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Development of biomimetic antimicrobial platelet-like particles comprised of microgel nanogold composites

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Abstract

A blood clot is formed in response to bleeding by platelet aggregation and adherence to fibrin fibers. Platelets contract over time, stabilizing the clot, which contributes to wound healing. We have developed platelet-like particles (PLPs) that augment clotting and induce clot retraction by mimicking the fibrin-binding capabilities and morphology of native platelets. Wound repair following hemostasis can be complicated by infection; therefore, we aim to augment wound healing by combining PLPs with antimicrobial gold to develop nanogold composites (NGCs). PLPs were synthesized with N-isopropylacrylamide (NIPAm)/co-acrylic acid in a precipitation polymerization reaction and conjugated to a fibrin-specific antibody. Two methods were employed to create NGCs: 1) noncovalent swelling with aqueous gold nanospheres, and 2) covalent seeding and growth. Since the ability of PLPs to mimic platelet morphology and clot retraction requires a high degree of particle deformability, we investigated how PLPs created from NGCs affected these properties. Cryogenic Scanning Electron Microscopy (cryoSEM) and atomic force microscopy (AFM) demonstrated that particle deformability, platelet-mimetic morphology and clot retraction were maintained in NGC-based PLPs. The effect of NGCs on bacterial adhesion and growth was assessed with antimicrobial assays. These results demonstrate NGCs fabricated through noncovalent and covalent methods retain deformability necessary for clot collapse and exhibit some antimicrobial potential. Therefore, NGCs are promising materials for preventing hemorrhage and infection following trauma.

Lay Summary:

Following injury, a blood clot is formed by platelets aggregating and binding to fibrin fibers. Platelets contract over time, stabilizing the clot, which contributes to wound healing. We have developed platelet-like particles (PLPs) that enhance clotting and stimulate clot retraction by

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Supporting Information

Supporting Information is available online or from the author.

mimicking the fibrin binding capabilities and morphology of native platelets. Wound repair following hemostasis can be complicated by infection; therefore, we aim to amplify wound healing by combining PLPs with antimicrobial gold to develop nanogold composites (NGCs). These NGC PLPs mimic platelet morphology, generate clot retraction, demonstrate some antimicrobial potential, and are promising materials for preventing blood loss and infection following trauma. Future work will include exploring the application of these particles to treat hemorrhage and infection following traumatic injury.

Keywords

Synthetic platelet; fibrin; hemostatic material; trauma; antimicrobial material; microgels

Introduction

Traumatic hemorrhage remains a significant clinical problem despite decades of research. According to the CDC, injury is the leading cause of death for men and women between the ages of 1 and 44 [1], and many trauma victims often exsanguinate before reaching the hospital. Even in patients who achieve hemostasis, subsequent wound repair can be impeded by several complicating factors including an increased risk of infection [2–4], which can be fatal. Native platelets play a critical role in initial hemostasis, modulation of immune responses, and healing following trauma [5]. In a healthy body, injury initiates coagulation to stop bleeding by forming a platelet-rich fibrin clot. This is achieved immediately following injury, when platelets activate, aggregate, bind to fibrin fibers through $\alpha_{IIb}\beta_3$ integrins and augment fibrin formation. Platelets then spread within the fibrin network and actively modify network properties over time by retracting the clot and increasing fibrin density. Platelet-mediated clot retraction stabilizes the fibrin network and contributes to wound healing outcomes [5–7]. Platelets also contribute to healing following hemostasis via their role in the body's innate immune response to pathogens: platelets are involved in several mechanisms that comprise the first line of defense against pathogens, including the release of antimicrobial peptides upon binding of bacteria to platelet surface receptors [8,9]. Here, we use platelets as inspiration to create injectable biomimetic hemostatic materials that stop hemorrhage, fight infection, and promote wound healing following injury. In particular, we describe the creation of platelet-like particles (PLPs) that bind to fibrin, induce clot retraction, and possess antimicrobial activity.

Due to platelets' role in hemostasis, there has been interest in mimicking their biological properties with synthetic particles. These typically comprise a nanoparticle platform surface-decorated with ligands that interact with various components of the coagulation system [10]. These particles recreate specific biological characteristics of platelets, including targeting to injury and disease sites and/or facilitating platelet aggregation and adhesion under flow to augment coagulation [11–13]. Other injectable hemostatic nanoparticle materials that augment coagulation have been described, such as injectable, shear-thinning composite hydrogels containing gelatin and silicate nanoplatelets and self-propelling nanoparticles loaded with procoagulants [14–16].

We have previously described the development of fibrin-binding microgel-based platelet mimetic particles to augment hemostasis. Our specific design employs a deformable micron-sized hydrogel coupled to a fibrin-binding motif to target the wound environment. This allows us to mimic natural platelet functions to bind the wound site, stabilize and enhance clot formation, and subsequently stimulate clot retraction [17]. Clot retraction is an important feature for clot stability and wound repair. The clot retraction feature of our PLPs is the result of the high degree of microgel deformability and high fibrin-binding ability imparted by a fibrin binding antibody. If particle deformability is decreased by increasing particle crosslinking, or in the absence of fibrin binding antibody, this clot retraction feature is lost [18]. In this project, we aim to combine PLPs with antimicrobial agents to mimic the antimicrobial action of platelets and improve healing outcomes following hemostasis. Because microgel deformability and high fibrin affinity are both critical to obtaining PLP-mediated clot retraction, the primary objective of these studies was to develop antimicrobial gold nanoparticle-microgel composites that inhibit bacterial growth while maintaining microgel deformability.

Gold in its bulk form is chemically inactive but has been shown to be antimicrobial against gram-positive and gram-negative bacteria when in smaller nanoparticle sizes. Synthesized gold nanoparticles have specifically shown antimicrobial effects against known human antigens *E. coli* and *Streptobacilli* [19]. Gold nanoparticles in combination with laser have also shown enhanced therapeutic antimicrobial effects against *C. pseudotuberculosis* [20], likely due to gold's photothermal combined effect. Conversely, gold has low toxicity to animals and humans [21]. The antimicrobial effect of metallic nanoparticles is dependent upon concentration, size (surface area), and surface modification of particles, with a large surface area to volume ratio correlating to increased antimicrobial effect [22]. In this manuscript, we combine gold with ULC pNIPAm microgels, previously utilized to create PLPs, to develop antimicrobial PLPs.

Microgel gold composites have been previously fabricated through pNIPAm assembly on an etched gold core [23]. Additionally, gold nanoparticles have been embedded into swollen hydrogel films by a simple immersion method wherein the Coulombic interactions between the anionic Au particles and the polycation are likely important [24]. In this project, we aim to incorporate gold nanoparticles into previously described ULC pNIPAm microgel platelet-like particles (PLPs) that have been shown to augment hemostasis [17]. We want to build upon this previous design and incorporate antimicrobial elements while maintaining the ability of our particles to generate clot retraction through maintenance of particle deformability. This will be achieved through: 1) a noncovalent method, through hydrogel swelling with gold nanospheres or 2) covalently, with gold chloride hydrate incorporation. The goal of the project is to assess how gold incorporation affects 1) particle deformability/morphology; 2) the ability to induce clot retraction; and 3) antimicrobial properties.

Materials and Methods

Ultra-low crosslinked microgel (ULC) synthesis

ULC microgels were synthesized using a precipitation polymerization reaction [25,26]. N-Isopropylacrylamide (NIPAm) (Sigma) was recrystallized with hexanes. NIPAm (95% of a

100 mM monomer solution by weight) was dissolved in ultrapure water then filtered into a 3-neck reaction vessel heated in silicon oil, stirred at 450 rpm, and fluxed at 70 °C for 1 hr under nitrogen. Acrylic acid (AAc) (Sigma; 5% by weight) was added prior to initiation with 1mM ammonium persulfate (APS). The reaction proceeded for 6 hrs and was cooled afterwards. Microgels were purified by filtering over glass wool to remove large aggregates and by dialysis (1000 kDa MWCO cellulose ester dialysis tubing (Spectrum)) against ultrapure water.

Nanogold composite (NGC) fabrication

A covalent and non-covalent method of gold incorporation were performed to create nanogold-microgel composites. The noncovalent NGC (ncNGC) synthesis method incorporates gold nanospheres into microgels via noncovalent swelling (Figure 1). Microgels were lyophilized and then rehydrated at a microgel concentration of 10 mg/mL with a 0.05 mg/mL aqueous suspension of gold nanospheres (5, 50, or 100 nm diameter) in 2 mM sodium citrate (nanoComposix, San Diego, CA) with shaking at room temperature overnight. The second method of NGC fabrication utilized a covalent nanogold composite (cNGC) fabrication method in which gold-microgel composites were synthesized in a two-step procedure modified from Chen et al [27]. Briefly, tetra(hydroxymethyl)phosphonium chloride (THPC)-mediated reduction of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ was used to form small Au NPs, which were then grown in size using hydroxylamine and Au^{3+} solution. The second step of the composite particle production process involves seeded growth of gold particles in order to covalently bond them within the microgel crosslinking network. Different cNGC formulations were synthesized by varying the total mass of gold incorporation during synthesis at both the initial gold seeding and subsequent gold particle expansion and designated as 1X, 2X, and 3X. Gold (III) chloride hydrate (20 mM; 1X: 150 μL , 2X: 300 μL , 3X: 450 μL) and NaOH (1M; 50, 100, or 150 μL) were added and stirred at room temperature for 30 min. THPC (80%; 2.45 μL , 4.90 μL , or 7.35 μL) was added to reduce the gold ions and covalently bond them within the microgel particle network. Particles were purified via centrifugation, resuspended into ultrapure water and then hydroxylamine (80 mM; 50 μL , 100 μL , or 150 μL) was added immediately before 100mM gold (III) chloride hydrate (60 μL , 120 μL , or 180 μL) to increase the size of seeded gold nanoparticles. Resulting cNGCs were purified by centrifugation.

Characterization of NGC size and deformability

Microgel size and deformability as a measure of the ability to spread on a glass surface [28] was determined with AFM using an MFP-3D BIO AFM (Asylum Research, Santa Barbara, CA). Glass coverslips were cleaned by submerging in a series of solutions in an ultrasonic bath for 10 min each: alconox detergent, water, acetone, absolute ethanol, and isopropyl alcohol. Microgel suspensions were diluted in ultrapure water, pipetted onto coverslips, and dried. AFM imaging was performed in tapping mode with a tip frequency of 95.4 kHz, drive amplitude of 316 mV, a set point of 500 mV, and a scan rate of 0.5 Hz with silicon probes (ARROW-NCR, NanoAndMore, Watsonville, CA). Average particle diameter and height $\pm$ standard deviation were determined through analysis of at least 30 microgels per condition from at least 3 different images using ImageJ image analysis software (National Institutes of Health, Bethesda, MD). Aspect ratio was also determined. NGC distribution of gold

nanospheres within the microgels were also visualized through Transmission Electron Microscopy (TEM) performed on a JEOL JEM-2000FX TEM at 200 kV. TEM samples were prepared by pipetting 3 μ L of diluted NGCs on copper-coated side of TEM grids (Carbon Type-B, 200 mesh (TedPella, Redding, CA)) and allowed to dry overnight.

Noncovalent nanogold composite stability studies

To characterize the stability of ncNGCs, ncNGCs were sampled immediately after fabrication and sampled after longer time periods of storage (>2 months). ncNGCs were suspended at 0.02 mg/mL in ultrapure water and incubated with TEM grids. TEM grids were then allowed to air dry at room temperature for up to 24 hours. All TEM images were obtained on a JEOL 2000FX at a voltage of 200 kV. Nanogold particle content was then characterized by counting the number of nanogold particles enclosed inside each ncNGC using Image J.

Cryogenic scanning electron microscopy (cryoSEM)

Microgel morphology was characterized *in situ* using cryoSEM with a JEOL 7600F. Microgels suspended in water were diluted to approximately 10 μ g/mL and then prepared for imaging by flash freezing in liquid nitrogen under vacuum. Samples were fractured, etched, and sputter coated with gold for visualization. For a comparison to native platelets, blood was acquired from the New York Blood Center (New York City, NY) and platelets were isolated by centrifugation at 150 g's for 15 min with no deceleration. A second spin at 900 g for 5 min was then performed and then isolated platelets were washed and resuspended in Tyrode's albumin buffer containing 0.35% human serum albumin (Fisher Scientific, Hampton, NH) [29]. Platelets were activated by the addition of 0.25 U/mL human α -thrombin (Enzyme Research Laboratories, South Bend, IN) immediately before imaging. At least 5 fields were imaged at 50000X magnification per condition.

Production of fibrin-specific NCGs

ncNGC platelet-like particles (ncNGC PLPs) were created by adding fibrin-binding antibody to microgels and subsequent rehydration with gold nanosphere solution. A fibrin-binding IgG antibody (Sheep anti-human Fibrin fragment E, Affinity Biologicals, Ancaster, ON) was first conjugated to the AAc residues of microgels through EDC/NHS coupling. Purification of PLPs was performed either by dialysis against 200X volume ultrapure water over 48 hrs with 3 diluent exchanges or by centrifuging at 3,700g x 30 min and washing 3 times with 30X volume ultrapure water. ncNGC PLPs were then lyophilized and loaded with gold by rehydration, as described above. cNGC PLPs (cNGC PLPs) were also created through EDC/NHS coupling, but after covalent gold incorporation. cNGC PLPs were similarly purified by dialysis or centrifugation and washing.

Analysis of fibrin clot structure

The effect of ncNGC and cNGC PLPs on clot structure was analyzed using cryoSEM. Fibrin clots were prepared with a final fibrinogen concentration (FIB 3, Enzyme Research Laboratories, South Bend, IN) of 2 mg/mL in HEPES buffer (25 mM HEPES, 150mM NaCl, 5 mM CaCl_2). The clot was polymerized by adding 0.1 U/mL final concentration

human α -thrombin in the presence or absence of 1 mg/mL of ncNGC and cNGC PLPs. Fibrin clots were fractured, etched, and imaged via cryoSEM as described above at 5,000X. Quantification of connectivity and porosity were performed with BoneJ plugin through Image J. At least three clots were imaged and quantified per condition.

Microgel Thin Film Fabrication

Microgel thin films were fabricated from ULCs, cNGCs, and ncNGCs to assess antimicrobial activity. Thin films were created on cleaned 12 nm diameter glass coverslips that were functionalized with (3-aminopropyl)trimethoxysilane (APTMS) before actively depositing microgels (1 mL of 0.5 mg/mL). Films were rinsed with water and allowed to dry overnight before UV sterilization. Gram-negative *Escherichia coli* (*E. coli*) was overlaid on each film (10^5 CFU/mL x 0.5 mL/well in a 24-well plate) and cultured in a 37 °C humidified incubator. *E. coli* cultured on unmodified glass or ULC microgel thin films in the presence of 100 ug/mL ampicillin served as a control. After 12 hr, wells were rinsed and then either prepared for a Live/Dead assay or fixed for visualization with Scanning Electron Microscopy (SEM), as described below.

Live/Dead Assay

Antimicrobial effects of microgel thin films were assessed by staining for viability. Films were incubated with *E. coli* for 12 hrs, as described above, before supernatant was removed and films gently washed with PBS pH 7.4. Films were incubated with a green fluorescent nucleic acid stain, 10 μ M SYTO 9 dye (Thermo Fisher Scientific) and 60 μ M Propidium Iodide (0.5 mL/well) x 10 min at room temperature in the dark. Films were washed 2X with water and mounted in *BacLight* mounting oil on glass slides and imaged on an EVOS FL Auto Imaging System with a 40X objective. Quantification of total corrected fluorescence was calculated for each image with ImageJ; (Corrected Total Fluorescence = Integrated density - (Area of rect. Selection $\times$ Mean fluorescence of 2 background selections per image)). The corrected fluorescence intensity values shown in Figure 5B represent green fluorescence. At least 4 films were analyzed per condition and a minimum of 3 different regions were imaged per film.

Scanning Electron Microscopy

Microgel thin films incubated for 12 hr with *E. coli* were washed with PBS and fixed in 4% paraformaldehyde x 10 min at room temperature. Films were washed 2X in water and dried completely before sputtercoating with 14 nm gold and imaging at 5000X magnification with a FEI Verios 460L high resolution field emission Scanning Electron Microscope. At least 4 films were analyzed per condition and a minimum of 3 images of different regions were imaged per film.

Colony-Forming Unit Assay

The antimicrobial effects of NGCs in suspension was evaluated using a colony-forming unit assay. Gram-negative *Escherichia coli* (*E. coli*) and gram-positive *Staphylococcus aureus* (*S. aureus*) were each cultured in a 37 °C humidified incubator at 180 rpm. After 6 hours, 10^5 CFU/mL x 0.2 mL/sample of each bacteria were diluted 1:10000 to create a testing solution;

this testing solution was then incubated 1:1 with a 10 mg/ml suspension of ncNGCs (5 nm, 50 nm, or 100 nm) or cNGCs (1X, 2X, or 3X). Resulting suspensions were incubated at 37 °C at 180 rpm. 50 µL aliquots were taken from each sample at t = 0, 3, 6, 12, and 24 hours after initial bacteria-microgel suspensions were created and spread on agar plates. Plates were kept in a 37 °C humidified incubator; after 15 hours, plates were removed from the incubator and the number of colonies formed were counted. Colonies counted were multiplied by the dilution factor used for plating in order to determine total number of colony-forming units per mL.

Statistical analysis

All statistical analyses were performed with Prism software program (GraphPad, San Diego CA). To compare the nanogold content of TEM images an unpaired t-test with Welch's was used to determine statistical significance. CFU assays were analyzed using a Two-way analysis of variance (ANOVA) with Tukey's posthoc tests. All other data was analyzed using a one-way ANOVA with a Tukey's post hoc test at a 95% confidence interval. Means $\pm$ standard deviation are reported. For all analyses, a minimum of three samples were analyzed per group. Any specific preprocessing of data (such as normalization) and specific sample sizes for each experimental are detailed in specific experimental sections above.

Results

NGC size, deformability, and morphology characterization

Microgels containing gold were formed through noncovalent and covalent methods to obtain nanogold composites (NGCs) as illustrated in Figure 1. In the noncovalent fabrication method, lyophilized microgels are rehydrated at 20 mg/mL with solution containing 0.02 mg/mL gold nanospheres (5 nm, 50 nm, or 100 nm diameter) in 2 mM sodium citrate. Covalent NGCs were formed by first seeding hydrated microgels with 20 mM gold (III) chloride with tetrakis(hydroxymethyl)phosphonium chloride (THPC) and NaOH and then growing the gold with hydroxylamine at 3 different concentrations of gold (III) chloride (1X, 2X, and 3X). We first analyzed how incorporation of varying size/amounts of gold in each method effected particle size and deformability using AFM (Figure 2). Unmodified ULC microgels had an average diameter of 1.6 ± 0.2 µm and heights of 8.5 ± 2.7 nm. After gold loading via the noncovalent method with either 5, 50 or 100 nm nanospheres, ncNGCs were found to have mean diameters of 1.3 ± 0.1 µm, 1.7 ± 0.2 µm, or 1.7 ± 0.1 µm and mean heights of 25.0 ± 3.5 nm, 13.7 ± 2.5 nm, or 13.3 ± 1.6 , respectively. After gold loading via the covalent method with either 1X, 2X, or 3X gold, cNGCs were found to have mean diameters of 2.3 ± 0.3 µm, 2.2 ± 0.2 µm, or 2.0 ± 0.2 µm and mean heights of 12.3 ± 1.9 nm, 13.1 ± 2.7 nm, or 16.0 ± 2.2 nm, respectively. Aspect ratios (width: height) for 5, 50, and 100 nm nanosphere ncNGCs were found to be 52.7 ± 34.0 , 120.0 ± 73.5 , and 130.4 ± 56.1 , respectively, while aspect ratios for 1X, 2X, and 3X cNGCs were found to be 188.8 ± 131.0 , 168.3 ± 90.8 , and 123.8 ± 69.7 , respectively. TEM images reveal a homogenous distribution of gold throughout microgels for NGCs created either through noncovalent or covalent gold incorporation (Supplemental Figure 1). Nanogold content for ncNGCs are as follows: 5 nm ncNGC composites contain 87 ± 29 nanogold particles per microgel, 50 nm ncNGC composites contain 39 ± 18 nanogold particles per microgel, and 100 nm ncNGC composites contain

1.5±0.8 particles per microgel. For cNGCs, nanogold content are as follows: 1x cNGCs contain 44±14 particles per microgel, 2x cNGCs contain 380±139 particles per microgel, and 3x cNGCs contain particles 677±253 per microgel (Supplemental Figure 1). In addition, we also analyzed nanogold content for ncNGCs >2 months after fabrication. TEM images of ncNGCs show that little difference in ncNGC structure or nanogold content was observed over the course of longer storage periods (Supplemental Figures 1, 2), indicating that ncNGCs are relatively stable over time. Nanogold content of ncNGCs after >2 months after fabrication is as follows: 5 nm ncNGC composites contain 89±30 nanogold particles per microgel, 50 nm ncNGC composites contain 34±9 nanogold particles per microgel, and 100 nm ncNGC composites contain 1.6±0.6 particles per microgel.

We next analyzed how gold incorporation into ULC microgels influenced microgel morphology using cryoSEM (Figure 3). Native circulating platelets display an ovoid morphology and form spindle-like projections upon activation. Due to their high degree of deformability, unmodified ULC microgels are capable of spreading and display morphology similar to native activated platelets. Noncovalent incorporation of gold nanospheres of varying diameters (5–100 nm) did not significantly affect morphology, and ncNGCs continued to display morphologies similar to activated platelets. cNGCs were also found to display a spindle-like morphology at different synthesis formulations (1X-3X), however, cNGCs appeared less spread than ncNGCs. This could be because covalent incorporation reduces polymer mobility within the microgel to a greater degree than non-covalent incorporation of nanogold. Nonetheless, even with covalent incorporation of gold, the ULC microgels are still deformable enough to change shape and mimic activated platelet morphology. Overall, AFM and cryoSEM characterization of NGCs demonstrates ULC deformability, and corresponding ability to mimic activated platelet morphology, is largely maintained following incorporation of gold nanoparticles.

Effect of gold incorporation on PLP-mediated clot retraction.

Our previous studies have demonstrated microgel deformability is critical to obtaining PLP-mediated clot retraction [17]. Having established that NGCs maintain similar levels of deformability to ULC microgels, we next generated NGC PLPs and compared their ability to produce fibrin network deformation to that of ULC PLPs. CryoSEM demonstrated that when both covalent and noncovalent NGCs were conjugated to fibrin-binding antibodies to create NGC PLPs and then polymerized into a fibrin clot, NGCs significantly increased fibrin network density and decreased porosity compared to fibrin alone or non-fibrin-binding ULCs. This fibrin clot collapse can be seen at 24 hrs in the ultrastructure image of a fibrin clot at 5000X (Figure 4). These results were similar to those observed in the presence of unmodified PLPs, seen in Figure 4C. Because inclusion of the gold nanoparticles in the majority of the NGC microgels did not greatly influence deformability, and because particle deformability is a major feature required for induction of clot retraction by fibrin binding microgels, it is not surprising that major differences were not observed between these groups. The exception to this observation was the 3X cNGC PLPs, which, while significantly increasing connectivity density, did not significantly decrease porosity, compared to control clots. Indeed, the micrographs of 3X cNGC PLPs appear more porous

and have a less dense fibrin structure compared to all other PLP conditions (Figure 4C–H), which correlates with the quantification.

Antimicrobial Film Assays

Antimicrobial assays demonstrate that all formulations of NGCs inhibit bacterial growth and attachment when fabricated into thin films. A modified BacLight Live/Dead assay demonstrated significantly reduced *E. coli* attachment and growth on NGC thin films compared to non-gold-containing ULC microgel films (Figure 5). SEM images confirmed Live/Dead assay results by replicating similar reduction in *E. coli* attachment to NGC thin film compared to ULC microgel films or glass (Supplemental Figure 3).

Colony-Forming Unit (CFU) Assay

CFU assays were performed to evaluate the effect of NGCs on bacterial growth in solution. CFU assays demonstrate that while cNGCs demonstrate some inhibition of growth of both *E. coli* and *S. aureus* in suspension, ncNGCs do not have antibacterial effects in suspension (Figure 6). cNGCs were shown to inhibit growth of both *E. coli* and *S. aureus* to some degree at early time points (0–6 hours). 3X cNGCs were found to significantly reduce growth of gram-positive *S. aureus* at longer time points (24 hours) in culture relative to control *S. aureus* cultures. 2X cNGCs in particular demonstrated inhibition of growth in *S. aureus* cultures at early time points (0–3 hours in culture), however, these effects were not statistically significant. 3X cNGCs demonstrated significant inhibition of growth after 24 hours in culture. When compared to ampicillin, however, cNGCs do not inhibit bacterial growth as strongly. Ampicillin completely reduced growth in both *E. coli* and *S. aureus* until after 12 hours and, even then, continued to inhibit growth to much greater extents than cNGCs after 24 hours (Figure 6).

Discussion

Due to their roles in hemostasis, enhancement of fibrin network properties through clot retraction, and prevention of infection, platelets are an ideal source of inspiration for designing materials to stem hemorrhage and promote wound healing following injury. Here we demonstrate that fibrin-binding NGC PLPs, created either through noncovalent or covalent incorporation of gold nanoparticles into ULC microgels and coupled to fibrin-binding antibodies to create PLPs, are able to induce clot retraction and inhibit bacterial attachment and growth when presented as films. Our previous studies with ULC microgel-based PLPs demonstrated that microgel deformability and fibrin binding ability is critical to mimicking the platelet function of clot retraction [17]. In this manuscript, we demonstrate that ncNGC and cNGCs remain deformable, as evidenced by their ability to spread extensively on a glass surface and through their ability to mimic activated platelet morphology, however, it does appear that covalent incorporation results in a slight diminishment of overall particle deformability. However, most formulations of both ncNGC PLPs and cNGC PLPs appear to have sufficient deformability to generate fibrin network deformations to similar levels as that seen in the presence of ULC PLPs (Figure 4), as evidenced by significantly increased connectivity density and decreased porosity, compared to fibrin only controls. The cNGC PLPs with the highest amount of gold incorporation (3X)

altered fibrin structure to the least degree of all formations and, though porosity was increased compared to control clots, connectivity density was not altered significantly. It is likely at these high levels of gold incorporation that overall particle deformability is diminished enough to stall or hinder particle-mediated clot retraction.

We recently published that our first generation PLPs bind fibrin clots and augment hemostasis both *in vitro* and *in vivo* [17]. Using an endothelialized microfluidics device which recapitulates the physiology and hemodynamics of microvasculature to evaluate clotting, we found that PLPs promote clot formation to levels similar to native platelets. We also evaluated the ability of PLPs to augment clotting *in vivo* in a rodent traumatic injury model. Bleeding times were significantly decreased in the presence of PLPs compared to saline or non-binding ULC controls and PLPs were found to localize to sites of fibrin deposition at the injury site. Together, these data demonstrated that PLPs home to injury sites, enhance fibrin formation, and promote hemostasis. While particle deformability was critical to clot retraction, only fibrin binding was required to augment hemostasis, as even non-deformable fibrin-binding microgels promoted hemostasis, therefore, we expect that all formulations of NGC PLPs to augment hemostasis. The focus of these studies was to characterize the ability of NGC PLPs to induce retraction and prevent infection. The ability of promising formulations to augment hemostasis will be explored in future studies.

All formulations of NGCs were found to significantly decrease bacterial adhesion/growth compared to unmodified ULC microgels (Figure 5) when fabricated into thin films. For ncNGCs, gold nanoparticle size used in fabrication influenced these responses, such that the smallest size particles (5 nm) resulted in the greatest inhibition of bacterial adhesion and growth, likely due to an increased gold surface area compared to larger gold particles. A similar trend was seen with cNGCs, such that NGCs synthesized in the presence of the lowest amount of gold (1X), resulted in the greatest inhibition of bacterial adhesion and growth. We additionally evaluated the antimicrobial activity of the NGCs in solution using a CFU assay. The results obtained from those studies indicated that cNGCs inhibit bacterial growth in suspension to some degree, while ncNGCs do not have significant antibacterial effects in suspension (Figure 6). Additionally, we found that ncNGCs are extremely stable and do not appear to release nanogold particles even after two months of storage (Supplemental Figure 2). This suggests that the physical entrapment of the nanogold particles within the microgel network is robust and that the nanogold particles are not being released in solution. Because our AFM analysis (Figure 1) demonstrates that the ncNGCs retain a high degree of deformability, and spread extensively on glass surfaces even after nanogold incorporation, it is possible that when ncNGCs are deposited on surfaces into films, that gold is exposed upon flattening of the microgel particle, thereby facilitating the antimicrobial events observed when the ncNGCs are utilized in film formats. While 3X cNGCs do demonstrate significant antimicrobial effects in *S. aureus* cultures at 24 hours, it is possible that both cNGCs and ncNGCs display fewer antimicrobial properties in suspension culture than on films due to particle settling, which reduces the available gold surface area that can come into contact with bacteria. These results overall suggest that the when used as films NCGs are effective at inhibiting bacterial attachment and growth, however, the effect of NGCs on inhibiting bacterial growth in suspension are minimal and should be optimized in future studies. Because these results show that inclusion of

nanometals do induce clot retraction when used to create PLPs to similar levels as that observed with ULC-based PLPs, other antimicrobial metals with greater antimicrobial potential, such as nanosilver, could be useful in future antimicrobial PLP design. Future work will investigate the application of optimized particle formulations to treat hemorrhage and infection following traumatic injury.

Conclusions

In conclusion, we demonstrate NGC PLPs can be fabricated through both noncovalent and covalent methods to incorporate nanogold into ULC microgels. These metallic composites maintain deformability, mimic activated platelet morphology, and when coupled to a fibrin-binding motif, mimic platelet-mediated clot retraction. Furthermore, these NGC PLPs demonstrate antimicrobial effects when applied as thin films. Therefore, these NGC PLPs are a promising material to prevent infection and hemorrhage following trauma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A. Noncovalent Hydrogel Swelling

B. Covalent Fabrication

Figure 1. Microgels containing gold were formed through noncovalent and covalent methods to obtain nanogold composites (NGCs).

In the noncovalent fabrication method, **A**, lyophilized microgels are rehydrated with solution containing gold nanospheres. **B**, Covalent NGCs are formed in a 2-step process to seed gold (III) chloride and then grow gold in to larger size nanoparticles.

Figure 2. AFM characterization of NGC size and spreading.

Microgel size and deformability as a measure of the ability to spread on a glass surface was determined with AFM using an MFP-3D BIO AFM (Asylum Research, Santa Barbara, CA). Diameter and height traces were generated with Asylum AFM software for at least 30 microgels per condition from at least 3 different images. Representative images and height traces are shown. Aspect ratios (width: height) were calculated for at least 30 microgels based on the diameter and height trace measurements. *p<0.05; **p<0.01; ****p<0.0001

Figure 3. CryoSEM analysis of NGC morphology.

Platelet and microgel morphology was imaged with a JEOL 7600F CryoSEM at 50,000X (scale bar = 500 nm) magnification. Native circulating platelets display an ovoid morphology (A) that upon activation with 0.25 U/mL thrombin, forms spindle-like projections (B). Unloaded microgels (C) illustrate a morphology similar to native platelets which remains largely unaffected by noncovalent incorporation of gold nanospheres of varying diameters including 5 nm (D), 50 nm (E), and 100 nm (F). Covalent NGCs also display a spindle-like morphology at different synthesis formulations: 1X (G), 2X (H), and 3X (I). Representative images are shown from at least 13 images per condition.

Figure 4. CryoSEM analysis of fibrin ultrastructure demonstrates NGC platelet-like particles (PLPs) induce clot retraction.

Fibrin clots were prepared in the presence or absence of 1 mg/mL of ULC, ncNGC and cNGC PLPs. Clot structure 24 hr after polymerization is shown for control clots (A) and clots formed in the presence non-fibrin-binding microgels (B), ULC PLPs (C), 5 nm (D), 50 nm (E), or 100 nm (F) ncNGC PLPs, and 1X (G), 2X (H), or 3X (I) cNGC PLPs imaged via cryoSEM at 5,000X magnification. Representative images are shown from at least 3 images per condition. Quantification of connectivity (J) and porosity (K) was performed with BoneJ plugin of ImageJ. Statistical analysis was performed with a one-way ANOVA and post hoc Tukey's multiple comparisons test. ***p<0.001

Figure 5. NGCs inhibit bacterial growth.

Microgel thin films were created on functionalized glass by actively depositing suspended ULC and NGC microgels with centrifugation (3700 g x 10 min). Films were rinsed and UV sterilized before culturing with 0.5 mL of *E. coli* (10^5 CFU/mL) for 12 hours at 37°C. **A**, A modified BacLight live/dead stain was performed on washed films to observe the adhered *E. coli* (n=4). A distinct reduction in *E. coli* growth was observed in NGC films compared to unloaded ULC microgel films demonstrated in **C** showing mean corrected fluorescence $\pm$ standard deviation for a minimum of 3 images per film (n=4 films/condition) as quantified with ImageJ (Corrected Total Fluorescence = Integrated density – (Area of rect. Selection x Mean fluorescence of 2 background selections per image)). Statistical analysis was performed with a one-way ANOVA and post hoc Tukey's multiple comparisons test. *p<0.5.

Figure 6: Effect of NGCs on *E. Coli* and *S. aureus* growth in suspension.

A) cNGCs and ncNGCs were cultured in suspension with *E. coli* or *S. aureus* over a period of 24 hours. Aliquots of suspension cultures were taken at $t = 0, 3, 6, 12$, and 24 hours, diluted, and spread on agar plates to observe colony growth ($n = 3/\text{group}$). **B)** cNGCs and ncNGCs do not have a significant impact on *E. coli* growth in suspension culture. **C)** cNGCs demonstrate inhibition of *S. aureus* growth after 24 hours in suspension culture. Statistical analysis was performed using a two-way ANOVA with a post hoc Tukey's multiple comparisons test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.