



Published in final edited form as:

Adv Healthc Mater. 2025 June ; 14(15): e2404584. doi:10.1002/adhm.202404584.

Development of DNase-1 Loaded Polymeric Nanoparticles Synthesized by Inverse Flash Nanoprecipitation for Neutrophil-Mediated Drug Delivery to *in vitro* Thrombi

Sophie Maiocchi^{1,2,3,4,7,#}, Erica E. Burnham⁴, Ana Cartaya^{1,2,3,5,8}, Veronica Lisi⁷, Nancy Buechler⁷, Rachel Pollard⁴, Danial Babaki^{1,2,3}, Wolfgang Bergmeier⁶, Nathalie M. Pinkerton^{4,*}, Edward Bahnson^{1,2,3,*}

¹Department of Cell Biology and Physiology, University of North Carolina at Chapel Hill, NC, 27599, USA.

²Curriculum in Toxicology and Environmental Medicine, University of North Carolina at Chapel Hill, NC, 27599, USA.

³Center for Nanotechnology in Drug Delivery, University of North Carolina at Chapel Hill, NC, 27599, USA.

⁴Department of Chemical and Biomolecular Engineering, Tandon School of Engineering, New York University, NY, 11201, USA.

⁵Department of Pharmacology, University of North Carolina at Chapel Hill, NC, 27599, USA.

⁶Department of Biochemistry and Biophysics, University of North Carolina at Chapel Hill, NC, 27599, USA.

⁷Department of Biomedical Engineering, Wake Forest University School of Medicine, NC, 27101, USA.

⁸Light Microscopy Core Facility, Duke University, NC, 27710, USA.

Abstract

#Correspondence can be sent to smaiocchi@wakehealth.edu. *NMP and EMB contributed equally and share senior authorship. For NMP correspondence can be sent to: nathalie.pinkerton@nyu.edu. For EMB correspondence can be sent to: edward_bahnson@med.unc.edu.

Corresponding Author

Sophie Maiocchi current affiliation: Department of Biomedical Engineering, 3E-018, Suite 310, 3rd Floor, Biotech Place, 575 Patterson Ave, Wake Forest University School of Medicine, Winston-Salem., NC, 27101, USA.

Nathalie Pinkerton: Chemical and Biomolecular Engineering Department 6 MetroTech Center, Rogers Hall 702K, New York University, Brooklyn, NY 11201

Edward Moreira Bahnson: Department of Cell Biology and Physiology, 5109D Neuroscience Research Building, 125 Mason Farm Rd., University of North Carolina, Chapel Hill, NC, 27599.

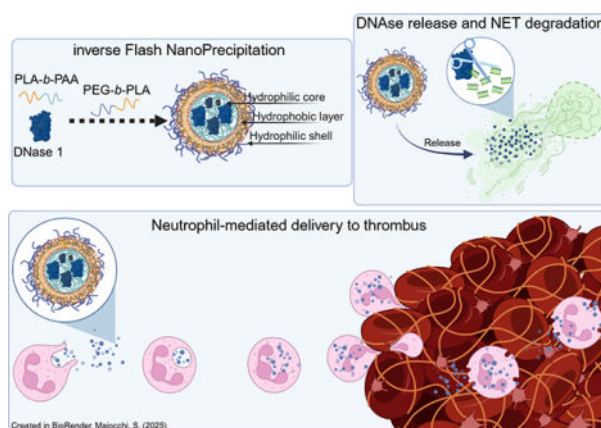
Author Contributions – CRediT Author statement

Sophie Maiocchi: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing-Original Draft, Visualization, Funding Acquisition. **Erica E. Burnham:** Methodology, validation, formal analysis, investigation, Data curation. **Veronica Lisi:** Investigation, Data Curation. **Nancy Buechler:** Investigation, Data Curation. **Rachel Pollard:** Investigation, Data Curation. **Ana Cartaya:** Visualization, data curation. **Danial Babaki:** Investigation. **Wolfgang Bergmeier:** Methodology, Supervision, Funding acquisition. **Nathalie M. Pinkerton:** Conceptualization, Methodology, Resources, Writing-review and editing, Supervision, Funding acquisition. **Edward Bahnson:** Conceptualization, Methodology, Formal Analysis, Resources, Writing-review and editing, Supervision, Project Administration, Funding acquisition.

Any additional relevant notes should be placed here.

Activated neutrophils release Neutrophil Extracellular Traps (NETs), comprising decondensed chromatin, peroxidases, and serine proteases, which aid in host defense but are also implicated in thrombosis and resistance to thrombolysis. Recombinant DNase 1, which degrades NETs, may aid in thrombus dissolution synergistically with fibrinolytics. However, its short half-life and susceptibility to plasma proteases limit its therapeutic applicability. To address these limitations, we encapsulated DNase1 into polymeric nanoparticles (DNPs) using inverse Flash Nanoprecipitation (iFNP), a scalable nanoparticle synthesis technique. Previously only used in model proteins, our study demonstrates for the first time the feasibility of extending iFNP to the encapsulation of therapeutic proteins. We detail conditions that promote DNase1 solubility, preserve activity, and demonstrate release resulting in *ex vivo* NET degradation. Furthermore, we investigated using neutrophils, the source of NETs, as carriers for DNPs to enhance targeted delivery. Our findings confirm that DNP-loaded neutrophils maintain key functionalities, including viability and oxidative burst, and associate with *in vitro* blood clots to deliver nanoparticles, and DNase1 protein. This study not only extends the feasibility of applying iFNP to encapsulate therapeutic proteins into polymeric nanoparticles, a promising alternative to lipid nanoparticles, but also contributes to the emerging literature on neutrophils as delivery vectors for nanocarriers..

Graphical Abstract



DNase I nanoparticles (DNPs) can be efficiently synthesized using inverse Flash NanoPrecipitation, with a loading efficiency of 95%. DNPs preserved DNase enzymatic activity. The DNPs release enzyme capable of degrading DNA in Neutrophil Extracellular Traps, a structural component of blood clots. Neutrophils loaded with DNPs *ex vivo* can deliver the cargo to pre-formed clots *in vitro*.

Keywords

Inverse Flash Nanoprecipitation; DNase 1; Protein delivery; Neutrophil nanomaterial interaction; Neutrophil Extracellular Traps; Cell Mediated Drug Delivery

INTRODUCTION

Neutrophil Extracellular Traps (NETs) are an extracellular network of decondensed chromatin that are critical in host defense against fungal infections and virulent bacteria

which disrupt alternative neutrophil-mediated antimicrobial strategies.¹ However, NETs also exhibit complex roles contributing to pathology in various disease contexts such as autoimmune disorders,² diabetes³ and cancer.⁴ In addition, recent experimental and clinical data have increasingly shown that NETs are present in large quantities in human venous thrombus samples,^{5, 6} contributing to pathological thrombus organization⁵ and fibrosis.⁷ NETs are reported to be pro-thrombotic as well as anti-fibrinolytic,^{8–12} rendering thrombi resistant to lysis.^{12–16} Deoxyribonuclease 1 (DNase 1); an endonuclease that cleaves DNA in the extracellular space, is a promising therapy to target the destruction of NETs in the context of venous thrombosis,¹⁷ however its clinical utility is limited by its short *in vivo* half-life.^{18–21}

Nano therapeutic delivery technologies offer strategies to extend the circulating lifetime of biologic therapies.²² We describe the encapsulation of DNase 1 in polymeric nanoparticles, utilizing a next generation nanoparticle synthesis technology: inverse Flash Nanoprecipitation (iFNP).^{23–28} Current complexity and low-throughput manufacturing methods are challenges for the clinical translation of lipid and polymeric nanoparticles and liposomes.²⁹ Moreover, most nanoparticle synthesis techniques cannot achieve reproducible nanoparticle characteristics across production scales, exhibit poor drug-loading efficiency, and poor homogeneity of nanoparticle production. iFNP uniquely addresses these challenges as it offers a scalable manufacturing technique that preserves nanoparticle characteristics across microliter to liter volumes of production.^{23, 26} In this process, nanoparticles are generated that contain biologic therapies in the hydrophilic hydrogel-like core of the particle, surrounded by a hydrophobic slow-release layer and an outer stabilizing, hydrophilic polymeric corona that can also be functionalized to exploit ligand-receptor interactions (Scheme 1A). The nanoparticles are assembled in a multi-step process that can be run in batch or continuous fashion (Scheme 1B). Overall, the technology involves the rapid micro-mixing of organic streams, containing the polymers and therapies, in a milli-fluidic micro-mixing device (either a multi-inlet vortex mixer (MIVM)^{27, 30} or confined impinging jet mixer (CIJ)^{31–33}. Turbulent mixing homogenizes the distribution of components prior to self-assembly, resulting in a robust generation of homogenous nanoparticles. The nanoparticle size and drug-loading can be easily tuned by adjusting the concentrations and ratios of polymer to therapeutic in the organic streams.^{34, 35} To scale-up production, the mixer size and flow rates can be increased.^{36, 37} Alternatively, to scale-out production, the mixers can be run in parallel, as is done in the production of the mRNA-containing lipid nanoparticle, Comirnaty, the Pfizer-BioNTech Covid-19 vaccine.³⁸ FNP is well established for the encapsulation of hydrophobic pharmacologic drugs,^{39, 40} whilst for the nano-encapsulation of weakly hydrophobic and ionizable drug molecules via FNP, the hydrophobic ion pairing solubility engineering approach can be used.^{41–44} However, for the encapsulation of highly water-soluble biologics, one must use the novel iFNP process. At the time of writing, iFNP has been employed to encapsulate horse radish peroxidase, the antibiotic peptide Vancomycin, RNA and other peptides.²⁶ Notably, cargo loading of these biologics was 5–15x higher than typical values obtained for liposomes and polymerosomes (9–27 wt% vs <2%).²⁶ To the best of our knowledge, iFNP has not been reported to encapsulate any therapeutic biologic for a biotherapeutic application. We present an iFNP protocol to generate nanoparticles encapsulating and retaining the activity

of DNase 1 (DNase-1 nanoparticles; DNPs). We characterize these particles for their size, polydispersity, encapsulation efficiency, loading capacity and release of active DNase 1. We show that these nanoparticles can release active DNase 1 to the extracellular space where it can degrade NETs *in vitro*.

Inadequate localization of DNase 1 at sites of NET production may also hinder its therapeutic efficacy. Neutrophils, which infiltrate thrombi and are the source of NETs, may serve as carriers of DNPs for cell-mediated delivery. Neutrophils are among the first cells recruited to a growing thrombus and play a central role in its progression.^{17, 45, 46} They are actively recruited and migrate into the milieu of thrombi via platelet-receptor interactions as well as signaling molecules.^{17, 47, 48} Moreover, the density of neutrophils inside a thrombus increases steadily with thrombus age.^{17, 45} Prior efforts to target therapies to thrombi have focused on (1) ligand-coated nanoparticles, (2) red blood cell and platelet membrane coating of nanoparticles and (3) responsive cargo release, for example activated by shear.⁴⁹ However, neutrophils, rapidly internalize NPs up to 200 nm in size through various endocytic pathways, including phagocytosis and clathrin-dependent and -independent endocytosis.^{50, 51} This interaction, influenced by NP properties, has implications for immune responses and therapeutic applications, as neutrophils may serve as carriers for nanodrugs to target tumors or inflammation.⁵² Our study explores the use of neutrophils as cell carriers of DNPs to an *in vitro* thrombus. We present data demonstrating the rapid internalization of DNPs by murine bone marrow derived neutrophils (BMDNs), without significant effects on their viability or oxidative burst. Moreover, we demonstrate that DNP-loaded neutrophils retain their ability to associate with *in vitro* blood clots and that neutrophils can effectively release nanoparticles and DNase 1 protein within these clots. Our studies support the next-generation nanoparticle synthesis technique, iFNP, coupled with cell-mediated delivery as an innovative tool to address poor therapeutic accumulation. This approach is promising for a new range of targeted therapeutics in the context of venous thrombosis.

RESULTS

1. Inverse Nanocarrier (iNC) Formation

a. Solvent Choice and Stream Composition: We sought to harness the next-generation nanoparticle synthesis technology, iFNP,^{23, 26} to encapsulate the protein DNase 1, with the goal of achieving (1) high encapsulation efficiency, (2) high loading capacity, and (3) employing a nano-formulation synthesis technique with proven scalability.³⁶ An initial requirement of iFNP is the dissolution of the protein in an organic solvent/aqueous mixture. We tested several organic solvent/aqueous mixtures for both solubility and maintenance of DNase 1 activity (Table 1). Calcium Chloride (CaCl₂) was included a priori in the aqueous portion of all mixtures tested, as not only does previous literature indicate that the inclusion of salts may enhance solubility of proteins in organic solvents,⁵³ but calcium binding could aid in maintaining DNase 1 structure.⁵⁴ Our results indicated that, although DNase 1 was readily soluble in mixtures of DMSO/water, enzyme activity was completely lost. Moreover, other pure solvents such as acetonitrile (ACN) and tetrahydrofuran (THF) precipitated the protein easily. When dissolved in ACN/water or THF/water mixtures, a

significant loss of enzyme activity was observed. Alternatively, we found that dissolution in 1,4-dioxane was feasible. Additionally, it was found that prior dissolution of DNase 1 in 0.1 M Tris-HCl promoted greater activity as seen in trends with THF and 1,4-dioxane in Table 1. Indeed, dissolution of DNase 1 in organic/aqueous mixtures of 1,4-dioxane with 0.1M Tris-HCl resulted in preservation of DNase 1 activity. As we were utilizing CaCl_2 to enhance dissolution and help maintain activity, we tested increasing the CaCl_2 content in the aqueous matrix and its effect upon solubility and DNase 1 activity in a 1,4-dioxane mixture (Table 2). We found that CaCl_2 concentrations of 20 mM and higher resulted in a complete loss of DNase 1 activity. Moreover, CaCl_2 concentrations above 40 mM resulted in phase separation of the aqueous phase from the organic phase. Thus, to form the iNC a solvent stream of 90% dioxane containing a 10% aqueous portion of 0.1 M Tris-HCl at pH 8, and 100 mM CaCl_2 , and 10 mg/mL DNase 1 protein (final concentration: 0.01 M Tris-HCl, 10 mM CaCl_2 , 1 mg/mL DNase 1) was chosen.

To form the iNC, hydrophilic cargo is initially encapsulated using an amphiphilic di-block co-polymer (BCP).^{23, 26} In the present study, we utilized Poly(D,L-lactide-*block*-acrylic acid (PLA-PAA, Sigma Aldrich, Cat #802190). It must be noted that we performed characterization (NMR and GPC) of the polymer and found that it did not correspond to the manufacturer's reported composition and molecular weight. Characterization results can be found in the Appendix, Supplementary Figures 1, 2. As the PAA block can be cross-linked by divalent cations such as Ca^{2+} , we separated the protein and polymer into two streams, a protein stream with CaCl_2 and a polymer stream without divalent cations. To accommodate the additional stream, we used a micro multi-inlet vortex mixer (μMIVM), which has four stream inlets. Hence, to initially encapsulate DNase 1 in an iNC, stabilized by a PLA corona, PLA-PAA (20 mg/mL) was dissolved in 90% 1,4-dioxane, containing 10% 0.1 M Tris-HCl buffer at pH 8 to create stream 1. Meanwhile, DNase 1 was also dissolved in 90% dioxane at a final concentration of 1 mg/mL in 10% aqueous volume of 0.1 M Tris-HCl buffer at pH 8 containing 100 mM CaCl_2 to create stream 2. These two streams were turbulently micro-mixed in the MIVM against the antisolvent, chloroform, in streams 3 and 4 (Scheme 1B, **Step 1**). The resulting dispersion was quenched in a chloroform bath. A typical intensity-weighted size distribution for this formulation is shown in Figure 1A. Additionally, the iNC correlogram is shown (Figure 1B) alongside a control without the BCP stabilizer, which had afforded visible precipitation of DNase 1, and no particle formation. This highlights that the particles are not simply DNase 1 aggregates but are core-shell nanoparticles encapsulating DNase-1 and stabilized by BCP.

b. Cross-linking of iNC Core: To crosslink the iNC core and stabilize the iNC for the further processing steps, we employed ionic complexation with the polyamine, spermine (Scheme 1B, **Step 2**). Spermine could be introduced either in the chloroform antisolvent stream or in the quench bath. We found that iNC size and distribution was best preserved by including 10 mM spermine in the chloroform quench bath. We hypothesize that when the spermine was included in the anti-solvent stream, rapid crosslinking interfered with the iNC assembly. To assess the strength of the cross-linking, we employed a previously reported dynamic light scattering method, relying on comparing the correlation function of the iNC with and without spermine added.²⁶ As shown in the correlation function in Figure 2A,

when the iNCs were diluted 10-fold into 1,4-dioxane, those that were not stabilized by ionic complexation with spermine did not persist. On the other hand, the spermine crosslinked iNCs were stable upon dilution. Additionally, we could disperse these stabilized iNCs in either DMSO or THF (data not shown) without significant swelling or adverse change to the iNC correlation function. Thus, the inclusion of spermine allowed us to successfully maintain nanoparticle size and polydispersity from the initial generation of the iNC, and throughout extraction and solvent exchange with 1,4-dioxane, as shown in Figure 2B. We opted to continue to disperse the nanocarriers in 1,4-dioxane to minimize potential contact of DNase 1 with adverse enzyme-inactivating solvents such as THF or DMSO.

c. pH Control of iNC Core: As DNase 1 enzyme activity is pH sensitive, we sought to mimic the core conditions to approximate the pH of the iNC core and quantify the impact of this pH on DNase 1 activity (Table 3). We dissolved an equimolar amount of PAA homopolymer as that calculated to be in our iNC core in water, and included 10 mM spermine to this solution, which resulted in an initial pH of ~3.5, and a loss of DNase 1 activity of ~60%. Raising the pH with increasing concentrations of triethylamine resulted in concomitant increases in DNase 1 activity. Based on these core condition approximation studies, we chose to include a final concentration of 0.5 M triethylamine in the quench bath, with the goal of raising the pH of the iNC core to favor conditions that preserve DNase 1 activity.

2. Water-dispersible (or PEGylated) nanocarrier formation

To form water-dispersible nanoparticles, the iNC must be coated with a PEGylated amphiphilic block co-polymer. To achieve this, we employed the well-established technique of Flash Nanoprecipitation (FNP),²³ which requires the dispersion of the nanocarrier in a water miscible organic solvent (such as 1,4-dioxane). As the formation of the initial iNC results in nanoparticles dispersed in chloroform along with excess spermine and triethylamine, we first performed an aqueous extraction with 150 mM sodium chloride to remove the residual 1,4-dioxane, spermine and triethylamine, and any unincorporated DNase 1. Secondly, we performed a solvent exchange with a rotary-evaporator to disperse the nanoparticles in 1,4-dioxane (Scheme 1B, **Step 3 & 4**). Representative intensity distributions of the iNC prior to extraction, following extraction, and the solvent exchange steps are shown in Figure 2B. Minor changes in the size distribution are observed, which may be due to the changes in the solvent quality for the polymer corona. The monodispersed particle size distribution is maintained.

To coat the iNC with PEG, the iNC was dispersed in 1,4-dioxane alongside dissolved methoxy-PEG-PLA (20 mg). This polymer/nanocarrier mixture was rapidly mixed with an antisolvent water, in a CIJ to generate PEG-coated DNase 1 nanoparticles (DNPs). A representative, monomodal trace of the final PEGylated nanoparticles is shown in Figure 2C. We show that the nanoparticles detected by DLS is not afforded by simple mixing of the free PEG-PLA micelles and iNCs as shown in Figure 2C. When mixed, the free micelle population (~20 nm) is distinguishable from the DNP particles on DLS. To make fluorescent particles, we added ~1 weight% of rubrene relative to the PEG BCP (0.2 mg), which did not significantly alter size or polydispersity of the PEG-coated DNase 1 nanoparticles, as

shown in Figure 2D. Stability of the particles was measured using the correlation function via DLS over 48 hrs of incubation with the nanoparticles in 10 mM Tris-HCl buffer (Figure 2E). A summary of the sizes and polydispersity indices and physiochemical characterization of DNPs at each processing step of iFNP is shown in Table 4 and Table 5, respectively. A release assay was then performed employing Tris-HCl buffer at 37°C (Figure 2F). This assay demonstrated an initial release of ~10% of the DNase 1 from the DNPs within the first 10 h, followed by a sustained release of DNase 1 over 8 days resulting in a release of up to 80% of the content of the DNPs. Finally, to confirm particle morphology, transmission electron microscopy (TEM) images demonstrated production of spherical nanoparticles (Figure 3A).

3. Encapsulation Efficiency and Loading Capacity

The aqueous extraction step in the processing of the iNC allows for the extraction of unencapsulated DNase 1 to be solubilized in the aqueous phase. Thus, we can measure DNase 1 content in the aqueous phase to quantify encapsulation efficiency.²⁴ We performed control experiments where DNase 1 was mixed in the MIVM in the absence of stabilizing BCP, such that no nanoparticles were formed. Thus, we confirmed that DNase 1 could be quantitatively extracted into the aqueous phase. Overall, we employed two protein quantification methods (micro-BCA and protein absorbance at 280nm) to quantify that the encapsulation efficiency was $94 \pm 1\%$, and $96 \pm 5\%$, respectively. These results are summarized in Table 6. Combining the micro-BCA results with measurements by thermogravimetric analysis, we were able to measure the loading capacity of the iNC. This was determined to be $3.7 \pm 0.3\%$. Thermogravimetric analysis also allowed us to confirm that there was no detectable loss of nanoparticle mass during the extraction step, as masses stayed the same before and post extraction (data not shown). This result, combined with the stability in the size and polydispersity of the particle through the processing steps, support that DNase 1 that is encapsulated at the iNC stage was carried through to the PEG-coated nanoparticle.

To quantify DNase 1 protein content in water-dispersible PEGylated DNPs we performed denaturing gel electrophoresis, and subsequently stained the gel with the highly sensitive protein stain, Silver Stain (Figure 3B). We found that the Silver Stain process allows for the quantitation of DNase 1 down to ~20 ng. Employing a combination of Silver Staining and thermogravimetric analysis of the total mass content of the DNP we determined that the final loading capacity was $1.09 \pm 0.29\%$, with an average total amount of DNase 1 yield in a formulation being $169 \pm 69 \mu\text{g}$ of DNase 1. To demonstrate that DNase 1 protein is present in the final preparation of aqueous stabilized DNase 1 nanoparticles, we employed a zymography assay to measure DNase activity, with a denatured DNA-containing SDS-gel. In this assay, DNA degradation will appear as a dark band and can only be achieved in the presence of an active DNA degrading enzyme. Briefly, the nanoparticles were degraded by incubating them with 0.1M NaOH, and 5% SDS over 48 hours, and then loaded into the denatured DNA-containing SDS-gel. Following protein refolding, we showed that DNase 1 activity could be demonstrated in aqueous stabilized DNase 1 nanoparticle samples (Figure 3C).

4. DNase 1 nanoparticles degrade neutrophil extracellular traps *in vitro*

As a release assay demonstrated that DNase 1 is released by DNP (Figure 2F), we then demonstrated that the released DNase 1 is capable of degrading NETs *in vitro*. We generated NETs *ex vivo* from bone-marrow derived murine neutrophils, stimulated with PMA (Figure 4A, **left panel**). The NETs were then incubated with DNPs (1 U/mL of DNase 1), and NET degradation was quantified over 6 hours (Figure 4) compared to time-matched buffer only conditions. SYTOX green nucleic acid stain was used to visualize NETs and measure their degradation (Figure 4A). Impressively, we found that DNPs could achieve NET degradation within 1 hr of incubation (Figure 4B). Collectively, this provides compelling evidence demonstrating that DNase 1 is not only released from the DNPs, but that the protein has preserved functionality and can perform a therapeutic function. This finding underscores the dual achievement that DNase 1 is successfully released and retains enzymatic activity, demonstrating a potent and timely therapeutic response, holding significant implications for the therapeutic potential of this delivery system.

5. Murine Neutrophils Rapidly Internalize DNase1-Nanoparticles and Can Associate With *in vitro* Blood Clots

We next explored the potential of neutrophils as carriers for depositing DNP cargo and NPs in *in vitro* thrombi. Here we used both fluorescent DNP to confirm internalization by neutrophils, and rubrene/quantum-dot containing nanoparticles (QD-NP, and Ru-QD-NPs) with equivalent size and surface chemistry to DNPs for NP visualization by immunofluorescence. The synthesis and characterization of these quantum-dot containing NP are detailed in the supplementary (Supplementary Table 2, Supplementary Figures 3 & 4). Representative characterization of murine bone marrow derived neutrophils employed for these studies is shown in Supplementary Figure 6. Our findings revealed that neutrophils effectively internalize fluorescent DNPs, with greater than 80% of neutrophils being positive for fluorescent DNPs at 1.5 h post-administration (Figure 5A). Notably, the viability of neutrophils remained unaffected, indicating the non-toxic nature of the DNPs at the tested concentration and exposure time (Figure 5A). To confirm the observed signal as internalization, we compared the percentage of cells positive for fluorescent DNP at low temperatures, where active processes like internalization are hindered (Figure 5A). Confocal microscopy further validated internalization, as the DNP signal localized within the neutrophils, which bounds were delineated by the cell membrane marker CD45 (Figure 5B, 5C). This can also be visualized in Supplementary Movie 1. Similarly, Ru-QD-NP are efficiently and rapidly internalized by both murine and human neutrophils (Supplementary 7 & 8, respectively). We next showed that internalization of DNPs did not induce activation of the respiratory burst, indicated by a lack of reactive oxygen species production, measured with the de-hydro ethidium fluorescence assay (Figure 5D). Moreover, the internalization of DNPs by neutrophils did not hinder their ability to perform the oxidative burst following stimulation with either PMA or ionomycin and PMA (Figure 5D). Finally, CD11b expression in murine neutrophils remained unchanged over 90 minutes of incubation with Ru-QD-NPs (Supplementary Figure 7). Together, these data indicate that internalization of DNPs, and parameter-matched Ru-QD-NP, does not perturb important neutrophil features: viability and activation.

Next, we assessed whether DNP internalization would affect neutrophils' capacity to bind and penetrate *in vitro* thrombi (Figure 6A). BMDNs, labeled with either Cell Tracker Deep Red or Green CMFDA dyes to distinguish DNP-loaded from empty cells, respectively, exhibited equivalent clot association, as quantified by flow cytometry of single cell suspensions of clot (Figure 6B) and observed with immunofluorescence (Figure 6C). To more easily visualize thrombus margins and tissue morphology, directly adjacent tissue sections of the representative thrombi in Figure 6C were stained with H&E (Supplementary Figure 9). The gating strategy and representative controls for flow cytometry can be found in the Appendix (Supplementary Figure 10). To validate whether the Cell Tracker dye itself influenced the number of neutrophils detected by flow cytometry in the *in vitro* clot, we performed the experiment two ways by crossing over the BMDN dyes and DNP treatment, thus: (1) Green CMFDA/DNP-loaded, Deep Red/empty; and (2) Deep Red/DNP-loaded and Green CMFDA/empty. In Supplementary Figure 11, we observe that Cell Tracker Deep Red labelling alone can result in a significant overrepresentation of the number of neutrophils detected via flow cytometry, whereas DNP internalization has no significant influence on the number of neutrophils in the *in vitro* clot. Next, these *in vitro* thrombi were stained with an anti-bovine DNase 1 antibody and imaged with both wide-field and confocal fluorescence microscopy (Figure 6D & E). These representative images demonstrated positive signal adjacent to the DNP-loaded neutrophils, indicating delivery of DNase 1 cargo to *in vitro* thrombi and exocytosis of DNP from the DNP-loaded neutrophil, as the signal was outside the cellular membrane boundaries (Figure 6D & E, Supplementary Figure 12).

To visualize nanoparticle delivery to *in vitro* clots, quantum dot-containing nanoparticles (QD-NPs) with equivalent size and surface chemistry to DNPs were employed (see Supplementary Table 2). Human peripheral neutrophils (characterized in Supplementary Figure 13) internalized QD-NPs within one hour (Supplementary Fig. 8). *In vitro* human clots incubated with QD-NP-loaded neutrophils demonstrated significant QD delivery compared to clots without QD-NP-loaded neutrophils (Figure 7A, C, D), confirming neutrophil-mediated nanoparticle transport to *in vitro* thrombi. We also corroborated that QD-NP internalization did not impact neutrophils' association with *in vitro* thrombi compared to empty neutrophils (Figure 7B). Further representative fluorescent images of thrombus sections and directly adjacent thrombus sections stained with H&E, to more easily visualize tissue morphology and thrombus margins, are shown in Supplementary Figure 14. Thus, our results demonstrate that neutrophils loaded with DNPs retain their clot-binding capabilities and neutrophils can serve as efficient vehicles for nanoparticle delivery into *in vitro* thrombi.

DISCUSSION

Since the discovery of NETs, these structures have been implicated in the pathogenesis of multiple inflammatory diseases, preparation of the metastatic niche in several cancers, and more recently in promoting thrombosis in both arterial and venous contexts.¹⁻³ DNase 1 degrades extracellular DNA, and as such represents a desirable therapeutic protein to mitigate the deleterious impact of NETs. Clinically, DNase 1 is currently the only approved mucolytic agent to treat cystic fibrosis⁵⁵, and experimentally has been employed to reduce NET burden in models of venous thrombosis¹⁷. However, challenges for its clinical utility

include both its short *in vivo* half-life^{18–21} and inadequate co-localization at sites where NETs are produced. We sought to utilize iFNP for the first time for the encapsulation of DNase 1, and to explore the feasibility of neutrophils as delivery vehicles for these DNPs to thrombi. The results of this study show that we successfully optimized conditions that allowed for the preservation of DNase 1 activity and its encapsulation with ~95% efficiency, resulting in a final loading capacity of ~1% in polymeric nanoparticles utilizing bio-compatible polymers. These nanoparticles released DNase 1 *in vitro* over 8 days and the released DNase 1 was capable of degrading NETs *in vitro*. We then performed experiments to explore the hypothesis that neutrophils may deliver DNPs to thrombi *in vitro*. We showed that murine neutrophils internalized DNPs without significant effects upon their viability, activation status or ability to perform the oxidative burst. Our results then showed that these DNP-loaded neutrophils were capable of associating with *in vitro* formed thrombi to the same degree as un-loaded neutrophils, quantified by flow cytometry and confirmed by fluorescence imaging.

In line with the increasing evidence supporting the varied pathophysiological role of NETs in human disease, in the last decade there has been increasing interest in nano-formulations and modification of recombinant DNase 1, mainly with the goal of enhancing its serum half-life (mice: 105 min⁵⁶, humans: 4–5 h)^{18–21}. Various approaches have been explored including PEGylation of the recombinant protein^{57, 58} and incorporation of DNase 1 onto the surface of nanoparticles^{59–62}. One drawback of DNase1-coated nanoparticles is potential inhibition of DNase 1 activity in circulation through interaction with extracellular circulating actin that can be present in inflammatory contexts^{63, 64}. Additionally, DNase-1 coated polymeric nanoparticle formulations demonstrated a loss of DNase 1 activity beginning at 24 h of incubation in phosphate buffered saline¹⁸. On the other hand, encapsulation of DNase 1 has also been explored with liposomal formulations^{65, 66} and polymeric nanoparticles⁶⁷. With a liposomal formulation of DNase 1, the authors reported an encapsulation efficiency of 7–9% for recombinant DNase 1, with this improving to 50% for hydrophobically modified DNase⁶⁵. Whereas, for polymeric nanoparticles, employing the solvent evaporation method, Osman et al. optimized a formulation to achieve 87% encapsulation, with 2% loading capacity, although the conditions resulted in a 33% loss in DNase 1 activity⁶⁷. In reports where DNase 1 has been incorporated in the surface of nanoparticles, most publications do not report on the loading capacity, however Park *et al* reported an 83% binding efficiency⁶⁸. In line with the predicted advantages of iFNP, we demonstrated a ~95% encapsulation efficiency, resulting in a final loading capacity of ~1%, and our modeled iNC core conditions indicated that we preserve DNase 1 activity at native protein activity levels. Overall, this indicates that our scalable formulation can encapsulate DNase 1 more efficiently than other currently available approaches and maintains DNase 1 activity.

To encapsulate proteins with iFNP, they must be dissolved in organic solvent (typically ≥90%) and will be in contact with a highly acidic aqueous core environment²⁶, both of which threaten protein activity. We explored solvents and conditions that not only result in the efficient encapsulation of DNase 1, but also demonstrate DNase 1 protein activity both under modeled nanoparticle core conditions (Table 2) and following its release from the nanoparticles (Figure 4). Initially we examined solubility of the enzyme in organic solvents

that are compatible with the initial step of iFNP (formation of the iNC) (Table 1). Our findings were that DMSO provided high levels of solubility but led to complete enzyme inactivation, indicative of protein denaturation. THF and 1,4-dioxane both sustained some DNase 1 activity, which in the case of 1,4-dioxane, when combined with Tris-HCl buffer in the aqueous phase resulted in near complete preservation of DNase 1 activity. These results are consistent with literature reports where highly polar solvents, such as DMSO and DMF, cause protein denaturation through several mechanisms^{53, 69–72}. On the other hand, increasing solvent hydrophobicity is associated with protein rigidity and increased protein activity^{53, 72, 73}. With regards to enhancing protein activity in organic solvents, it has become evident that the aqueous environment in which the enzyme is first dissolved can play a dramatic role in subsequent enzymatic activity in organic solvent^{54, 72, 74, 75}. For this reason, in our studies, we added CaCl₂ a priori to aid in preserving DNase 1 protein activity as it is critical to DNase 1 structure⁷⁶. We then showed that indeed the addition of CaCl₂ was important for enzyme activity in organic solvent (Table 2). We additionally found that the initial dissolution of the enzyme in 0.1M Tris-HCl buffer provided substantial increases in enzyme activity measurements in 1,4-dioxane (Table 1), in line with previous literature that indicated that enzyme activity may be improved by first dissolving the enzyme at its optimal pH⁷⁷. Finally, we mimicked the core conditions by using the predicted concentration of poly(acrylic acid) and found that the addition of 0.5M triethylamine raised the pH sufficiently to approach the optimal pH of 6.5 to 8⁷⁸, resulting in preservation of DNase 1 activity. Overall, our results were consistent with previous literature describing appropriate methods to enhance the activity of proteins in organic solvents and demonstrates preservation of a therapeutic protein activity for the first time in the iFNP process.

As neutrophils marginate and infiltrate into sites of inflammation and cancer tumors,¹ they are of increasing interest as a target for both therapeutic modulation of their activity, and also as delivery vehicles for nanocarriers,² or as endogenous targets.³ Here, we performed proof-of-concept studies examining whether internalization of DNPs by murine bone marrow derived neutrophils affected their viability, ROS production and oxidative burst activity, and ability to associate with pre-formed *in vitro* thrombi. In the latter case, this was also extended to human neutrophils and thrombi. Our data demonstrated that nanoparticles enter cells within 30 min (Figure 5A, B) and do not affect viability nor induce ROS production, and neutrophils maintain their capacity to perform the oxidative burst in response to stimuli (Figure 5A, C). We additionally examined internalization, viability and activation status (CD11b expression) by murine neutrophils incubated with parameter-matched Ru-QD-NPs over 90 minutes and found no change in viability or Cd11b expression (Supplementary Figure 7). A more extensive characterization of neutrophil activation and apoptosis is warranted in future work, such as more thoroughly characterizing CD11b expression and CD62L shedding, as well as performing each assay in human neutrophils. However our current results are in line with prior studies by Bisso *et al.* who demonstrated rapid internalization of a range of nanoparticles (polymeric, gold, and liposomes, 50–200 nm) by human neutrophils which did not affect viability, activation status and apoptosis as indexed by CD16 and CD62L expression². In contrast to Bisso and colleagues,² work by the Eniola-Adefeso group^{4, 5}, has shown that internalization of nano-micro particles (0.5 and 2µm polystyrene particles) negatively affects neutrophil capacity for adhesion to the

activated endothelium both *in vitro*⁵ and *in vivo*⁴, possibly through induction of apoptosis and neutrophil activation, as incubation with 2µm particles led to an increase in CD11b expression⁵, and a corresponding reduction in CD62L. On the other hand, previous reports have found that infusion of 200 nm sized particles, even at relatively high concentrations, had minimal effects upon neutrophil adhesion *in vitro*^{5, 6}. Notably, the DNPs and RuQDNPs in the present study are substantially smaller (<120nm) than those investigated by Fromen et al.⁴, and Kelley et al.⁵, and have a highly PEGylated, sterically stabilized surface rather than a charged stabilized polystyrene surface.

To preliminarily assess the impact of DNP internalization on the association of neutrophils with a pre-formed thrombus and neutrophil-mediated delivery of NP to *in vitro* thrombi, we used an *in vitro* thrombus generated through clot contraction of whole murine or human blood, adapted from Kattula *et al.*⁷ We quantified neutrophil-thrombus association and NP delivery via flow cytometry of a single cell suspension of thrombi (Figure 6) and via quantification of serial thrombus sections (Figure 7), respectively, including NP exocytosis into the surrounding clot. With this assay, we demonstrated that prior incubation of exogenous neutrophils with DNPs for 2 h, did not alter the degree to which neutrophils associated with pre-formed thrombi *in vitro* (Figure 6). We additionally showed that neutrophils could deliver DNase 1 protein cargo to *in vitro* murine thrombi (Figure 6C,D) and QD-NP to *in vitro* human thrombi (Figure 7). However, our current studies with neutrophil-mediated delivery to *in vitro* thrombi are proof-of-concept. Future work will include firstly, establishing mechanisms of DNP exocytosis by neutrophils and quantifying efficacy of neutrophil-DNP-mediated degradation of NETs *in vitro* and in NET-containing *in vitro* thrombi. Secondly, we will probe mechanisms of neutrophil-mediated delivery of DNP to venous thrombi *in vivo*. Overall, we envision that DNase 1 therapy will be delivered in a co-therapeutic manner with the thrombolytic enzyme, tissue plasminogen activator (tPA), to enhance thrombus dissolution *in vivo*. Several independent studies show that the presence of NETs, or alternatively DNA and histones together, delay clot lysis *in vitro*.⁸⁻¹³ Accordingly, the combination of DNase 1 and thrombolytic enzymes, synergistically promote clot dissolution *ex vivo* in thrombi from stroke,^{14, 15} coronary,¹⁶ and chronic thromboembolic pulmonary hypertension patients.¹¹ Thus a co-therapeutic approach *in vivo* is expected to promote clot dissolution.

CONCLUSIONS

In conclusion, the presented study extends the feasibility of iFNP (a promising translatable manufacturing method) by applying it for the first time to encapsulate a therapeutic protein, DNase 1. We contribute to the emerging literature on the use of neutrophils as delivery vehicles for nanocarriers. Our presented methodology involves a multi-step process, encompassing the formation of Inverse Nanocarriers (iNCs) and subsequent coating with PEGylated amphiphilic block co-polymers to produce water-dispersible DNase 1 nanoparticles (DNPs). We demonstrate that DNase 1 is effectively encapsulated with an efficiency of 94±1%, and 96±5%, as measured by either micro-BCA or protein absorbance at 280nm, respectively. Additionally, we found that for the iNC the loading capacity was 3.7 ± 0.3%, with the loading capacity of the final PEGylated DNP being 1.1 ± 0.3%. We confirmed the presence of DNase 1 in aqueous stabilized DNase-1 nanoparticles

both by SDS-gel electrophoresis with Silver Stain protein detection and gel zymography measurements. The subsequent release assay demonstrates sustained release of DNase 1 from DNPs over 8 days. Notably, the released DNase 1 demonstrated enzymatic activity, as evidenced by its ability to degrade NETs generated *ex vivo* by murine neutrophils.

Furthermore, our study explores the interaction of DNPs and parameter-matched RuQDNPs with murine and human neutrophils. We demonstrate that neutrophils internalize DNPs and RuQDNPs without compromising their viability, activation status (as indexed by CD11b), or functional attributes, such as the ability to produce reactive oxygen species. Our investigation extends to proof-of-concept studies regarding the association of DNP- and Ru-QDNP-loaded neutrophils with *in vitro* thrombi, demonstrating the preserved ability of these NP-loaded cells to associate with thrombi comparably to naïve neutrophils. We additionally demonstrated neutrophil-mediated delivery of DNase 1 cargo and QD-NPs to *in vitro* mouse and human thrombi, respectively, including NP exocytosis. This aspect suggests that neutrophils may be employed as delivery vehicles for nanocarriers to thrombi, thereby directing DNPs to sites of NET formation and enhancing a localized therapeutic effect. Further *in vivo* studies are warranted to determine the ultimate feasibility of this approach.

MATERIALS AND METHODS

General Materials

Poly(D,L-lactide-*block*-acrylic acid) (sigma Aldrich, 802190–1G), Methoxy poly(ethylene glycol)-*b*-poly(D,L-lactide) (5kDa-5kDa, Sigma Aldrich, 900658–500MG), Poly(ethylene oxide)-*block*-poly(D,L lactide) (5kDa-5kDa, Evonik Industries), Poly acrylic acid homopolymer (Sigma Aldrich, 9003-01-04), Poly(L-lactide)-PEG-Maleimide (PLLA-PEG-Mal, 5k-5k, Nanosoft Polymers), Rubrene (Sigma Aldrich, 554073–100MG), Quantum dots (580nm emission, stabilized with oleic acid, suspended in toluene were obtained from NNCrystal US), Triethylamine (Thermo Fisher, O4884100), DNase 1 (Sigma Aldrich, 10104159001), spermine (Sigma Aldrich, AAL11956203), Chloroform (CHCl₃, Thermo Fisher, AC423550025), 1,4-dioxane (Fisher Scientific, AC408820025), DMSO (Fisher Scientific, D128–1), Methanol (HPLC Grade, VWR, EM-MX0475–1), THF (Fisher Scientific, T425–4), Glacial Acetic Acid (Fisher Scientific, A38S-500), Molecular biology grade nuclease free water (VWR, 97062–794), RPMI 1640 media (11875135, Gibco), Heat-inactivated fetal bovine serum (FBS) (16140071; Gibco), Penicillin-Streptomycin 10,000U/mL (15140122, Gibco), Phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich P8139–5MG), Paraformaldehyde (158127; Sigma-Aldrich), 10X PBS (20–134; Apex Bioresearch Products, San Diego, CA), Calcium Chloride dihydrate (Fisher Scientific, C79–500), Magnesium Chloride Hexahydrate (Fisher Scientific, M33–500), Tris-HCl (Sigma Aldrich, T3253–1KG), Citric Acid (Sigma Aldrich, 251275–500G), Bovine Serum Albumin (Sigma Aldrich, A9418–5G), HBSS, no calcium, no magnesium, no phenol red (Thermo Fisher, Gibco, 14175095), Deoxyribonucleic acid sodium salt from salmon testes (Sigma-Aldrich, D1626–5G), 40% Acrylamide (Sigma Aldrich, A2792), TEMED (Sigma Aldrich, 50-197-8483), Ammonium persulfate (Sigma Aldrich, A3678).

Animal studies

All animal handling and experimental procedures were approved by both the Institutional Animal Care and Use Committee (IACUC) at the University of North Carolina – Chapel Hill (IACUC ID: 20–148 and 23–116) and the IACUC at Wake Forest University Health Sciences (IACUC ID: A23–148). C57Bl/6 mice (strain number:000664, age: 8–12 wk old, male) were purchased from Jackson Laboratory and were fed standard chow. Mice were allowed ad libitum access to food and water throughout the study.

Human studies

This study was approved by the Institutional Review Board (IRB) of Wake Forest University Health Sciences (IRB protocol number: IRB00104255). All procedures were conducted in accordance with the 1964 Declaration of Helsinki and its later amendments. All participants provided written informed consent prior to participation. Inclusion criteria were healthy adults over the age of 18 with a weight > 110lb or 50 kg. Exclusion criteria included a history of cardiovascular disease, cancer, liver cirrhosis, diabetes, current pregnancy, chronic inflammatory conditions, or the use of anti-inflammatory or anticoagulant medications and smoking.

Venipuncture Procedure

Venipuncture was performed by a certified nurse in the Teammate Clinic at the Biotech Place (Wake Forest University Health Sciences) using a sterile technique under standardized conditions. For each participant, approximately 10 mL of venous blood was collected from the antecubital vein using a 21-gauge butterfly needle (BD Vacutainer, Becton Dickinson, USA) and a standard vacuum collection system. Blood was drawn into an EDTA-coated tube (BD Vacutainer K2 EDTA, USA). Tubes were inverted gently 8–10 times immediately after collection to ensure proper mixing of anticoagulant with the blood.

Generation of DNase 1 nanoparticles (DNPs)

DNase1 nanoparticles (DNPs) were synthesized via inverse flash nanoprecipitation (iFNP) with methods adapted from previous literature^{23, 26}.

Step 1. Inverse nanocarrier (iNC): Briefly, a micro multi inlet vortex mixer (MIVM) was fitted with a total of 4 1 mL syringes: 2 syringes containing 500 μ L of Chloroform (CHCl_3 , Thermo Fisher, AC423550025), 1 syringe containing 500 μ L of 20mg/mL of Poly(D,L-lactide-*block*-acrylic acid) (Sigma Aldrich, 802190–1G) dissolved in 90% 1,4-dioxane (Fisher Scientific, AC408820025) and 10% 0.1M Tris-HCl pH 7.8 buffer, and finally 1 syringe containing 50 μ L of DNase 1 (10 mg/mL in 0.1 M Tris-HCl, pH 7.8, containing 10mM CaCl_2), and 450 μ L of 1,4-Dioxane. The syringes were depressed rapidly into a quench bath (8 mL volume containing 2mg/mL spermine, 635 μ L Triethylamine and CHCl_3) and stirred rapidly for 5 minutes. The nanoparticle solution in CHCl_3 was then extracted into 4mL of 150 mM NaCl, pH 6 for 30 minutes at a slow rocking speed. The aqueous layer was removed, and the organic layer was centrifuged at 1000xg for 10 minutes at 4°C. Emulsions of the nanoparticle with aqueous layer that were difficult to remove were left aside. The organic layer was transferred to a 50 mL glass round bottom flask and 20

mg of Methoxy poly(ethylene glycol)-*b*-poly(D,L-lactide) (5k-5k, Sigma Aldrich, 900658–500MG) was added. The solution was then solvent-swapped into 1,4-dioxane by adding 10mL of 1,4-dioxane and removing the solvent by rotary-evaporation till 1mL remained. This was repeated three times.

Step 2. Aqueous stabilized nanocarrier: 1mL of the iNC in 1,4-dioxane and containing 20 mg of methoxy-PEG-PLA polymer was drawn into a 1mL syringe and fitted to a confined impinging jet mixer (CIJ). 1 mL of water was fitted to the other inlet. The syringes were depressed rapidly into 8 mL of water and stirred rapidly for 5 minutes. The nanoparticle suspension was then dialyzed overnight against DI water (Biotech CE Dialysis Tubing 300 KD 16 mm, VWR, 89068–798). To generate fluorescent nanoparticles, 2mg of rubrene (sigma Aldrich 554073–100MG) was added to the organic solvent just prior to injecting the solvent streams through the CIJ.

DNase 1 Activity Assay

DNase 1 activity in solution was measured with the commercially available fluorometric plate-reader assay from Abcam (ab23406). Briefly, solutions containing 5mg/mL of DNase 1 were diluted to 0.1–0.5 µg/mL in molecular biology grade water to be used as samples. In the case where organic solvents were used for the original suspension, a control was run containing a similar percentage of final organic solvent with control DNase 1 to ensure that this did not adversely affect the plate reader assay. Preparation of the samples and a standard curve were performed as per the protocol instructions in the product insert. Fluorescence was measured (Ex/Em = 651/681 nm) in kinetic mode, every 30 seconds, for 90 minutes at 37°C. DNase 1 activity from DNase 1 dissolved in organic solvent mixtures, or poly acrylic acid / spermine / triethylamine mixtures, was recorded as a percentage of the activity for DNase 1 from the same lot, dissolved in water.

Measurements with Dynamic Light Scattering

Measurements for nanoparticle size with intensity distribution and correlation function were performed with Dynamic Light Scattering (DLS) and were carried out with a Zetasizer NanoZS (Malvern Panalytical, Ltd, UK). Size measurements were conducted at a 173-degree scattering angle, with the appropriate settings for the solvent. Measurements were conducted at least 3 times with 30 s to 240 s of equilibration time. For measurements in organic solvents, we employed the Malvern Panalytical Sq Cell W Cap 12 mm O.D. glass cuvette (Fisher scientific, NC0631452), whilst measurements in aqueous solvents were recorded in Malvern Panalytical Inc 40µL Cuvettes (Fisher Scientific, NC0628994). Samples were typically diluted 10-fold for measurements into appropriate solvents (Chloroform, 1,4-dioxane or 0.02 µm filtered water or 1X PBS).

Transmission Electron Microscopy (TEM) measurements

Conventional TEM images were captured on a JEOL JEM 1230 TEM at 80 kV, tungsten filament, with a Gatan Orius SC1000 CCD camera and Gatan Microscopy Suite 3.0 software. Rubrene-DNase1-NPs in water were prepared for TEM by pipetting 8 µL atop Formvar on carbon mesh TEM grids that were treated with glow discharge (PELCO easiGlow™ Glow Discharge Cleaning System). After two minutes, samples were rinsed

with deionized water and stained with 2% uranyl acetate negative stain for two minutes prior to imaging.

Encapsulation Efficiency

Encapsulation Efficiency was broadly determined by measuring the amount of DNase 1 protein recovered into the aqueous extract phase following formation of the iNC. INCs containing DNase 1 were synthesized as described, but in the absence of spermine and triethylamine, and then extracted with 0.15M NaCl solution. Controls included: iNCs without DNase 1 added, DNase 1 alone with no stabilizing polymer added, aqueous extract buffer alone. The Encapsulation Efficiency was calculated using the following equation:

$$Encapsulation\ Efficiency = 100 - \left(100 \times \frac{DNase\ 1_{inverse}}{DNase\ 1_{max}} \right)$$

DNase 1_{inverse} = the amount of DNase 1 recovered into the aqueous extract phase following formation of DNase 1 containing iNCs

DNase 1_{max} = the amount of DNase 1 recovered into the aqueous extract phase following DNase 1 passing through the MIVM with no stabilizing BCP added (no iNC formed).

Micro BCA assay

The aqueous extraction phase was measured for protein using the commercially available micro BCA assay (Thermo Fisher, 23235). Instructions as per the user's manual were followed and sample absorbances were measured at 562 nm. Measurements were made in triplicate with three independent batches of nanoparticles synthesized.

Protein absorbance at 280 nm

A second, orthogonal, measure was utilized to confirm Encapsulation Efficiency. We measured protein absorbance at 280 nm using the Duetta Fluorescence and Absorbance Spectrometer (Horiba Scientific). Measurements were made in triplicate with three independent batches of nanoparticles synthesized.

Loading Capacity and loss to extraction of the INC

To determine the Loading Capacity and loss to extraction of the iNC we used a Thermogravimetric Analyzer (TGA, TA instruments). INCs containing DNase 1 were synthesized as described, but in the absence of spermine and triethylamine, and then extracted with 0.15M NaCl solution. 90 µL of the iNC solution in chloroform was added to the TGA pan prior to and following the extraction step, and the dry mass recorded. Loading capacity was determined by employing the following equation:

$$Loading\ Capacity\ (inverse\ nanocarrier) = \left(\frac{DNase1_{max} \times EE}{Nanoparticle\ Mass} \right) \times 100$$

DNase 1_{max} = the amount of DNase 1 recovered into the aqueous extract phase following DNase 1 passing through the MIVM with no stabilizing BCP added (no iNC formed).

EE = Encapsulation Efficiency

Nanoparticle Mass = total dry mass of the nanoparticle as calculated from measurements with the TGA. Mass in 90µL was extrapolated to calculate the total mass based on the total volume of the nanoparticle suspension.

DNase 1- nanoparticle loading in aqueous stabilized PEG-coated nanoparticle

Loading of the final aqueous stabilized PEG-coated nanoparticle was confirmed by gel electrophoresis followed by Silver Stain. Briefly, following dialysis, nanoparticles were diluted with 6X SDS loading buffer (Fisher Scientific, 50-196-785) and boiled (10 min, 90°C). A series of DNase 1 standards were similarly treated. These were loaded into a Sodium dodecyl sulfate (SDS)—polyacrylamide gel (4% (v/v) stacking gels and 10% (v/v) running gels). Electrophoresis was carried out at 120 V using Tris/glycine electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.7), just until the protein band had surpassed the stacking gel. To stain for the protein content, we used the commercially available Silver Stain Plus Kit, and followed the protocol instructions supplied (Biorad, 1610449). Gels were imaged using an Azure Biosystems Gel Doc imaging system.

Loading capacity of PEG-coated DNase1-containing nanoparticle

$$\text{Loading Capacity (DNP)} = \left(\frac{\text{DNase 1(silver stain)}}{\text{Nanoparticle mass}} \right) \times 100$$

DNase 1_(silver stain) = the amount of DNase 1 as measured by silver stain of a dispersion of DNPs in water. Nanoparticle mass = total solid mass of a dispersion of DNPs in water via thermogravimetric analysis

Detection of DNase 1 activity via denaturing polyacrylamide gel electrophoresis zymography

We performed this assay as previously described in Jimenez-Alcázar and co-authors⁸³, and Rosenthal and Lacks⁸⁴, with adaptations. Briefly, nanoparticles were degraded by diluting them 2-fold into a solution with a final concentration of 5% SDS and 0.1M NaOH, and then stirred rapidly at room temperature for 48 hrs. This sample was then diluted in water to represent ~100 pg of DNase 1. DNase 1 standards were prepared in water representing 51, 102 and 204 pg of DNase 1. The sample and standards were diluted with 6X SDS sample loading buffer, and then boiled for 10 minutes before loading onto the gels.

Sodium dodecyl sulfate (SDS)—polyacrylamide gels were prepared with 4% (v/v) stacking gels without DNA and 10% (v/v) running gels containing 100 µg/ml of denatured salmon testes DNA. DNA was first dissolved in water (30–60' sonication) then boiled (10 min, 90°C), and placed on ice before addition to gel reagents. Electrophoresis was carried out at 120 V using Tris-glycine electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.7). After electrophoresis, SDS was removed by washing the gel twice with 10 mM Tris/HCl pH 7.8 for 30 min at 50°C. Proteins were refolded by incubating the gels overnight at 37°C in a re-folding buffer containing 5% (w/v) milk powder, 10 mM Tris/HCl

pH 7.8, 3 mM CaCl₂, 3 mM MgCl₂, 100 U/ml penicillin, and 100 µg/ml streptomycin. The gels were transferred to a refolding buffer without milk powder (10 mM Tris/HCl pH 7.8, 3 mM CaCl₂, 3 mM MgCl₂, 100 U/ml penicillin, and 100 µg/ml streptomycin) and incubated for additional 24 hrs at 37°C. The gels were then stained with for DNA with SYBRTM Safe DNA Gel Stain in 1X TAE (Life Technologies, S33111). Fluorescent images of the gels were recorded using a molecular gel imager.

Release Assay

DNPs were dispersed into 10 mM Tris-HCl buffer containing 3 mM MgCl₂ and CaCl₂ and 2–3 mL placed into Tube-O-Dialyzer (G Biosciences, VWR, Cat#: 95057–606, MWCO 50K). They were then dialyzed while shaking at 37°C for up to 8 days against a volume of 30 mL of buffer. The dialysate was then reduced to 0.5mL using a passivated Pall Centrifugal Filter (MWCO 3K). The centrifugal filters were passivated overnight with 5% Tween-20 in water (0.22µm filtered) and then washed copiously (10x volume) with 0.22µm filtered water, then left in 1X PBS until use. The dialysate volume was measured with a mass balance to determine an accurate volume, and the protein content measured with the microBCA assay. The protein content in the dialysate was calculated as a % of the initial amount of protein in the DNPs prior to the release assay.

Isolation of bone marrow derived neutrophils

Bone marrow derived neutrophils were isolated as previously described with minor alterations. Briefly, the femur and tibia of 10–14-week-old C57bl/6 mice were excised, and the bone marrow collected into cold HBSS prior to red blood cell lysis with hypotonic sodium chloride (0.2%), followed by addition of NaCl solution (1.6%). Cells were strained (40 µm nylon cell strainer) and then pelleted (300 g, 5min) and resuspending in HBSS. Cells were counted and then neutrophils were isolated by magnetic separation with a mouse neutrophil isolation kit (Miltenyi, 130-097-658). The instructions were followed as per the kit protocol. A Midi Macs separator (Miltenyi, 130-042-302) with LS columns (Miltenyi, 130-042-401) was employed. Neutrophils were finally resuspended at $\sim 1 \times 10^6$ cells/mL in 1X RPMI (no serum or antibiotics added). The typical yield from 1 mouse was $\sim 30 \times 10^6$ bone marrow cells and $\sim 5 \times 10^6$ neutrophils, with 98% viability. Cells were checked for initial cell count and viability with the Muse Count and Viability kit (Sigma Aldrich, Luminex MCH100104). Neutrophils were always used fresh and within 2 hrs of isolation, they were kept at 4°C until use.

Isolation of human total blood derived neutrophils

Human neutrophils were isolated directly from venous blood by magnetic separation with the EasySepTM Direct Human Neutrophil Isolation Kit. The instructions were followed as per the kit protocol. An Easy 50 magnet (Catalog #18002) with 50 ml tube was employed. Cell count and viability were measured with the Nexcelom ViaStain AO/PI Solution (CS2-0101-5ml). Following isolation, neutrophils were maintained in 1X RPMI media. Neutrophils were used fresh, within 2 hr of isolation. They were kept 37°C until use.

Flow Cytometry of Murine Neutrophils

Flow Cytometry was performed on the Thermo Fisher Attune NxT instrument and data acquired on the Attune NxT 2.6 software. Data was analyzed using FlowJo software. For staining we employed Anti mouse CD45-PeCy5 (Fisher Scientific, 15-0451-81, 1:3000, final ~0.07 μ g/mL) and the Violet live dead stain (Thermo Fisher, L34963, 1:5,000). Cells were gated in the order of cell granularity/size, singlet events, live cells, CD45⁺, rubrene⁺.

For single cell suspensions of blood clots, cells were gated in the order of singlet events, DAPI+ve events, Ly6G positivity (PE), and finally either Green-CMFD fluorescence or Deep Red Fluorescence. Exogenous neutrophils had been stained with Anti-ly6G PE (Thermo Fisher, Clone 1RB6-8C5, Cat#: 12-5931-85, 1:1500 dilution, 0.13 μ g/mL).

Nanoparticle Internalization by Murine Neutrophils

This method was adapted from previous literature⁵⁰. Briefly, 96 well U-bottom microplates (08-772-54, Fisher scientific) were treated overnight with 1% BSA/1XPBS. On the day of the experiment, 100,000 murine neutrophils in RPMI media (no serum) at 1×10^6 cells/mL were added to the treated wells. 10 μ L of rubrene-DNase 1-NPs (final concentration of ~0.2 μ g/mL rubrene) were added to the neutrophils and incubated either at 37°C with 5% CO₂ and humidity or 4°C or up to 2 hours. Neutrophils were pelleted (300 g, 5min), and washed with flow cytometry staining buffer (0.5% BSA in 1X PBS). Cells were pelleted and resuspended in 1X PBS and stained with the violet live/dead stain for 30 minutes, protected from light at room temperature. Cells were washed and then stained with CD45 antibody for 30 minutes at 4°C. Cells were washed and then fixed with 1% PFA in 1X PBS for 15 minutes at 4°C. Cells were stored overnight in 1X PBS at 4°C and analyzed by flow cytometry within 48 hours.

Stimulation of oxidative burst in Neutrophils

Oxidative burst of bone marrow derived neutrophils was measured using the Abcam DHE ROS kit (Cat#Ab236206). Neutrophils (1×10^6 cells/mL, RPMI) were incubated in the presence or absence of DNase1-nanoparticles (3.4U/mL) for 2 hrs at 37°C, 5% CO₂, and then pelleted and washed to remove unincorporated nanoparticles. These neutrophils were subsequently stimulated with PMA (100 nM) or PMA (100 nM) and ionomycin (1 μ M) for 30 minutes at 37°C, 5% CO₂. The instructions for the Abcam kit were then followed to stain these cells for ROS production.

Generation of Neutrophil Extracellular Traps

NET degradation was analyzed as previously described^{83, 85}, with minor alterations. 50,000 purified murine neutrophils (250,000 cells/mL) in serum-free F12 media were seeded onto sterile 96 well glass bottom plates (VWR, 655891). To induce NET formation, neutrophils were treated with 100 nM PMA for 4 hrs at 37°C with 5% CO₂ and humidity. Wells were then washed three times with 1X PBS and stored in 1X PBS overnight at 4°C. DNase 1 or DNase 1-NPs at ~1U/mL of DNase 1 were added to wells in Tris-HCl, 10mM pH 7.8, containing 3mM CaCl₂ and 3 mM MgCl₂. Control wells were incubated with buffer alone. NET degradation was allowed to occur for up to 4 hrs. To stop NET degradation, wells were washed and treated with cold 2mM EDTA for 15 min at 4°C. Well were washed, and

then NETs fixed with 2% PFA in 1X PBS for 15 min in the fridge. Wells were washed and DNA stained with 2 μ M Sytox Green (Thermo Fisher, S7020) for 15 min at room temperature. Wells were washed and stored until imaging in 1X PBS, protected from light at 4°C. Entire wells were imaged for GFP fluorescence with High Content Analysis Nikon Elements software using an inverted fluorescence microscope (Nikon Ti2 eclipse, Objective: Plan Apochromat Lambda 10X, NA = 0.45, WD = 4mm). Analysis was performed in Image J. Firstly, a standardized well area was drawn that excluded the very edges of the well, and this region was duplicated. The same area was applied to all wells. The fluorescence was thresholded and the area of the thresholded fluorescence was measured. To restrict measurements to the filamentous NET structures, circular particles were counted, and the area of the particles measured. Area from spherical particles was deducted from the total initial area. The remaining area was considered to be that of filamentous NET structures.

Association of bone-marrow derived neutrophils with pre-formed blood clot

To generate the pre-formed blood clot, the method of Kattula et al. was modified slightly⁸⁶. Briefly, venous blood was drawn from healthy male C57bl/6 mice (8–12 wk old) into 4% sodium citrate. Blood was then recalcified (final concentration 30mM) and treated with tissue factor (TF; Innovin, diluted 1:9000 [1 pM TF]) and clot formation proceeded in siliconized wells for 1 h at 37°C. Bone marrow derived neutrophils from C57bl/6 mice (8–12 wk old) were isolated and labelled with 1 μ M Cell Tracker Deep Red (Thermo Fisher, Catalog #: C34565) or Green-CMFD (Thermo Fisher, Catalog #: C7025) according to manufacturer's instructions. Green or Deep Red labelled neutrophils were then loaded with DNPs (5U DNase 1/mL) for 2h at 37°C, 5% CO₂. Following incubation and washing, neutrophil count was standardized so that empty and DNP-loaded neutrophils were at 12 million cells/mL. Subsequently both empty and DNP-loaded neutrophils were added to the same pre-formed clot at equal concentrations and allowed to associate with the clot overnight (18 h). *Preparation of single cell suspension:* The clot was washed with 1X cold HBSS, and then ground into 10 mL of 1X cold HBSS and pelleted. The pellet was resuspended in warm DNase 1 digest solution (60U/mL DNase 1, 1X PBS 3mM MgCl₂) and incubated for 30 min at 37°C, 5% CO₂. The digest of free extracellular DNA was halted by the addition of EDTA to a final concentration of 2 mM in PBS. Following pelleting and washing with flow cytometry staining buffer, the cells were then stained with Anti-ly6G PE (Thermo Fisher, Clone 1RB6–8C5, Cat#: 12-5931-85, 1:1500 dilution, 0.13 μ g/mL). for 30 min at 4°C. Red blood cells were then lysed and the remaining single cell suspension cells were fixed using RBC Lyse-&-Fix (Thermo Fisher, Catalog #: 00-5333-54). The single cell suspension was then stained with DAPI (300 nM) for 15 min, room temperature. *Histological sections:* The clot was washed with 1X cold HBSS, and then fixed with 4%PFA/1X PBS for 1 h at 4°C. The clot was then placed in 30% sucrose/1XPBS for 1–2 h at 4°C, and then frozen in OCT freezing medium over liquid N₂ (Fisher Scientific, Catalog #: 23-730-625). 10 μ m sections were taken with a cryostat at -20°C. Tissue sections were washed with 1X PBS and stained with DAPI (300 nM) for 5 min, room temperature, and subsequently mounted in ProLong Gold AntiFade Mounting Medium (ThermoFisher Scientific, Catalog #: P36930), with #1 thickness cover slips (VWR, Catalog #: 16004–098). Fluorescent microscopy images were acquired on a Nikon Ti2 eclipse with Nikon NIS Elements AR software (Objective: Plan Apochromat Lambda 10X, NA = 0.45, WD = 4mm;

and Plan Fluor 40X, NA = 0.75, WD = 0.66mm, Camera: pco.edge 4.2Q High QE sCMOS). Fluorescence was detected on the DAPI, GFP and Narrow Cy5 channels. Image analysis was performed on Image J (FIJI).

Statistical Analysis

Data was graphed using Graphpad Prism 10 software (v10.2.2, Graphpad Software LLC). Data was analyzed with Graphpad Prism 10 software or SAS (9.4). To compare the means between multiple groups we employed factorial ANOVA. Ratios, which cannot be normally distributed, were analyzed using a beta-distributed generalized linear model. Percentages were log transformed and then analyzed via factorial ANOVA followed by Sidak Multiple Comparisons post-hoc test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We would like to acknowledge the Nanomedicines Characterization Core Facility, Center for Nanotechnology in Drug Delivery, UNC School of Pharmacy. Fluorescence microscopy was performed at the UNC Neuroscience Microscopy Core (RRID:SCR_019060), supported, in part, by funding from the NIH-NINDS Neuroscience Center Support Grant P30 NS045892 and the NIH-NICHD Intellectual and Developmental Disabilities Research Center Support Grant U54 HD079124. The Zeiss LSM 980 microscope was funded with support from NIH grant S10 OD032388. Transmission electron microscopy was performed at the Microscopy Services Laboratory, Department of Pathology and Laboratory Medicine. They are supported in part by P30 CA016086 Cancer Center Core Support Grant to the UNC Lineberger Comprehensive Cancer Center. Flow Cytometry was performed at the UNC Flow Cytometry Core Facility which is supported in part by P30 CA016086 Cancer Center Core Support Grant to the UNC Lineberger Comprehensive Cancer Center. We thank, in particular, Dr Ramiro Diz (Director of the UNC Flow Cytometry Facility) who provided invaluable advice in planning flow cytometry experiments. Research reported in this publication was supported in part by the North Carolina Biotech Center Institutional Support Grant 2017-IDG-1025 and by the National Institutes of Health 1UM2AI30836-01. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The authors would like to thank the reviewers for their insightful questions and suggestions, both of which inspired further experiments, overall helping to increase the impact of the manuscript.

Funding Sources

Burroughs Wellcome Fund Collaborative Travel Grant, NCTracs 2K pilot grant, NIH K99 1 K99 HL157690-01 & 4R00HL157690-03, F31HL156427.

ABBREVIATIONS

ACN	Acetonitrile
BMDNs	Bone Marrow Derived Neutrophils
CD62L	Cell Differentiation 62L, Leukocyte L-Selectin
CIJ	Confined Impinging Jet Mixer
CMFDA	5-Chloromethylfluorescein diacetate
FNP	Flash NanoPrecipitation
iFNP	inverse Flash NanoPrecipitation

GPC	Gel Permeation Chromatography
iNC	inverse NanoCarrier
DMSO	Dimethyl sulfoxide
DNase 1	Deoxyribonuclease 1
DNP	DNase 1 nanoparticle
MIVM	Multi-Inlet Vortex Mixer
µMIVM	Micro Multi-Inlet Vortex Mixer
NETs	Neutrophil Extracellular Traps
NMR	Nuclear Magnetic Resonance
PAA	Poly Acrylic Acid
PEG	Polyethylene Glycol
PLA	Poly Lactic Acid
PMA	Phorbol Myristate Acetate
THF	Tetrahydrofuran

REFERENCES

- (1). Papayannopoulos V Neutrophil extracellular traps in immunity and disease. *Nat Rev Immunol* 2018, 18 (2), 134–147. DOI: 10.1038/nri.2017.105 From NLM. [PubMed: 28990587]
- (2). Wigerblad G; Kaplan MJ Neutrophil extracellular traps in systemic autoimmune and autoinflammatory diseases. *Nat Rev Immunol* 2023, 23 (5), 274–288. DOI: 10.1038/s41577-022-00787-0 From NLM. [PubMed: 36257987]
- (3). Klopff J; Brostjan C; Eilenberg W; Neumayer C Neutrophil Extracellular Traps and Their Implications in Cardiovascular and Inflammatory Disease. *Int J Mol Sci* 2021, 22 (2). DOI: 10.3390/ijms22020559 From NLM.
- (4). Masucci MT; Minopoli M; Del Vecchio S; Carriero MV The Emerging Role of Neutrophil Extracellular Traps (NETs) in Tumor Progression and Metastasis. *Front Immunol* 2020, 11, 1749. DOI: 10.3389/fimmu.2020.01749 From NLM. [PubMed: 33042107]
- (5). Savchenko AS; Martinod K; Seidman MA; Wong SL; Borissoff JI; Piazza G; Libby P; Goldhaber SZ; Mitchell RN; Wagner DD Neutrophil extracellular traps form predominantly during the organizing stage of human venous thromboembolism development. *Journal of Thrombosis and Haemostasis* 2014, 12 (6), 860–870, Article. DOI: 10.1111/jth.12571 Scopus. [PubMed: 24674135]
- (6). Thålin C; Hisada Y; Lundström S; Mackman N; Wallén H Neutrophil Extracellular Traps. *Arteriosclerosis, thrombosis, and vascular biology* 2019, 39 (9), 1724–1738, Article. DOI: 10.1161/ATVBAHA.119.312463 Scopus. [PubMed: 31315434]
- (7). Sharma S; Hofbauer TM; Ondracek AS; Chausheva S; Alimohammadi A; Artner T; Panzenboeck A; Rinderer J; Shafran I; Mangold A; et al. Neutrophil extracellular traps promote fibrous vascular occlusions in chronic thrombosis. *Blood* 2021, 137 (8), 1104–1116. DOI: 10.1182/blood.2020005861 From NLM. [PubMed: 33512471]
- (8). Fuchs TA; Brill A; Duerschmied D; Schatzberg D; Monestier M; Myers DD Jr; Wroblewski SK; Wakefield TW; Hartwig JH; Wagner DD Extracellular DNA traps promote thrombosis.

- Proceedings of the National Academy of Sciences of the United States of America 2010, 107 (36), 15880–15885, Article. DOI: 10.1073/pnas.1005743107 Scopus. [PubMed: 20798043]
- (9). Brill A; Fuchs TA; Savchenko AS; Thomas GM; Martinod K; de Meyer SF; Bhandari AA; Wagner DD Neutrophil extracellular traps promote deep vein thrombosis in mice. *Journal of Thrombosis and Haemostasis* 2012, 10 (1), 136–144, Article. DOI: 10.1111/j.1538-7836.2011.04544.x Scopus. [PubMed: 22044575]
- (10). Semeraro F; Ammollo CT; Morrissey JH; Dale GL; Friese P; Esmon NL; Esmon CT Extracellular histones promote thrombin generation through platelet-dependent mechanisms: Involvement of platelet TLR2 and TLR4. *Blood* 2011, 118 (7), 1952–1961, Article. DOI: 10.1182/blood-2011-03-343061 Scopus. [PubMed: 21673343]
- (11). Jansen MPB; Emal D; Teske GJD; Dessing MC; Florquin S; Roelofs JJTH Release of extracellular DNA influences renal ischemia reperfusion injury by platelet activation and formation of neutrophil extracellular traps. *Kidney International* 2016, Article in Press. DOI: 10.1016/j.kint.2016.08.006 Scopus.
- (12). Varjú I; Kolev K Networks that stop the flow: A fresh look at fibrin and neutrophil extracellular traps. *Thrombosis Research* 2019, 182, 1–11, Review. DOI: 10.1016/j.thromres.2019.08.003 Scopus. [PubMed: 31415922]
- (13). Longstaff C; Varjú I; Sótónyi P; Szabó L; Krumrey M; Hoell A; Bóta A; Varga Z; Komorowicz E; Kolev K Mechanical stability and fibrinolytic resistance of clots containing fibrin, DNA, and histones. *Journal of Biological Chemistry* 2013, 288 (10), 6946–6956, Article. DOI: 10.1074/jbc.M112.404301 Scopus. [PubMed: 23293023]
- (14). Gould TJ; Vu TT; Stafford AR; Dwivedi DJ; Kim PY; Fox-Robichaud AE; Weitz JI; Liaw PC Cell-Free DNA Modulates Clot Structure and Impairs Fibrinolysis in Sepsis. *Arteriosclerosis, thrombosis, and vascular biology* 2015, 35 (12), 2544–2553, Article. DOI: 10.1161/ATVBAHA.115.306035 Scopus. [PubMed: 26494232]
- (15). Laridan E; Denorme F; Desender L; François O; Andersson T; Deckmyn H; Vanhoorelbeke K; De Meyer SF Neutrophil extracellular traps in ischemic stroke thrombi. *Ann Neurol* 2017, 82 (2), 223–232. DOI: 10.1002/ana.24993 From NLM. [PubMed: 28696508]
- (16). Li T; Peng R; Wang F; Hua L; Liu S; Han Z; Pei J; Pei S; Zhao Z; Jiang X; Chen X Lysophosphatidic acid promotes thrombus stability by inducing rapid formation of neutrophil extracellular traps: A new mechanism of thrombosis. *J Thromb Haemost* 2020, 18 (8), 1952–1964. DOI: 10.1111/jth.14839 From NLM. [PubMed: 32291893]
- (17). von Brühl ML; Stark K; Steinhart A; Chandraratne S; Konrad I; Lorenz M; Khandoga A; Timiceriu A; Coletti R; Köllnberger M; et al. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. *Journal of Experimental Medicine* 2012, 209 (4), 819–835, Article. DOI: 10.1084/jem.20112322 Scopus. [PubMed: 22451716]
- (18). Lee YY; Park HH; Park W; Kim H; Jang JG; Hong KS; Lee JY; Seo HS; Na DH; Kim TH; et al. Long-acting nanoparticulate DNase-1 for effective suppression of SARS-CoV-2-mediated neutrophil activities and cytokine storm. *Biomaterials* 2021, 267, 120389. DOI: 10.1016/j.biomaterials.2020.120389 From NLM. [PubMed: 33130319]
- (19). Davis JC Jr.; Manzi S; Yarboro C; Rairie J; McInnes I; Averbely D; Sinicropi D; Hale VG; Balow J; Austin H; et al. Recombinant human Dnase I (rhDNase) in patients with lupus nephritis. *Lupus* 1999, 8 (1), 68–76. DOI: 10.1191/096120399678847380 From NLM. [PubMed: 10025601]
- (20). Johnson AJ; Goger PR; Tillett WS The intravenous injection of bovine crystalline pancreatic desoxyribonuclease into patients. *J Clin Invest* 1954, 33 (12), 1670–1686. DOI: 10.1172/jci103048 From NLM. [PubMed: 13211824]
- (21). Angeletti A; Volpi S; Bruschi M; Lugani F; Vaglio A; Prunotto M; Gattorno M; Schena F; Verrina E; Ravelli A; Ghiggeri GM Neutrophil Extracellular Traps-DNase Balance and Autoimmunity. *Cells* 2021, 10 (10). DOI: 10.3390/cells10102667 From NLM.
- (22). Blanco E; Shen H; Ferrari M Principles of nanoparticle design for overcoming biological barriers to drug delivery. *Nat Biotechnol* 2015, 33 (9), 941–951. DOI: 10.1038/nbt.3330 From NLM. [PubMed: 26348965]

- (23). Markwalter CE; Pagels RF; Wilson BK; Ristroph KD; Prud'homme RK Flash NanoPrecipitation for the Encapsulation of Hydrophobic and Hydrophilic Compounds in Polymeric Nanoparticles. *J Vis Exp* 2019, (143). DOI: 10.3791/58757 From NLM.
- (24). Markwalter CE; Prud'homme RK Inverse Flash NanoPrecipitation for Biologics Encapsulation: Understanding Process Losses via an Extraction Protocol. In *Control of Amphiphile Self-Assembling at the Molecular Level: Supra-Molecular Assemblies with Tuned Physicochemical Properties for Delivery Applications*, ACS Publications, 2017; pp 275–296.
- (25). Markwalter CE; Pagels RF; Hejazi AN; Ristroph KD; Wang J; Chen K; Li J; Prud'homme RK Sustained release of peptides and proteins from polymeric nanocarriers produced by inverse Flash NanoPrecipitation. *J Control Release* 2021, 334, 11–20. DOI: 10.1016/j.jconrel.2021.04.002 From NLM. [PubMed: 33823220]
- (26). Markwalter CE; Pagels RF; Hejazi AN; Gordon AGR; Thompson AL; Prud'homme RK Polymeric Nanocarrier Formulations of Biologics Using Inverse Flash NanoPrecipitation. *Aaps j* 2020, 22 (2), 18. DOI: 10.1208/s12248-019-0405-z From NLM. [PubMed: 31897899]
- (27). Markwalter CE; Prud'homme RK Design of a Small-Scale Multi-Inlet Vortex Mixer for Scalable Nanoparticle Production and Application to the Encapsulation of Biologics by Inverse Flash NanoPrecipitation. *J Pharm Sci* 2018, 107 (9), 2465–2471. DOI: 10.1016/j.xphs.2018.05.003 From NLM. [PubMed: 29772223]
- (28). Pagels RF; Prud'Homme RK Inverse flash nanoprecipitation for biologics encapsulation: Nanoparticle formation and ionic stabilization in organic solvents. In *ACS Symposium Series*, 2017; Vol. 1271, pp 249–274.
- (29). Kis Z; Kontoravdi C; Shattock R; Shah N Resources, Production Scales and Time Required for Producing RNA Vaccines for the Global Pandemic Demand. *Vaccines (Basel)* 2020, 9 (1). DOI: 10.3390/vaccines9010003 From NLM.
- (30). Shen H; Hong S; Prud'homme RK; Liu Y Self-assembling process of flash nanoprecipitation in a multi-inlet vortex mixer to produce drug-loaded polymeric nanoparticles. *Journal of Nanoparticle Research* 2011, 13 (9), 4109–4120. DOI: 10.1007/s11051-011-0354-7.
- (31). Pinkerton NM; Gindy ME; Calero-Ddel CV; Wolfson T; Pagels RF; Adler D; Gao D; Li S; Wang R; Zevon M; et al. Single-Step Assembly of Multimodal Imaging Nanocarriers: MRI and Long-Wavelength Fluorescence Imaging. *Adv Healthc Mater* 2015, 4 (9), 1376–1385. DOI: 10.1002/adhm.201400766 From NLM. [PubMed: 25925128]
- (32). Han J; Zhu Z; Qian H; Wohl AR; Beaman CJ; Hoyer TR; Macosko CW A simple confined impingement jets mixer for flash nanoprecipitation. *J Pharm Sci* 2012, 101 (10), 4018–4023. DOI: 10.1002/jps.23259 From NLM. [PubMed: 22777753]
- (33). Johnson BK; Prud'homme RK Flash NanoPrecipitation of Organic Actives and Block Copolymers using a Confined Impinging Jets Mixer. *Australian Journal of Chemistry* 2003, 56 (10), 1021–1024. DOI: 10.1071/CH03115.
- (34). Fu Z; Li L; Wang M; Guo X Size control of drug nanoparticles stabilized by mPEG-b-PCL during flash nanoprecipitation. *Colloid and Polymer Science* 2018, 296 (5), 935–940. DOI: 10.1007/s00396-018-4311-1.
- (35). Pagels RF; Edelstein J; Tang C; Prud'homme RK Controlling and Predicting Nanoparticle Formation by Block Copolymer Directed Rapid Precipitations. *Nano Lett* 2018, 18 (2), 1139–1144. DOI: 10.1021/acs.nanolett.7b04674 From NLM. [PubMed: 29297690]
- (36). Feng J; Markwalter CE; Tian C; Armstrong M; Prud'homme RK Translational formulation of nanoparticle therapeutics from laboratory discovery to clinical scale. *J Transl Med* 2019, 17 (1), 200. DOI: 10.1186/s12967-019-1945-9 From NLM. [PubMed: 31200738]
- (37). Armstrong M; Wang L; Ristroph K; Tian C; Yang J; Ma L; Panmai S; Zhang D; Nagapudi K; Prud'homme RK Formulation and Scale-Up of Fast-Dissolving Lumefantrine Nanoparticles for Oral Malaria Therapy. *J Pharm Sci* 2023, 112 (8), 2267–2275. DOI: 10.1016/j.xphs.2023.04.003 From NLM. [PubMed: 37030438]
- (38). Warne N; Ruesch M; Siwik P; Mensah P; Ludwig J; Hripcsak M; Godavarti R; Prigodich A; Dolsten M Delivering 3 billion doses of Comirnaty in 2021. *Nature Biotechnology* 2023, 41 (2), 183–188. DOI: 10.1038/s41587-022-01643-1.

- (39). Pustulka KM; Wohl AR; Lee HS; Michel AR; Han J; Hoyer TR; McCormick AV; Panyam J; Macosko CW Flash nanoprecipitation: particle structure and stability. *Mol Pharm* 2013, 10 (11), 4367–4377. DOI: 10.1021/mp400337f From NLM. [PubMed: 24053447]
- (40). Wang ML,S; Wang J; Liu L; Zhou W; Ahmed R; Hu A; Guo X; Cohen Stuart MA. Controlling morphology and release behavior of sorafenib-loaded nanocarriers prepared by flash nanoprecipitation. *Industrial & Engineering Chemistry Research* 2018, 57 (35), 8.
- (41). Pinkerton NM; Grandeury A; Fisch A; Brozio J; Riebesehl BU; Prud'homme RK Formation of stable nanocarriers by in situ ion pairing during block-copolymer-directed rapid precipitation. *Mol Pharm* 2013, 10 (1), 319–328. DOI: 10.1021/mp300452g From NLM. [PubMed: 23259920]
- (42). Ristroph KD; Prud'homme RK Hydrophobic ion pairing: encapsulating small molecules, peptides, and proteins into nanocarriers. *Nanoscale Advances* 2019, 1 (11), 4207–4237, 10.1039/C9NA00308H. DOI: 10.1039/C9NA00308H. [PubMed: 33442667]
- (43). Lu HD; Rummaneeethorn P; Ristroph KD; Prud'homme RK Hydrophobic Ion Pairing of Peptide Antibiotics for Processing into Controlled Release Nanocarrier Formulations. *Mol Pharm* 2018, 15 (1), 216–225. DOI: 10.1021/acs.molpharmaceut.7b00824 From NLM. [PubMed: 29206046]
- (44). Fergusson AD; Zhang R; Riffle JS; Davis RM Encapsulation of PI3K Inhibitor LY294002 within Polymer Nanoparticles Using Ion Pairing Flash Nanoprecipitation. *Pharmaceutics* 2023, 15 (4), 1157. [PubMed: 37111642]
- (45). Novotny J; Chandraratne S; Weinberger T; Philippi V; Stark K; Ehrlich A; Pircher J; Konrad I; Oberdieck P; Titova A; et al. Histological comparison of arterial thrombi in mice and men and the influence of Cl-amidine on thrombus formation. *PLoS ONE* 2018, 13 (1), Article. DOI: 10.1371/journal.pone.0190728 Scopus.
- (46). Kapoor S; Opneja A; Nayak L The role of neutrophils in thrombosis. *Thrombosis Research* 2018, 170, 87–96, Review. DOI: 10.1016/j.thromres.2018.08.005 Scopus. [PubMed: 30138777]
- (47). Darbousset R; Thomas GM; Mezouar S; Frère C; Bonier R; Mackman N; Renné T; Dignat-George F; Dubois C; Panicot-Dubois L Tissue factor-positive neutrophils bind to injured endothelial wall and initiate thrombus formation. *Blood* 2012, 120 (10), 2133–2143, Article. DOI: 10.1182/blood-2012-06-437772 Scopus. [PubMed: 22837532]
- (48). Ghasemzadeh M; Kaplan ZS; Alwis I; Schoenwaelder SM; Ashworth KJ; Westein E; Hosseini E; Salem HH; Slattery R; McColl SR; et al. The CXCR1/2 ligand NAP-2 promotes directed intravascular leukocyte migration through platelet thrombi. *Blood* 2013, 121 (22), 4555–4566. DOI: 10.1182/blood-2012-09-459636 From NLM. [PubMed: 23550035]
- (49). Russell P; Esser L; Hagemeyer CE; Voelcker NH The potential impact of nanomedicine on COVID-19-induced thrombosis. *Nat Nanotechnol* 2023, 18 (1), 11–22. DOI: 10.1038/s41565-022-01270-6 From NLM. [PubMed: 36536042]
- (50). Bisso PW; Gagliione S; Guimarães PPG; Mitchell MJ; Langer R Nanomaterial Interactions with Human Neutrophils. *ACS Biomaterials Science and Engineering* 2018, 4 (12), 4255–4265, Article. DOI: 10.1021/acsbiomaterials.8b01062 Scopus. [PubMed: 31497639]
- (51). Sahay G; Alakhova DY; Kabanov AV Endocytosis of nanomedicines. *J Control Release* 2010, 145 (3), 182–195. DOI: 10.1016/j.jconrel.2010.01.036 From NLM. [PubMed: 20226220]
- (52). Vishnevskiy DA; Garanina AS; Chernysheva AA; Chekhonin VP; Naumenko VA Neutrophil and Nanoparticles Delivery to Tumor: Is It Going to Carry That Weight? *Adv Healthc Mater* 2021, 10 (9), e2002071. DOI: 10.1002/adhm.202002071 From NLM. [PubMed: 33734620]
- (53). Mattos C; Ringe D Proteins in organic solvents. *Curr Opin Struct Biol* 2001, 11 (6), 761–764. DOI: 10.1016/s0959-440x(01)00278-0 From NLM. [PubMed: 11751059]
- (54). Chen WJ; Liao TH Structure and function of bovine pancreatic deoxyribonuclease I. *Protein Pept Lett* 2006, 13 (5), 447–453. DOI: 10.2174/0929866060776819475 From NLM. [PubMed: 16800797]
- (55). Suri R The use of human deoxyribonuclease (rhDNase) in the management of cystic fibrosis. *BioDrugs* 2005, 19 (3), 135–144. DOI: 10.2165/00063030-200519030-00001 From NLM. [PubMed: 15984899]
- (56). Macanovic M; Sinicropi D; Shak S; Baughman S; Thiru S; Lachmann PJ The treatment of systemic lupus erythematosus (SLE) in NZB/W F1 hybrid mice; studies with recombinant

murine DNase and with dexamethasone. Clin Exp Immunol 1996, 106 (2), 243–252. DOI: 10.1046/j.1365-2249.1996.d01-839.x From NLM. [PubMed: 8918569]

- (57). Guichard MJ; Patil HP; Koussoroplis SJ; Wattiez R; Leal T; Vanbever R Production and characterization of a PEGylated derivative of recombinant human deoxyribonuclease I for cystic fibrosis therapy. Int J Pharm 2017, 524 (1–2), 159–167. DOI: 10.1016/j.ijpharm.2017.03.057 From NLM. [PubMed: 28356227]
- (58). Mahri S; Rondon A; Wilms T; Bosquillon C; Vanbever R Biodistribution and elimination pathways of PEGylated recombinant human deoxyribonuclease I after pulmonary delivery in mice. J Control Release 2021, 329, 1054–1065. DOI: 10.1016/j.jconrel.2020.10.034 From NLM. [PubMed: 33091532]
- (59). Filipczak N; Li X; Saawant GR; Yalamarty SSK; Luther E; Torchilin VP Antibody-modified DNase I micelles specifically recognize the neutrophil extracellular traps (NETs) and promote their degradation. J Control Release 2023, 354, 109–119. DOI: 10.1016/j.jconrel.2022.12.062 From NLM. [PubMed: 36596341]
- (60). Tasia W; Lei C; Cao Y; Ye Q; He Y; Xu C Enhanced eradication of bacterial biofilms with DNase I-loaded silver-doped mesoporous silica nanoparticles. Nanoscale 2020, 12 (4), 2328–2332. DOI: 10.1039/c9nr08467c From NLM. [PubMed: 31808770]
- (61). Park J; Wysocki RW; Amoozgar Z; Maiorino L; Fein MR; Jorns J; Schott AF; Kinugasa-Katayama Y; Lee Y; Won NH; et al. Cancer cells induce metastasis-supporting neutrophil extracellular DNA traps. Sci Transl Med 2016, 8 (361), 361ra138. DOI: 10.1126/scitranslmed.aag1711 From NLM.
- (62). Tan Y; Ma S; Leonhard M; Moser D; Ludwig R; Schneider-Stickler B Co-immobilization of cellobiose dehydrogenase and deoxyribonuclease I on chitosan nanoparticles against fungal/bacterial polymicrobial biofilms targeting both biofilm matrix and microorganisms. Mater Sci Eng C Mater Biol Appl 2020, 108, 110499. DOI: 10.1016/j.msec.2019.110499 From NLM. [PubMed: 31923978]
- (63). Dinsdale RJ; Hazeldine J; Al Tarrah K; Hampson P; Devi A; Ermogenous C; Bamford AL; Bishop J; Watts S; Kirkman E; et al. Dysregulation of the actin scavenging system and inhibition of DNase activity following severe thermal injury. Br J Surg 2020, 107 (4), 391–401. DOI: 10.1002/bjs.11310 From NLM. [PubMed: 31502663]
- (64). Hazeldine J; Dinsdale RJ; Naumann DN; Acharjee A; Bishop JRB; Lord JM; Harrison P Traumatic injury is associated with reduced deoxyribonuclease activity and dysregulation of the actin scavenging system. Burns Trauma 2021, 9, tkab001. DOI: 10.1093/burnst/tkab001 From NLM. [PubMed: 33834079]
- (65). Penaloza Arias LC; Huynh DN; Bability S; Marleau S; Brambilla D Optimization of a Liposomal DNase I Formulation with an Extended Circulating Half-Life. Mol Pharm 2022, 19 (6), 1906–1916. DOI: 10.1021/acs.molpharmaceut.2c00086 From NLM. [PubMed: 35543327]
- (66). Wang Z; Chen C; Shi C; Zhao X; Gao L; Guo F; Han M; Yang Z; Zhang J; Tang C; et al. Cell membrane derived liposomes loaded with DNase I target neutrophil extracellular traps which inhibits colorectal cancer liver metastases. J Control Release 2023, 357, 620–629. DOI: 10.1016/j.jconrel.2023.04.013 From NLM. [PubMed: 37061194]
- (67). Osman R; Kan PL; Awad G; Mortada N; El-Shamy AE; Alpar O Enhanced properties of discrete pulmonary deoxyribonuclease I (DNaseI) loaded PLGA nanoparticles during encapsulation and activity determination. Int J Pharm 2011, 408 (1–2), 257–265. DOI: 10.1016/j.ijpharm.2011.02.013 From NLM. [PubMed: 21335080]
- (68). Park HH; Park W; Lee YY; Kim H; Seo HS; Choi DW; Kwon HK; Na DH; Kim TH; Choy YB; et al. Bioinspired DNase-I-Coated Melanin-Like Nanospheres for Modulation of Infection-Associated NETosis Dysregulation. Adv Sci (Weinh) 2021, 8 (19), e2103748. DOI: 10.1002/adv.202103748 From NLM. [PubMed: 34623037]
- (69). Gorman LA; Dordick JS Organic solvents strip water off enzymes. Biotechnol Bioeng 1992, 39 (4), 392–397. DOI: 10.1002/bit.260390405 From NLM. [PubMed: 18600960]
- (70). Yang L; Dordick JS; Garde S Hydration of enzyme in nonaqueous media is consistent with solvent dependence of its activity. Biophys J 2004, 87 (2), 812–821. DOI: 10.1529/biophysj.104.041269 From NLM. [PubMed: 15298890]

- (71). Knubovets T; Osterhout JJ; Klivanov AM Structure of lysozyme dissolved in neat organic solvents as assessed by NMR and CD spectroscopies. *Biotechnol Bioeng* 1999, 63 (2), 242–248. From NLM. [PubMed: 10099601]
- (72). Serdakowski AL; Dordick JS Enzyme activation for organic solvents made easy. *Trends Biotechnol* 2008, 26 (1), 48–54. DOI: 10.1016/j.tibtech.2007.10.007 From NLM. [PubMed: 18037518]
- (73). Zaks A; Klivanov AM Enzyme-catalyzed processes in organic solvents. *Proc Natl Acad Sci U S A* 1985, 82 (10), 3192–3196. DOI: 10.1073/pnas.82.10.3192 From NLM. [PubMed: 3858815]
- (74). Dai L; Klivanov AM Striking activation of oxidative enzymes suspended in nonaqueous media. *Proc Natl Acad Sci U S A* 1999, 96 (17), 9475–9478. DOI: 10.1073/pnas.96.17.9475 From NLM. [PubMed: 10449717]
- (75). Ke T; Klivanov AM On enzymatic activity in organic solvents as a function of enzyme history. *Biotechnol Bioeng* 1998, 57 (6), 746–750. DOI: 10.1002/(sici)1097-0290(19980320)57:6<746::aid-bit12>3.0.co;2-5 From NLM. [PubMed: 10099254]
- (76). Guérout M; Picot D; Abi-Ghanem J; Hartmann B; Baaden M How cations can assist DNase I in DNA binding and hydrolysis. *PLoS Comput Biol* 2010, 6 (11), e1001000. DOI: 10.1371/journal.pcbi.1001000 From NLM. [PubMed: 21124947]
- (77). Ru MT; Dordick JS; Reimer JA; Clark DS Optimizing the salt-induced activation of enzymes in organic solvents: effects of lyophilization time and water content. *Biotechnol Bioeng* 1999, 63 (2), 233–241. DOI: 10.1002/(sici)1097-0290(19990420)63:2<233::aid-bit12>3.0.co;2-s From NLM. [PubMed: 10099600]
- (78). Lauková L; Konečná B; Janovičová L; Vlková B; Celec P Deoxyribonucleases and Their Applications in Biomedicine. *Biomolecules* 2020, 10 (7). DOI: 10.3390/biom10071036 From NLM.
- (79). Kolaczowska E; Kubes P Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol* 2013, 13 (3), 159–175. DOI: 10.1038/nri3399 From NLM. [PubMed: 23435331]
- (80). Fromen CA; Kelley WJ; Fish MB; Adili R; Noble J; Hoenerhoff MJ; Holinstat M; Eniola-Adefeso O Neutrophil-Particle Interactions in Blood Circulation Drive Particle Clearance and Alter Neutrophil Responses in Acute Inflammation. *ACS Nano* 2017, 11 (11), 10797–10807. DOI: 10.1021/acsnano.7b03190 From NLM. [PubMed: 29028303]
- (81). Kelley WJ; Onyskiw PJ; Fromen CA; Eniola-Adefeso O Model Particulate Drug Carriers Modulate Leukocyte Adhesion in Human Blood Flows. *ACS Biomater Sci Eng* 2019, 5 (12), 6530–6540. DOI: 10.1021/acsbiomaterials.9b01289 From NLM. [PubMed: 33417805]
- (82). Banka AL; Guevara MV; Brannon ER; Nguyen NQ; Song S; Cady G; Pinsky DJ; Uhrich KE; Adili R; Holinstat M; Eniola-Adefeso O Cargo-free particles divert neutrophil-platelet aggregates to reduce thromboinflammation. *Nature Communications* 2023, 14 (1), 2462. DOI: 10.1038/s41467-023-37990-z.
- (83). Jiménez-Alcázar M; Rangaswamy C; Panda R; Bitterling J; Simsek YJ; Long AT; Bilyy R; Krenn V; Renné C; Renné T; et al. Host DNases prevent vascular occlusion by neutrophil extracellular traps. *Science* 2017, 358 (6367), 1202–1206. DOI: 10.1126/science.aam8897 From NLM. [PubMed: 29191910]
- (84). Rosenthal AL; Lacks SA Nuclease detection in SDS-polyacrylamide gel electrophoresis. *Anal Biochem* 1977, 80 (1), 76–90. DOI: 10.1016/0003-2697(77)90627-3 From NLM. [PubMed: 18945]
- (85). Hakkim A; Fürnrohr BG; Amann K; Laube B; Abed UA; Brinkmann V; Herrmann M; Voll RE; Zychlinsky A Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. *Proc Natl Acad Sci U S A* 2010, 107 (21), 9813–9818. DOI: 10.1073/pnas.0909927107 From NLM. [PubMed: 20439745]
- (86). Kattula S; Byrnes JR; Martin SM; Holle LA; Cooley BC; Flick MJ; Wolberg AS Factor XIII in plasma, but not in platelets, mediates red blood cell retention in clots and venous thrombus size in mice. *Blood Adv* 2018, 2 (1), 25–35. DOI: 10.1182/bloodadvances.2017011890 From NLM. [PubMed: 29344582]

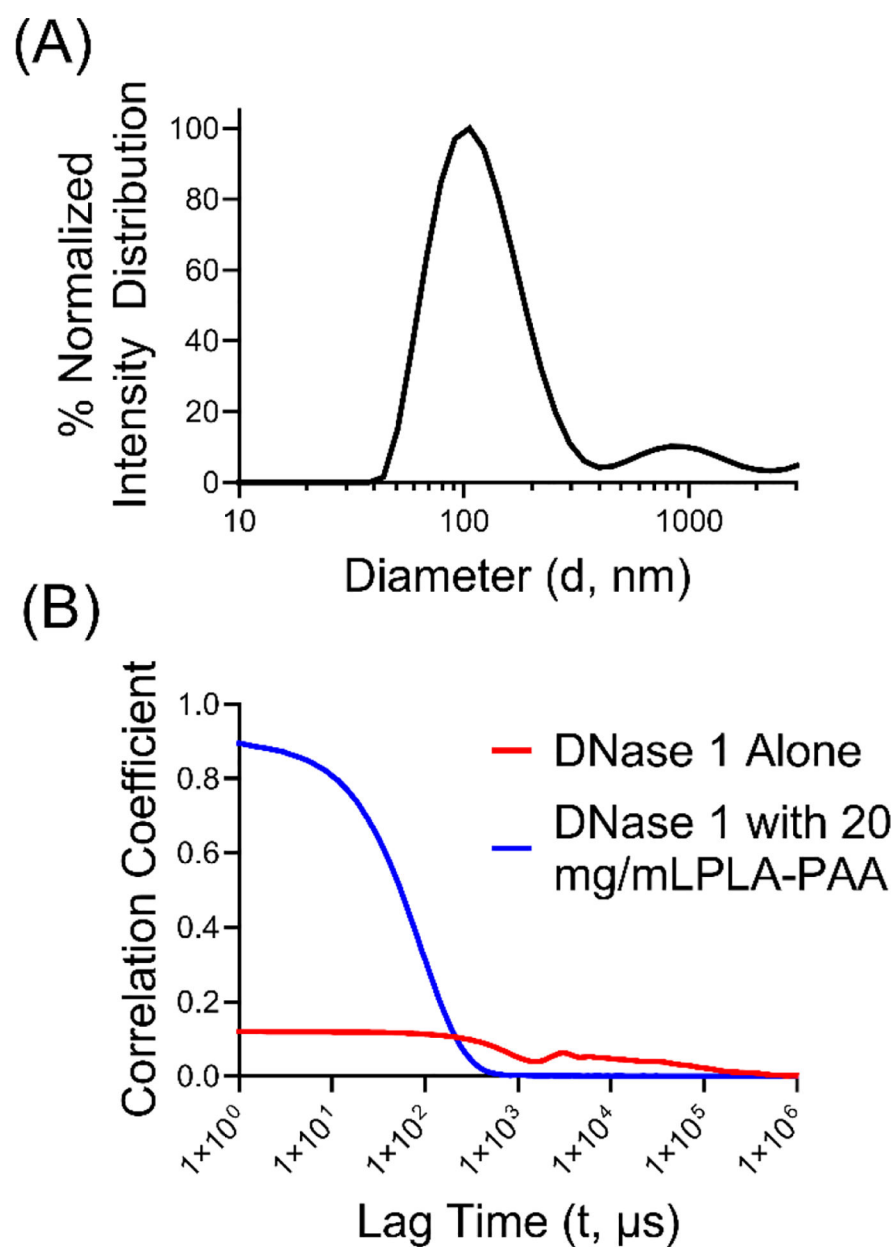


Figure 1. Formation of inverse nanocarrier of DNase 1.

(A) Normalized intensity distribution size measurement via dynamic light scattering of the inverse nanocarrier of DNase 1 in chloroform. **(B)** Correlation function of DNase 1 with (solid blue line) and without (solid red line) the addition of the stabilizing poly(D,L-lactide-*block*-acrylic acid) (PLA-PAA) block copolymer.

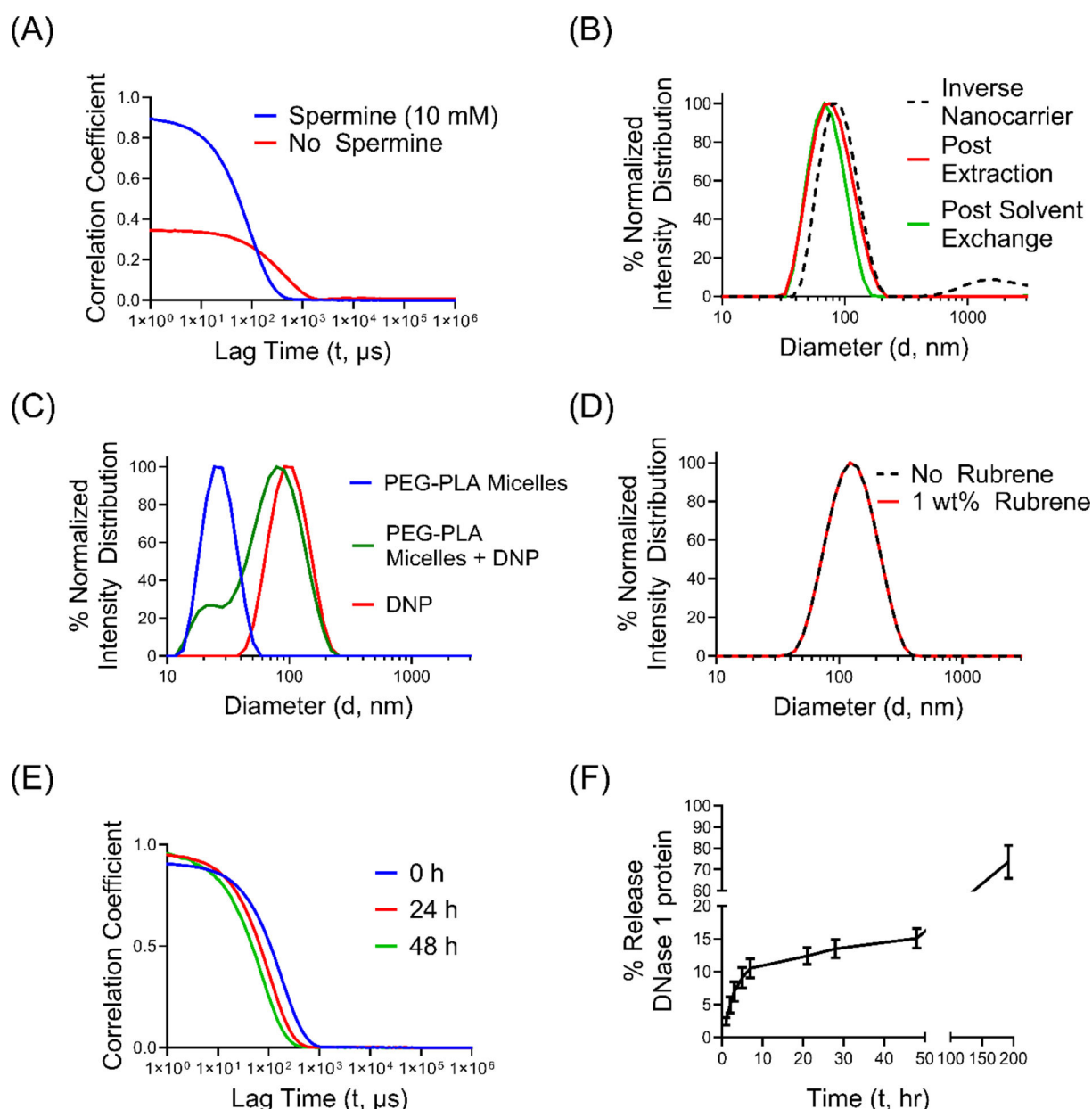


Figure 2. Formation of the PEGylated DNase 1 Nanoparticle.

(A) Spermine stabilizes the inverse nanocarrier by ionic complexation. Correlation function of the inverse nanocarrier diluted 10-fold into 1,4-dioxane solvent with (solid blue line) and without (solid red line) the addition of spermine (10 mM) in the chloroform quench bath.

(B) Normalized intensity distribution size measurement via dynamic light scattering (DLS) of the inverse nanocarrier of DNase 1 (black dashed line), followed by the processing steps of extraction (solid red line) and solvent exchange (solid green line). (C) DLS intensity weight traces of the PEG-PLA micelles alone (blue solid line), the full DNase-1 NPs (DNP) alone (red solid line) and a mixture of the micelles and DNPs (green solid line).

(D) Normalized intensity distribution size measurement via DLS of the PEG-coated DNase 1 nanoparticle (black dashed line), compared to the same nanoparticle but including the

addition of 1wt% of rubrene, relative to the amount of PEG block copolymer added (red solid line). **(E)** Stability of DNPs in 10 mM Tris-HCl buffer pH 8, 37°C represented by changes to the correlation function over 48 h. As the correlation function shifts left, this indicates smaller nanoparticles. **(F)** Release of DNase 1 protein into 10 mM Tris-HCl buffer pH 8 at 37°C as measured by microBCA assay from DNPs over 8 days.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

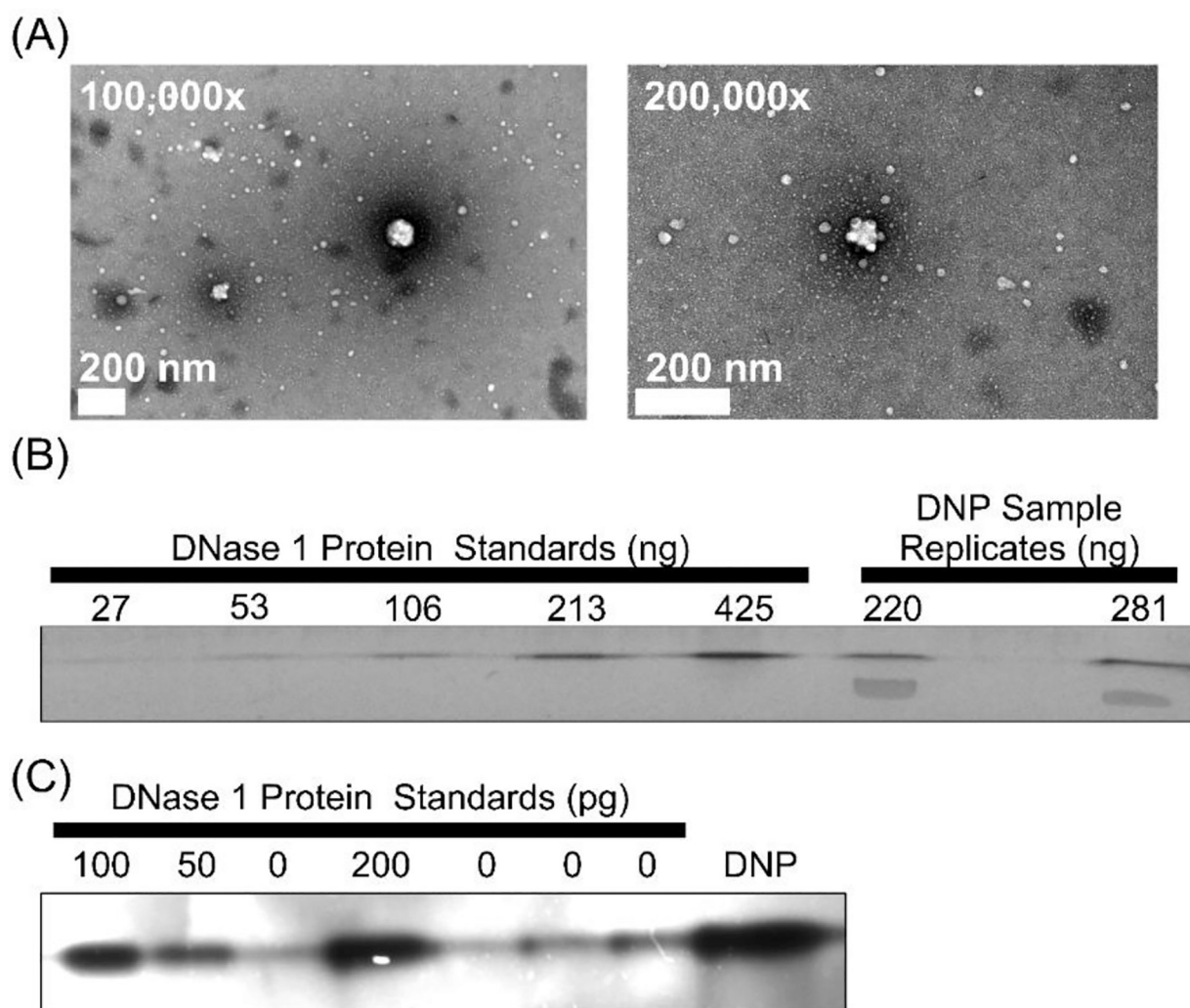


Figure 3. Characterization of the PEG-coated DNase 1 nanoparticle (DNP) and detection of DNase 1 protein and recovered activity.

(A) TEM of Rubrene-containing DNPs at 100,000x and 200,000x magnification (scale bar = 200 nm). (B) Representative protein detection of DNase 1 via silver stain. DNase 1 protein content in the nanoparticles was quantified via extrapolating from a linear regression of DNase 1 protein standards. (C) Detection of recovered DNase 1 activity in DNPs via Denatured Gel Zymography Assay.

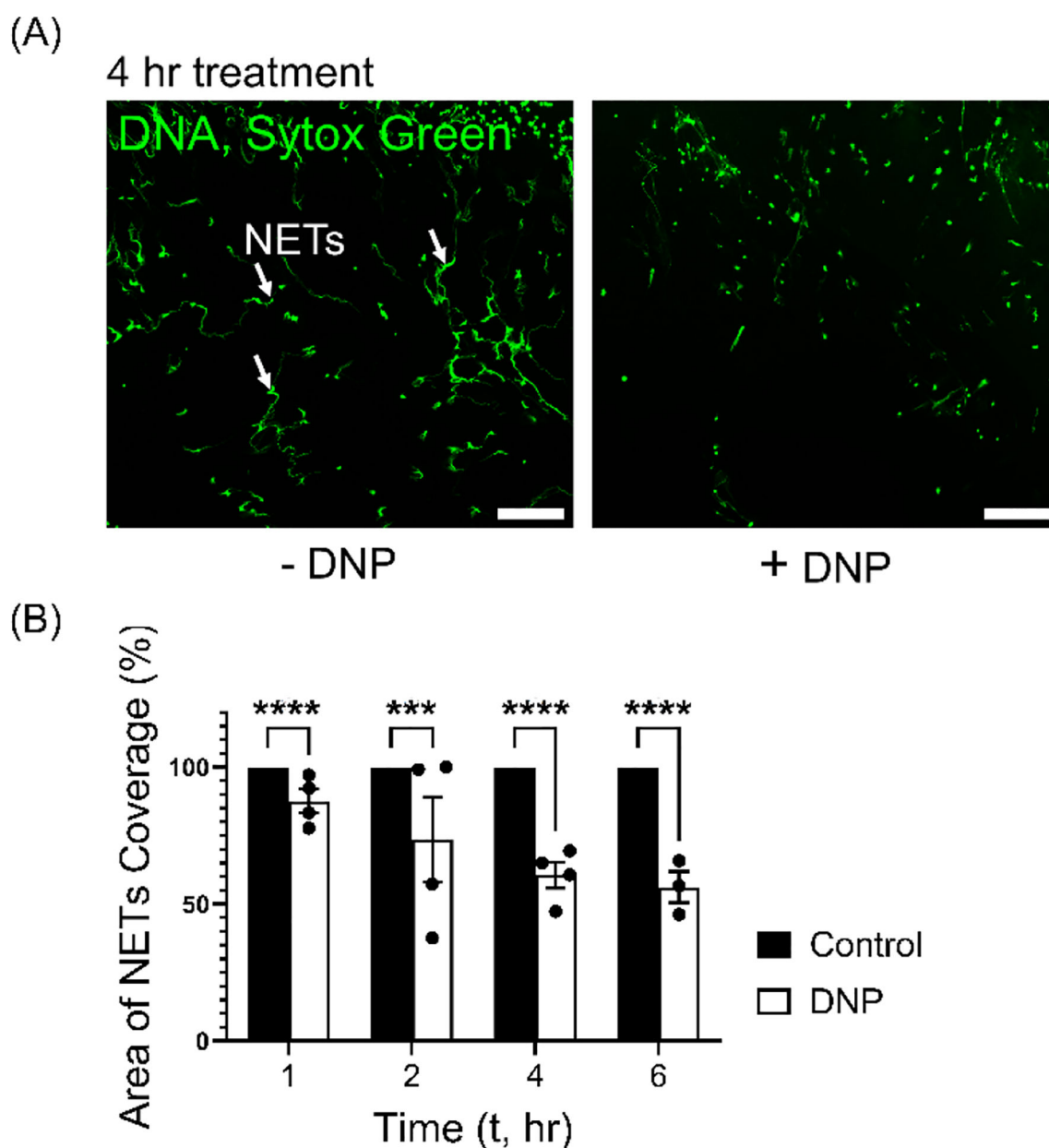


Figure 4. Degradation of Neutrophil Extracellular Traps (NETs) by DNPs.

(A) Representative fluorescence images of SYTOX-green stained NETs (Scale bar = 200μm). NETs were generated by stimulating murine bone marrow derived neutrophils with 100 nM PMA for 4 hours at 37°C with 5% CO₂ and humidity. NETs were then incubated with buffer (10 mM Tris-HCl, pH 7.8, 3mM CaCl₂, 3mM MgCl₂) (left panel) or DNPs (equivalent to 1U/mL of DNase 1 cargo) (right panel). Each condition was incubated in a time-matched manner. The reaction was stopped with 2mM EDTA. NETs were fixed with 2% PFA/1XPBS and the DNA was stained with SYTOX Green (2 μM) and imaged fluorescently. White arrows indicate NETs, which are distinguished by elongated, string-like structures, as opposed to the globular shape. **(B)** Quantification of NETs by Area coverage of NETs following treatment with DNase 1 alone or DNase 1 nanoparticles. 3–4

independent biological replicates are shown with 4–8 technical replicates per experiment. Data are presented as the mean $\pm$ the S.E.M. The ratios of area in DNP treated conditions over area in time-matched untreated conditions were analyzed using a beta-distributed generalized linear model ($F(1,22)=75.63$, $P<0.0001$). *** = $P < 0.001$, **** = $P < 0.0001$.

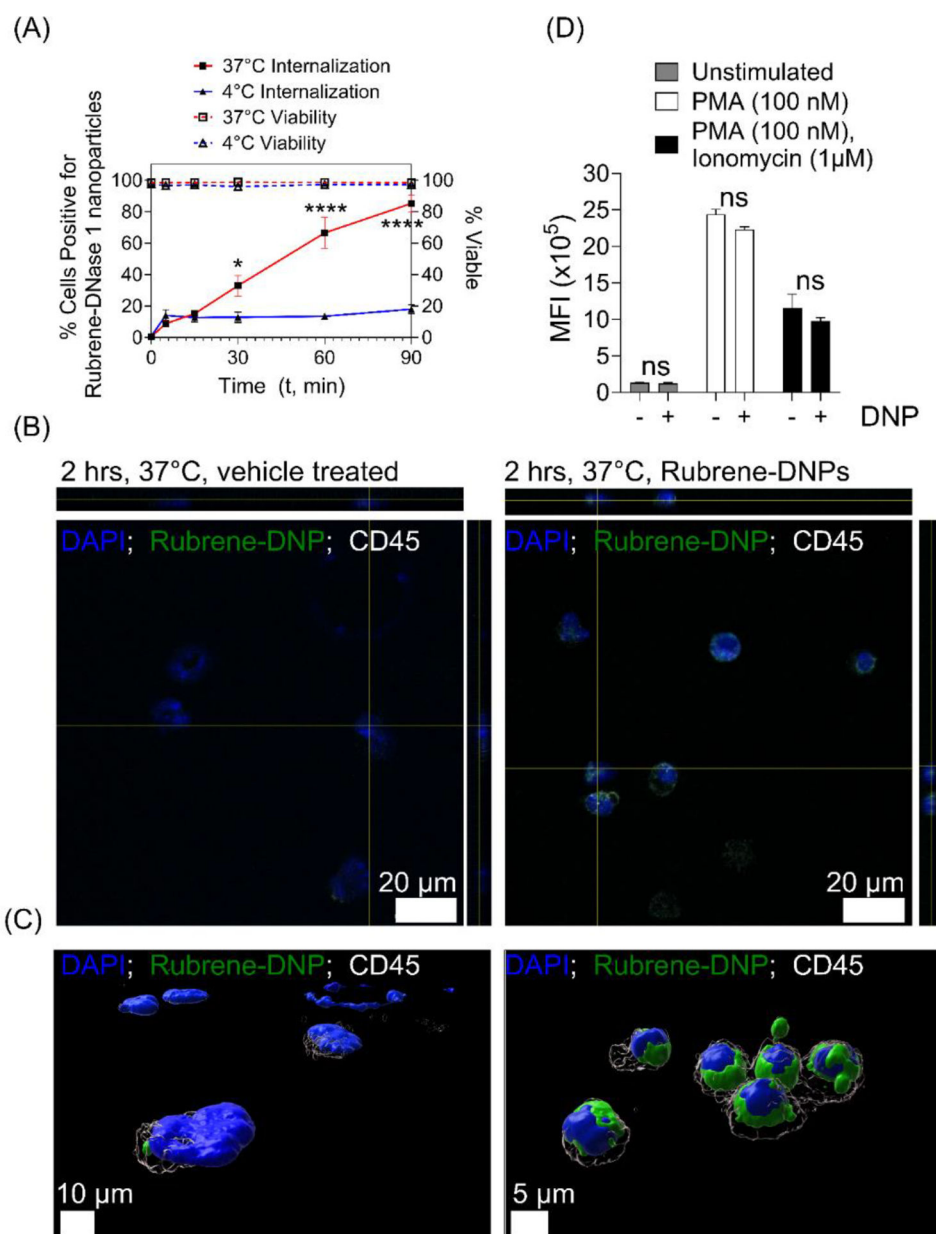


Figure 5. Internalization of rubrene-DNPs by murine bone marrow derived neutrophils. Rubrene-DNPs were incubated for up to 1.5 hr with murine bone marrow derived neutrophils with a final concentration of $\sim 0.2 \mu\text{g/mL}$ of rubrene at either 4°C or 37°C. Neutrophils were then labeled with live/dead violet stain and CD45 antibody. **(A)** Quantification of % positive cells for rubrene-DNPs (left Y-axis, solid lines) and viability of neutrophils (right y-axis, dashed lines) over 1.5 hrs at either 4°C or 37°C. Data represents $N = 3-4$ biological replicates with $N = 1-2$ technical replicates. Data is presented as the mean $\pm$ S.E.M. % Cells positive were log transformed and the interaction between time and temperature was analyzed via factorial ANOVA ($F(5,35)=10.088$, $P<0.001$, $\omega_p^2 = 0.55$), followed by Sidak multiple comparisons ($* = P < 0.05$, $**** = P < 0.0001$). **(B)** Representative confocal fluorescence microscopy images and orthogonal views of murine

neutrophils incubated for 2 hr at 37°C either in the absence or presence of rubrene-DNase 1 nanoparticles at a concentration of ~0.4 µg/mL of rubrene. The cell nucleus is shown in blue, the cell wall labelled with CD45 in white and the rubrene nanoparticles in green (scale bar = 20 µm). **(C)** 3D reconstructed images of internalized rubrene-DNase 1 nanoparticles, derived from fluorescence confocal microscopy images. The cell nucleus is shown in blue, the cell wall labelled with CD45 in grey and the rubrene-DNase1 nanoparticles in green (scale bar = 5µm in right panel, 10 µm in left panel). **(D)** Effect of internalization of DNPs on neutrophil stimulation. Murine bone marrow derived neutrophils were incubated with DNPs for 2 hr (~1U/mL), and subsequently were stimulated with PMA (100 nM), or PMA (100 nM) and ionomycin (1 µM). The oxidative burst was measured by the Abcam DHE ROS kit (Cat#Ab236206). Data represents 2 biological replicates conducted with 2 technical replicates. Data is presented as mean ± S.E.M, and was analyzed by factorial ANOVA.

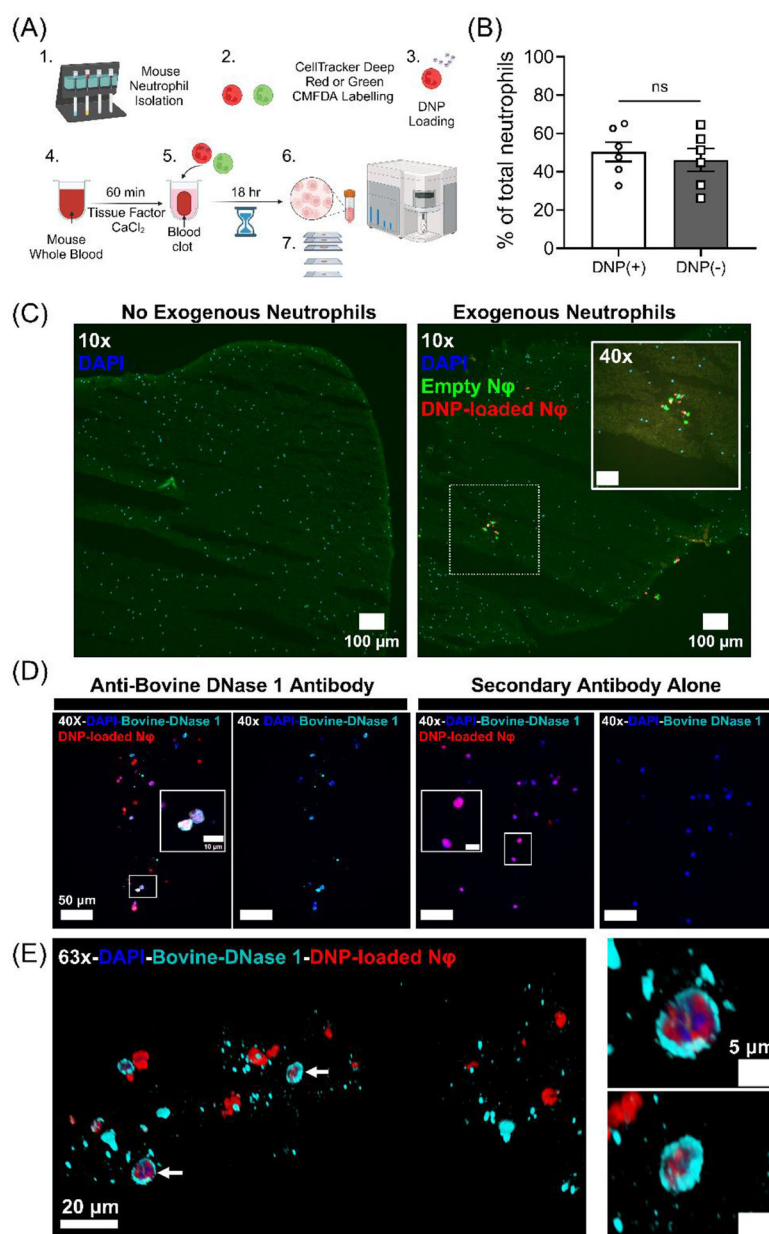


Figure 6. DNP-loaded and empty neutrophils associate with a murine blood clot *in vitro*. **(A)** Schematic overview of experimental protocol. (1) murine bone marrow derived neutrophils were isolated through magnetic enrichment. (2) BMDN ϕ s were labelled with either Cell Tracker Far Red or Green-CMFD. (3) BMDN ϕ s were incubated with DNPs (~10U/mL DNase 1) for 2 hrs at 37°C (4) murine venous blood was isolated and an *in vitro* blood clot was formed via recalcification and treatment with Tissue Factor. (5) 60 min post clot formation, exogenous DNP loaded and empty BDM ϕ s were added to the blood clot and incubated overnight. (6) The clot was then retrieved and prepared as a fixed, individual cell suspension for analysis by flow cytometry or (7) as histological slides for immunofluorescence. **(B)** Exogenous BMDN ϕ populations were detected in blood clot single cell suspensions by flow cytometry. The single cell suspension was gated to

include DAPI+ve events (cellular), then Ly6G +ve staining (N ϕ s), and then gated for positive populations of either green or red events. Data represents 6 independent biological replicates. Data is presented as the mean $\pm$ S.E.M. Data was confirmed to be normal with a Shapiro-Wilk test and the difference between the two groups was compared with a two-tailed t-test. NP internalization was not found to have any significant effect on neutrophil-clot association. **(C)** Representative fluorescent tissue sections of either clot with no exogenous BMDN ϕ s (left panel) or of a clot with exogenous empty and DNP-loaded BMDN ϕ s (right panel). A 10x image is shown (scale bar = 200 μ m) with corresponding 40x inset (scale bar = 50 μ m), for the exogenous BMDN ϕ section. **(D)** Representative fluorescent tissue sections stained with anti-bovine DNase 1 antibody and secondary antibody (left two panels), or secondary antibody alone (right two panels). Scale bar for 40x images is 50 μ m, and scale bar for insets are 10 μ m. **(E)** Representative fluorescent maximum projection z-stack 3D images captured at 63x with confocal microscopy. Images show nuclear stain (DAPI, blue), DNP-loaded neutrophils labelled with CellTracker Deep Red (Red) and anti-bovine DNase 1 antibody stain (cyan). Anti-Bovine DNase 1 antibody stain can be seen associated with these cells, and coating the exterior of DNP-loaded neutrophils, indicative of release of DNase 1 cargo. Scale bar for main image is 20 μ m, scale bar for 2 right panels is 5 μ m.

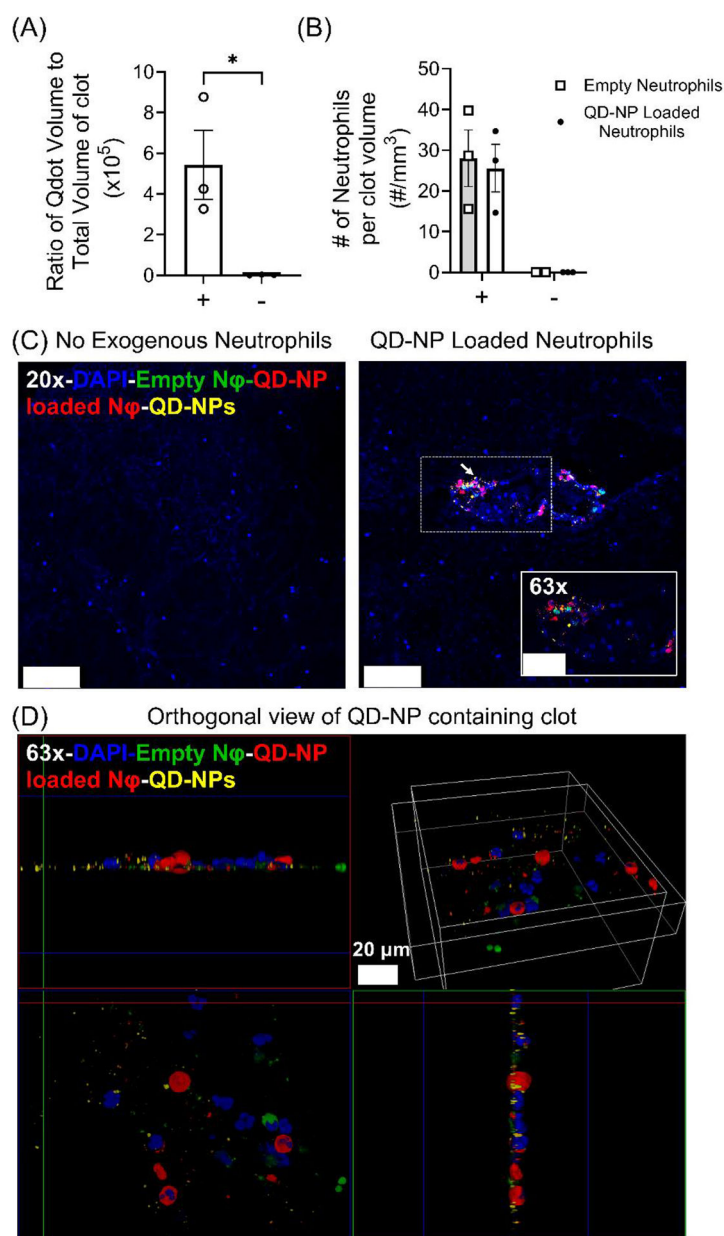


Figure 7. QD-NP loaded human Nφ associate with *in vitro* human blood clots and deliver QD-NP. QD-NP loaded human Nφ were labelled with Cell Tracker Deep Red, and empty human Nφ labelled with Cell Tracker Green CMFDA. **(A)** Quantification of QD delivery to *in vitro* human blood clots as measured by QD fluorescence volume as a ratio of total clot volume. At least 5, and up to 10 sections (each 100 μm apart) were analyzed for total area, and QD fluorescence area. Volume was determined by area under the curve. Data is expressed as means $\pm$ S.E.M, and means were compared by the Mann-Whitney test, (* = $p < 0.05$); **(B)** QD-NP-loaded and empty neutrophils associate equally with *in vitro* human blood clots. Green and Red neutrophils were manually counted across at least 5 sections (each 100 μm apart). The total number of neutrophils counted were then divided by the total clot volume in mm^3 . **(C)** Representative fluorescent images captured at 20x magnification of

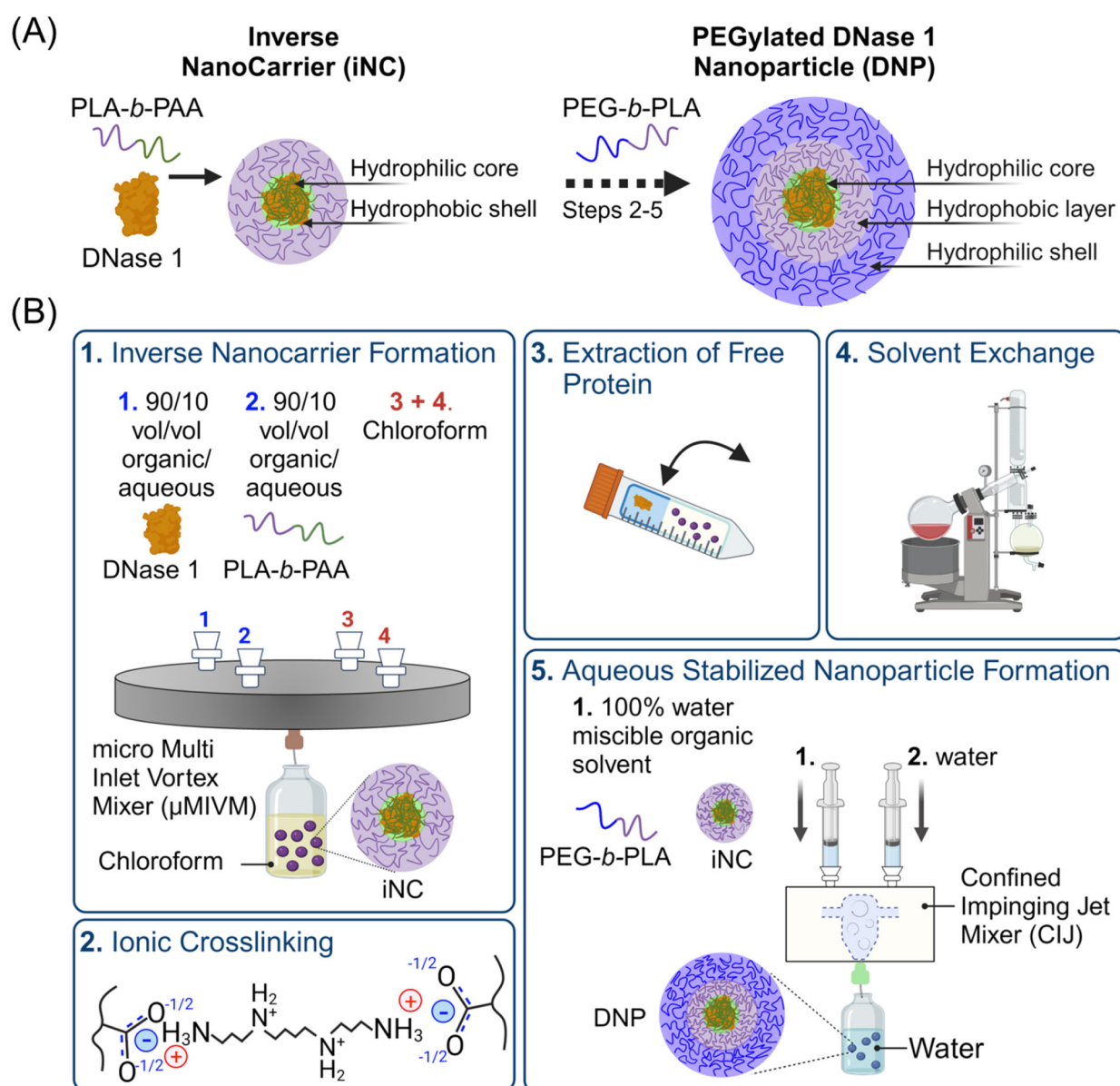
tissue sections of human *in vitro* thrombi either without exogenous neutrophils added (left panel) or containing exogenous neutrophils loaded with QD-NPs (right panel). Scale bar for 20x images is 100 μm , scale bar for 63x inset is 50 μm . **(D)** Representative orthogonal 63x image of a clot with exogenous empty (Green CMFDA-labelled) and QD-NP-loaded human N ϕ (Deep Red labelled). Two additional 6x images are shown from other slides for the clot with exogenous empty and QD-NP-loaded N ϕ . Scale bar is 20 μm .

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript



Scheme 1.

(A) Overview of the layers comprising the inverse nanocarrier and subsequently the PEG-ylated final DNase 1 nanoparticle. **(B)** Inverse Flash Nanoprecipitation processing steps.

Step 1. An amphiphilic block co-polymer and DNase-1, both dissolved in water-miscible organic solvent containing 10% aqueous solvent are rapidly mixed ($Re > 2000$) against an organic anti-solvent stream using a micro Multi Inlet Vortex Mixer (μ MIVM) to generate a stable protein-rich hydrophobic inverse nanocarrier (iNC). **Step 2.** The core of the iNC is ionically crosslinked via the use of multi-valent cationic species, either present in the quench bath or in one the non-polymer containing inlets of step 1. **Step 3.** The chloroform dispersion of inverse nanocarrier is extracted with 150 mM NaCl solution for 30 minutes, followed by centrifugation at 1000xg for 10 minutes to separate the phases. This allows for the removal of unincorporated biologic cargo. **Step 4.** The chloroform dispersion of

inverse nanocarriers is swapped to an organic water miscible solvent using repeated steps of solvent evaporation by rotary evaporator to yield ~0.5–1mL of final inverse nanocarrier dispersion. **Step 5.** The inverse nanocarrier is coated with amphiphilic PEG di-block copolymer using a confined impinging jet mixer (CIJ), where the anti-solvent is aqueous solvent, and the quench bath is also aqueous solvent, and where the volume allows only 10% maximum remaining of water miscible organic solvent. This results in a stabilized nanoparticle dispersion. Created with BioRender.

Table 1.

Dissolution of DNase 1 in organic solvent/aqueous mixtures and its residual activity.

Solvent	Organic (vol%)	CaCl ₂ (mM)	Aqueous matrix	% of Maximum Activity
DMSO	40–75	10, 20	H ₂ O	~0
	40–75	10	0.1M Tris pH 8	~0
Acetonitrile	50	10	H ₂ O	~0
	50	10	0.1M Tris pH 8	*
	50	10	H ₂ O	~0
	70	10, 20		~0
	80	10, 20		*
	50	10		16.1 ± 4.8
	70	10		*
	80	10		*
Tetrahydrofuran	80	10	0.1M Tris pH 8	*
1,4-Dioxane	50	10	H ₂ O	11.2 ± 3.8
			0.1M Tris pH 8	98.9 ± 20.5

* Protein precipitated, pre-empting measurement of DNase 1 activity.

DNase 1 was prepared to be a final concentration of 5mg/mL in these organic solvent/aqueous mixture preparations. DNase 1 activity was measured using the fluorometric DNase 1 activity assay (ab23406), and activity represented as a percentage of the activity of DNase 1 dissolved at a similar concentration but in water or 0.1M Tris pH 8 buffer and incubated for similar periods of time at room temperature (15 min). The data represents 2–5 independent assays ± standard deviation.

Table 2.
Influence of CaCl₂ on DNase 1 activity in Dioxane/aqueous mixture

Solvent	Organic (vol%)	CaCl ₂ (mM)	% of Maximum Activity
1,4-Dioxane	90	0	25.3
		4	107.8
		10	102.3
		20	0
		40	0

DNase 1 was prepared to be a final concentration of 1mg/mL in 90% 1,4-dioxane with 0.1M Tris-HCl, pH 8 as the aqueous solution (10% of final volume). DNase 1 activity was measured using the fluorometric DNase 1 activity assay (ab23406), and activity represented as a percentage of the activity of DNase 1 dissolved at a similar concentration but in water. Data represents the average of 3 technical triplicates in one assay.

Table 3.

Modeling optimal conditions for DNase 1 activity in the inverse nanocarrier core

PAA mg/mL	Spermine (mM)	TEA (M)	% Max activity	pH
50	0	0	18.90	3
	10	0	48.20	3.3
		0	39.3 ± 3.9	3.5
		0.2	38.5 ± 1.4	4
		0.35	82.2 ± 2.4	4.5
		0.5	102.2 ± 5	5
100	10	0.5	37.7 ± 0.6	4.5
200	10	0.75	89.5 ± 13.4	5

PAA is Poly-acrylic acid, TEA is triethylamine. DNase 1 activity was measured using the fluorometric DNase 1 activity assay (ab23406), and activity represented as a percentage of the activity of DNase 1 dissolved at a similar concentration but in water. pH was measured using pH strips. Data represents 1–3 independent assays ± standard deviation, where technical duplicates were performed and averaged for each assay.

Table 4.
Summary of diameter and polydispersity of DNase 1 nanoparticles

Stage of Processing	Solvent	Size (diameter, nm)	Polydispersity Index (PDI)
Inverse Nanocarrier	Chloroform	108 ± 8	0.237 ± 0.084
Post Extraction	Chloroform	87 ± 6	0.138 ± 0.067
Post Solvent Exchange	1,4-dioxane	72 ± 6	0.182 ± 0.012
PEG-Coated Nanoparticle	Water	120 ± 16	0.192 ± 0.051
Post-Dialysis in Water	Water	112 ± 13	0.192 ± 0.034
PEG-Coated Nanoparticle with Rubrene	Water	103 ± 7	0.201 ± 0.032

Data represents 20 independent batches of DNase-1 nanoparticles and 3 independent batches of rubrene-containing DNase-1 nanoparticles.

Table 5.
Properties of the PEG-coated DNase 1 Nanoparticle

Size (diameter, nm)	Polydispersity Index (PDI)	Zeta Potential (mV)
120 ± 16	0.192 ± 0.051	-13.1 ± 0.1 ^a

^a3 independent batches of DNase-1 nanoparticles.

Table 6.
Encapsulation Efficiency and Loading Capacity of the Inverse Nanocarrier and PEG-coated DNP

Encapsulation Efficiency	Loading Capacity		Final DNase 1 content (µg)
Inverse Nanocarrier	Inverse Nanocarrier	PEG-coated DNP	PEG-coated DNP
96.4 ± 5.5 ^a	3.7 ± 0.3% ^c	1.1 ± 0.3% ^d	169 ± 69 ^e
94.2 ± 1.1 ^b			

^aDetermined by protein absorbance measurements of the extract at A280nm (N = 3 separate formulations, ± standard deviation).

^bDetermined by micro-BCA assay of the extract (N = 3 separate formulations, ± standard deviation).

^cQuantified by thermogravimetric analysis (TGA) of the inverse nanocarrier and micro-BCA assay (N = 3–4 separate formulations, mean ± standard deviation).

^dQuantified by calculating the percentage of the DNase 1 content of the PEG-coated DNP dispersed in water determined by silver staining (See Fig. 4B), relative to the total mass of the same DNP as measured by TGA. (N = 5 separate formulations, mean ± standard deviation).

^eData represents the mean of 13 independent formulations of DNP ± standard deviation. DNase 1 content quantified by silver staining.