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Multifunctional Nanoparticles for Doxycycline Delivery Towards Localized Elastic Matrix Stabilization and Regenerative Repair

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Abstract

Abdominal aortic aneurysms (AAAs) are abnormal expansions of the aortic wall, typically characterized by chronic upregulation of matrix metalloproteases (MMPs) -2 and -9. These MMPs degrade elastin and elastic matrix within the aortic wall, leading to a progressive loss of elasticity of the abdominal aorta as the condition progresses. Doxycycline (DOX) is tetracycline-based antibiotic which has shown significant promise in delaying and slowing the growth of AAAs in clinical studies and in animal models. However, it has been found to inhibit elastic matrix deposition by vascular cells at dosages in the $\mu\text{g/mL}$ range which is typically observed in the circulation, in addition to systemic side effects, following oral dosage. In this paper, we describe the development of DOX-loaded poly(lactic-co-glycolic acid) (PLGA) nanoparticles for localized, controlled and sustained DOX delivery towards AAA therapy. Further, we demonstrate that surface-functionalization of these nanoparticles with cationic amphiphiles, not only impart them with a positive charge for potentially enhanced aortic uptake, but also enabled enhanced elastin binding via hydrophobic interactions, as well as upregulating activity of the elastin crosslinking enzyme lysyl oxidase (LOX). In addition to the DOX released from the nanoparticles being effective in inhibiting MMP-2 production and activity, we also demonstrate that surface-functionalization of the nanoparticles cationic amphiphiles may also play a role in MMP-2 inhibition via (i) electrostatic interactions with negatively-charged residues in the active-site of MMP-2, or (ii) steric blockade of the active site on account of the presence of two dodecyl chains in the DMAB molecule. Thus, in addition to enhanced aortic uptake and retention illustrated in studies by other groups, we have demonstrated that cationic functionalization of PLGA nanoparticles enhances elastogenic outcomes, by targeted binding to elastin, as well as their potential to inhibit elastolysis. These results establish their multifunctionality as a localized delivery system for AAA therapy. Overall, this delivery system has potential in enhancing regenerative outcomes at sites of proteolytic matrix disruption/degradation by enabling targeted, controlled, and long-term release of therapeutic agents.

Keywords

Nanoparticles; drug delivery; elastic matrix; regenerative matrix repair; matrix metalloproteases (MMPs)

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1. Introduction

Abdominal aortic aneurysms (AAAs) are a disease condition primarily afflicting senior males [1]. It involves localized dilatation of the abdominal aorta due to progressive elastic matrix loss, leading to weakening of the vessel wall and potentially culminate in fatal rupture [2]. More than 90% of the detected human AAAs are small (maximal diameter < 5.5 cm) [3], and grow very slowly (~10% per year) [4] over 5 or more years. During this period, AAAs are typically monitored passively via ultrasound until they attain a critical size (diameter > 5.5 cm) beyond which they are prone to rupture. At this point they are treated via surgical or minimally invasive methods, which may frequently be unsuitable for senior patients. Presently there are no non-surgical AAA treatment strategies applicable during this 5-year window of opportunity to treat patients with small, asymptomatic AAAs, or those high-risk patients who may not withstand surgery.

AAA growth has been linked to progressive elastolysis in the aortic wall [5, 6]. This is mediated by matrix metalloproteinases (MMPs), especially MMP-2 and -9, overexpressed by inflammatory cells recruited to the AAA site following initial causative injury. MMP-9 in particular, has been shown to play a critical role in AAA growth in experimental animal models of AAA, in that targeted deletion of the MMP-9 gene has been shown to suppress AAA development [7]. On this basis, pharmacological inhibition of MMP production and/or activity is a potent strategy for inhibiting elastic matrix degradation to attenuate or even suppress AAA growth.

Doxycycline (DOX), a tetracycline derivative, is known to non-specifically inhibit a variety of MMP-types via direct co-ordination with the catalytic site, and by transcriptional inhibition of MMP mRNA [8–10]. It has been shown to be useful in delaying the growth of induced AAAs in animal models [7, 11–15], as well as in clinical studies in human AAAs [16–18]. Further, DOX has also been shown to directly inhibit MMP-2 and -9 activity or pro-MMP activation in some cultured cell types [19], resulting in loss of their enzymatic activity [20].

To date, treatment of AAAs in humans and animal models has been achieved via the oral route. However, oral administration of DOX has been shown to lead to dose-dependent systemic side effects such as gastrointestinal tract disturbances, dental discoloration and cutaneous photosensitivity in some patients [16]. Moreover, since MMPs are also involved in remodeling of healthy tissues, their system-wide inhibition can have an adverse impact. Hence, it is imperative to deliver DOX for effective AAA treatment to the aortic wall in a localized and sustained manner, to avoid or minimize any systemic side-effects associated with its oral or parenteral administration.

Recent studies have reported on localized periaortic delivery of DOX via implanted micropump-systems [11, 21], and demonstrated the efficacy of DOX delivered in this manner, in suppressing the development of experimental AAAs at doses 100-fold lower than that achievable by systemic DOX administration [11]. While these studies suggest that localized DOX delivery over a limited time can also inhibit AAA dilatation, at least in part due its inhibition of matrix elastolysis, the side-effects of long-term implantation of micro-osmotic pumps is unknown, and likely adverse.

As an alternative, in this study, we investigated a localized DOX delivery approach for treating AAAs via its controlled, long-term release from poly(lactic-co-glycolic acid) nanoparticles (PLGA NPs). We specifically sought to (a) examine the release of DOX from the NPs as a function of DOX loading and NP concentration, and (b) analyze the impact of DOX released from NPs on elastic matrix deposition and MMP-inhibition in aneurysmal smooth muscle cell (SMC) cultures. The latter aspect is particularly relevant since DOX has

been implicated in inhibition of matrix synthesis in a variety of cell types [22], including vascular SMCs [23], and thus can interfere with new elastic matrix deposition or accumulation in AAA tissue. Additionally, cationic amphiphiles which are used to impart a positive surface charge on NPs to enhance their aortic uptake and retention [24], are also known to have been found to bind elastin and stimulate activity of lysyl oxidase (LOX) [25, 26], the enzyme that mediates crosslinking of elastin precursors into a mature matrix. Based on this, we evaluate the effects of surface modification of NPs with cationic amphiphiles from the standpoint of targeted binding to elastin when delivered to AAAs, and enhancement of LOX activity. This work will guide localized, controlled delivery of DOX within AAAs, or more generally at sites of tissue proteolytic disease not only from the standpoint of inhibiting MMPs, but also enhance regenerative elastic matrix repair towards slowing, arresting and even regressing tissue disruption.

2. Materials and methods

2.1 Isolation and culture of SMCs from elastase perfusion-induced rat AAAs

All animal procedures were conducted with approval of the Institutional Animal Care and Use Committee (IACUC) at the Cleveland Clinic (ARC # 2010-0299). The Clinic animal facility is AAALAC-approved and has animal assurance (#A3145-01; Expires 04/30/15). Aneurysmal rat aortic SMCs (EaRASCs) were isolated from adult male Sprague-Dawley rats ($n = 3$) at 14-days post-AAA induction via elastase infusion, as described in an earlier study by our group [27]. The aortae were cut open longitudinally and the intimal layer scraped off gently with a scalpel. The medial layer was then dissected from the underlying adventitial layer, chopped into ~0.5 mm long slices, and washed twice with warm sterile phosphate buffered saline (PBS). These were pooled and enzymatically digested in DMEM-F12 cell culture medium (Invitrogen, Carlsbad, CA) containing 125 U/mg collagenase (Worthington Biochemicals, Lakewood, NJ) and 3 U/mg elastase (Worthington Biochemicals) for 30 min at 37 °C, centrifuged (400 g, 5 min), and cultured over 2 weeks in T-75 flasks. The cells were cultured in DMEM-F12 medium (Invitrogen) supplemented with 10% v/v fetal bovine serum (FBS; PAA Laboratories, Etobicoke, Ontario) and 1% v/v PenStrep (Thermo Fisher, South Logan, UT). The primary EaRASCs obtained from these tissue explants were propagated over 2 weeks, and passaged when they attained confluence.

Primary healthy rat aortic smooth muscle cells (RASCs) were similarly isolated from aortae excised from healthy adult Sprague-Dawley rats ($n = 3$), and propagated for cell culture experiments as described in previous studies by our group [28, 29].

2.2 Determination of appropriate PLGA NP size for delivery to SMC cultures

An acceptable PLGA NP size based on their relative exclusion by cells in the extracellular space was determined by incubating fluorescent PLGA NPs (FITC-PLGA; Fluorophorex™, Phosphorex, Inc., Fall River, MA) of three different sizes (100 nm, 200 nm, and 500 nm hydrodynamic diameter) with cultured EaRASCs. Briefly, the EaRASCs were seeded at 15×10^3 cells/well in sterile, 2-well Permax® chamber slides (Nalge Nunc International, Rochester, NY) and cultured in DMEM-F12 cell culture medium supplemented with 2% v/v FBS and 1% v/v PenStrep. The FITC-PLGA NPs were added to the EaRASCs at a concentration of 0.2 mg/mL and incubated at 37 °C for 48 h. The cells were then fixed with 4% v/v formaldehyde, labeled with the lipophilic membrane stain DiI (Invitrogen, Carlsbad, CA), and then mounted with Vectashield containing the nuclear dye 4', 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Imaging to visualize NP uptake or exclusion was performed using a spectral laser scanning confocal microscope (Leica TCS SP2, Leica Microsystems, Inc., Buffalo Grove, IL). To visualize and confirm the intracellular presence or extracellular exclusion of NPs, z-stack maximum projections

(overlays) were created from images acquired at 1 μm intervals across the thickness of the cell layers, using Leica LAS AF software. Outcomes were deduced from a minimum of 10–12 images per NP size.

2.3 Formulation of DOX-loaded PLGA NPs

Poly (*D*-lactic-co-glycolic acid) nanoparticles (PLGA; 50:50 lactide: glycolide; inherent viscosity 0.95–1.20 dL/g in hexafluoroisopropanol; Durect Corporation, Birmingham, AL) loaded with DOX (doxycycline hyclate; Sigma-Aldrich, St. Louis, MO) were prepared by a double emulsion-solvent evaporation technique [24, 30, 31]. Briefly, PLGA was dissolved in chloroform (Fisher Scientific, Fair Lawn, NJ) at a concentration of 2.5–3.0 % w/v. An aqueous DOX solution at three different loadings (2%, 5% and 10% w/w ratios of DOX:PLGA) was emulsified into the PLGA solution using a probe sonicator (Q500; QSonica LLC, Newtown, CT) for 30 s on ice, at an amplitude setting of 20 %. The water-in-oil emulsion thus formed, was further emulsified into an aqueous solution of 1% w/v didodecyldimethylammonium bromide (DMAB; Sigma-Aldrich) using the probe sonicator for 30 s on ice at 20 % amplitude setting, to form a water-in-oil-in-water emulsion. This second emulsion was stirred for 16 h at room temperature, and then desiccated for 1 h under vacuum, to remove any residual chloroform. The NPs formed were recovered by ultracentrifugation at 35,000 rpm (Beckman L-80, Beckman Instruments, Inc., Palo Alto, CA). The NPs were washed two times with nanopure water to remove residual DMAB and unencapsulated DOX, and then lyophilized for 48 h to obtain a dry powder. Suitable precautions were taken to ensure minimal exposure to light during the formulation and release process, as tetracyclines are known to be sensitive to light [32, 33].

2.4 Efficiency of DOX encapsulation within nanoparticles

The supernatants from the washing/ultracentrifugation steps were pooled for each individual NP formulation. The unencapsulated DOX was quantified via high-performance liquid chromatography (Waters Separation Module 2690 HPLC, Waters Corporation, Milford, MA) coupled with a triple quadrupole mass spectrometer (Micromass Quattro Ultima; Waters Corporation). The total amount of DOX encapsulated in the NPs and the overall encapsulation efficiencies were determined by subtracting the total amount of unencapsulated DOX from the total weighed amounts of DOX added during NP formulation.

2.5 Size and surface charge measurements on DOX-loaded PLGA nanoparticles

Mean hydrodynamic diameters of the NPs were determined using a dynamic light scattering technique, and mean zeta potentials (i.e., surface charge) of these NPs were determined 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 [34].

2.6 Determining cytotoxicity of DOX-loaded PLGA NPs

Cytotoxicity of PLGA NPs was assessed using a LIVE/DEAD® viability assay (Invitrogen, Inc.) applied to EaRASCs that were incubated with the NPs. Briefly, the EaRASCs were seeded at 15×10^3 cells/well in sterile, 2-well Permax® chamber slides (Nalge Nunc International) and allowed to adhere over a 48 h period in DMEM-F12 cell culture media supplemented with 2% v/v FBS and 1% v/v PenStrep. The DOX-PLGA NPs (2%, 5%, 10% DOX) were added to the EaRASCs at concentrations of 0.1, 0.2 and 0.5 mg/mL and incubated for 48 h prior to assessing their viability. PLGA NPs containing no DOX were tested as a control. Stained cells were viewed using an Olympus IX51 fluorescence

microscope (Olympus America, Center Valley, PA). Six different regions were assessed for each replicate culture.

2.7 Characterizing DOX release from PLGA NPs *in vitro*

DOX release from NPs was carried out in phosphate buffer saline (PBS, pH 7.4; Sigma-Aldrich) at 37 °C on a shaker at 100 rpm. Briefly, 1.5 mL polypropylene microcentrifuge tubes ($n = 3$ per formulation) were filled with 1.0 mL of NP suspensions containing 0.2, 0.5 and 1.0 mg/mL of NPs, respectively. Release in each case, was carried out over 40 days. At each analysis time point, the samples were centrifuged (14,000 rpm, 30 min, 4 °C) in a microcentrifuge (Beckman Microfuge 16®, Beckman Coulter, Inc.), the supernatants withdrawn to quantify DOX content, and volume-replenished with fresh PBS. DOX released was quantified by UV-spectrophotometry (SpectraMax M2, Molecular Devices, Inc., Sunnyvale, CA). DOX absorbance at 270 nm [35–37], was calibrated to its concentration using serial dilutions of a 1.0 mg/mL DOX solution. The DOX standards were incubated under the same conditions as the NP samples, to avoid any time- and temperature-dependent degradation of DOX [38, 39].

2.8 Experimental design for cell culture

For cell culture experiments intended to investigate the impact of DOX-PLGA NPs on cellular matrix synthesis, EaRASCs (passages 2–5) were seeded at 3×10^4 cells/well and cultured over 21 days in 6-well plates ($A = 10 \text{ cm}^2$; BD-Biosciences, Franklin Lakes, NJ). The cells were cultured in DMEMF12 supplemented with 2% v/v FBS, 1% v/v PenStrep and 100 ng/mL TNF- α (PeproTech, Inc, Rocky Hill, NJ) to simulate an aneurysmal or activated microenvironment in culture. NPs loaded with 2% w/w of DOX were cultured with cell layers at concentrations of 0.2, 0.5 and 1.0 mg/mL of NPs ($n = 6$ per concentration) for 21 days. EaRASCs cultured with DOX-unencapsulated NPs (0.5 mg/mL of NPs) served as the active-agent (DOX) control. Healthy, passage-matched RASCs were similarly seeded, without any NPs, as the cell type controls. Fresh medium was added to the cultures weekly, with the spent medium slowly pipetted out from individual wells pooled at each time point and stored at $-20 \text{ }^\circ\text{C}$. The NPs were almost all bound to the cell layer, and were not removed along with the spent medium. The pooled spent medium aliquots were biochemically assayed along with their corresponding cell layers which were harvested after the 21 days of culture.

For western blots and zymography to evaluate the effect of DOX released from the NPs on the proteolytic response, EaRASCs were seeded at 2×10^5 cells/well in a 6-well plate and cultured for 7 days in DMEM-F12 supplemented with 5% v/v FBS and 1% PenStrep, along with 100 ng/mL TNF- α . PLGA NPs (0% w/w DOX active-agent control, 2%, 5% and 10% w/w DOX-loading at 0.2 mg/mL of NPs) were added to the EaRASCs at day 1 after seeding ($n = 6$ per treatment).

2.9 DNA assay for cell proliferation

The DNA content of the cell layers was measured via a fluorometric assay, as described earlier [40], to determine the effects of DOX released from the NPs on RASC and EaRASC proliferation. The cell layers were harvested at 1 day 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/cell [40].

2.10 Fastin assay for elastin

The total elastin content in the cell layer (alkali-soluble and insoluble fractions), as well as the tropoelastin (soluble elastin precursor) released in the cell culture medium, was

quantified using a Fastin assay (Accurate Scientific and Chemical, Westbury, NY), as described in recent publications by our group [28, 29, 41]. The cell layers were harvested after 21 days of culture, resuspended in NaCl-Pi buffer, and sonicated on ice to homogenize the cell layer. This suspension thus obtained was digested with 0.1N NaOH (1 h, 98 °C) and then centrifuged to yield a pellet containing mature, highly cross-linked alkali-insoluble elastin, with the supernatant fraction containing less cross-linked alkali-soluble elastin. The alkali-insoluble elastin was then converted into a soluble form prior to quantification, as the Fastin assay can only quantify soluble α -elastin. The pellet obtained after the NaOH digestion step was dried and solubilized with 0.25 M oxalic acid (1 h, 95 °C), after which they were pooled and centrifuge-filtered (3000 rpm, 10 min) in microcentrifuge tubes (Amicon® Ultra, 10 kDa molecular weight cut-off; Millipore, Inc., Billerica, MA). These soluble and insoluble matrix elastin fractions, as well as the tropoelastin fraction in the cell culture medium were then measured using the Fastin assay. The amounts of elastin measured were also normalized to their corresponding DNA amounts, so as to provide an accurate comparison between the different treatments.

2.11 Western blots for MMP-2 and MMP-9 protein expression

MMP-2 and -9 production by activated EaRASCs in the absence and presence of the PLGA NPs and NP-released DOX was semi-quantitatively assessed using western blots. After 7 days of culture, as described in Section 2.8, the cell layers were harvested in RIPA buffer (Thermo Scientific) containing Halt™ protease inhibitor (Thermo Scientific), and assayed for total protein content using a bicinchonic acid (BCA) assay kit (Thermo Scientific) [41]. Maximum volumes of sample protein (15.6 μ L) were then loaded under reduced conditions into each lane of a 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Invitrogen), along with a BenchMark™ prestained molecular weight ladder (Invitrogen). The gels were then transferred dry onto nitrocellulose membranes (iBlot® Western Blotting System, Invitrogen). The membranes were blocked with the Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) for 1 h, following which they were immunolabeled for 16 h at 4 °C with a rabbit polyclonal antibody against MMP-2 (1:500 dilution; Abcam, Cambridge, MA) and 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). Fluoro-luminescent detection of the protein bands was then carried out using a LI-COR Odyssey laser-based scanning system. The intensities of active MMP-2 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-2 for the PLGA NP-treated and other test cultures were further normalized to those of the NP-untreated control cultures to determine the fold-change(s) in production of active MMP-2, and the statistical significance of the differences between them. The results presented in this manuscript were averaged from 5 replicate gels run per culture treatment.

2.12 Gel zymography for MMP-2 and MMP-9 activity

Treatment-specific effects on cellular MMP-2 and -9 activities were analyzed via gel zymography, as we have described previously [27, 41]. 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 BenchMark™ 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 overnight 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 measured using ImageJ software, and normalized to those obtained for the NP-untreated control cultures to determine fold changes in MMP-2 activity. Data was acquired from 3 independent replicate gels.

2.13 Immunofluorescence detection of elastic matrix proteins

For immunofluorescence studies, EaRASCs were seeded in sterile, 2-well Permanox® chamber slides (Nalge Nunc International) at a cell density of 5×10^3 cells/well, in DMEM-F12 cell culture media supplemented with 5% v/v FBS and 1% v/v PenStrep. DOX-loaded PLGA NPs (2% and 10% w/w to PLGA; 0.2 mg/mL NP concentration) were added to the cultures 1 day later, with 0% DOX-PLGA NPs added as the active-agent control. EaRASCs cultured without any NPs served as the treatment control. After 21 days of culture, the cell layers were fixed with 4% w/v paraformaldehyde and treated with rabbit anti-rat polyclonal antibodies against elastin (Millipore) and LOX (Abcam), both at 1:100 v/v dilutions. The detected proteins were visualized with Alexa633-conjugated IgG secondary antibodies (1:1000 v/v dilution; Invitrogen). Spatial extent of the cell bodies were defined by labeling filamentous actin using Alex Fluor 488 Phalloidin (1:50 dilution; Invitrogen). The cell layers were mounted with Vectashield containing DAPI 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 confocal microscopy (Leica TCS SP5 II, Leica Microsystems, Inc.). To visualize and confirm the intracellular or extracellular localization of elastin and LOX, z-stack maximum projections (overlays) were created from images acquired at 0.5 μ m intervals across the thickness of the cell layers, using Leica LAS AF software.

2.14 Impact of surface modification of NPs on their binding affinity to elastin

PLGA NPs were formulated using a protocol similar to that described earlier (in Section 2.3) with 1% w/v poly(vinyl) alcohol (PVA; Sigma-Aldrich) as the surfactant. The NPs were lyophilized, resuspended in nanopure water and then functionalized with 1 % w/v of dodecyltrimethyl ammonium bromide (DTAB; Sigma-Aldrich) or dodecylamine hydrochloride (DAH; Acros Organics, Fair Lawn, NJ) by sonication for 30 s on ice [24]. This alternative method for surface-modification of PLGA NPs was necessary, as DTAB and DAH did not form nanoparticles at the 1% w/v concentration previously used with DMAB (see Section 2.3), reasons for which are described in the results and discussion section. Surface-modified PLGA NPs were ultracentrifuged to remove residual traces of stabilizer, and then lyophilized. The size and charge of these NPs prepared via surface functionalization with DTAB and DAH, as well as those prepared using 1% w/v PVA alone was determined, as described in Section 2.3.

To enable their fluorometric detection, PLGA NPs were formulated with the three different cationic amphiphile surface modifiers (DMAB, DTAB and DAH), with 1.0 mg of BSA-Cy5 (NanoCS, New York, NY) encapsulated within. Negatively-charged PLGA NPs prepared using PVA as the sole surfactant, were also tested as controls.

To compare the elastin binding capacities of the different surface-modified PLGA NPs, bovine aortic elastin strips (Elastin Products Company, Owensville, MO) were hydrated in PBS, punched into 4.5 mm diameter pieces and placed in 180 μ L PBS in individual wells of a 96 well plate. Aliquots of each of the surface-modified NPs (20 μ L) were added to each well at three different NP concentrations (0.1, 0.2 and 0.5 mg of NPs; $n = 3$ per NP type) and

incubated with the elastin strips for 30 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, and all wells (elastin and empty blanks) were washed four times with PBS, with the wash solutions being collected and added to the supernatant for fluorometric analysis for NP content. The dilution of the supernatant due to the addition of solution from the four wash steps was taken into account in the subsequent quantification of NP binding.

NP binding to elastin strips and empty wells, was quantified from the fluorescence of the supernatant ($\lambda_{\text{excitation}} = 650 \text{ nm}$, $\lambda_{\text{emission}} = 690 \text{ nm}$) based on a calibration curve prepared from serial dilutions of suspensions containing known concentrations of the different NPs. The total NP adherence to the elastin was obtained by subtracting the amount of NPs adherent to the empty wells from the amount adherent in the wells containing the elastin strip, and is expressed as a percentage of the amount added.

2.15 Effect of surface-modified PLGA NPs on cellular LOX activity

EaRASCs were seeded in 6-well plates at a seeding density of 6×10^4 cells per well, and cultured with DMEM-F12 supplemented with 5% v/v FBS and 1% v/v PenStrep. PLGA NPs surface-modified with DMAB, DTAB, DAH and PVA, formulated as described in earlier sections, were then added to the cell layers at a nanoparticle concentration of 0.2 mg/mL of NPs. The cells were cultured with the NPs for 21 days. The cell culture media was changed every week and the spent cell culture medium aliquots were collected from each individual well, pooled in individual tubes and frozen at -20°C . The cell layer was harvested at the end of the culture in RIPA buffer, and assayed for LOX activity along with the pooled, spent culture medium.

Hydrogen peroxide released upon deamination of diamino compounds by LOX was detected using a fluorometric Amplex[®] Red assay (Invitrogen) [42], using a modified protocol that has been used previously in our laboratory [29]. Briefly, 50 μL of samples from cell culture media or cell layers were mixed with 50 μL of a 50 mM sodium phosphate reaction buffer (pH 7.4) containing 1.2 M urea, 10 mM 1,5-diaminopentane (Sigma-Aldrich), 0.2 U/mL horseradish peroxidase (HRP; Invitrogen), and 10 μM Amplex[®] Red (Invitrogen). Control samples (50 μL) were mixed with the sodium phosphate reaction buffer contained only 0.2 U/mL HRP and 10 μM Amplex[®] Red. A calibration curve was constructed based on the absorbance of serial dilutions (0–10 μM) of hydrogen peroxide standards (Invitrogen) treated with the sodium phosphate buffer containing 0.2 U/mL HRP and 10 μM Amplex[®] Red, measured at 560 nm. The absorbance of the controls samples was subtracted from that of their individual test case samples to obtain a measure of the hydrogen peroxide released in response to the deamination of urea and 1,5-diaminopentane by the LOX in the media and cell layer samples.

2.16 Isolating the effects of cationic NP surface modifiers on MMP-2 production and activity

The cell layers isolated (in RIPA buffer) for the LOX activity assay from EaRASC cultures treated with DMAB, DTAB, DAH and PVA NPs, along with untreated controls were also examined for MMP-2 expression (western blots) and activity (gel zymography), as described earlier in Sections 2.11 and 2.12. The intensity of the active MMP-2 bands in western blots were normalized to the intensity of their corresponding β -actin bands and compared to those of NP-untreated control cultures to determine the fold-change in MMP-2 expression. For gel zymography, all lanes were loaded with sample volumes equivalent to 12 μg protein, and the intensities of the MMP-2 bands from zymograms were directly

normalized to that of the NP-untreated controls to ascertain fold-change in MMP-2 activity for the NP-treated cultures. All data was acquired from 3 independent replicate gels.

2.17 Statistical analysis

All experimental data presented ($n = 6$ /condition, unless stated otherwise) are 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

3.1 Determination of optimal PLGA NP size for delivery to SMC cultures

We examined the intracellular uptake of FITC-PLGA NPs of different sizes to determine the NP size or size-range which would preferably not be internalized by the SMCs, but instead would predominantly remain in the extracellular space for effecting functional improvements to cell-mediated elastic matrix assembly, which occurs extracellularly. Representative confocal micrographs of EaRASC cultures incubated with FITC-PLGA of 100, 200 and 500 nm are shown in Figure 1. The FITC-PLGA NPs of 100 nm and 200 nm sizes (Figure 1A and B, respectively) were internalized by the EaRASCs, while NPs of 500 nm size (Figure 1C) were excluded in the extracellular space. Thus, we conclude that the PLGA NPs must be of sizes > 200 nm to enable them to be excluded by cells in the extracellular space.

3.2 Formulation and characterization of DOX-loaded PLGA NPs

The physical characteristics of PLGA NPs, such as size and zeta potential, are typically controlled by the formulation method(s) [43, 44], with the single or double emulsion solvent evaporation methods being the most common method used for PLGA NP formulation. DOX-free PLGA NPs formulated via a single emulsion solvent evaporation technique using 1% w/v DMAB as the stabilizer, exhibited a mean hydrodynamic diameter (or hydrated particle size) of 332.7 ± 8.3 nm, and a ζ -potential of $+33.8 \pm 0.4$ mV (shown in Table 1). As DOX is a water-soluble drug, we utilized a double emulsion solvent evaporation technique for its encapsulation within the PLGA NPs. DOX encapsulation at 2, 5, and 10% (w/w ratios to PLGA) did not drastically affect the size or surface charge of these PLGA NPs, as indicated in Table 1. Overall, these NPs exhibited a relatively uniform size distribution and surface charge, as indicated in Table 1. The overall encapsulation efficiency of DOX in the PLGA NPs was in the range of 39–42 %.

3.3 *In vitro* determination of concentration-specific cytotoxicity of DOX-loaded PLGA NPs

The effect of DOX-loaded PLGA NPs on the viability of EaRASCs *in vitro* was assessed using a LIVE-DEAD® assay. As gauged by the absence of any dead (red) cells in the fluorescence images seen in Figure 2, the DOX-PLGA NPs and the control 0% DOX-PLGA NPs were not cytotoxic at any of the provided concentrations.

3.4 DOX release from PLGA NPs *in vitro*

The *in vitro* release profiles for DOX from the PLGA NPs are shown in Figure 3. Since DOX released at a 0.1 mg/mL concentration of NPs was close to the threshold of detection, release studies were performed with the higher NP concentrations of 0.2, 0.5 and 1.0 mg/mL. The DOX release curves obtained exhibit a characteristic biphasic release profile, characterized by an initial burst phase that spans the first day [24, 45, 46], followed by a slower exponential release phase similar to that observed for other drugs released from PLGA NPs [24, 45, 47].

The cumulative release of DOX over 40 days was less than 30% of the total amount of drug encapsulated for the 2 and 5% DOX-loaded PLGA NPs. The 10% DOX-loaded NPs on the other hand, demonstrated a lower percentage of cumulative release (3% to 11%) of the total DOX encapsulated, with the release continuing gradually up to 60 days.

3.5 Effects of DOX released from NPs on SMC proliferation and elastic matrix synthesis

Based on previous studies by our group (unpublished data) which demonstrated that an exogenous DOX dosage of 5.0 $\mu\text{g/mL}$ limited the viability of aneurysmal RASMC cultures, we chose the 2% DOX loading condition to evaluate the functional effects of these DOX-loaded NPs on cell proliferation and elastic matrix synthesis, as the overall cumulative release over 21 days was below this threshold of 5 $\mu\text{g/mL}$. As observed in Figure 4, the DOX released from the PLGA NPs did not inhibit EaRASC proliferation. In the present study, we found EaRASC proliferation to be significantly higher ($p < 0.05$) for the DOX NP treated cultures compared to the untreated and blank NP controls.

From an elastogenic standpoint, DOX released from the NPs did not affect tropoelastin (elastin precursor) synthesis on a per cell basis (Figure 5A), as evidenced by the fact that there was no significant difference ($p > 0.05$) between the values obtained for EaRASC cultures treated with 2% DOX-loaded NPs at 0.2 mg/mL NP concentration and that of untreated EaRASC cultures. Although we observed a decrease in tropoelastin production with increasing NP concentration of 2% DOX-loaded PLGA NPs, we also observed that DOX released induced a significant increase in total matrix elastin deposition (on a per cell basis), relative to non-NP additive controls (Figure 5C). Interestingly, EaRASCs treated with 0% DOX-PLGA NPs also showed enhanced elastin matrix deposition compared to the non-additive controls. Nevertheless, increases in elastic matrix deposition induced by the DOX-loaded NPs was significantly greater ($p < 0.05$) than that induced by the unloaded NPs.

3.6 Impact of DOX released from PLGA NPs on MMP-2 production and activity in EaRASC cultures

Figure 6A shows a representative western blot for MMP-2 produced by EaRASCs subject to the different NP-treatment conditions, along with the NP-untreated control. It must be noted that the intensity of the active MMP-2 band was normalized to the intensity of its corresponding β -actin band (loading control) for each test condition, in order to compare the effects between them. This normalized value for five biological replicates was plotted in Figure 6B for each test case, and clearly shows that DOX released from the PLGA NPs is functional in causing an ~50% inhibition of the active form of MMP-2, relative to the NP-untreated control. Surprisingly, even the 0% DOX-PLGA NPs caused a ~25% decrease in expression of active MMP-2, and this was deemed to be statistically significant ($p < 0.05$) compared to NP-untreated control. In all cases, MMP-9 bands were absent or too faint to be quantified reliably via densitometry [23, 41], and hence those outcomes have not been included presently.

Gel zymography data shown in Figure 6C demonstrates that DOX-loaded NPs significantly inhibit ($p < 0.05$) MMP-2 activity, with the samples treated with 2% DOX-PLGA NPs causing an almost 10-fold decrease. Interestingly, EaRASCs cultured with 0% DOX-PLGA NPs caused a ~4-fold attenuation in MMP-2 activity.

3.7 Immunofluorescence detection of elastin and LOX

Figure 7 shows confocal images for EaRASC layers immunolabeled for elastin and LOX (red fluorescence) at the end of 21 days of culture with DOX-loaded PLGA NPs, as well as 0% DOX-PLGA NPs as active-agent controls and the treatment controls without any NPs. A

representative image of the immunofluorescence control (untreated with primary antibodies) is included, which indicates the absence of any false fluorescence on account of any non-specific binding of the fluorescent secondary antibodies to the cell layer. The images of all culture groups show that there is significant deposition of elastin and LOX in the extracellular space surrounding the cells, and only limited elastin within cells. Although there is no apparent evidence of fiber formation, this is not entirely unexpected in a static, 2-D culture system, and it remains to be determined whether functional elastin fibers will be formed *in vivo*.

3.8 Impact of choice of cationic amphiphilic surfactant on PLGA NP characteristics

Although PLGA NPs were imparted with a positive surface charge by using 1% w/v DMAB directly as the stabilizer during the NP formulation process, similar use of 1% w/v DTAB and DAH as the stabilizers failed to generate any NPs. Therefore, PLGA NPs were first formulated using PVA as the stabilizer, and then functionalized with 1% w/v DTAB and DAH.

As observed in Table 2, PLGA NPs prepared using PVA as the surfactant were similar in diameter to those prepared with DMAB which had a diameter of 332.7 ± 8.3 nm (see Table 1), but exhibited a strong negative surface charge, comparable to that obtained in an earlier study by Labhasetwar *et al.* (-27.8 ± 0.5 mV) [24]. Modification of these NPs with 1% w/v DTAB and DAH led to the neutralization of this strong negative charge on the NPs, without any significant alteration in NP size, as also indicated in Table 2. However, we have shown that it is possible to still formulate NPs using 5% w/v DAH directly as the surfactant and impart them with a positive surface charge, by increasing the DAH concentration.

3.9 Binding of modified PLGA NPs to elastin

We utilized BSA-Cy5 encapsulated PLGA NPs functionalized with the different amphiphilic surface-modifiers to quantitatively compare their binding to elastin using a fluorometric method. As shown in Table 3, the sizes and zeta potential of the BSA-Cy5 loaded surface-modified PLGA NPs were comparable to unloaded (blank) surface-modified PLGA NPs listed in Table 2. Regardless of added amounts, PLGA NPs functionalized with DMAB demonstrated significantly greater binding to the elastin ($p < 0.05$), compared to PVA-functionalized NPs, as seen in Figure 8. Cationic DTAB-PLGA NPs also demonstrated enhanced elastin binding compared to negatively charged PVA-PLGA NPs, although their binding was lower than that observed for DMAB-PLGA NPs.

3.10 Effect of surface-functionalized PLGA NPs on lysyl oxidase (LOX) activity in SMC cultures

We determined LOX activity in the EaRASMC cultures to be significantly enhanced ($p < 0.05$) when treated with PLGA NPs which were surface-modified with the cationic amphiphiles DMAB, DTAB and DAH (Figure 9B), relative to cultures treated with NPs that were surface-modified with PVA. Importantly, such increases were only detected in the cell layer fraction and not in the culture medium (Figure 9A).

3.11 Analysis of effects of cationic surface modification of NPs on MMP-2 production and activity

Based on the results obtained for the DOX-loaded NPs in EaRASMC cultures (Figure 6), which demonstrated that the cationic surface charge on the NPs potentially plays a role in inhibiting MMP-2 production and activity, we compared the effects of DOX-free DMAB-, DTAB-, DAH- and PVA-modified PLGA NPs on MMP-2 synthesis and activity. The representative western blot for MMP-2 production by EaRASMCs for the different NP

treatments (Figure 10A) clearly illustrates that the functionalization of the DOX-free PLGA NPs with the cationic amphiphiles DMAB, DTAB and DAH, led to inhibition of active MMP-2 synthesis, while PVA functionalization appears to upregulate active MMP-2 production. Comparison of the β -actin normalized intensities of the MMP-2 bands for the different NP treatment conditions (Figure 10B) demonstrated that the cationic functionalization of the PLGA NPs with DMAB led to a ~2.5 fold downregulation of MMP-2 production compared to the NP-untreated control. The DTAB and DAH-functionalized NPs, both of which exhibit a near neutral mean ζ -potential, caused a ~1.5 fold decrease in MMP-2 production compared to the NP-untreated control. On the other hand, the PVA-functionalized NPs, which exhibit a negative surface charge (mean ζ -potential = -32 mV), were found to cause a ~2.5 fold enhancement of MMP-2 production compared to the NP-untreated controls. Results from gel zymograms shown in Figure 10C confirm that the cationic surface charge on the NPs led to a significant inhibition of MMP-2 activity, as is illustrated by the ~4 fold inhibition of MMP-2 activity by the DMAB-functionalized NPs. Although there was no significant difference in MMP-2 activity for the EaRASC cultures treated with PVA-functionalized NPs compared to NP-untreated control cultures, both the DTAB- and DAH-functionalized NPs led to a 2-fold inhibition of MMP-2 activity.

4. Discussion

The overall goal of this study was to develop a NP-based delivery system for AAA therapy, which would enable localized, controlled, and sustained release of DOX over an extended duration (> 3 weeks when ultimately delivered within AAA tissue), so as to avoid systemic action observed with oral DOX delivery [16]. We further sought to surface-functionalize these NPs with cationic amphiphiles to impart them with a positive surface charge, for potentially enhancing their arterial uptake, while improving their elastogenic benefits due to increased elastin binding and upregulation of elastin crosslinking by LOX.

PLGA 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 [43, 44]. For this study we investigated NPs formulated with PLGA containing a 50:50 lactide:glycolide ratio, since it is the predominant form of PLGA used in nanotechnology applications including targeted, controlled drug delivery [44]. 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 [31].

Our studies indicated that NP sizes greater than 200 nm substantially remain in the extracellular space, with minimal intracellular uptake by EaRASC, as shown in Figure 1. Although NPs of 500 nm size were almost completely excluded by the cells, polymeric NPs of this size and greater have been shown to undergo phagocytic uptake [48], which is undesirable. This suggests that an optimal NP size range for the proposed application lies between 200 and 500 nm. PLGA NPs formulated in this study with DMAB as the stabilizer were within this size range (mean hydrodynamic diameter = 300–350 nm, as listed in Table 1). While NPs of this size exhibited some degree of internalization within EaRASCs (Supplementary Figure 1), this was significantly less pronounced than that observed with the 100 and 200 nm NPs (Figure 1). Further, we observed considerable NPs continued to remain localized in the extracellular space and on top of the cells. Coupled with our observations that these NPs (a) significantly improved elastin crosslinking and other facets of elastic matrix deposition (discussed later in this section), which occur in the extracellular space, and (b) did not adversely impact cell viability, it may be concluded that NP sizes of

~350 nm would likely be suitable for the proposed application. Further, DOX encapsulation within these NPs did not affect either their size or surface charge.

These NPs did not affect EaRASC MC viability, as illustrated in Figure 2, demonstrating their noncytotoxic nature. From the perspective of drug release from PLGA NPs, in some situations, the initial burst release phase during the first 24 h of release can be of concern [49, 50], particularly from the aspect of the drug release exceeding toxic limits, and also from the inability of the formulation to provide sustained release of the agent(s). In our study, however, DOX released during this initial phase accounted for less than 5% of the total DOX encapsulated, and was less than 1.5 $\mu\text{g/mL}$, which was well below an exogenously delivered dose of 5.0 $\mu\text{g/mL}$ which we have observed to inhibit proliferation as well as elastic matrix synthesis by EaRASC MCs (unpublished outcomes). Hence, the burst release of DOX is likely not a concern. In our release curves (Figure 3), the DOX release from the 10% DOX-loaded NPs was lower than that observed for 5% DOX-loaded NPs. This could be explained by our hypothesis that there exists a loading threshold (likely between 5 and 10% w/w DOX loading) beyond which DOX molecules are so compactly packed within the polymeric NPs, such that they hinder infiltration of aqueous medium, DOX solubilization and diffusion out. Thus, the 5% DOX-loaded NPs wherein DOX loading falls below the above defined threshold, are still be able to release DOX without any hindrance, which is higher (in terms of absolute amount) than the 2% DOX-loaded NPs. Regardless, our results clearly demonstrate the suitability of DOX-loaded PLGA NPs for controlled DOX release over a duration of more than 40 days, at a neutral (near physiologic) pH.

Standard oral doses of DOX greater than 30 mg/kg/day have been found to slow the growth of rat AAAs [12], but have also been shown to significantly inhibit SMC proliferation and migration, as well as decrease arterial elastin content in balloon-injured rat carotid arteries [51]. The DOX concentrations equivalent to these oral doses as measured within AAA tissues in mice and in human patients has been shown to be ~50 ng/mL, while levels in the circulation are between 2 and 10 $\mu\text{g/mL}$ [15, 52]. DOX and other tetracyclines have also been found to inhibit cell proliferation, migration and matrix synthesis by vascular cells [23, 51, 53]. Exogenously delivered DOX doses in the range of 16–54 $\mu\text{g/mL}$ have been shown to inhibit proliferation and migration of SMCs isolated from rat carotid arteries, and concurrently inhibit elastic matrix synthesis [23]. In this present study, we observed that EaRASC MC proliferation was not inhibited (Figure 4), as the DOX released from the 2% DOX-PLGA NPs was below the 5 $\mu\text{g/mL}$ threshold which was found to limit the viability of rat aneurysmal SMCs in culture, in previous (unpublished) studies by our group. In fact we found that the proliferation was significantly higher for DOX-NP treated EaRASC MC cultures compared to those which received blank NPs, and also untreated controls. Therefore, based on our results with EaRASC MCs, as well as those of other groups with tumor cells [54, 55], chondrocytes [56] and vascular SMCs [23, 51, 53], we suggest that DOX may have biphasic effects on cell proliferation as a function of the delivered dose. Future studies will provide further insights into the nature of these effects.

From an elastogenic perspective, although we observed a decrease in tropoelastin production (normalized to cellular DNA content) with increasing NP concentration for the DOX-loaded NPs (Figure 5A), this could be attributed to (a) increased DOX release with increasing NP concentration potentially causing a decrease in tropoelastin production, (b) increased macromolecular crowding due to increasing NP concentration, or (c) both a & b. For example, recent studies have shown that macromolecular crowding within fibroblast cultures interfere with the release of procollagen (the collagen precursor molecule) into the cell culture medium [57, 58], leading to enhanced collagen deposition in the cell layer. However, it should also be noted that while tropoelastin is a key player in the elastic matrix deposition

process, its levels in the cell culture medium may not be indicative of the extent of elastic matrix deposition. The pericellular matrix also plays a critical role in the formation and crosslinking of elastic fibers from tropoelastin molecules. Proteoglycans such as versican have been shown to impede elastic matrix assembly via binding of their negatively-charged chondroitin sulfate (CS) chains with the EBP, which leads to the premature release of EBP-bound tropoelastin molecules [59, 60], preventing their interactions with the scaffold of microfibrils. We hypothesize that increased elastic fiber assembly may occur as a result of positively charged NPs interacting with the negatively charged cell membrane of SMCs to form a sheath around, which in turn serves to shield the EBP from (negatively charged) CS, both by spatial exclusion and by electrostatic repulsion. As a result of this potential decrease/attenuation of CS/versican interactions with EBP, as well as their interactions with the crosslinking enzyme LOX which is negatively charged at physiological pH, the positively charged NPs may promote a microenvironment conducive for greater recruitment and crosslinking of tropoelastin precursors towards enhanced elastic matrix assembly.

Treatment with NPs caused an overall enhancement in elastic matrix deposition by EaRASMCs (as seen in Figures 5B and C), compared to NP-untreated healthy RASMCs and EaRASMC controls. Treatment with 0% DOX NPs significantly enhanced total matrix elastin on a per cell basis compared to untreated EaRASMCs, as seen in Figure 5C, which may suggest that the macromolecular crowding effect due to the NPs, in addition to their positive surface charge, mediate enhanced elastic matrix deposition. We also observed a significant upregulation of elastic matrix deposition in EaRASMC cultures treated with 2% DOX-loaded NPs at 0.2 mg/mL NP concentration compared to untreated control cultures and cultures treated with 0% DOX-loaded NPs, which can be attributed to the effects of DOX. However, with an increase in NP concentration to 0.5 mg/mL, the normalized elastic matrix deposition in EaRASMC cultures treated with 2% DOX-loaded NPs was not significantly different from cultures treated with 0% DOX-loaded NPs at 0.5 mg/mL, suggesting that the DOX likely does not have any effect or may potentially be inhibitory towards elastic matrix deposition at this NP dose. However, upon closer analysis, it must be noted that the DOX released from the PLGA NPs significantly enhanced EaRASMC proliferation (Figure 4) and total matrix elastin production (Figure 5B), at all NP concentrations relative to the 0% DOX-loaded NPs at 0.5 mg/mL. Hence, the enhanced elastic matrix deposition observed for the treatment condition of 2% DOX-loaded NPs at 0.5 mg/mL was balanced by the concurrent increase in cell proliferation upon normalization of the matrix elastin to the cell number. This would potentially explain the lack of significant difference between the 0% and 2% DOX-loaded NPs at 0.5 mg/mL NP concentration. Based on this, it would be expected that 2% DOX-loaded NPs at an even higher NP concentration of 1.0 mg/mL would show lower elastic matrix content on a per cell basis. However, the macromolecular crowding effect mediated by the NPs likely offsets the effect of increased DOX concentration, leading to higher matrix elastin levels, and this concurs with Figure 5A which showed lower tropoelastin in the medium for this treatment.

The surface charge and hydrophobicity of the NPs, on account of their functionalization with the cationic amphiphile DMAB likely play an important role in the enhanced elastic matrix deposition in the NP-treated EaRASMC cultures. The positive charge on the NPs potentially mediates LOX recruitment in the extracellular space via electrostatic interactions, as LOX is negatively charged at physiological pH [26]. Additionally, as demonstrated in earlier studies with DTAB and DAH [25, 26, 61], which are chemical analogs of DMAB, one of the hydrophobic dodecyl chains of DMAB may bind to hydrophobic domains of tropoelastin molecules. This facilitates the nucleation of these tropoelastin molecules and exposes their lysine side chains to lysyl oxidase (LOX) [62], thereby enhancing their ability to be crosslinked into a matrix. Further, the net positive charge on the elastin-surfactant complex may also serve to improve or preserve the quality of newly deposited or pre-

existing elastic matrix, and enhance the net accumulation of new elastic matrix by repulsion of elastase, which exhibits a net positive charge at physiological pH [62]. Thus, the overall enhanced elastic matrix deposition observed in these studies can be attributed to (i) the charge and hydrophobicity of the NPs due to their surface functionalization with DMAB, as well as (ii) the macromolecular crowding effect due to the presence of these NPs in proximity to the cells, and (iii) the effects of DOX released from the NPs. It must be noted that the primary goal of the studies carried out for this manuscript was to examine the effects of DOX released from NPs on elastic matrix stabilization and (re)generation in EaRASMC cultures, with greater focus on the combinatorial effects of the DOX along with the NPs, rather than delineating their individual effects. Based on these results, we therefore propose to delineate the roles of these three critical parameters in mediating elastic matrix synthesis in SMC cultures in future studies.

DOX has been shown to be effective in suppressing aneurysmal dilatation in animal models of AAAs [11, 12, 14], as well as in humans [17] by its inhibition elastolytic gelatinases MMP-2 and MMP-9 [9, 17], which are overexpressed within AAAs and contribute to their growth [63, 64]. DOX-based inhibition of MMP production and activity is achieved via its chelation of the Zn^{2+} or Ca^{2+} ions in the catalytic domain of the active site of these enzymes [65], which causes their structural destabilization [55], leading to their denaturation and degradation [66]. While the DOX released from the PLGA NPs was effective in inhibiting the synthesis and activity of MMP-2 (Figure 6), the ability of DOX-free blank NPs to attenuate MMP-2 expression and activity was surprising. Based on these results, we hypothesized that the ability of DOX-free PLGA NPs to inhibit MMP-2 synthesis and activity is due to their cationic surface charge. A recent study by Mendis *et al.* [67] which showed the effects of a cationic glucosamine derivative in attenuating expression of MMP-2 and MMP-9 at the transcriptional level, while also inhibiting their activities, lends credence to this hypothesis.

From a mechanism-based perspective, our results could be attributed to charge-based interactions of these cationic NPs with MMPs, particularly their active site(s). The catalytic domain of MMPs contains adjacent pairs of glutamic acid and histidine residues, which are essential for MMP activity, and mediate their binding to their substrates [68]. At physiological pH, glutamic acid has a free carboxyl group, and thus remains negative charged. It has been hypothesized that cationic compounds, such as quarternary ammonium metacrylates, can bind to these glutamic acid residues via electrostatic interactions [69], to alter the configuration of the active site, and render it unrecognizable to complementary peptide sequences present in matrix proteins (e.g., elastin and collagen). Additionally, they may also sterically prevent the active site from interacting with matrix proteins, thereby inhibiting MMP activity. Chlorhexidine, a cationic compound has also been shown to chelates cations to inhibit MMP-2 and -9 activity at lower doses, while at higher concentrations, it has been shown to inactivate MMP-2 by denaturation [66]. Similarly, cationic surfaces have also been shown to inhibit MMP-7 activity [70]. Based on this, we hypothesize that the inhibitory effect of 0% DOX-PLGA NPs on MMP-2 activity is due to their cationic surface charge. Their positive charge potentially enables them to bind to the negatively-charged glutamic acid residues in the catalytic domain of the active site of MMPs, leading to a decrease in their proteolytic activity. Further, on account of its large size, due to the presence of two dodecyl chains, it is also possible that DMAB may block the active site of MMP-2 via steric hindrance to exert its inhibitory effect. Thus, in addition to the effect of DOX released from the PLGA NPs, the cationic surface functionalization of the NPs with DMAB likely plays an important role in mediating the inhibition of MMP-2 activity.

We formulated DAH- and DTAB-functionalized PLGA NPs, to compare their functional effects with those of DMAB-functionalized NPs in binding to elastin and also mediating LOX activity in EaRASMC cultures. Although DTAB- and DAH-modified NPs do not possess the high positive surface charge observed with DMAB-functionalized PLGA NPs, the long hydrophobic chains of DTAB and DAH may bind to the hydrophobic domains in elastin and 'anchor' the NPs to elastin/elastic fibers. The enhanced binding observed with DMAB-PLGA NPs (Figure 8) can be attributed to the fact that it has the greatest hydrophobicity of the surfactants used [34, 71–73]. Additionally, its binding capacity may also be enhanced due to the presence of two dodecyl chains in its structure, unlike DTAB and DAH, which have only a single dodecyl chain. PVA on the other hand, is a hydrophilic polymer which is used as a surfactant on account of its amphiphilic nature [65]. Further, the surface charge on these NPs may also mediate their binding to elastin, as is evidenced by the fact that PLGA NPs directly formulated with DMAB exhibit a stronger cationic surface charge compared to their counterparts which were formulated with PVA as the stabilizer and then functionalized with DTAB or DAH (Tables 1 and 2). These results demonstrate the improved elastin binding capabilities achieved upon surface functionalization of PLGA NPs with cationic amphiphiles.

Based on our results with 5% w/v DAH leading to the formation of NPs with improved cationic surface charge, we expect that increasing DTAB or DAH concentrations during NP formulation would lead to the formation of more positively-charged NPs which would potentially stimulate enhanced elastic matrix deposition and elastin binding, to an extent as observed for DMAB. On the other hand, increasing the DMAB concentration would likely lead to its saturation on the NP surface, with steric hindrance of the dodecyl chains on adjacent molecules, causing no further enhancement of elastin binding or matrix deposition. However, it must be noted that other factors such as DOX release and macromolecular crowding by the NPs mediate elastic matrix deposition. Hence, we cannot make comprehensive conclusions on the role of enhanced surface modifier concentration in mediating elastic matrix production, and on cell proliferation. These aspects will be examined in future studies.

Cationic PLGA NPs also exhibit enhanced binding interactions with SMCs [74–76]. This is primarily on account of SMCs exhibiting a net negative charge (resting membrane potential = -55 mV) [77]. Further, since LOX is a negative charged enzyme at physiological pH [25, 78], it is likely to bind via electrostatic interactions with the positively-charged PLGA NPs that would be expected to be attached similarly to the cell surface of SMCs. This is a potential explanation for the significantly higher levels of LOX activity observed for the cationic PLGA NPs modified with DMAB, DTAB and DAH, compared to the negatively-charged PVA-modified PLGA NPs (as seen in Figure 9B). Electrostatic repulsion of the negatively-charged LOX molecules by the negatively-charged PVA-modified NPs would thus explain the decreased LOX activity. Further, the enhanced LOX activity observed in the cultures treated with cationic NPs also likely illustrate that the effect of their positive surface charge is maintained over the 21 day duration of the culture. Thus, surface modification of PLGA NPs using cationic amphiphiles improves LOX activity in EaRASMC cultures.

Additionally, we also demonstrated the ability of the DMAB-, DTAB-, and DAH-functionalized NPs to inhibit MMP-2 expression and activity in EaRASMC cultures (Figure 10), compared to untreated control cultures. DMAB-functionalized NPs exhibited a greater extent of inhibition of MMP-2 expression and activity compared to DTAB- and DAH-functionalized NPs, which could be attributed to the higher cationic surface charge on the DMAB NPs. The fact that negatively-charged PVA-functionalized PLGA NPs caused a significant upregulation of MMP-2 expression, without any significant inhibition of its activity, confirms the critical role of the cationic surface charge on these NPs in attenuating

MMP-2 expression and activity. However, at the same time, it must be noted that other studies [68, 79] have reported decreased MMP activity due to increased repulsion of negatively charged drug-conjugates by the negatively-charged active site of MMP-2. Thus, it is essential for future studies to examine the exact nature of the mechanisms underlying charge-based interactions in the attenuation of MMP activity.

5. Conclusions

Our results demonstrate the efficacy of controlled, long-term release of DOX from cationic PLGA NPs in inhibiting MMP-2 production and activity in EaRASMC cultures. Further, the DOX released did not inhibit cell proliferation and contrary to expectations, enhanced elastic matrix synthesis and accumulation. Along with the effects of the cationic surface charge in attenuating MMP-2 production and activity, these positively-charged NPs were also demonstrated to bind strongly to elastin, and also improve LOX activity in EaRASMC cultures. Thus, the cationic functionalization of these NPs clearly imparts them with a multifunctional nature, particularly in the context of the preservation and stabilization of elastic matrix towards regenerative repair in AAAs. The effects/benefits of the NP surface charge (and hydrophobicity), as well as the potential macromolecular crowding effect(s) from the pro-elastogenic standpoint warrant further examination in future work. Further studies will also be essential to evaluate and optimize the delivery and retention of DOX-loaded PLGA NPs *in vivo* in animal models of AAA, as well as examine their functional effects in inhibiting matrix proteolysis and enhancing elastin regenerative repair, to stabilize the condition. If so demonstrated to be effective, this system may potentially be combined with endovascular repair techniques to develop a potent therapeutic strategy for stabilizing or even regressing AAAs. Overall, considering their potential ability to inhibit MMP and their enhanced ability for binding to elastin, these results demonstrate the multifunctional potential and utility of these cationic NPs in mediating improved elastogenic outcomes in aneurysmal SMC cultures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Representative confocal microscopy images of uptake of fluorescent FITC-PLGA NPs (green) by EaRSMCs (red). Images are z-stack overlays of single EaRSMCs that show internalization of FITC-PLGA NPs of size 100 nm (A) and 200 nm (B), but not 500 nm (C).

Figure 2. Representative images illustrating the viability of EaRASMCs upon exposure to DOX-loaded (2%, 5%, 10%) and unloaded (0%) PLGA NPs for 48 h. LIVE-DEAD[®] assay stains live cells green and dead cells red. (20× magnification; scale bar equivalent to 100 μ m).

Figure 3.

In vitro DOX release profiles from the PLGA NPs, at the three different DOX loadings (2%, 5% and 10% w:w ratio to PLGA), and the three NP concentrations of 0.2, 0.5 and 1.0 mg/mL. ($n = 3$ per group; mean $\pm$ SD).

Figure 4.

Proliferation of aneurysmal SMCs (EaRSMCs) in response to DOX released from PLGA NPs at 0.2, 0.5 and 1.0 mg/mL NP concentrations. Healthy SMCs (RASMCs) were used as the cell-type control, while EaRSMCs treated with blank NPs (0% DOX) as the treatment control. The cell number was calculated based on an estimate of 6 pg of DNA per cell, via a DNA assay, at 0 and 21 days post-seeding, (mean $\pm$ SD; $n = 3$ per case, * denotes $p < 0.05$ compared to untreated EaRSMCs; # denotes $p < 0.05$ compared to 0% DOX-PLGA treatment controls).

Figure 5.

Effects of DOX released from PLGA NPs on (A) tropoelastin synthesis, and (B) synthesis of alkali-soluble and alkali-insoluble crosslinked matrix elastin by EaRSMCs. (C) Total matrix elastin was normalized to DNA content per cell. RSMCs were used as cell-type controls and EaRSMCs treated with blank (0% DOX) NPs as treatment control. (mean $\pm$ SD; $n = 6$ /case for tropoelastin, $n = 3$ /case for tropoelastin, * denotes $p < 0.05$ compared to untreated EaRSMCs; # denotes $p < 0.05$ compared to 0% DOX-PLGA treatment controls). Note: Total matrix elastin for each well per test case was normalized to the average DNA content per test case.

Figure 6.

Effect of DOX released from NPs on MMP-2 synthesis and activity in EaRASMC cultures, as analyzed with western blots and gel zymography. (A) Representative image of western blot, with β -actin bands as loading controls. (B) Fold-change in production of active MMP-2 compared to control cultures without NPs. (C) Fold-change in MMP-2 activity compared to NP-untreated control EaRASMC cultures (representative zymogram in inset). Data represented (mean $\pm$ SD; $n = 5$ /case for western blots, $n = 3$ /case for gel zymograms, * denotes $p < 0.05$ compared to controls with no NPs, # denotes $p < 0.05$ compared to 0% DOX-PLGA treatment controls). Notes: Values in Figure 6B represent the fold-change in normalized band intensities of the active MMP-2 band 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 untreated (no NP) controls. The normalized band intensity of MMP-2 for the NP-untreated control test condition was set to unity to determine fold-change in MMP-2 expression or activity for the different NP-treated test cases.

Figure 7. Confocal microscopy images for immunofluorescence detection of elastin and lysyl oxidase (LOX) within EaRASC layers following 21 days of culture in the presence of DOX-loaded PLGA NPs. 0% DOX-PLGA NPs were added as the active-agent control, while EaRASCs cultured without any NPs served as the treatment control. Immunolabeling controls received no primary antibodies and exhibited no background when treated with the Alexa633-conjugated secondary antibody. Nuclei were stained with DAPI, and filamentous actin stained with Alexa Fluor 488 phalloidin.

Figure 8.

Percentage of PLGA NPs functionalized with the different amphiphilic surface-modifiers (with BSA-Cy5 encapsulated within) bound to elastin. (mean $\pm$ SD; $n = 3/\text{case}$, * denotes $p < 0.05$ compared to corresponding amount of PVA-functionalized PLGA NPs) Note: The percentage retention was calculated after subtracting the amount of NPs adhering to empty wells.

Figure 9.

Fold-change in lysyl oxidase (LOX) activity in EaRASC cultures at 21 days, treated with the different surface-functionalized PLGA NPs. LOX activity was measured in (A) the pooled cell culture medium and (B) in the cell layer by the quantity of hydrogen peroxide released upon oxidative deamination of alkyl monoamines and diamines and normalized to that of the PVA-modified PLGA NP controls. (mean $\pm$ SD; $n = 6$ /case; * denotes $p < 0.05$ compared to PVA-functionalized PLGA NP controls).

Figure 10.

Effect of surface functionalization of PLGA NPs on MMP-2 synthesis and activity in EaRASMC cultures, as analyzed with western blots and gel zymography. (A) Representative image of western blot, with β -actin bands as loading controls. (B) Fold-change in production of active MMP-2 compared to control cultures without NPs. (C) Fold-change in MMP-2 activity compared to untreated control EaRASMC cultures. Data represented (mean $\pm$ SD; $n = 3$ /case, * denotes $p < 0.05$ compared to controls with no NPs). Notes: Values in Figure 10B represent the fold-change in normalized band intensities of the active MMP-2 band 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 NP-untreated controls. The normalized band intensity of MMP-2 for the NP-untreated control test condition was set to unity to determine fold-change in MMP-2 expression or activity for the different NP-treated test cases.

Table 1

Size and surface charge of DOX-encapsulated PLGA NPs. ($n = 6$ replicate formulations, mean $\pm$ 95% CI).

	DOX loading [%; w/w ratio to PLGA]			
	0	2	5	10
Size [nm]	332.7 $\pm$ 8.3	357.9 $\pm$ 79.1	379.4 $\pm$ 14.4	274.0 $\pm$ 6.7
ζ -potential [mV]	33.8 $\pm$ 0.4	34.2 $\pm$ 6.6	25.3 $\pm$ 2.5	31.1 $\pm$ 3.7
DOX encapsulation [%]	N/A	40.1 $\pm$ 5.7	42.3 $\pm$ 7.4	38.7 $\pm$ 7.4

Table 2

Size and surface charge of PLGA NPs formulated with PVA as surfactant, and functionalized with DTAB and DAH. ($n = 6$, mean $\pm$ S.D.).

Surfactant	1% w/v PVA	1% w/v PVA	1% w/v PVA	5% w/v DAH
		+	+	
		1% w/v DTAB	1% w/v DAH	
Size [nm]	336.0 $\pm$ 13.3	337.4 $\pm$ 15.6	330.6 $\pm$ 14.6	298.3 $\pm$ 26.9
ζ -potential [mV]	-35.4 $\pm$ 3.6	1.3 $\pm$ 2.1	2.5 $\pm$ 4.4	20.1 $\pm$ 4.0

Table 3

Size and surface charge of PLGA NPs encapsulating BSA-Cy5, formulated with PVA as surfactant, and functionalized with DTAB and DMAB. ($n = 6$, mean $\pm$ SD).

Surfactant	1% w/v DMAB	1% w/v PVA	1% w/v PVA	1% w/v PVA
			+	+
			1% w/v DTAB	1% w/v DAH
Size [nm]	275.0 $\pm$ 25.1	354.4 $\pm$ 19.0	382.9 $\pm$ 51.3	357.9 $\pm$ 56.7
ζ -potential [mV]	25.6 $\pm$ 2.3	-32.3 $\pm$ 3.6	-0.4 $\pm$ 1.0	0.9 $\pm$ 0.5