin clot and empty blanks) washed with PBS, and the wash solutions then collected and added to the supernatant for fluorometric analysis for NP content. Dilution of the supernatant upon addition of wash solution was taken into account in subsequently quantifying NP binding.

Efficiency of fibrin clot binding of the NPs was estimated from the decrease in fluorescence of the supernatant of the NP suspension ($\lambda_{\text{excitation}} = 580 \text{ nm}$, $\lambda_{\text{emission}} = 650 \text{ nm}$) based on a calibration curve prepared from serial dilutions of suspensions containing known concentrations of the two NP formulations. The total NP binding to the clots was obtained by subtracting the amount of NPs adherent to the empty wells from the amount adherent in the wells containing the clot, and is expressed as a percentage of the amount of NPs added to the fibrin clot in the wells.

2.6 Isolation and culture of SMCs from elastase infusion-induced rat AAAs

EaRASCs were isolated from aneurysmal aortae excised from adult male Sprague-Dawley rats ($n = 3$) at 14-days post-AAA induction via elastase infusion, as we have described previously [4]. The primary EaRASCs obtained from these tissue explants were expanded over 2 weeks in DMEM-F12 cell culture medium (Invitrogen; Carlsbad, CA) supplemented with 10% v/v fetal bovine serum (FBS; Invitrogen) and 1% v/v PenStrep (Thermo Fisher, South Logan, UT), and passaged upon attaining confluence. In a prior study [4], we have thoroughly characterized the cells to confirm their identity and shown them to maintain a diseased phenotype in culture over several passages.

2.7 Experimental design for cell culture

We evaluated the effects of fibrinolytic byproducts generated upon lysis of fibrin clots by PVA- and DMAB-FNPs, and separately by exogenous tPA ($0.2 \mu\text{g/mL}$), on cell proliferation, elastic matrix synthesis/content, MMP production & activity, and plasmin activity in EaRASC cultures. This was carried out in a transwell assay system, which comprised EaRASCs cultured in a well plate, with the fibrin clot and FNPs (or exogenous tPA) present within a transwell insert placed within the well. EaRASCs (passages 2–5) were seeded at 3×10^4 cells/well and cultured over a total of 21 days in 6-well plates ($A = 10 \text{ cm}^2$; USA Scientific, Ocala, FL) in DMEM-F12 cell culture medium supplemented with 10% v/v FBS and 1% v/v PenStrep. Following seeding, the EaRASCs were allowed to proliferate for 10 days, to allow them to deposit measurable amounts of elastic matrix. On day 10 post-cell seeding, the fibrin clot was formed for (1 h at 24°C , as described in Section 2.4) in a 6-well cell culture insert (ThinCert™, Greiner Bio-One, Monroe, NC) and DMAB- or PVA-FNPs (encapsulating $10 \mu\text{g}$ tPA) were then added to the clot at a concentration of 0.5 mg/mL . The clot and NPs/exogenous tPA (in the insert) were exposed to the EaRASCs (cultured in the wells) for a further 11 days. EaRASCs cultured without exposure to a clot served as the cell control, while those cultured with the clot alone (no FNPs) served as the unlysed clot control. The EaRASCs were harvested after 21 days of culture. For

immunofluorescence detection of elastic matrix proteins, cells were cultured on 42 mm × 42 mm glass cover slips (VWR Scientific) within the 6-well plate.

2.8 DNA assay for cell proliferation

A fluorometric assay using the Hoechst 33258 dye (Invitrogen) was utilized to determine the DNA content of the cell layers, as described in recent studies by our group [30, 31], towards assessing the effects of tPA released and fibrinolytic products on EaRASMC proliferation. The cells were seeded as described in Section 2.7 and the cell layers were harvested at 1 day, 10 days (prior to clot exposure) and 21 days of culture in NaCl-Pi buffer, sonicated on ice and assayed for DNA content. The cell density was calculated, assuming 6 pg DNA per cell [33].

2.9 Fastin assay for elastin

The total elastin content in the harvested EaRASMC cell layers was quantified using a Fastin assay (Accurate Scientific and Chemical, Westbury, NY), as described in recent publications by our group [30, 31]. Cell layers harvested in NaCl-Pi buffer, were sonicated on ice to homogenize the cell layer. Following this, the cell layer suspension was digested with oxalic acid, as per the manufacturer's protocol, to extract elastin. Briefly, a 300 µL aliquot of the cell layer suspension was first mixed with 100 µL of 1.0 M oxalic acid, then digested (90 min, 95 °C), centrifuged at 14,000 rpm, and the supernatant (containing solubilized elastin) extracted. 400 µL of 0.25 M oxalic acid was then added to the remainder of the cell layer, and further digested for 60 min at 95 °C. The extracts from the two digestion steps were pooled, and the elastin content within these cell layers was assayed using the Fastin assay. The amounts of elastin measured were also normalized to their corresponding DNA amounts (see Section 2.8), so as to provide an accurate comparison between the different treatments.

2.10 Western blots for MMP-2 and MMP-9 protein synthesis

The impact of the NP-released and exogenous tPA, and the fibrinolytic products following clot lysis on MMP-2 and -9 synthesis by EaRASMCs was semi-quantitatively assessed by western blot analysis, as described in our previous papers [30, 31]. After 21 days of culture, the cell layers were harvested and assayed for total protein content using a bicinchonic acid (BCA) assay kit (Thermo Scientific). Maximum volumes of sample protein (15.6 µL) were then loaded under reduced conditions into each lane of a 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Invitrogen). Following electrophoresis, the gels were transferred onto nitrocellulose membranes (iBlot® Western Blotting System, Invitrogen), blocked with Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) for 1 h at room temperature, and finally immunolabeled for 16 h at 4 °C with a rabbit polyclonal antibody against MMP-2 (1:500 dilution; Abcam, Cambridge, MA) or a rabbit monoclonal antibody against MMP-9 (1:500 dilution; Millipore, Inc.), with a mouse monoclonal antibody against β-actin (1:1000 dilution; Sigma-Aldrich) as the loading control. Secondary antibody labeling was carried out for 1 h at room temperature using IRDye® 680LT goat-anti-rabbit (1:15,000 dilution) and IRDye® 800CW goat-anti-mouse (1:20,000 dilution) polyclonal antibodies (LI-COR Biosciences). Fluoroluminescence detection of protein bands for MMP-2 (zymogen = 72 kDa; active = 62–66 kDa) and MMP-9 (zymogen = 92 kDa;

active = 82 kDa) was then carried out using a LI-COR Odyssey laser-based scanning system. The intensities of MMP bands on all gels were quantified using ImageJ software, expressed in terms of relative density units (RDU) and normalized to the intensity of their respective β -actin bands to enable comparison between the different test cases within the same blot. The normalized band intensities of active MMP for the FNP- or tPA-treated and NP-untreated EaRASC cell control cultures were further normalized to those of the NP-untreated unlysed clot control cultures to determine the fold-change(s) in production of active MMP, and the statistical significance of the differences between them. The results presented in this manuscript were averaged from 3 replicate gels run per culture treatment.

2.11 Gel zymography for MMP-2 and MMP-9 enzyme activities

The effects of NP-released and exogenous tPA and of the products of fibrinolysis on MMP-2 and -9 activities in EaRASC cell layers were analyzed via gel zymography, as we have previously described [30]. Briefly, volumes of the cell layer samples (harvested in RIPA buffer containing protease inhibitor) equivalent to 10 μ g of protein, were loaded into each lane of a 10% zymogram gel (Invitrogen), along with a SeeBlue[®] pre-stained molecular weight ladder (Invitrogen). The gel was run for 2 h at 125 V. The gels were then washed in a buffer containing 2.5% v/v Triton-X-100 for 30 min to remove SDS, and then incubated for 48 h in a substrate/development buffer to activate the MMPs. The gels were stained with Coomassie Brilliant Blue solution for 45 min, and destained for 90 min, until clear bands appeared visible against the blue background of the gel. Band intensities (RDU) of the bands obtained for test cultures were quantified using ImageJ software, and normalized to those obtained for the unlysed clot control cultures to determine fold changes in MMP-2 and -9 activities. Data was acquired from 3 replicate gels.

2.12 Plasmin activity within EaRASC cultures

The chromogenic plasmin synthetic substrate S2403 (Diapharma, Inc., West Chester, OH) was utilized to measure plasmin activity in EaRASC layers. Volumes of cell layer samples equivalent to 20 μ g of protein were incubated with S2403 (final concentration = 0.3 mM) [34] for 24 h at 24 °C. A calibration curve was constructed based on the absorbances of serial dilutions of plasmin incubated similarly with S2403. Plasmin activity within the cell layers was calculated from the absorbance of the cell layer samples at λ = 405 nm, based on the plasmin calibration curve, and expressed in terms of ng of plasmin per 20 μ g of protein in the cell layer.

2.13 Immunofluorescence-based visualization of elastic matrix in cell layer

For immunofluorescence studies, EaRASCs were seeded and cultured on glass cover slips placed within wells of a 6-well plate, as described earlier in Section 2.7. On the tenth day after EaRASC seeding, a fibrin clot was first formed within a cell culture insert placed within these wells, and then lysed with PVA- and DMAB-FNPs, as well as exogenous tPA (0.2 μ g/mL). The cultured EaRASCs were exposed to the clot lysis products generated by tPA release from the FNPs for the next 11 days. At this time (i.e., a total of 21 days of culture of the EaRASCs), the cell layers were fixed with ice-cold methanol and treated with a rabbit anti-rat polyclonal antibody against elastin (1:100 v/v dilution; Millipore) at. The detected proteins were visualized with Alexa488-conjugated IgG secondary antibodies

(1:1000 v/v dilution; Invitrogen). The cell layers were mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA), which labels the cell nuclei. A minimum of 6 regions per cell layer from a total of $n = 3$ replicate treatments were imaged to assess representative outcomes. Imaging was carried via immunofluorescence microscopy using an Olympus IX51 fluorescence microscope (Olympus America).

2.14 Statistical analysis

All experimental data presented ($n = 6$ /condition, unless stated otherwise) represent mean values with standard deviation (SD). Statistical significance of differences between mean values for different samples and conditions was evaluated using a Student's t -test, with $p < 0.05$ considered as statistically significant.

3. RESULTS AND DISCUSSION

3.1 Formulation and characterization of tPA-loaded PLGA NPs

The overall goal of this study was to develop a NP-based delivery system for slow and predictable lysis of fibrin-rich ILTs at AAA sites, to render them porous for transmural delivery of drugs to AAA tissue from circulation, and while continuing to avail of the mechanical stability they provide to the weakened AAA wall. Our NP formulations were formulated using PLGA, which has been approved by the FDA for drug delivery applications on account of its biodegradability, biocompatibility with a variety of drugs, appropriate biodegradation characteristics, as well as its ease of formulation [35, 36]. For this study we investigated NPs formulated with PLGA containing a 50:50 lactide:glycolide ratio, since it is the primary form of PLGA used in nanoparticle applications involving controlled drug delivery [36]. Additionally, the PLGA had an inherent viscosity of 0.95–1.2 dL/g (molecular weight equivalent to 117,700 Da, as specified by the company), as high molecular weight PLGA exhibits slower degradation compared to low molecular weight PLGA [37]. We used a double emulsion-solvent evaporation technique to encapsulate tissue plasminogen activator. An emulsion stabilizer or surfactant was added to enable nanoparticles to form from the tPA-PLGA (water-oil) emulsion and to prevent their coalescence into larger aggregates. In essence, the surfactant reduces the interfacial tension to reduce laplace pressure (pressure difference between the inside and outside of a formed nano-droplet) [38]. In addition, when the zeta potential, which is a measure of NP surface charge, is high (either positive or negative), the NPs are stable and prevented from aggregating [39]. Accordingly, in this study, we prepared two different tPA-releasing NP formulations based on use of a non-ionic emulsion stabilizer, polyvinyl alcohol (PVA) and DMAB, a cationic amphiphile, which are known to impart NPs negative and positive zeta potentials respectively [39]. An additional rationale to create and evaluate both negatively- and positively-charged tPA-releasing NPs is that they could potentially generate different clot lysis profiles, since the anionic PVA-FNPs would likely be distributed throughout the clot to enable homogenous clot lysis [40, 41], and the cationic DMAB-FNPs likely to strongly bind negatively-charged fibrin within the clot [27, 28], thereby enhancing their longer-term retention for lytic activity within the clot. Additionally, cationic NPs have also been shown to a) exhibit enhanced arterial uptake [42], and as we have earlier published [30], to b) repel cationic elastases [43], c) improve elastin crosslinking, and d) inhibit

MMPs, which could portend NP-derived benefits to improved preservation of existing elastic matrix within the AAA wall and augmented elastic matrix regenerative outcomes.

The surface charges (ζ -potentials) of the NPs were -36 ± 2 mV and $+23 \pm 3$ mV for PVA-FNPs and DMAB-FNPs, respectively (Table 1). Encapsulating tPA (10 μ g) within these NPs did not alter their surface charge. The NPs formulated with both PVA and DMAB exhibited mean hydrodynamic diameters of 358 ± 5 nm and 446 ± 20 nm, respectively (Table 1); tPA encapsulation did not affect their sizes. We previously showed that NP sizes < 500 nm remain excluded within the extracellular space, and are not taken up by EaRSMCs [30]. These sizes are also significantly smaller than the typical pore size (~ 4 μ m) of a fibrin clot [44], which would allow non-clustered NPs to easily permeate into the clot interior. However, it must be noted that the ILT pore sizes *in vivo* within AAAs tends to be highly variable (from a few nm to μ m) [45], with clots within large AAAs (diameter > 5.5 cm) being denser with smaller pores compared to those with small AAAs, rendering them more resistant to lysis [14]. Thus, the NPs we have formulated would be expected to permeate to some extent within the ILT, and thereafter render the ILT more porous via clot lysis following their release of tPA.

3.2 Encapsulation and release of tPA from PLGA NPs

Since our goal was to prolong fibrinolysis, the amounts of tPA we loaded within our NPs (10 μ g) were ~ 10 -fold lower than that encapsulated by other groups [27, 28, 46]. The supernatant solutions generated during the centrifugal pelleting of the formulated NPs contained less than 5 ng/mL of f-tPA, which based on the calibration curve of serial dilutions of f-tPA, indicates that $\sim 90\%$ of f-tPA was encapsulated within the NPs. Our tPA encapsulation efficiencies were much higher than the 55–71% obtained by these other groups [27, 28], and may be attributed to the fact that at lower tPA loadings, the amount of tPA included within the emulsion during the formulation process is higher and thus results in more efficient encapsulation [47].

The cumulative release of f-tPA from NPs is shown in Figure 1. Consistent with prior observations [27, 28], significant release of f-tPA occurred during the initial 2 h, following which a release plateau was attained by ~ 48 h. The burst release of tPA can be attributed to the rapid out-diffusion of tPA near the NP surface upon solubilization by the infiltrating aqueous medium, a process driven by the high concentration gradient (of tPA). Differently, the steady state release phase corresponds to slow diffusion driven by a much-reduced concentration gradient, with the tPA release rate being limited by the slow hydrolysis of the PLGA matrix.

Our results also showed that in addition to the burst release of tPA (Figure 1), the steady-state concentrations for f-tPA release from PVA-FNPs (0.2 μ g/mL) was significantly higher ($p < 0.05$) than that generated by DMAB-FNPs (0.1 μ g/mL). Since the encapsulation efficiencies for both formulations were very similar, the higher f-tPA release from the PVA-FNPs is likely due to their smaller size (compared to DMAB-FNPs), and resultant higher surface area per unit volume available for out-diffusion of tPA. Additionally, we also hypothesize that hydrophobic interactions between the hydrophobic chains of DMAB and hydrophobic domains in tPA [48] may have resulted in its slower release from DMAB-

FNPs, compared to FNPs formulated with PVA, which exhibit hydrophilic surface functionality. It must also be noted here that the total amount of f-tPA released (at 48 h) represents ~20% of the total amount of tPA initially encapsulated.

3.3 Fibrinolytic efficacy of tPA-loaded PLGA NPs

ILTs present at the AAA site have been shown to be fibrin-rich [14, 49]. Fibrin plays a critical role in mediating thrombus growth via its strong affinity for platelets (and plasma proteins involved in the coagulation cascade), while providing the thrombus with structural definition and mechanical stability [14, 50]. Since our proposed localized therapies target primarily small AAAs (<5.5 cm diameter), in this study we seek to specifically target the ILTs in AAAs of this size, which tend to be patchy and nascent, with fewer platelets, and which lie adjunct to the aortic wall wherein matrix disruption is only partial. This is unlike in large AAAs in which the much greater ILT thickness correlates strongly with high levels of matrix disruption [9, 10, 14]. On this basis, the fibrin component of the clot has a greater relative significance in small AAAs than in large AAAs wherein platelets form a major component of the more established ILTs. Therefore, in this first study, we have utilized the fibrin clot model to evaluate different modalities for controlled/gradual clot lysis. Future studies will gradually increase the complexity of clot composition, to more closely mimic a fully-developed clot, for more rigorous assessment of our FNPs, which will help assess the extension of their use to treating ILTs in large AAAs.

Preliminary experiments utilizing PLGA NPs encapsulating 50 µg of tPA (Supplementary Figure S1) showed them to successfully lyse fibrin clots *in vitro*. Clot lysis time, as defined as the duration for the clot absorbance to decrease to 50% of its initial value [32], was longer for tPA released from the NPs, compared to exogenous tPA (2 µg/mL). PVA-FNPs demonstrated more rapid fibrinolysis compared to DMAB-FNPs, with the former exhibiting lysis times between 105 min (for 0.2 mg/mL NP concentration) and 145 min (for 0.5 mg/mL NP concentration). DMAB-FNPs, on the other hand, exhibited clot lysis times of 960 min (for 0.2 mg/mL NP concentration) and 420 min (for 0.5 mg/mL NP concentration).

Towards achieving more gradual clot lysis, we investigated the fibrinolytic efficacy of NPs encapsulating still lower amounts (10 µg) of tPA. The exogenous control of 0.2 µg/mL tPA solution (equivalent to the steady state concentration of tPA released from the NPs; Figure 1) generated a clot lysis time of ~20 min. Differently, as seen in Figure 2A, the absorbance of fibrin clots treated with 0.5 and 1.0 mg/mL of PVA-FNPs decreased to approximately 36% of its initial value after 5 h, and that of clots incubated with 0.2 mg/mL PVA-FNPs was ~89% of the initial clot absorbance after the same duration. However, at the 24 h time-point, the absorbance of the clots treated with 0.2 mg/mL PVA-FNPs decreased significantly to 55% of its initial value, while those of clots incubated with 0.5 and 1.0 mg/mL PVA-FNPs decreased further only marginally to 26% and 30% of their initial value. Due to the discrete sampling events being spread over the entire extended period of lysis, the exact time point at which the absorbance of the clot decreased to 50% of its initial value could not be specifically identified. However, interpolating between our data points, we estimate that the clot lysis time for 0.2 mg/mL of PVA-FNPs is > 24 h, while that for 0.5 and 1.0 mg/mL of PVA-FNPs is > 5h and < 24 h.

Fibrinolysis was even slower for DMAB-FNPs loaded with the same 10 µg of tPA, with the absorbances of clot treated with 0.2, 0.5 and 1.0 mg/mL NP concentrations decreasing marginally to ~88% of their initial value after 5 h. However, even after 24 h of incubation with the clot, only the DMAB-FNPs at a concentration of 0.5 mg/mL decreased clot absorbance below 50% of its initial value (36%), while those at 0.2 and 1.0 mg/mL NP concentrations showed moderate decreases to 67 and 70% of their initial values. We therefore estimate that the clot lysis time for 0.5 mg/mL of DMAB-FNPs lies between 5–24 h, while that for 0.2 and 1.0 mg/mL of DMAB-FNPs is longer than 24 h. The longer lysis time observed for the 0.2 mg/mL NP concentration (for both PVA – and DMAB-FNPs) may be attributed to the lower amounts of tPA being released from the NPs at this NP concentration relative to higher NP concentrations. On the other hand, at the much higher 1.0 mg/mL NP concentration, we hypothesize that at this higher FNP concentration, steric crowding effects of the NPs hinder tPA release and subsequent fibrinolysis.

Overall, our results indicate that the DMAB-FNPs cause more gradual clot lysis compared to the PVA-FNPs. Although this could be due to the slower release of tPA from DMAB-FNPs compared to their PVA counterparts (Figure 1), we hypothesize that the more dominant effect underlying the more gradual fibrinolysis may be attributed to the positively charged DMAB-FNPs binding electrostatically to fibrin, which is negatively charged at pH 7.4, leading to their localization at the clot boundary/interface. PVA-FNPs on the other hand would be expected to permeate and become distributed throughout the clot, mediating more rapid intra-clot lysis unlike DMAB-FNPs which would primarily cause clot lysis to proceed as a front (i.e., outside to inside), in a top-down manner.

3.4 Effects of surface charge of FNPs on their clot binding affinities

We evaluated the binding efficiency of DMAB- and PVA-surface functionalized NPs to fibrin clots using AF633-encapsulated NPs. As shown in Figure 3, the DMAB-functionalized NPs exhibited a greater binding to the fibrin clots, compared to the PVA-functionalized NPs, likely due to opposite charge interactions. For DMAB-NPs, $77.6 \pm 2.9 \%$, $81.7 \pm 4.2 \%$ and $83.2 \pm 5.9 \%$ of NPs bound to the fibrin clot at NP concentrations of 0.2, 0.5 and 1.0 mg/mL, respectively; the lack of dependence of binding of the DMAB-functionalized NPs on NP concentration suggests that their association with the fibrin clot is primarily dictated by these strong electrostatic interactions rather than by physical trapping within the fibrin clot. The corresponding figures for PVA-NPs, were significantly lower at $34.8 \pm 9.4 \%$, $49.6 \pm 3.4 \%$ and $58.9 \pm 7.0 \%$ respectively. This differential ability of cationic NPs to bind fibrin within clots may thus represent a means of enabling or enhancing targeting of NPs from blood circulation to ILTs at the AAA site as we had initially hypothesized.

3.5 Effects of clot lysis products on proliferation and elastic matrix content within EaRASC cultures

We demonstrated that FNPs (both PVA and DMAB-functionalized) loaded with 10 µg of tPA effectively extend clot lysis time, compared to an exogenous dose of tPA (equivalent to steady state release of 0.2 µg/mL tPA; Figure 1). Accordingly, we formulated NPs with this level of tPA loading to investigate how the clot lysis products generated impact elastic

matrix preservation and assembly by EaRASCs. We have also previously shown that EaRASCs in low passage culture a) replicate numerous aspects of human AAA etiology and pathology [4, 51], b) maintain their diseased, MMP-overexpressing, and hyper-proliferative phenotype, c) demonstrate poor elastogenic potential relative to healthy aortic SMCs, and d) respond to pro-elastogenic stimulation with growth factors [4, 51]. On this basis, we deemed it appropriate to investigate functional effects of the FNPs and their clot lysis products on cultured EaRASCs.

EaRASCs were first allowed to proliferate for 10 days post-seeding, in order for them to deposit measurable quantities of pre-existing elastic matrix (to replicate existing elastic matrix in the AAA wall). They were then cultured for a further 11 days with exposure to a fibrin clot in the absence or presence of clot-lysing FNPs. While there was no significant increase in cell number at day 21 in cell control cultures (no clot, no NP) compared to day 10 of culture (Figure 4), EaRASC proliferation during the same 11-day period was increased in the presence of the unlysed fibrin clot control (clot, no NP), as well as the test cases with fibrin clots lysed by DMAB and PVA-FNPs. EaRASCs cultured in the presence of a fibrin clot lysed by DMAB-FNPs demonstrated significantly higher levels of proliferation ($p < 0.05$) compared to those cultures exposed to the unlysed clot control. This result is similar to that obtained by other groups who observed that fibrin degradation products [52], [53] enhance vascular SMC proliferation. However, the exact mechanisms by which these biomolecules mediate this effect remain unclear, and must be explored in a future study. We hypothesize that the enhanced proliferation of EaRASCs exposed to clot lysis product released following fibrinolysis with DMAB-FNPs may be attributed to the slower tPA release profile from DMAB-FNPs, coupled with their ability of achieve more gradual fibrinolysis (compared to PVA-FNPs).

Although studies have demonstrated that tPA overexpression increases SMC proliferation [54], we did not observe a significant increase in EaRASC proliferation upon exposure to a clot lysed with exogenous tPA (0.2 $\mu\text{g/mL}$) compared to the cell control or unlysed clot control cultures (data not shown). Based on studies that have indicated that the activation of plasminogen to plasmin leads to pericellular proteolysis and SMC apoptosis [55, 56], we hypothesize that the relatively rapid plasmin generation (initially) following clot lysis with exogenous tPA may cause some degree of SMC apoptosis. However, the post-fibrinolytic release of fibrin degradation products likely enhances cell proliferation to counter this cell loss by restoring cell counts to levels observed for cell and unlysed clot control EaRASC cultures. This potentially explains the lack of higher levels of EaRASC proliferation following exposure to a clot rapidly lysed by exogenous tPA compared to the cells exposed to clots more gradually lysed with FNP-released tPA.

We observed a significant increase ($p < 0.05$) in total elastic matrix content in cell control EaRASC cultures at day 21 compared to day 10 (at the time of initiating clot formation and lysis, used in this study as a baseline level for pre-existing elastic matrix), both on an absolute basis (Figure 5A) and a per cell basis (Figure 5B). There was no significant difference ($p > 0.05$) in elastic matrix content at day 21 between the cell control cultures and the EaRASCs cultured in the presence of an unlysed fibrin clot (clot, no NP), both on an absolute and per cell basis. However, fibrinolysis with exogenous tPA (0.2 $\mu\text{g/mL}$;

equivalent to maximal steady state release from the FNPs) caused a significant decrease in net elastic matrix content within EaRASC cultures, compared to the unlysed clot control, both on an absolute and per cell basis (Figures 5A and B). In fact, these levels were even lower than the baseline levels in EaRASC cultures at day 10 (prior to clot formation and lysis), suggesting that rapid clot lysis via exogenously dosed tPA leads to the degradation of pre-existing elastic matrix, and also likely compromises the ability of EaRASCs to synthesize new elastic matrix. When cultured with a fibrin clot lysed with DMAB- and PVA-FNPs, there were no increases in absolute elastic matrix amounts within control EaRASC cultures, over the baseline elastic matrix levels observed in day 10 cultures, though in EaRASC cultures exposed to DMAB-FNP lysed clots alone, the DNA-normalized elastic matrix amounts measured at 21 days of culture was significantly lower ($p < 0.05$) than the corresponding baseline levels measured in the cell layers at 10 days post-seeding (Figure 5B). There were no significant differences in elastic matrix content (absolute and DNA-normalized) in cultures exposed to DMAB-FNP-lysed clots vs. those exposed to PVA-FNP-lysed clots ($p > 0.05$).

Since exposure to unlysed clots increased, and not decreased elastic matrix content relative to the same cultures at the time of initiating clot exposure (at 10 days post seeding), our results suggest that tPA released from the FNPs and the clot lysis products cause decrease in net elastic matrix content within EaRASC cultures. The mechanisms underlying these effects, i.e., whether decreased elastic matrix content following clot lysis (vs. baseline levels at 10 days post-seeding) is only due to degradation of preexisting and newly synthesized elastic matrix, or instead/additionally due to inhibition of de novo elastin synthesis is unclear. Regardless, the results indicate net decrease in elastic matrix content may be due to proteolysis by MMPs-2 and/or -9, or by post-fibrinolytic release of plasmin from the clot. Overall, these results clearly illustrate the benefit of gradual clot lysis via controlled release of tPA from PLGA NPs, over its equivalent exogenous delivery, in the preservation/maintenance of elastic matrix content within these EaRASC cultures.

3.6 Effects of fibrinolytic products on MMP synthesis and activity in EaRASC cultures

As a first step towards delineating the causes for the observed decrease in net elastic matrix content in EaRASC cultures following exposure to FNP-lysed clot, we evaluated the effects of the fibrinolytic products on MMP-2 synthesis (via western blotting) and MMP-2 and -9 activities (via gel zymography). Western blots showed synthesis of MMP-2 and -9 in the cell control EaRASC cultures (no clot, no NP) to be significantly lower (1.7 ± 0.2 fold for MMP-2 and 1.6 ± 0.2 fold for MMP-9; $p < 0.05$) than in cultures exposed to unlysed fibrin clots (Figures 6A–D). A 3.1 ± 0.3 fold increase in MMP-2 synthesis (compared to the unlysed clot controls; $p < 0.01$), was observed in EaRASC cultures exposed to fibrinolytic products following clot lysis with exogenous tPA ($0.2 \mu\text{g/mL}$). Again, compared to the unlysed clot control, we observed a 4.5 ± 0.7 fold increase in MMP-9 synthesis by EaRASCs, following exposure to a clot lysed with exogenous tPA. Differently, tPA released from the FNPs and the generated clot lysis products did not induce any significant increase in active MMP-2 protein synthesis compared to cell layers cultured in the presence of an unlysed clot ($p > 0.05$). In all FNP-treated cases, MMP-9 bands were absent or too faint to be quantified reliably via densitometry (Figure 6C and D) despite concentrating the

samples, suggesting that MMP-9 synthesis was likely actively decreased by FNP released tPA and/or its clot lysis products, besides possibly the FNP degradation products themselves. While this aspect needs to be investigated further, it is nevertheless clear that tPA release from FNPs provides significant advantages over exogenous tPA dosing in terms of not stimulating production of elastolytic MMPs 2 and 9 by aneurysmal SMCs located adjacent to the clot.

Densitometric analysis of bands in gel zymograms (Figure 6E) showed activities of MMP-2 and -9 in EaRASC cultures exposed to unlysed clots to be similar to that in cell control EaRASC cultures ($p > 0.05$). On the other hand, upon exposure to clot lysis products following exogenous dosage of tPA, we observed a 2.0 ± 0.5 and 1.6 ± 0.3 fold increase in MMP-2 and -9 activities ($p < 0.01$), respectively, compared to the unlysed clot control, suggesting that exogenous tPA and the products of its rapid lysis of a fibrin clot cause an upregulation of MMP activity within EaRASC cultures. However, MMP-2 and -9 activities in EaRASC layers exposed to DMAB- and PVA-FNP-released tPA and its generated clot lysis products were significantly lower ($p < 0.05$), compared to the unlysed clot controls. Thus, the lack of any increase in MMP-2 synthesis (Figure 6A, B), as well as the decrease in MMP-9 synthesis (Figure 6C, D), and significant decreases in MMP-2 and -9 activities (Figure 6E, F) in EaRASC cultures exposed to FNP-lysed clots relative to those exposed to unlysed clots (which mimics a 'pre-treatment' scenario), suggests that the moderate decrease in elastic matrix content in EaRASC cultures following exposure to FNP-lysed clots (Figures 5A and B) is not effected by MMPs-2 and -9. On the other hand, our results suggest that the significant net decrease in elastic matrix content observed within EaRASC cultures exposed to exogenous tPA-lysed clots is at least in part attributable to increased synthesis and activities of both MMP-2 and -9, a likely outcome of exposure of the EaRASCs to much higher concentrations of free tPA, and to rapidly generated products of clot lysis.

3.7 Effects of clot lysis products on plasmin activity in EaRASC cultures

We evaluated the effects of the fibrinolytic products on plasmin activity within the EaRASC cultures. As seen in Figure 7, exposure to an unlysed clot (Clot, no NPs) significantly enhanced plasmin activity ($p < 0.05$) in EaRASC cultures relative to cell control EaRASCs (no clot, no NP). Plasmin activity was dramatically higher in EaRASC cultures exposed to clots lysed with exogenous tPA, relative to control EaRASC cultures and even those exposed to an unlysed clot. Though exposure to FNP-lysed fibrin clots induced significant increases in plasmin activity within EaRASC cultures relative to the unlysed clot-exposed cultures, the plasmin activity levels remained significantly lower than that in cultures exposed to exogenous tPA-lysed clots.

There were no significant differences ($p > 0.05$) in plasmin activity between cultures exposed to clots lysed with DMAB-FNPs, and with PVA-FNPs. These results show that post-fibrinolytic release of plasmin following exposure to a clot lysed by tPA (both exogenous and released from FNPs) is significant, and likely mediates the elastic matrix breakdown and net decrease in elastic matrix content in the cell cultures during the period of clot exposure. This is not unexpected, as studies by other groups have shown plasmin to play

a key role in enhancing MMP synthesis and activity [22], as well as degradation of ECM (especially elastic matrix) [23, 56]. Importantly, the decreases in elastic matrix content in cell layers exposed to the FNP-lysed clots and exogenous tPA-lysed clots directly correlate with the level of increases in plasmin activity within these cultures, which in turn appear to be likely determined by the bioavailability of free tPA and rapidity of generation of clot lysis products.

3.8 Immunofluorescence detection of elastin in matrix

Figure 8 shows representative immunofluorescence (IF) micrographs of elastic matrix in control EaRASMC (no clot, no NPs) layers at 21 days of culture, and in EaRASMC layers also at 21 days of total culture, but exposed to unlysed fibrin clots, FNP-lysed clots, and exogenous tPA-lysed clots respectively for the last 11 days, as described in Section 2.7. IF labeling controls showed no fluorescence, thus demonstrating lack of non-specific binding of the secondary antibody (Figure 8A). Relative to EaRASMC layers exposed to the cell control (no clot, no NP; Figure 8B) and the unlysed clot control (**clot, no FNP**; Figure 8C), those cultured in the presence of clots lysed by PVA- and DMAB FNPs (Figures 8D and E) showed significantly lower elastic matrix content, which concurs with biochemical data obtained using a Fastin assay (Figure 5). Additionally, the EaRASMC layers exposed to a clot lysed with exogenous tPA (0.2 µg/mL; Figure 8F) demonstrated relatively minimal immunofluorescent staining for elastic matrix compared to those exposed to clot lysis products following fibrinolysis with DMAB- and PVA-FNPs.

4. CONCLUSIONS

The studies described in this manuscript represent the first step(s) towards evaluating NPs releasing tPA for slow fibrinolysis as a strategy for revascularization of AAAs towards endoluminal delivery of therapeutics. We conclusively demonstrate the benefits of NP-based delivery of tPA over its exogenous delivery, in attenuation of the degradation of elastic matrix, as well as the synthesis and activities of proteolytic enzymes, such as MMP-2, MMP-9 and plasmin, within EaRASMC cultures. The use of a simple fibrin clot system enables us to evaluate the effects of individual components of the fibrinolytic system on elastic matrix and MMP outcomes *in vitro* within EaRASMC cultures. Our results suggest that our FNPs will be able to permeate into and bind to fibrin within the clot *in vivo*, following endoluminal delivery via a catheter, and mediate its lysis in a gradual, controlled fashion in order to enable subsequent delivery of AAA therapeutic agents.

However, we acknowledge that the *in vivo* environment will be much more complex, with a significantly higher extent of inflammatory activity than can be achieved in vitro culture, due to the chronic presence of inflammatory cells (such as macrophages), as well as overexpressed matrix proteases (such as MMPs and plasmin). Further, ILTs present within small AAAs are likely to be also likely to be associated with platelets and red blood cells besides a fibrin clot, which can influence fibrinolytic NP binding and clot lysis outcomes. Thus, future studies will need to be performed to assess FNP efficacy in permeating and lysing ILTs *in vivo*. These studies will seek to examine options to suitably optimize NP surface charge and hydrophobicity, as well as tPA loading, towards improving the FNP

design for suitable therapeutic effect(s) *in vivo*. Additionally, the *in vivo* targetability of these NPs can be enhanced drastically by conjugating them with anti-fibrin [57] or anti-platelet [58] antibodies or moieties. Overall, our results demonstrate the feasibility of developing NP-based systems for controlled release of tPA towards gradual clot lysis as a strategy for AAA therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Formulated tPA-releasing PLGA NPs for slow lysis of fibrin-rich clots within small AAAs
- NP-delivered tPA achieves much slower clot lysis versus equivalent dose of exogenous tPA
- NP-released tPA attenuates proteolysis and limits elastic matrix loss versus exogenous tPA
- NPs with cationic surfaces show improved clot binding and slowed clot lysis versus anionic NPs

Figure 1.

(A) *In vitro* release profiles for tissue plasminogen activator (tPA) from PLGA NPs (10 µg of tPA loading) functionalized with PVA and DMAB, at an NP concentration of 3.0 mg/mL. (n = 6, mean ± SD). (B) Inset of the release profile of tPA over the initial 2h of release. At all assay time points, the measured levels of tPA release from PVA-PLGA NPs were significantly higher than that from DMAB-PLGA NPs ($p < 0.05$).

Figure 2.

Lysis profiles of fibrin clots incubated with (A) PVA- and (B) DMAB-FNPs (loaded with 10 μg of tPA), at 0.2, 0.5 and 1.0 mg/mL NP concentrations, with 200 ng/mL tPA solution as the exogenous control. The absorbance of the NP-lysed clot (at $\lambda = 405 \text{ nm}$), calculated by subtracting the absorbances of control wells (containing only FNPs) from that of wells containing the NPs with clot, was plotted as a fraction of its initial absorbance as a function of time. All values plotted are mean values of three different clots.

Figure 3. Percentage of PLGA NPs functionalized with PVA and DMAB (with AF633 encapsulated within) bound to fibrin clots. (mean $\pm$ SD; $n = 6$ /case, * denotes $p < 0.05$ compared to corresponding amount of PVA-functionalized PLGA NPs) # indicates significance of differences in clot binding of PLGA NPs (PVA or DMAB functionalized) when provided at different concentrations, deemed for a p value < 0.05 . Note: The percentage retention was calculated after subtracting the amount of NPs adhering to empty wells.

Figure 4.

Proliferation of aneurysmal SMCs (EaRSMCs) in response to tPA and clot lysis products released following lysis of a fibrin clot by PVA- and DMAB-FNPs (loaded with 10 μ g of tPA) at 0.5 mg/mL NP concentration in a transwell culture (Clot+NPs in insert, EaRSMCs in wells). The cell number was calculated based on an estimate of 6 pg of DNA per cell, via a DNA assay, at 0, 10 and 21 days post-seeding, (mean $\pm$ SD; $n = 6$ per case, * denotes $p < 0.05$ compared to unlysed clot control (Clot, No NP); @ denotes $p < 0.05$ compared to day 10).

Figure 5.

Effects of tPA and fibrinolytic products released following lysis of a fibrin clot by PVA- and DMAB-FNPs (loaded with 10 μ g of tPA; 0.5 mg/mL NP concentration) and exogenous tPA (0.2 μ g/mL; equivalent to steady state release of tPA from FNPs) in a transwell culture (Clot +NPs/tPA in insert, EaRASCs in wells) on net elastic matrix deposition and/or preservation (A) on an absolute basis and (B) normalized to DNA content. (mean $\pm$ SD; $n = 6$ /case, * denotes $p < 0.05$ compared to unlysed clot control (No Clot, No NP), @ denotes $p < 0.05$ compared to day 10 baseline). Note: EaRASCs were cultured for a total of 21 days – the initial 10 days to allow them to deposit measurable amounts of elastic matrix (which we used as a baseline), followed by 11 days in the presence of a fibrin clot and FNPs/tPA.

Figure 6.

(overleaf). Effects of tPA and fibrinolytic products released following lysis of a fibrin clot by PVA- and DMAB-FNPs (loaded with 10 μ g of tPA) at 0.5 mg/mL NP concentration in a transwell culture (Clot+NPs in insert, EaRASCs in wells) on (A, B) MMP-2 synthesis, (C, D) MMP-9 synthesis and (E, F) MMP-2 and -9 activity in EaRASC cultures, as analyzed with western blots and gel zymography, respectively. Representative images of western blots are shown for (A) MMP-2 and (b) MMP-9, with β -actin bands as loading controls. Fold-change in production of (B) MMP-2 and (D) MMP-9 compared to control cultures without NPs. (E) Representative image of gel zymogram, showing MMP-2 and -9 bands. (F) Fold-change in MMP-2 and -9 activities compared to NP-untreated control EaRASC cultures. (mean $\pm$ SD; $n = 3$ /case for western blots and gel zymograms, * denotes $p < 0.05$ compared to unlysed clot control (No Clot, No NP)). Notes: Values in Figures 6B and D represent the fold-change in normalized band intensities of the MMP bands for each treatment test case (normalized to its corresponding β -actin loading control band to enable accurate comparison between the different test cases) compared to unlysed (No Clot, No NP) controls. The normalized band intensity of MMP-2 or -9 for the NP-untreated control test condition was set to unity to determine fold-change in MMP-2 or -9 expression or activity for the different NP-treated test cases.

Figure 7.

Plasmin activity in EaRASC layers following exposure to tPA and fibrinolytic products released following lysis of a fibrin clot by PVA- and DMAB-FNPs (loaded with 10 µg tPA) at 0.5 mg/mL NP concentration in a transwell culture (Clot+NPs in insert, EaRASCs in wells). (mean $\pm$ SD; $n = 3$ /case, * denotes $p < 0.05$ compared to unlysed clot control (No Clot, No NP)).

Figure 8.

Immunofluorescence micrographs showing extent of elastic matrix (green; white arrows) within EaRASC layers following 21 days of culture in the presence of PVA- and DMAB-FNPs (D and E, respectively). EaRASCs cultured without any FNPs served as the cell control (B), while those cultured in the presence of an unlysed clot served as the unlysed clot control (C). Immunolabeling controls (A) received no primary antibodies and exhibited no background when treated with the Alexa488-conjugated secondary antibody. Nuclei were stained with DAPI. Scale-bar: 200 μm . Note: EaRASCs were cultured for a total of 21 days – the initial 10 days to allow them to deposit measurable amounts of elastic matrix, followed by 11 days in the presence of a fibrin clot and FNPs/tPA.

Table 1

Size and surface charge of PLGA NPs encapsulating tPA, formulated with PVA and DMAB as the emulsion stabilizers ($n = 6$ replicate formulations, mean $\pm$ 95% CI).

Sample	Size (nm)	Zeta Potential (mV)
PVA – 0 μ g tPA	358 $\pm$ 5	– 36 $\pm$ 2
PVA – 10 μ g tPA	344 $\pm$ 16	– 33 $\pm$ 2
DMAB – 0 μ g tPA	446 $\pm$ 20	+ 23 $\pm$ 3
DMAB – 10 μ g tPA	476 $\pm$ 9	+ 21 $\pm$ 1