Inhibition of high shear arterial thrombosis by charged nanoparticles

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(Received 8 February 2018; accepted 7 May 2018; published online 29 May 2018)

Platelet accumulation under high shear rates at the site of atherosclerotic plaque rupture leads to myocardial infarction and stroke. Current antiplatelet therapies remain ineffective within a large percentage of the population, while presenting significant risks for bleeding. We explore a novel way to inhibit arterial thrombus formation by biophysical means without the use of platelet inactivating drugs. Our computational multi-scale dynamics model has predicted that charged particles of a specific size may entangle von Willebrand Factor (vWF) polymers and reduce the amount of elongation at high shear rates. We tested this hypothesis experimentally for negatively charged nanoparticles (CNP) to inhibit arterial thrombus formation. CNP of a particular size and charge inhibited thrombus formation, with a 10-fold peak inhibition over control conditions of thrombotic occlusion. Particles of differing material composition, size, and charge had little effect as predicted by computational studies. Surprisingly, the dose response curve was not sigmoidal, but exhibited a peak at 1.5 CNP:vWF proteins, which was not predicted by the model. This study describes a new antithrombotic agent that may have a different mechanism of action than current pharmaceutical therapies. *Published by AIP Publishing.* <https://doi.org/10.1063/1.5025349>

INTRODUCTION

Three factors are essential to arterial occlusive thrombosis: (1) high-shear flow, (2) a pro-thrombogenic surface (collagen), and (3) blood components of von Willebrand Factor (vWF) and platelets.¹ Under high shear flows, vWF unwinds from a shielded globular structure to a fibrillar structure with increased bond availability to exposed subendothelial collagens.^{2,3} Once bound to the arterial surface, vWF captures and tethers platelets initially by binding Glycoprotein Ib (GPIb), which is followed by platelet activation under high shear.⁴ More vWF is released from the platelet, and the platelet develops a stronger bond with vWF via $\alpha_{IIb}\beta_3$ binding.⁵ A stable platelet thrombus then begins to rapidly form, leading to serious embolic risks that could lead to myocardial infarction or stroke.⁶ Interfering with any of these steps in the thrombotic cascade may significantly reduce the risk of atherothrombosis from occurring.

Antiplatelet therapies, such as aspirin (ASA) and Plavix[®] (Clopidogrel), have been developed to inhibit platelet activation or binding (Fig. 1). ASA acts by irreversibly inhibiting COX-1, interrupting thromboxane (TXA₂) action that normally induces platelet aggregates.⁷ Clopidogrel is designed to irreversibly inhibit the P2Y₁₂ ADP receptor on platelets.⁸ However, these drugs do not work as intended for a large percentage of the population, as 5%–50% of patients exhibit an “antiplatelet resistance” under recommended drug doses.^{9–11} Other therapies, such as Abciximab, Eptifibatide, and PAR-1 antagonists, aim to inhibit platelet interaction with

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FIG. 1. Depiction of antiplatelet mechanisms of action for irreversible inactivation (ASA and Clopidogrel) or protein receptor inhibition (Abciximab and PAR-1 antagonists).

proteins such as vWF and thrombin. However, these drugs were found to not reduce the rates of mortality in patients.^{12,13} The observed resistances to leading antiplatelet agents proves the need to research and develop novel therapeutic agents. Recent efforts have been made to engineer nanoparticles for enhanced drug delivery for inhibition of thrombosis in those individuals that are resistant to therapy.¹⁴ ASA, eptifibatide, tissue Plasminogen Activator (tPA), and D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) conjugated nanoparticles are four of the most recently investigated ones for thrombotic inhibition.^{15–19} However, it is known that biochemical inactivation of platelets may lead to severe bleeding complications, while anti-coagulants do not attack the central mechanism of arterial clotting at all.^{20,21} Instead, prevention of the physical interactions between the main contributors of arterial clotting may provide a more effective and safe method of anti-thrombotic therapy.

In a study investigating the effects of environmentally toxic nanoparticles on the cardiovascular system, Nemmar *et al.* suggests a particle surface-dependent effect on venous clotting in a hamster.²² In their study, a reduction in the transmitted light intensity of a thrombus in the hamster femoral vein was observed following the intravenous injection of 60 nm polystyrene (PS) particles with carboxyl modification, while amine-modified and unmodified particles provided prothrombotic and negligible responses, respectively. However, these effects on hemostatic processes were only explored in venous flows. This present study has extended this concept of nanoparticle effects on blood clotting at low shear rates present in veins to evaluate the effects of carboxyl-modified charged nanoparticles (CNP) on arterial thrombosis in high shear rate flow. We hypothesize that the negatively charged CNP will reduce arterial thrombosis via physical interactions with either vWF or platelets under shear flow, as opposed to biochemical inhibition by current pharmaceutical antiplatelet agents. To test this hypothesis, we make use of the lattice-Boltzmann (LB) method with a Langevin dynamics (LD) approach to computationally assess the physical interaction between vWF and CNP under pathophysiological flows.^{23,24} Furthermore, we make use of a custom arterial thrombosis microfluidic assay mimicking pathophysiological flows to assess the effects of PS-CNP and a similarly charged biodegradable poly(lactic-co-glycolic acid) (PLGA) CNP on thrombotic inhibition.²⁵

METHODS

Computational modeling

We developed and validated the application of the lattice-Boltzmann (LB) method for direct numerical simulation (DNS) of the dense suspension of particles in viscous flow,^{26–29}

deformable capsules,^{30,31} and cellular blood flow.³² The LB method is highly scalable for parallel processing and enables complex flow simulations. However, the sizes of CNP (60 nm) and vWF dimers (~120 nm) are too small to resolve with the LB lattice for direct simulation. Therefore, a multiscale computation approach has been developed where the CNP and vWF are modeled with a Langevin dynamics (LD) approach fully integrated and coupled with the LB method (LB-LD).^{23,24} The vWF is modeled as a chain of N beads (each representing a dimer) as done in many previous studies.^{33–37} The total force on the particle or bead in LD consists of hydrodynamic, Brownian, and non-hydrodynamic forces which include any external body forces, such as the Lennard-Jones (LJ) potential (employed for long-range attraction and short-range repulsive force between beads) and electrostatic forces. The electrostatic force is modeled by a Coulomb potential as an effective pair potential that describes the interaction between two point-charges. The connectivity force between neighboring vWF beads is modeled through a finitely extensible nonlinear elastic (FENE) spring potential, U_s .^{36,38} The same LD approach is applied to charged nanoscale particles with $U_s = 0$. The LD method for modeling the vWF and CNP has been extensively validated including modeling the vWF transition from the globular to elongated phase at the critical shear rate.^{2,23,24,35,36}

As reported,³⁹ electrostatic interactions with vWF are induced because of large patches of positive charge at the A1 interface. Although the electrostatic charge is not constant, it can be estimated to be about on average 6 to 10 kT/e (150 to 250 mV).⁴⁰ In our simulations, we take an average of +8 kT/e for the A1 domain implemented as a point charge in each dimer. Among all domains in vWF, only A1 is strongly positively charged at physiological pH and therefore will orient toward the nearby negatively charged CNP more than other domains, similar to negatively charged GPIb on the platelet membrane.⁴¹ So, for negatively charged CNP and vWF bead interactions, there is an additional net attraction effect from electrostatic force. Modeling the charge of the A1 domain as a point particle in each dimer is an assumption that requires further experimental verification. The average charge of CNP for the simulations is -50 mV.⁴²

CNP acquisition, synthesis and characterization

Both PS and PLGA CNP were utilized in this study. 60 nm and 510 nm PS particles with carboxyl surface modification were purchased from a supplier at a concentration of 10 mg/ml (Bangs Laboratories). Similar PS-CNP have been utilized in venous flow experiments.²² Biodegradable PLGA-CNP of a similar size and surface charge were fabricated in house by nanoprecipitation, similar to previously published methods.²⁵ 100 mg of RG503H Resomer® (Sigma Aldrich) was dissolved in an 85:15 acetone to ethanol mixture. The dissolution was performed over 5 min at 150g stirring. Ultrapure water was added with continued stirring at 150g for 3 h to create a final PLGA-CNP concentration of 10 mg/ml. Neutral PLGA-Polyethylene Glycol (PEG) particles were also fabricated by addition of a 20% PEG (1500 MW) solution rather than ultrapure water.

PS-CNP and PLGA-CNP were stored at 4 °C until needed for characterization or whole blood treatment. Prior to use, the CNP were sonicated for 15 min and vortexed for 10 s to evenly disperse with minimal agglomeration. Characterization of the average diameter and zeta potential was performed by addition of 100 μ l of the 10 mg/ml CNP mixture to 900 μ l of deionized water for a final test concentration of 1 mg/ml. Diameter was measured by dynamic light scattering, while zeta potential was measured by photon correlation spectroscopy (ZetaSizer Nano-ZA, Malvern Instruments).

Blood collection and treatment

Porcine whole blood was obtained from a local abattoir (Holifield Farms, Covington, GA) and lightly heparinized at 3.5 USP units/ml, as previously described by Para *et al.*⁶ Blood was stored at room temperature on a rocker prior to testing. All testing was completed within 8 h after collection.

Blood samples were treated with varying concentrations of the PS-CNP and PLGA-CNP immediately before the flow assay. Estimations of the appropriate dose were based on the reported effective value of 500 μ g/kg body weight in the hamster model.²² Assuming an average body weight of 100 g and 7 ml of blood in the systemic circulation of the animal, the

systemic particle concentration for a 500 $\mu\text{g}/\text{kg}$ dose is approximately 7 $\mu\text{g}/\text{ml}$ blood.⁴³ The concentration of 7 $\mu\text{g}/\text{ml}$ was treated as the baseline concentration, with multiples of this concentration of 14, 21, 28, 35, and 70 $\mu\text{g}/\text{ml}$ also investigated for PS-CNP. The weight concentrations were converted to a stoichiometric CNP:vWF ratio, given the weight of an individual CNP and an approximated vWF molecular concentration of $1.4 \times 10^{11}/\text{ml}$ (assuming a mass concentration of 5 $\mu\text{g}/\text{ml}$). Equivalent CNP:vWF ratios were not investigated between 60 and 510 nm particles due to the large amount of PS necessary in the 510 nm case to match the particle concentration of the 60 nm group. Stoichiometric concentrations of PLGA-CNP were selected based on results of the PS-CNP investigation.

Microfluidic thrombosis assay

Microfluidic chips were created by casting Polydimethylsiloxane (PDMS) (Sylgard 184, Krayden) on a custom-etched silicon 3D stenotic mold with the channel geometry illustrated in Fig. 2. After plasma bonding the PDMS to a 25×75 mm glass slide, the devices were coated with type I fibrillar collagen (Chronopar, Chronolog, Inc.). The fibrillar coating method was detailed previously by Casa *et al.*, where microfluidic channels were filled with a 100 $\mu\text{g}/\text{ml}$ collagen type I solution in 0.9% saline and incubated in a humid environment at room temperature for 24 h.⁴⁴ The collagen-coated microfluidic chips were positioned on the stage of a light microscope (DM6000B, Leica Microsystems) with a $4\times$ objective and connected to an upstream reservoir with Tygon tubing, similar to that previously described.⁴⁴ Downstream tubing led to four discharge reservoirs, each placed on a precision balance (Ohaus Scout SPX222, Ohaus Corp) to measure total mass discharge. Flow was gravity fed with an initial wall shear rate of 6500 s^{-1} . Images of thrombus formation were acquired every 500 ms with a high-resolution CCD camera (Pixelfly, PCO). Image acquisition was facilitated by the $\mu\text{Manager}$ open-source microscopy software.⁴⁵ Occlusion time, t_{occ} , was measured as the time from first blood contact in the stenosis region of the channel to the time of the initial maximum mass reading. The average and standard deviation of t_{occ} was calculated for each concentration of PS-CNP and PLGA-CNP. Statistical analysis was performed between groups with a t-test (significance at a p-value < 0.01).

RESULTS

Computational vWF-CNP interaction

The conformational dynamics and motion of vWF proteins were simulated using the LB-LD method outlined above. The natural vWF exhibits periodic stretching, tumbling, and folding

FIG. 2. (Left) Schematic of the overall channel geometry with a single-inlet and dual-bifurcation to a four-outlet system. The four-channel system allows for immediate test replicates of each CNP treatment group. The inset describes the important dimensional characteristics of the microfluidic system, with a 250 μm nominal channel height leading to an 800 μm long stenosis region of 70 μm height. The width of each stenotic channel is 475 μm . (Right) High shear thrombosis experiment with single-inlet (blocked by objective) and four-outlet (three visible) tubes leading to mass balances (not shown).

FIG. 3. vWF (blue) without CNP in a pure shear flow (6500 s^{-1}) with arrows indicating the fluid direction. The two pictures illustrate two different time points for vWF in high shear flow. vWF undergoes dynamic elongation and folding and does not remain in a stable globular shape under high shear as indicated by the two screenshots above. Note that the elongation of vWF by high shear rates has been demonstrated to be less than 1 ms by Fu⁴¹ and estimated at less than $10 \mu\text{s}$ by Wellings and Ku.³

events under linear shear flow (Fig. 3). Without the CNP, the vWF re-elongates frequently, going through periodic cycles of stretching and relaxation. The patterns of elongation in Fig. 3 are representative of the elongation and folding model described previously by Schneider *et al.*² These patterns form independently of initial conditions.

Electrostatic attraction by charged particles were introduced to counteract the elongation of vWF from drag forces of high shear stress. The vWF-CNP interaction simulations demonstrate that CNP have a demonstrable effect on conformation of vWF under shear flow conditions. In the simulation, there are 20 CNP and 10 vWF proteins each consisting of 50 dimers. A ratio of two CNPs available per vWF filament is chosen to match the relative CNP:vWF concentration used in experiments. The addition of negatively charged CNP radically altered the behavior of the vWF in high shear flows.

Figure 4 illustrates the interaction of CNP (red) and vWF (blue) in a constant shear flow of 6500 s^{-1} . The elongated vWF wraps the charged CNP into the interior of the coiled form, stabilizing this conformation. When the simulation reaches dynamic equilibrium, each coiled vWF (6 out of 10 in the bottom snapshot, 2 strands have self-associated) contains one or more CNP. The vWF-CNP compound maintains the globular conformation and does not elongate again at this shear rate. The vWF-CNP effective length, i.e., the projected length in flow direction, drops by more than 2/3 to less than 30% of its maximum length, suggesting a significant drop in capture power of vWF or a lengthening of occlusion time when CNP are mixed in blood. The failure of vWF-CNP to elongate would be expected to severely reduce the ability of vWF to capture platelets under high shear rate conditions.

The computational simulation was then used to assess the importance of charge sign. The model predicts that CNP with a neutral or positive charge would not hinder the elongation of vWF nor would induce a preferred globular form of vWF. Only the negatively charged nanoparticle is predicted to have an effect on vWF elongation and, subsequently, less thrombosis.

FIG. 4. vWF (blue) immersed in experimental concentrations of CNP (red) in shear flow (6500 s^{-1}) with arrows indicating the fluid direction. The top picture is at the beginning of the simulation and the bottom picture shows the vWF after time. vWF polymers fold back into globular form (dense balls) after interaction with CNP and remain in the globular conformation under the same shear rate.

FIG. 5. Experimental results comparing the control (untreated, top two lanes) whole blood condition and the addition of 60 nm PS-CNP at a concentration of 1.75 CNP:vWF of blood (bottom two lanes). Each time point includes two side-by-side channels from the bifurcation as they run to full occlusion, with flow from left to right in each image. The comparison indicates that full occlusion occurs between 120 and 180 s for the control group, while the PS-CNP inhibit the rate of thrombus formation with no occlusion at 180 s.

EXPERIMENTAL FORMATION OF THROMBOSIS WITH CHARGED NANOPARTICLES

Polystyrene charged nanoparticles (PS-CNP)

Whole blood was then subjected to a high shear rate environment of 6500 s^{-1} with a fibrillar collagen coated surface in a microfluidics system previously described. This system of flowing porcine blood uses a minimal amount of heparin and no citrate to preserve native vWF and platelets and create occlusive thrombus with high consistency by gravity. The thrombosis experiments in the stenotic microfluidic chip were performed for concentrations of 0 to 4.25 CNP:vWF of 60 nm PS-CNP and 0 to 3.5×10^{-3} CNP:vWF of 510 nm PS-CNP, which represents equivalent mass concentrations.

Images acquired during the duration of the experiment made it possible to quantify the level of platelet accumulation (Fig. 5). Significant differences in the amount of light transmitted between the control and PS-CNP (60 nm) groups were found at time points of 90, 120, and 180 s (Fig. 6). The control group occluded quickly ($t_{\text{occ}} = 133 \text{ s}$), by forming rapid platelet accumulation (RPA). In contrast, the PS-CNP treated group remained in the initial platelet adhesion phase (lag phase), with accumulation occurring at a much slower rate than normal.

The 60 nm PS-CNP were found to have a narrow efficacy range in their ability to inhibit thrombus. The peak concentration was found to be between 1.0 and 2.0 CNP:vWF and with an approximately 5-fold increase in occlusion time compared to the control group (Fig. 7). However, the effect of the PS-CNP at 60 nm began to diminish as the concentration was increased beyond the 2.0 CNP:vWF.

FIG. 6. The 60 nm CNP significantly retarded thrombus formation as quantified by light transmittance. The overall light transmittance in the channels was found to be significantly different between the control and PS-CNP at times of 90, 120, and 180 s (*p-value < 0.01).

FIG. 7. Occlusion time dose response of 60 and 510 nm PS-CNP in the arterial thrombosis microfluidic assay. 60 nm PS-CNP had a narrow dose response, with a maximum effective concentration between 1.0 and 2.0 CNP:vWF. The 510 nm PS-CNP showed a smaller effect on occlusion time at similar mass concentrations, but at much smaller CNP:vWF ratios. Effectiveness increased linearly with concentration over the range studied. (Note that higher doses of 510 nm particles would be very expensive.) Statistical significance was determined from the treated group compared to the untreated (0 CNP:vWF) group (**p*-value < 0.01; *n* = 4 to 32).

A different behavior was found for the 510 nm PS-CNP group, as the effective inhibition increased linearly with dose (Fig. 7). Due to maintaining a low CNP:vWF ratio compared to the computational simulations and 60 nm PS-CNP experiments (although at comparable mass concentrations), a milder effect on *in vitro* thrombosis was observed. While the observed delay in occlusion continues to increase with increasing dose, a significantly higher mass concentration of CNP would be needed to attain the appropriate stoichiometric dose. Therefore, administration of smaller CNP (60 vs. 510 nm) will yield relevant CNP levels for peak effects at smaller mass concentrations.

Biodegradable PLGA-CNP

Biodegradable nanoparticles allow us to explore whether the base material is important and whether a more biocompatible particle has a similar therapeutic effect. A range of concentrations of PLGA-CNP were studied based on the peak CNP:vWF ratio observed in the 60 nm PS-CNP experiments. From those findings, the peak effective concentration of the PS-CNP is a CNP:vWF ratio of 1.0 to 2.0. The concentrations of PLGA-CNP tested in the arterial thrombosis assay was then varied around this space, with eight calculated ratios between 1.0 and 5.0, five of which were between 1.0 and 2.0 CNP:vWF.

The results of the arterial thrombosis assay indicated a similar peak in this range of CNP concentrations, with a maximum effective concentration found at approximately 1.5 PLGA-CNP:vWF (Fig. 8). At this concentration, the occlusion time was delayed by an even greater **10-fold** from the control case. However, this effect diminished to nearly baseline occlusion times at concentrations outside the optimal ratio similar to the 60 nm PS-CNP. The neutrally charged PLGA-PEG condition was found to have no significant antithrombotic effect.

Nanoparticle characterization

PS-CNP and PLGA-CNP characterization of the hydrodynamic diameter (D_{CNP}) and zeta potential (ZP_{CNP}) was performed with the use of a ZetaSizer instrument (ZetaSizer Nano-ZA, Malvern Instruments). The purchased PS-CNP were within the manufacturer's range of size distributions. While the desired PLGA-CNP size was on the order of 80 nm, multiple syntheses of bulk PLGA led to values similar to those listed in Table I, with the batch of 155.0 nm and -32.1 mV utilized in the thrombosis assay. PLGA-PEG particles were slightly larger and with near-zero zeta potential.

DISCUSSION

Computational simulations of proteins in shear flow are formed using the LB-LD method with an electrostatic charge model of vWF.^{23,24} Addition of CNP alters the vWF tertiary

FIG. 8. Experimental results comparing the effects of PS-CNP (60 nm), PLGA-CNP (155 nm), PLGA-PEG (230 nm), and volumetric equivalents of saline on thrombotic occlusion time inhibition with respect to the ratio of CNP:vWF. A similar peak dose response was observed in PLGA-CNP and PS-CNP at approximately 1.5 CNP:vWF. A dose of PLGA-PEG approximately at this ratio was found to have no significant effect over the saline control. All CNP groups were found to be significantly different from the non-treated control group and saline groups at the same ratio (p -value < 0.01 ; $n = 4$ to 32).

unfolding in the model, suggesting a possible change in thrombogenicity at high shear rates. We tested this prediction and demonstrated that negatively charged particles of a particular size and charge density have a profound effect on occlusion time by shear induced platelet aggregation.

Wellings and Ku estimated that >100 GPIb-vWF-A1 bonds acting simultaneously on the surface of platelets are necessary to strengthen and stabilize platelet adhesion at a shear rate of 6500 s^{-1} .³ Thus, the alteration of vWF length should have a major effect on the rapid accumulation of platelets and the length of time needed to create occlusive thrombus.

The A1 domain of vWF molecules has positively charged amino-acid residues that control the tertiary structure of the polymer into a globular form or an elongated form under high shear stress. We have predicted that the negative surface charge of the CNP may prevent shear induced unfolding in a computational model based on first-principles of physics. This mechanistic hypothesis of vWF-CNP interaction was then demonstrated in a whole blood assay of arterial-type thrombosis where vWF and billions of platelets can accumulate to hemodynamic occlusion.

The experiments show a specific optimal stoichiometric ratio between CNP and vWF. This same ratio was used in the LB-LD computational model showing a significant change in the conformation of the vWF under the same average experimental shear rate. CNP do not exhibit a typical sigmoid pharmaceutical dose-response curve in that higher concentrations do not continue the response. Instead, the 1.5–2.0 CNP:vWF ratio appears to balance the electrostatic and drag forces at a wall shear rate of $>6500 \text{ s}^{-1}$. This peak response curve in the experiments was not predicted by considering only the vWF and CNP in the computational model, and thus, a second mechanism may be at play that is yet undiscovered. In addition to direct visualization experiments, we plan to include platelets and red blood cells in future computational analyses to better understand the experimental observations.

TABLE I. Quantified characteristics of CNP utilized in the whole blood assay.

	Diameter _{CNP} (nm)	Zeta Potential _{CNP} (mV)
PS-CNP (60)	80.0	−48.0
PS-CNP (510)	514	−45.0
PLGA-CNP	155.0	−32.1
PLGA-PEG	230.0	−0.1

The two major factors in arterial thrombosis, vWF and platelets, may be likely targets for CNP interaction. While an interesting stoichiometric ratio has been found between vWF and CNP, it is also worth computationally and experimentally exploring the effects of CNP on arterial thrombosis in the context of a CNP and platelet interaction. Platelets circulate in the blood at an approximate concentration of $1.0 \times 10^9/\text{ml}$. Therefore, the stoichiometric ratio for platelets with respect to the maximum effective concentration of CNP is approximately 200 CNP per platelet.

CONCLUSIONS

This study describes the use of computational models and microfluidic assays to predict a novel therapeutic approach to modulating thrombosis at high shear rates common in heart attacks and strokes. Using physics models of vWF, we can predict that electrostatic interactions within the molecule can be altered by exogenous particles of a particular charge, size, and shape. We then tested this hypothesis on a validated microfluidics assay of thrombus formation under similar high shear hemodynamics with whole blood. The resulting effect was found to delay the development of an occlusive thrombus 10-fold without the introduction of platelet inactivating drugs. Further mechanistic and *in vivo* exploration into non-functionalized CNP may lead to a new class of antithrombotic agents useful for the prevention of myocardial infarction and stroke.

SUPPLEMENTARY MATERIAL

See [supplementary material](#) for further explanation of the computational method.

ACKNOWLEDGMENTS

The authors would like to acknowledge the contributions of Christopher Bresette and Courtney Smith for assistance in data collection. This work was performed in part at the Georgia Tech Institute for Electronics and Nanotechnology, a member of the National Nanotechnology Coordinated Infrastructure, which was supported by the National Science Foundation (Grant No. ECCS-1542174). M.T.G. was supported by the ARCS Scholar Award. C.K.A. acknowledges the computational grant (CTS100012) for use of the Extreme Science and Engineering Discovery Environment (XSEDE), which was supported by National Science Foundation Grant No. ACI-1548562.

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