Polycationic Nanofibers for Nucleic Acid Scavenging

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ABSTRACT: Dying cells release nucleic acids (NA) and NA-containing complexes that activate inflammatory pathways of immune cells. Sustained activation of these pathways contributes to chronic inflammation frequently encountered in autoimmune and inflammatory diseases. In this study, grafting of cationic polymers onto a nanofibrous mesh enabled local scavenging of negatively charged pro-inflammatory molecules in the extracellular space. Nucleic acid scavenging nanofibers (NASFs) formed from poly(styrene-alt-maleic anhydride) conjugated with 1.8 kDa bPEI resulted in nanofibers of diameters 486 ± 9 nm. NASFs inhibited the NF-κB response stimulated by the negatively charged agonists, CpG and poly(I:C), in Ramos-blue cells but not Pam3CSK4, a nonanionic agonist. Moreover, NASFs significantly impeded NF-κB activation in cells stimulated with damage-associated molecular pattern molecules (DAMPs) released from doxorubicin killed cancer cells. In vivo application of NASFs to open wounds demonstrated nucleic acid scavenging in wounds of diabetic mice infected with Pseudomonas aeruginosa, suggesting the in vivo efficacy of NASFs. This simple technique of generating NASF results in effective localized anti-inflammation in vitro and local nucleic acid scavenging in vivo.

INTRODUCTION

Extracellular nucleic acids can elicit pro-inflammatory responses by activating the innate immune system. Pattern recognition receptors (PRRs) such as the toll-like receptors (TLRs) are activated by pathogenic nucleic acids (e.g., viral RNAs, bacterial DNAs) as well as nucleic acids released by necrotic cells.1–3 Hindered clearance of extracellular nucleic acids from the blood has been linked to inappropriate activation of TLRs by endogenous nucleic acids, which is observed in various inflammatory and autoimmune diseases including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and multiple sclerosis (MS). In the case of autoimmune diseases, antibodies to extracellular DNA (eDNA) lead to the formation of immune complexes that increase cellular uptake, allowing for TLR activation to occur, leading to cytokine production and prolonged immune activation. Studies in lupus-prone MRL/lpr mice confirm that activation of TLR7 and TLR9, nucleic acid receptors, can mediate the pathogenesis of SLE.4–6 Current therapies (e.g., monoclonal antibodies) can reduce the symptoms of lupus, but fail in many patients due to serious side effects from widespread immunosuppression.7 In light of these side effects, researchers have shifted their focus to blocking antibody−DNA interactions using oligonucleotides, peptides, and small molecules to block the antibody binding site on DNA. However, these approaches are limited due to the expression of DNA antibodies that interact with diverse sites on the DNA molecule, therefore, limiting the efficacy of antibody blocking. Previous work from our laboratory has demonstrated the effectiveness of certain nucleic acid-binding polymers (e.g., PAMAM-G3, CDP, HDMBr, protamine, polyethylenimine) at inhibiting nucleic acid-mediated activation of nucleic acid-sensing PRRs, irrespective of whether they recognize ssRNA, dsRNA, or hypomethylated DNA.8

An alternative approach for nucleic acid scavenging of freely circulating NAs would be useful, as such NAs may present issues with regard to cytotoxicity and nonspecific cellular uptake. Here we show that a nucleic acid-scavenging nanofiber (NASF) serves as a highly cationic mesh to scavenge extracellular nucleic acids in a controlled and localized environment without compromising TLR responses to non-nucleic acid, pathogenic stimulators.

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The NASFs are synthesized by electrospinning of poly-(styrene-alt-maleic anhydride) (PSMA). The combination of polystyrene and maleic anhydride provides tunable mechanical properties and ease of chemical modification. Electrospun PSMA nanofibers have been applied for aptamer and gas sensing, while microdiameter fibers have been investigated for hydrogel formation and enzyme stabilization. In this study, we present the covalent modification of PSMA nanofibers with amine-containing polycations, namely branched polyethyleneimine (bPEI), for nucleic acid scavenging.

## MATERIALS AND METHODS

### Poly(styrene-alt-maleic anhydride) Electrospun Nanofiber Formation

Preparation of neutral fibers was optimized by testing various concentrations of poly(styrene-alt-maleic anhydride) (PSMA) with solvent combinations of one or more of the following: tetrahydrofuran, acetone, and dimethylformamide. Effective electrospinning occurred using PSMA (0.6 or 1 g) with an average molecular weight of 1900 Da (Sigma-Aldrich, St. Louis, MO) dissolved in a 1:1:1 (v/v/v) mixture of tetrahydrofuran/acetone/dimethylformamide (10 mL; Sigma-Aldrich) by constant mixing for 24 h at room temperature. PSMA nanofibers were electrospun using 2 mL of polymer solution in a 2 cm glass syringe (Cedence Science, Staunton, VA) at a dispensing rate of 1 mL/h, achieved by inserting the syringe into an automated syringe pump, with an applied voltage of +15 kV. The polymer fibers were collected on a grounded cylindrical mandrel (~6.4 cm wide with a ~2.16 cm circumference) spinning at ~130 rpm at a distance of 10 cm away from the tip of the syringe needle. The neutral electrospun PSMA fibers were soaked in a solution of 1.8 kDa branched polyethyleneimine (bPEI; 0.1 M; Polysciences, Inc., Warrington, PA) for 72 h at room temperature with constant shaking to form NASF. To form PAMAM-NASF, the neutral fibers were soaked in PAMAM-G3 (0.004 M; Sigma-Aldrich) at 4 °C for 24 h with constant shaking. Following conjugation, the NASF were washed for 10 min with deionized water a total of 5 times. NASF were sterilized for 30 min in 70% ethanol, the ethanol was removed, and the NASF were allowed to air-dry in a sterile environment. PAMAM-NASF were washed five times for 10 min each in sterile water and allowed to air-dry in a sterile environment, skipping the ethanol sterilization step. The levels of 1.8 kDa bPEI conjugated onto the fiber were determined by ninhydrin assay.

**Scanning Electron Microscopy (SEM) of Nanofibers.** Dry fibers were placed on aluminum foil and mounted on an SEM stub. The fibers were gold sputter-coated for 250 s using the Denton Vacuum Desk IV sputter unit (Denton Vacuum, Moorestown, NJ) and imaged using a FEI XL30 SEM-FEG (FEI, Hillsboro, OR). Images were analyzed in Scandium (ResAlta Research Technologies, Golden, CO).

**X-ray Photoemission Spectroscopy (XPS) of Nanofibers.** XPS measurements were taken on a Kratos Analytical Axis Ultra XPS (Kratos, Manchester, UK) using a monochromated Al Kα source. The source was operated at 15 kV and 10 mA (150 W). Electron collection was made at 90° to the sample surface. Survey scans were taken with a pass energy of 160 eV and region scans with a pass energy of 20 eV.

**Nucleic Acid Adsorption of NASF.** Alexa Fluor 488 labeled CpG ODN 1668 (3.33 x 10^-14 to 1 x 10^-3 ng/mL; IDT, Coralville, IA) was incubated with 0.08 mg of NASF for 4 h at room temperature under constant shaking. The NASFs were washed three times with deionized water, placed on a microscope slide, and mounted with SlowFade Diamond reagent (Life Technologies, Carlsbad, CA). Fluorescent images of adsorbed DNA onto NASFs were captured with an Upright Axiolmage A1 microscope powered by a Zeiss HBO100 power supply and lamp housing. To generate the DNA adsorption curve, salmon sperm DNA (25-100000 ng; Life Technologies) in 1xTE buffer was added to 0.08 mg of NASF for 4 h at room temperature (RT) with constant shaking. Total salmon sperm DNA concentration remaining in the 1xTE solution was determined using a PicoGreen assay (Life Technologies).

**Cell Culture.** All cell experiments were performed in complete growth media unless specifically stated otherwise, with cells incubated at 37 °C (5% CO₂). STO (ATCC, Manasses, VA) and RAW (ATCC) cells were cultured in DMEM (Gibco 11960-044) supplemented with 10% FBS and 1% Pen-Strrep. Ramos-Blue cells (InvivoGen, San Diego, CA) were cultured in IMDM (Gibco 12440-053) supplemented with 10% FBS, 1% Pen-Strrep, and 100 μg/mL Zeocin every 4 passages. NHDF (Lonza, Basel, Switzerland) cells were cultured in DMEM (Gibco 11960-044) with 10% FBS, 1% Pen-Strrep, 1% nonessential amino acids, 1% sodium pyruvate, 1% Glutamax, and 0.1% β-mercaptoethanol.

**Cell Viability.** STO cells (40000 cells/well) were plated 18–24 h before experiments to allow for cellular adhesion. Ramos-Blue cell viability experiments were performed by plating cells (200000 cells/well) immediately prior to adding the NASF into the medium. Both cell types were incubated with 0.107 mg of NASF for 4 h and cell viability was measured using CellTitre-Glo (Promega, Madison, WI). Proliferation studies were performed by using a 1.13 mg piece of NASF, cut to fit into the well, onto the bottom of a nontissue culture treated 48-well plate (Greiner Bio-One, Kremsmunster, Austria) using 10 μL of PBS to aid in NASF adhesion to the well. After the PBS dried, Normal Human Dermal Fibroblast cells (NHDF; 100000 cells/well) were added to the top of the NASF. Proliferation was determined using LIVE/DEAD viability/cytotoxicity kit (Life Technologies) at 24 and 48 h.

**Inhibition of Nuclear and Drived TLR Activation Using Polycationic NASFs.** For scaving studies, NASFs or neutral PSMA fibers (0.107 mg) were incubated in media for 10 min with one of the following TLR agonists at 10 μg/mL in 250 μL of complete media: CpG 1668 (TLR 9), PolyI:C (TLR 3), or Pam3CSK4 (TLR 1/2; InvivoGen). The fiber was removed and the fiber treated media (62.5 μL) was added to Ramos-Blue cells (200000 cells in 137.5 μL of complete media) for 18–24 h at 37 °C. For comparison, TLR agonists (2.5 μg/mL) were also applied directly to Ramos-Blue cells. For direct contact studies, NASFs were added directly to Ramos-Blue cells (200000 cells/well), followed by incubation with agonists (2.5 μg/mL) for 18–24 h at 37 °C. After incubation, the supernatant (40 μL) from the treated Ramos-Blue cells was added to the serum alkaline phosphatase colorimetric indicator Quanti-Blue (160 μL; InvivoGen), incubated for 5 h at 37 °C, and absorbance was measured at 650 nm.

**Inhibition of TLR Activation by DAMPs Using NASFs.** Doxorubicin (DOX) was used to cause cell death of RAW cells and release of damage-associated molecular pattern molecules (DAMPs) as described. Different doses of DOX were administered to RAW cells and the subsequent TLR activation was measured in Ramos-Blue reporter cells and the doses that generated DAMPs capable of activating the Ramos-Blue cells potently were used in subsequent studies. The DOX doses were incubated for 48 h in RAW cells (40000 cells/well, DOX (3, 6, or 9 μg/mL; Sigma-Aldrich)) and the supernatant from the DOX-treated cells (100 μL) was added to a 1.41 mg piece of NASF. The NASF and supernatant were incubated for 30 min and the entire volume was added to Ramos-Blue cells (200000 cells in 100 μL IMDM). After incubation for 18–24 h, Ramos-Blue supernatant (40 μL) was added to Quanti-blue (160 μL; InvivoGen) and the absorbance was read at 650 nm at 3 and 5 h.

**NASF Application to Open Wound Mouse Model Infected with Pseudomonas aeruginosa.** The murine diabetic wound model was created according to the protocol described by Zhao et al. Briefly, diabetic female mice, 8–12 weeks old (BKS.CgDock7m+/+Lepr−/−) from the Jackson Laboratory were given an 8 mm diameter excisional wound between the shoulder blades that was covered with Tegaderm Film (3M). NASF treatment was started 72 h following wounding in diabetic mice. All mice received 1.13 mg of NASF placed directly inside the open wound, which was then covered by Tegaderm film. Diabetic mice received NASF treatment for 7 consecutive days and the NASF sheets were changed every 24 h over the 7 day period. Following treatment, the NASF was removed from the mouse wound, placed in PBS, and vortexed in pulses at the highest level for 30 s to remove any mechanically adhered components. The NASF was then placed in 10 mg/mL of heparin sodium salt (Sigma) for 30 min with...
intermittent vortexing. After 30 min, the NASF was discarded and a TRIzol (ThermoFisher Scientific) extraction was performed on the solution to separate the RNA, DNA, and protein components extracted from the NASF.

For *Pseudomonas aeruginosa* infected wound studies, diabetic mice with an 8 mm diameter excisional wound had 50 μL of *Pseudomonas aeruginosa* (PA01, ATCC 15692) at a 10^4 CFUs administered to the open wound 72 h after initial wounding. NASF was administered and processed as described above.

**DNA Gels and PCR.** DNA extracted using the TRIzol reagent was resuspended in 8 mM NaOH. 200 ng of DNA was run on a 1% agarose gel at 100 V for 90 min and visualized using GelStar Nucleic Acid Stain (Lonza). Two primer sets for mitochondrial cytochrome c oxidase were used for PCR. Primer set 1 included primers: 5′-ACC AAG GCC ACC ACA CTC CT-3′ and 5′-AGG CTC AGA ATC CTG CAA AGA A- 3′, while primer set 2 included primers: 5′-TCC AAG TCC ATG ACC ATT AAC TG-3′ and 5′-TAT TGG TGA GTA GGC CAA GGG-3′, leading to fragment sizes of ~101 and ~115 bp, respectively. Taq 2x Master Mix (New England BioLabs) and 100 ng of DNA was used for all PCR reactions. Primer set 1 was done with an annealing temperature of 54 °C and an extension time of 10 s, while primer set 2 was done with an annealing temperature of 50 °C and an extension time of 10 s. All PCR products were run on a 3% agarose gel at 70 V for 60 min and visualized using GelStar Nucleic Acid Stain.

**RNA Sequencing.** RNA library preparation was done using the Strand RNA-Seq Kit (Kapa) with 10 ng of RNA. Following library preparation, the libraries were pooled in equimolar amounts, and sequenced with a 50 bp SR run on the Illumina HiSeq 2500 sequencer. RNA-seq data was processed using the TrimGalore toolkit, which employs Cutadapt to trim low quality bases and Illumina sequencing adapters from the 3′ end of the reads. Only reads that were 20 nt or longer were kept for further analysis. Reads were mapped to a custom genome and transcriptome that contained the mouse NCBIM38r73 data as well as the *Pseudomonas aeruginosa* PAO1 data using the STAR RNA-seq alignment tool. Reads were kept for subsequent analysis if they mapped to a single genomic location. Gene counts were compiled...
Figure 2. Fluorescent microscope images of polycationic nanofibers after 4 h interaction with (Ai) no AlexaFlour488-CpG or AlexaFlour488-CpG at (Aii) 3.33 × 10⁻⁴ µg/µL, (Aiii) 6.6 × 10⁻⁴ µg/µL, and (Aiv) 1 × 10⁻³ µg/µL; (B) quantification of average fluorescence after interaction with AlexaFlour488-CpG normalized to polycationic nanofiber alone and compared to the initial amount of CpG added (n = 3); (C) Salmon sperm DNA absorption onto 0.08 mg of polycationic nanofiber (n = 3), where the x-axis represents the amount of DNA added to the solution and the y-axis represents how much DNA the polycationic nanofiber removed from the solution.

using the HTSeq tool. Only genes that had at least 10 reads in any given library were used in subsequent analysis. Normalization and differential expression was carried out using the DESeq2 package with the R statistical programming environment. Statistical Analysis. All data were evaluated for statistical analysis with One Way Analysis of Variance (ANOVA) followed by secondary Tukey-HSD analysis in JMP. The average values and standard errors of the mean are presented with p < 0.05 being considered statistically significant.

RESULTS AND DISCUSSION

Unmodified PSMA nanofibers electrospun from two concentrations (6% and 10% w/v) resulted in randomly aligned nanofibers with average diameters of 297 ± 13 and 737 ± 26 nm, respectively (Figure 1A). Although electrospun PSMA fibers and polystyrene-PSMA fiber sheets can be found in the literature, there is no report on functionalizing these structures with a cationic moiety. The process used to form the NASF has some distinct advantages because it involves modular steps that allow conjugation of any amine-containing polymer onto the neutral fiber. This modular approach has been utilized for development of microfibers, but here we employ it to produce nanofibers to generate a higher surface area for cationic polymer attachment that are subsequently used for nucleic acid scavenging. Nucleic acid scavenging using fibers has been previously reported, however, in the aforementioned study, a biodegradable poly(ε-caprolactone) and bPEI block copolymer was used for electrospinning and involved a complicated synthesis step followed by electrospinning. This process would require reoptimization of electrospinning parameters for each different block copolymer synthesis, as opposed to our neutral fiber modification technique that allows for modification of already formed nanofibers. The PSMA nanofibers electrospun from 6% and 10% w/v polymer solutions demonstrated different physical characteristics after conjugation with 1.8 kDa bPEI. The 10% NASFs (NASFs made with 10% PSMA nanofibers) were brittle and difficult to handle whereas the 6% NASFs demonstrated a malleable tissue paper-like texture. After initial proof-of-concept in vitro experiments with both 6% and 10% NASFs, 6% PSMA fibers were chosen for preparation of the NASF sheets as they were more durable, easier to cut into various sizes and did not show reduced efficacy as compared to 10% NASFs. Upon conjugation of 1.8 kDa branched bPEI to the 6% PSMA nanofibers, the resulting polycationic nanofibers (NASFs) had an increased fiber diameter of 486 ± 9 nm (Figure 1B) as compared to the original at 297 ± 13 nm (Figure 1A) and contained ~11 µM bPEI per 1 mg of nanofiber. Successful conjugation of bPEI onto the nanofibers was confirmed by contact angle measurements, X-ray photoelectron spectroscopy (XPS), and DNA-binding affinity. The contact angle of neutral PSMA nanofibers was 122°, indicating high hydrophobicity, while the NASFs were so hydrophilic that the contact angle could not be determined, validating the conversion from hydrophobic neutral nanofibers to hydrophilic cationic nanofibers. Additionally, XPS data confirmed the abundance of nitrogen on the surface of the NASFs at an atomic percent of 10.57%, which indicated successful conjugation of amino groups onto the nanofiber surface. The unmodified PSMA fibers showed a surface nitrogen abundance of atomic percent <0.01% (Figure 1C).

Functionality of cationic NASFs to bind nucleic acids was validated using CpG ODN 1668 DNA and salmon sperm DNA. Alexa-Fluor 488 labeled CpG was used to demonstrate binding of nucleic acids by the NASFs (Figure 2A,B). As expected, increasing amounts of labeled CpG resulted in increased fluorescence as compared to background nanofiber fluorescence. The increased fluorescence of the NASFs following soaking with labeled DNA and subsequent washes to remove any unattached DNA confirmed the ability of NASFs to scavenge nucleic acids. Through adsorption analysis using salmon sperm DNA (Figure 2C), the maximum adsorption capacity of the NASFs was ~35.7 µg DNA/0.08 mg of fiber. SEM images showed that the initial modification of neutral PSMA nanofibers with bPEI resulted in swelling of the fibers and coalescing of the fibers at points where they overlapped (Figure 1A,B). However, interaction with salmon sperm DNA did not change the fiber morphology, thereby maintaining the NASF mechanical structure following DNA interaction (data not shown).

Previous studies indicate that the presence of highly charged moieties (e.g., soluble cationic polymers) lead to high cytotoxicity in some cell lines. A benefit of this insoluble NASF strategy is that scavenging activity happens entirely in the extracellular space, avoiding cellular uptake of the bound polycation-NA complex. In an in vivo application, this would allow the systemic circulation to be void of circulating polycations, bypassing the toxicity issue associated with soluble polycations. Additionally, the NASF utilized is not biodegradable, so there are no concerns of components slipping into circulation and causing systemic toxicity. When cell viability was tested with STO and Ramos-Blue cells in the presence of NASF, the cell viability remained over 70% (Figure 3A). To determine the effects of direct cellular interaction with the NASF, a LIVE/DEAD stain was performed after 24 and 48 h of plating NHDF cells directly onto NASFs (Figure 3B). The LIVE/DEAD assay in NHDF showed that viability remained
above 80% at all times when cells were grown directly on top of NASF. Taken together, these results indicate that the NASFs display minimal toxicity.

Next, the NASF were tested to determine if they could effectively reduce inflammatory cytokine production by scavenging NAs from solution. Initial tests in TLR reporter cells, Ramos-Blue cells, demonstrated the NASFs’ ability to scavenge CpG, prevent TLR9 activation, and concomitantly reduce NF-κB/AP-1-inducible secreted alkaline phosphatase (SEAP) levels down to baseline. To show that this inhibition was due to electrostatics-driven scavenging and not by nonspecific adsorption of fluid and the components in it, comparative experiments were done with neutral fibers (Figure 4A). Neutral, unmodified PSMA fibers have no effect on CpG TLR stimulation, therefore showing that nonspecific fluid interaction was not responsible for the scavenging of CpG.

Beyond CpG scavenging as was done by Kang and Yoo, the NASF were also tested for their ability to scavenge Poly(I:C), a synthetic double-stranded RNA molecule. Specificity for negatively charged TLR agonists is demonstrated in Figure 4B by comparing the ability of the NASFs to return SEAP levels to baseline after preincubation with CpG, Poly(I:C), and Pam3CSK4. CpG and Poly(I:C) are both nucleic acid agonists, while Pam3CSK4 is a synthetic triacylated lipoprotein agonist. NASFs blocked TLR3 and TLR9 activation by Poly(I:C) and CpG, respectively, both negatively charged nucleic acid agonists. However, they were not able to block activation by non-nucleic acid agonist, Pam3CSK4 (TLR2/1). To demonstrate that the NASFs can effectively scavenge NAs in the presence of cells and serum, Ramos-Blue cells were coincubated with the fibers, followed by administration of the TLR agonists (Figure 5). Co-incubation experiments showed the same results as preincubation with NASFs; both CpG and Poly(I:C) stimulation effects were reduced to baseline SEAP levels, while stimulation by Pam3CSK4 remained unaffected. To demonstrate the functional versatility of this scavenging strategy, a polyamido(amine) cationic dendrimer (PAMAM-G3) was grafted onto the neutral fibers to make PAMAM-NASF and tested for efficacy in the coincubation studies with Ramos-Blue TLR reporter cells. As expected, the PAMAM-NASFs mimicked the results seen with bPEI-grafted NASFs; nucleic acid TLR agonists CpG and Poly(I:C) were scavenged, therefore, significantly reducing SEAP expression; whereas, SEAP expression caused by non-nucleic acid agonist Pam3CSK4 was not affected. Together, these results prove that the NASFs can effectively prevent TLR activation by CpG and Poly(I:C) and present relevant evidence that this blocking by NASF can be extended to other stimulatory nucleic acids. Additionally, the nucleic acid scavenging effectiveness of NASFs is not limited to using bPEI as the grafted polycation. The neutral PSMA fibers can also be modified with different polycations (e.g., PAMAM-G3) while maintaining scavenging capabilities.

To test the NASF in a biologically relevant in vitro study, DOX-induced cell death was used to generate cell debris and damage-associated molecular patterns (DAMPs). Studies show
that DOX, a commonly used chemotherapeutic, induced cell death leads to transient NF-κB expression, which is associated with nucleic acid fragments that are released from dead and dying cells. Increased amounts of circulating nucleic acids resulting from excessive cell death caused by chemotherapy or radiation therapy initiates inflammatory responses. High doses of DOX cause apoptotic cell death resulting in abnormal DNA fragmentation, these endogenous DNA and RNA fragments can subsequently be uptaken into healthy cells leading to TLR activation. To model increased nucleic acid amounts as released from dying cells and to show the application of the NASFs in a biologically relevant inflammatory situation, nanofiber scavenging was tested using DOX-killed cell debris as the pro-inflammatory TLR stimulant. Administration of DOX to RAW 264.7 cells induced cell death that released various TLR and/or NOD1 agonists that stimulate NF-κB/AP-1 and subsequent SEAP secretion from Ramos-Blue B lymphocyte cells. RAW 264.7 cells were chosen for this application because DOX treatment for 48 h resulted in cell-death debris that promoted high TLR/NOD1 activation. When the cell-death debris was scavenged by the NASFs, a decrease in NF-κB/AP-1 secretion, as illustrated by SEAP amounts, was observed (Figure 6). The maximum blocking of 41.4% of TLR/ NOD1 activation by NASFs was achieved after interaction with the DOX-treated cell debris administered from the lowest DOX dose (3 μg/mL). The percent blocking by NASFs decreased to 26.9% and 28.3% after treatment with DOX-treated cell debris with initial DOX doses of 6 and 9 μg/mL, respectively. We hypothesize that this modest blocking of TLR activation is due to apoptosis-associated protein complexes that stimulate NOD1, which also induce NF-κB/AP-1 secretion. Yet another explanation is that the higher DOX concentrations resulted in more cell death consequentially causing saturation of the nanofibers and therefore reduced blocking. Exploring increased doses of NASF may allow for increased blocking of the immune response caused by the DOX-treated cell debris. Nevertheless, these results indicate that the NASFs are functional in a clinically relevant application.

An open wound mouse model with and without a Pseudomonas aeruginosa infection was used to validate the NASFs nucleic acid scavenging capabilities in an in vivo setting. NASFs were placed on uninfected and infected wounds for 24 h, the contents on the NASF were competed away using a heparin competition assay, and the components were separated into DNA and RNA extracts. In both infected and uninfected diabetic mice wounds treated with NASF, DNA extracted from the NASF and DNA gels run on the samples demonstrated two prominent DNA bands, an unidentified high base pair band and another band around ~16000 bp (data not shown). Amplification of mitochondrial DNA (mtDNA) with cytochrome-c oxidase primers validated that the ~16000 bp band was mtDNA. Figure 7A,B shows a sample of the PCR amplicons from the DNA samples extracted from the NASF. mtDNA is important because increased circulating mtDNA leads to increased TLR9 activation resulting in a prolonged inflammatory response.

The RNA extracts were analyzed using RNA sequencing. The Venn diagram in Figure 8B shows the number of detected RNAs that were extracted from mouse wounds that were either infected with P. aeruginosa or not infected. Overall, the NASF that was not exposed to P. aeruginosa bacteria had higher numbers of RNA at 18448 as compared to 12693 that were infected with P. aeruginosa. Figure 7A,B shows a sample of the PCR
A heatmap in Figure 8A shows how the RNAs extracted from uninfected wounds and *P. aeruginosa* infected wounds differ. Approximately 20−24% of the total RNAs detected in the infected wounds were found to be of bacterial origin, whereas in the uninfected wounds, all of the RNAs were of mouse origin. This demonstrates that the NASF can scavenge both bacterial and mouse RNA and that over a 24 h period, a greater percentage of the RNA scavenged is of mouse origin.

### CONCLUSION

In conclusion, we have built upon our previous findings that scavenging of extracellular nucleic acids can reduce inflammation. We report an insoluble, minimally toxic cationic nanofiber, NASF, which selectively scavenges polyanionic pro-inflammatory species (DNA, RNA) and blocks improper activation of nucleic-acid sensing TLRs without compromising the ability to respond to non-nucleic acid agonists. We also report that this nanofiber scavenging strategy may be useful in reducing pro-inflammatory side effects associated with chemotherapy induced cell death, which releases large amounts of pro-inflammatory nucleic acids and complexes into circulation. Finally, we validate the scavenging capacity of the NASF in vivo using an open wound model. If the utility of soluble polycations is limited due to the toxicity associated with cellular uptake of highly positively charged molecules, these insoluble, functional NASFs prevent cell internalization and minimize cytotoxic side effects, presenting an attractive strategy to reduce chronic inflammation due to extracellular nucleic acids.

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**Author Contributions**

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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