Published in final edited form as:

J Control Release. 2017 October 10; 263: 185–191. doi:10.1016/j.jconrel.2017.01.016.

Erythrocyte membrane-coated nanogel for combinatorial antivirulence and responsive antimicrobial delivery against *Staphylococcus aureus* infection

Yue Zhang¹, Jianhua Zhang^{1,2}, Wangsong Chen^{1,3}, Pavimol Angsantikul¹, Kevin A Spiekermann¹, Ronnie H. Fang¹, Weiwei Gao¹, and Liangfang Zhang^{1,*}

¹Department of Nanoengineering and Moores Cancer Center, University of California, San Diego, La Jolla, CA 92093, USA

²Department of Polymer Science and Engineering, School of Chemical Engineering and Technology, Tianjin University, Tianjin, 300072, China

³Department of Applied Chemistry, School of Chemistry and Chemical Engineering, Central South University, Changsha, Hunan 410083, China

Abstract

We reported an erythrocyte membrane-coated nanogel (RBC-nanogel) system with combinatorial antivirulence and responsive antibiotic delivery for the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infection. RBC membrane was coated onto the nanogel via a membrane vesicle templated in situ gelation process, whereas the redox-responsiveness was achieved by using a disulfide bond-based crosslinker. We demonstrated that the RBC-nanogels effectively neutralized MRSA-associated toxins in extracellular environment and the toxin neutralization in turn promoted bacterial uptake by macrophages. In intracellular reducing environment, the RBC-nanogels showed an accelerated drug release profile, which resulted in more effective bacterial inhibition. When added to the macrophages infected with intracellular MRSA bacteria, the RBC-nanogels significantly inhibited bacterial growth compared to free antibiotics and non-responsive nanogel counterparts. These results indicate the great potential of the RBC-nanogel system as a new and effective antimicrobial agent against MRSA infection.

Graphical abstract

*Corresponding author: Tel: 858-246-0999, zhang@ucsd.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

We synthesized redox-responsive hydrogel nanoparticles cloaked with red blood cell membranes, which integrate antivirulence therapy with responsive antimicrobial delivery for effective treatment against *Staphylococcus aureus* infection.

Keywords

nanomedicine; cell membrane coating; detoxification; antibiotic delivery; staph infection

1. Introduction

Staphylococcus aureus (*S. aureus*) is a prominent Gram-positive bacterium responsible for a wide range of human skin and wound infections [1, 2]. With the widespread use of antibiotics during the past a few decades, *S. aureus* bacteria have experienced several waves of antibiotic resistance and now display broad resistance to the entire β -lactam class of antibiotics, including penicillins, cephalosporins, and carbapenems [3–5]. To keep up with the pace of antibiotic resistance development, alternative antibiotics including vancomycin, linezolid, tedezolid, daptomycin, ceftaroline, and tigecycline have been introduced to the market in recent years [6–9]. However, the emergence of antibiotic-resistant *S. aureus* strains, especially toward vancomycin resistance, has greatly increased [10, 11]. Currently, virulent strains of methicillin-resistant *S. aureus* (MRSA) have become increasingly prevalent, imposing a major clinical challenge that threatens public health [12, 13]. Clearly, all these facts underscore an undisputed and urgent need to develop novel and effective therapeutic strategies for MRSA treatment.

To address antibiotic resistance, targeting and neutralizing virulence factors such as bacterial toxins offer an attractive alternative that is likely to lower the selective pressure on the bacterial pathogens [14]. Notably, MRSA infection features a critical element of virulence resulting from a diverse arsenal of pore-forming toxins (PFTs) secreted by the bacteria [15]. In this perspective, coating red blood cell (RBC) membrane onto synthetic nanoparticles for broad spectrum neutralization of PFTs has recently gained much attention [16, 17]. These RBC membrane-coated nanoparticles (denoted ‘nanosponges’) target the membrane-disrupting mechanism shared by all PFTs regardless of their molecular structures and sources, thereby offering a non-specific and all-purpose toxin decoy strategy. For example, in a subcutaneous MRSA infection mouse model, the nanosponges alone when applied at the infection site effectively neutralized tissue-damaging α -toxin and markedly reduced the lesion development caused by MRSA infection [18].

In addition to the antibiotic resistance development, *S. aureus* bacteria are also known to invade and survive inside phagocytic cells such as macrophages, further diminishing the efficacy of existing antibiotics [19, 20]. Incomplete clearance of intracellular MRSA infection facilitates their dissemination and subsequent invasion of various non-phagocytic cell types [21]. As a result, intracellular MRSA infection is often associated with a number of chronic or recurrent infections including recurrent rhinosinusitis, pulmonary infections, osteomyelitis and endocarditis [22]. To address the challenges, nanoparticle delivery systems have attracted much attention. These nanomedicines are designed to preferentially deliver antibiotics into the host cells to inhibit residue MRSA bacteria. For example, synthetic nanoparticles made from cationic polymers such as chitosan and polyhexamethylene biguanide exploit strong charge interactions to enhance uptake by the host cells [23, 24]. Modifying nanoparticles with targeting ligands including wheat germ agglutinin and mannose represents another effective approach to improving phagocytic uptake of antibiotic payloads and enhancing MRSA inhibition [25, 26]. Moreover, nanoparticles responsive to intracellular cues for on-demand antibiotic release have also gained much interest for targeting intracellular MRSA bacteria. In this perspective, nanoparticles capable of drug release in response to MRSA-associated enzymes such as penicillin Gamidase, β -lactamase, phosphatase, and phospholipase have been investigated for controlled release of antibiotics within the host cells [25].

Inspired by the unique toxin neutralization property of the ‘nanosponges’ and the ‘on-demand’ drug release capability of various anti-MRSA nanomedicines against intracellular infection, herein, we combined the two features into one nanoparticle system and engineered a redox-responsive RBC membrane-coated nanogel (denoted ‘RBC-nanogel’) platform, which consists of a responsive hydrogel core and an RBC membrane shell (Fig. 1A). The hydrogel core is used for redox-responsive antimicrobial delivery and release, while the RBC membrane shell is used for absorbing and neutralizing PFTs secreted bacteria. In the study, a cell membrane-templated polymerization method was utilized to prepare such RBC-nanogels [27]. Compared to the prior strategy of wrapping cell membranes onto pre-formed nanoparticle substrates, this approach forms cell membrane-derived vesicles first, followed by polymerizing hydrogel cores inside the vesicles. To make a redox-responsive hydrogel core, we synthesized a crosslinker with a disulfide bond, which is readily cleaved inside an endosome following nanoparticle phagocytosis [28]. As shown in Fig. 1B, in the extracellular environment, the RBC-nanogels can absorb and neutralize PFTs secreted by MRSA bacteria. Toxin neutralization counteracts the bacterial virulence and facilitates bacterial uptake by phagocytic cells. Once inside the cells, the RBC-nanogels respond to the reducing environment and rapidly release antibiotic payloads for the inhibition of intracellular MRSA bacteria.

2. Materials and methods

Materials

Reagents including L-cystine, methacrylic anhydride (MA), hydrochloride acid, Triethanolamine (TEA), *N,N'*-methylene bis-acrylamide (non-responsive crosslinker), acrylamide (monomer), tris(2-carboxyethyl)phosphine (TCEP), and Triton X-100 were

purchased from Sigma-Aldrich (St. Louis, USA) and used as received unless otherwise stated. Acetonitrile (ACN, HPLC grade) were purchased from EMD Chemicals (Gibbstown, USA). Succinimidyl succinate-poly(ethylene glycol)-succinimidyl succinate (NHS-PEG-NHS, average molecular weight = 3400 g/mol) was purchased from Laysan Bio, Inc. (Alabama, USA). The macromolecular inhibitor TEMPO-PEG-TEMPO was synthesized by conjugating HNS-PEG-NHS and 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (4-aimno-TEMPO) in tetrahydrofuran (THF) with the presence of triethylamine (TEA) following a published protocol [27]. The photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) was synthesized following a published protocol [29, 30].

Synthesis and characterization of redox-responsive crosslinker

To synthesize cystine dimethacrylate (CDA), a redox-responsive crosslinker, L-cystine (0.48 g, 2 mmol) was dissolved with 5 mL DI water and 1 mL TEA was added to the solution. After complete dissolution of L-cystine, 2 mL MA was added dropwise into the solution. The mixture was then stirred vigorously and the reaction was allowed for 48 h at room temperature. Following the reaction, TEA and unreacted MA were extracted with diethyl ether. Then the pH value of the resulted solution was adjusted to 2 with hydrochloric acid. The sample was then lyophilized and the final product was obtained as an off-white powder. Its molecular structure was confirmed by ¹HNMR (Varian Inova-500M, Varian Inc., Palo Alto, USA) with CO(CD₃)₂ (d-Acetone) as the solvent and tetramethylsilane (TMS) as the internal standard. Its molecular weight was confirmed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF, Bruker Biflex IV MALDI-TOFMS) mass spectrometry.

Preparation and characterization of RBC membrane-coated nanogels (RBC-nanogels)

RBC membranes were derived by following a previously published protocol [31]. Briefly, the whole blood withdrawn from male ICR mice (Charles River Laboratories, Wilmington, MA) was centrifuged (×800 g for 5 min at 4°C) to remove the plasma and the buffy coat. The resulting packed RBCs were washed and the RBC ghosts were prepared through a hypotonic treatment. To prepare RBC-nanogels, the collected RBC ghosts were mixed with acrylamide as monomer (0.71 mmol), CDA as redox-responsive crosslinker (0.03 mmol), and LAP (5 μmol) in PBS containing 1 mM Mg²⁺ and 1 mM Ca²⁺. Then the mixture was sonicated for 5 min using a bath sonicator (FS30D, Fisher Scientific) at a frequency of 42 kHz and power of 100 W. The mixture was then extruded serially through 400 nm and then 100 nm polycarbonate porous membranes with an Avanti mini extruder (Avanti Polar Lipids). Following the extrusion, macromolecular inhibitor, TEMPO-PEG-TEMPO (7 nmol), was added into the solution. For gelation, the solution was degassed by blowing nitrogen and then irradiated with ultraviolet (UV) light for 5 min (Black Ray® Lamp, 365 nm, model UVL-56, Upland, USA). To synthesize non-responsive RBC-nanogels (control nanogels), CDA was replaced with *N,N*-methylene bis-acrylamide (0.03 mmol) as a non-responsive crosslinker. For filtration study, rhodamine B (0.3 mg/mL) was included into the reactant mixture for encapsulation. Following the nanogel synthesis, samples were added with 0.25 mL 5% w/v Triton X-100 and 20 μg proteinase K (New England Biolabs, Inc., Beverly, USA) to dissolve RBC membranes. To dissolve hydrogel core, 10 μL TCEP (300

mM) was added to the solution with a final concentration of 10 mM. Samples were filtered through an Amicon ultra filter with a molecular weight cut-off of 100 kDa. The released rhodamine B from both samples was then quantified by a UV spectrophotometer (Lambda 850 spectrophotometer, Perkin Elmer Inc., Waltham, MA, USA). Further characterizations with dynamic light scattering (DLS) were carried on a Zetasizer Nano ZS (Malvern Instruments). The nanogel concentrations of all samples were quantified by using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) and following the manufacturer's protocol.

In vitro toxin neutralization with RBC-nanogels

Alpha toxin neutralization with RBC-nanogels was examined by first mixing a fixed amount of RBC-nanogels (3 μ L, 0.8 μ g/ μ L protein) with various amount of α -toxins, followed by incubating the mixture at 37°C for 30 min. After the incubation, 500 μ L 5% purified mouse RBCs was added and the samples were incubated for another 30 min. The released haemoglobin was then quantified by UV-vis spectrometer at 576 nm to determine the percentage of RBCs being lysed.

Bacterial culture

MRSA USA300 bacteria were cultured on tryptic soy broth (TSB) agar overnight at 37°C. Then a single colony was inoculated in TSB medium and the medium was cultured in a rotary shaker at 37°C. Overnight culture was refreshed in TSB medium at 1:100 dilution and under shaking for another 3 h until the OD₆₀₀ of the culture medium reached approximately 1.0 (logarithmic growth phase). The bacteria were then harvested by centrifugation at 5000 $\times$ g for 10 min, and then washed with sterile PBS twice. After the removal of PBS by centrifugation, the obtained bacteria pellet was suspended in an appropriate amount of sterile PBS for future use.

Cellular uptake of MRSA bacteria

MRSA USA300 bacteria were labelled by mixing 10 μ L calcein-AM (1 mg/mL, excitation/emission = 495/516 nm) with 0.5 mL bacterial suspension (1.5×10^9 CFU/mL). The mixture was incubated at 37°C for 30 min. The free Calcein-AM was removed by washing the bacteria with PBS solution twice. THP-1 cells were cultured in RPMI1640 medium (Gibco Life technologies) supplemented with 2 mM L-glutamine, 1% penicillin, 1% streptomycin and 10% fetal bovine serum (HyClone Laboratories). THP-1 cells were differentiated with phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, St. Louis, MO, USA) at the concentration of 100 ng/mL in RPMI1640 medium with all the aforementioned supplement for 4 days. For uptake study, 8×10^5 differentiated THP-1 cells were seeded to each well of a 12-well plate. After cell adhesion, different concentrations of free α -toxin or α -toxin mixed with RBC-nanogels were added. To each well, 8×10^7 Calcein-AM labelled USA300 bacteria were added. The plate was cultured for 2 h with gentle shaking. Following the incubation, cells were washed, detached, and fixed with 10% formalin for intracellular bacterial quantification using flow cytometry (BD FACSCanto-II).

Vancomycin loading and release study

Vancomycin was encapsulated into the RBC-nanogels by mixing 5 mg/mL drug solution with the reactant mixture during the nanogel synthesis process described above. Nanogels were lyophilized and weighted for total mass. To quantify encapsulated vancomycin, nanogels were resuspended with DI water and the suspension was stirred for 24 h to allow for equilibrium. Vancomycin concentration was quantified with high-performance liquid chromatography (HPLC, Perkin Elmer Flexar series 200, Waltham, MA, USA) equipped with a Brownlee SPP C18 column (100 mm × 4.6 mm and 2.7 μm beads). The mobile phase contained 25 mM KH₂PO₄ solution and ACN (9:1, volume ratio) with a flow rate of 1.0 mL/min. The detection wavelength was set at 230 nm. The loading yield was calculated as the weight percentage of vancomycin to the total nanogels. Vancomycin release was measured by loading 1 mL RBC-nanogel suspension into a dialyzer (Harvard Apparatus, Holliston, USA). Dialysis membranes with pores of 50 nm in diameter (Isopore membrane filters, Merck Millipore Ltd. Billerica, USA) were used. The dialyzer was immersed into 1000 mL PBS, PBS with Triton X-100 (5% w/v), or PBS with Triton X-100 (5% w/v) and TCEP (10 mM) at 37°C under agitation. The released vancomycin at pre-determined time intervals was quantified with the same HPLC method.

In vitro MRSA inhibition activity of vancomycin-loaded RBC-nanogels

Bacterial suspensions (1 mL) containing 1×10^8 CFU/mL ($OD_{600} = 0.1$) MRSA USA300 bacteria in TSB were added to UV-transparent cuvettes. To each cuvette, 100 μL TECP (10 mM) and 2 μL Triton X-100 (5% w/v) were added, followed by the addition of vancomycin-loaded RBC-nanogels, vancomycin-loaded control nanogels, or free vancomycin at various concentrations. The cuvettes were incubated at 37°C for 24 h and then the values of OD_{600} were measured.

Vancomycin-loaded RBC-nanogels against intracellular MRSA infection

THP-1-derived macrophages were seeded onto 6-well plates at a density of 2×10^6 cells/well. The cells were allowed to adhere for overnight. To infect the cells, 2×10^7 bacteria (100 μL at a concentration of 2×10^8 CFU/mL) were added to each well and the plates were incubated at 37°C for 2 h. Following the incubation, the cells were gently washed with warm PBS and then medium containing 100 μg/mL gentamicin was added. The plates were incubated for another 3 h to eliminate any extracellular bacteria. After establishing the intracellular infection, PBS, empty RBC-nanogels, control-nanogels, vancomycin-loaded RBC-nanogels, vancomycin-loaded control-nanogels, and free vancomycin (with an equivalent vancomycin concentration of 5 μg/mL) were added to the wells. The plates were incubated for 6 h followed by macrophage disruption and bacterial enumeration. The viable intracellular bacteria were quantified by first erupting the macrophages with hypotonic treatment. Then a series of 10-fold dilution from the suspension was inoculated to TSB agar plate. The agar plate was cultured at 37°C for 24 h and the colony formation was counted.

3. Results and discussion

In the study, we first synthesized cystine dimethacrylate (CDA) as a redox-responsive crosslinker through a reaction between methacrylic anhydride (MA) and L-cystine in

tetrahydrofuran (THF) with the presence of triethylamine (TEA). The product was purified by repeated extraction with ethyl acetate followed by evaporating the solvent. The molecular structure of the product was first analyzed with ^1H NMR (Fig. 2A). The spectrum shows the characteristic resonance signals of vinyl hydrogens adjacent to the C=C double bond (*a* and *b*), methyl (*c*), methine (*d*, hydrogens adjacent to the -COOH carboxyl bond), and methylene (*e*, hydrogens adjacent to the S-S disulfide bond). The matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to further confirm the conjugation reaction and analyze the purity of the product (Fig. 2B). The mass spectrum of CDA was dominated by the deprotonated ions $[\text{M}-\text{H}]^-$ at m/z 375.0687 and exhibited negligible fragmentation. In addition, the mass spectrum also shows other ions of lower intensity (m/z 376.0716 and m/z 377.0667 ions), attributable to the presence of the ^{13}C isotopic analog of the peak of m/z 375.0687. The close match between the measured and the theoretical values of CDA confirms the successful synthesis of the crosslinker.

Following the synthesis of the crosslinker, we proceeded to prepare RBC-nanogels by using RBC membrane-derived vesicles (RBC-vesicles) as templates for *in situ* polymerization [27]. Herein, the RBC vesicles were used not only as nanoreactors to encapsulate the reactants including monomers, crosslinkers, initiators, and drug payloads, but also as a template to control the final nanogel size. After forming vesicles of approximately 100 nm in diameter using an extrusion method, we added a water-soluble macromolecular inhibitor, TEMPO-PEG-TEMPO, to the mixture. As the inhibitor is membrane impermeable, it selectively inhibits the polymerization reaction outside of the vesicles while keeping the reaction inside alive. To confirm the nanogel formation, we loaded rhodamine B to the precursor solution. Following the synthesis, the samples were treated with Triton X-100 and proteinase K, followed by filtration through Amicon ultra filters with a molecular weight cutoff of 100 kDa. Triton X-100 was shown to disrupt the RBC membrane coating on the nanogels; however, samples treated with Triton X-100 did not release rhodamine B into the solution, suggesting the hydrogel cores were formed and entrapped the dye (Fig. 3A). When a reducing agent, TCEP, was added to the solution, it effectively cleaved the disulfide bonds, resulting in quick release of the encapsulated rhodamine B. This result indicates the potential of redox-responsive drug release from the RBC-nanogels. The resulting RBC-nanogels were also stained with uranyl acetate and visualized with transmission electron microscopy (TEM). As shown in Fig. 3B, the nanogels exhibited an inner core region enclosed by a thin shell of approximately 10~15 nm in diameter. Such morphology is consistent with the core-shell structure of the nanogels.

To further confirm the RBC-nanogel formation, we examined nanogel size with dynamic light scattering (DLS). Prior to the UV irradiation, the RBC-vesicles showed a hydrodynamic size of 100.8 ± 0.3 nm in diameter with a unimodal size distribution (Fig. 3B). When added with Triton-X and proteinase K, the measured size of the vesicles decreased to 10.2 ± 0.5 nm in diameter. This peak was likely attributed to small lipid micelles because of the surfactants solubilizing the phospholipid bilayers. The RBC-nanogels showed a diameter of 104 ± 0.4 nm after UV irradiation. However, when Triton-X and proteinase K were added to the solution, two particle peaks were observed: a small micelle peak at 10.7 ± 0.1 nm and a large particle peak at 93.2 ± 0.6 nm. The presence of the large peak suggests the formation of crosslinked hydrogel cores within the RBC membrane

coating as the crosslinked cores remained insoluble in the surfactant. The diameter of the insoluble hydrogel cores was approximate 12 nm smaller as compared to the RBC-nanogels; the diameter decrease was consistent with the dissolution of the RBC membrane bilayer. Notably, the observed large peak disappeared after the addition of TCEP, a reducing agent that can break the disulfide bonds and thus cause core dissolution. To confirm the role played by the disulfide bonds, we also synthesized a non-responsive RBC-nanogels (control nanogels) by replacing CDA with methacrylic anhydride (MA). Following the synthesis, we added Triton-X and proteinase K and observed a small micelle peak at 10.1 ± 0.2 nm and a large particle peak at 93.7 ± 0.4 nm. However, the large peak remained after adding TCEP, indicating the non-responsive nature of the control nanogels. Together, these results confirm the successful formation of the RBC-nanogels with a redox-responsive property.

Following the synthesis, we examined the bioactivity of RBC-nanogels in neutralizing α -toxin, a major hemolytic virulence factor released by MRSA bacteria. In the study, α -toxins with amounts ranging from 0.3 to 3.6 μ g were either directly added to 5% purified mouse RBCs or first incubated with 2.4 μ g (protein amount) of RBC-nanogels, followed by the addition to the RBCs. RBC hemolysis was quantified by measuring the released hemoglobin percentage. As shown in Fig. 4A, free α -toxin exhibited a concentration-dependent hemolytic activity and the hemolysis reached maximum at 3.6 μ g of α -toxin. On the other hand, RBC-nanogels were able to completely inhibit the hemolytic activity of up to 1.2 μ g of α -toxin. Above this concentration, hemolysis was observed, indicating the presence of excessive free α -toxin. Based on these results, 1 μ g (protein amount) of RBC-nanogels was able to neutralize about 0.5 μ g of α -toxin.

Neutralization of toxins, especially α -toxin, has been shown to increase proinflammatory cytokine production and enhance bacterial clearance [32, 33]. Therefore, by neutralizing toxins, RBC-nanogels may facilitate bacterial uptake by the immune cells. To test this hypothesis, we used THP-1 derived macrophages as a model cell line and compared their ability to take up MRSA USA300 bacteria with or without the presence of RBC-nanogels. In the study, we first added 20 μ g free α -toxins or the mixture of 20 μ g α -toxins with 50 μ g RBC-nanogels to macrophages. Then to the cell culture, calcein-labeled MRSA bacteria were added. The bacterial uptake by the cells was quantified by measuring the fluorescence intensity from the phagocytosed MRSA USA300 in each cell using flow cytometry. As shown in Fig. 4B, macrophages treated with α -toxin showed a decreased bacterial uptake when compared to the untreated macrophages. In contrast, macrophages treated with the mixture of α -toxin and RBC-nanogels demonstrated a similar uptake level to that of the untreated macrophages. The mean fluorescence intensity based on the histograms was further quantified (Fig. 4C). The comparison showed that macrophages treated with the mixture of α -toxin and RBC-nanogels had taken up significantly more bacteria compared to those treated with α -toxin alone. These results indicate that the RBC-nanogels can neutralize α -toxin and promote bacterial uptake by the immune cells.

To demonstrate a redox-responsive drug release property, we first encapsulated vancomycin into RBC-nanogels and control nanogels and quantified their loading yield (the weight percentage of vancomycin to total nanogels) to be 2.6 wt% and 2.4 wt%, respectively. We then compared their drug release profiles under various conditions (Fig. 5). In PBS (no

treatment), RBC-nanogels and control nanogels released approximately 30% and 40% of the total encapsulated vancomycin in 24 h, respectively. To simulate the degradation condition in endosome, we first added Triton X-100 to the nanogel suspensions. RBC membrane coating was shown to act as a diffusion barrier to hinder drug release. With the presence of Triton X-100, vancomycin release increased in both groups: RBC-nanogels and control nanogels released approximately 40% and 50% of the total encapsulated vancomycin in 24 h, respectively. Next, we added both Triton X-100 and TCEP to the nanogel suspensions. In this case, vancomycin release from RBC-nanogels was significantly accelerated, reaching a cumulative release of approximately 65% of the total encapsulated drug in 0.5 h and 95% in 24 h, indicating a clear responsiveness to the reducing environment. However, such responsive release of vancomycin was not observed for the control nanogel sample. In fact, similar vancomycin release profiles were observed from the control nanogels in the absence or presence of TCEP. Such responsive antibiotic release is expected to benefit MRSA treatment. Most of the loaded vancomycin will remain in the RBC-nanogels for a considerable time when the nanogels stay in the extracellular space under normal physiological conditions. However, the drug will be quickly released when the RBC-nanogels are taken up by the infected macrophages.

After having confirmed the redox-responsive vancomycin release from RBC-nanogels, we proceeded to evaluate their anti-MRSA efficacy. We first incubated MRSA USA300 (1×10^6 CFU/mL) with free vancomycin, vancomycin-loaded RBC-nanogels, and vancomycin-loaded control nanogels with vancomycin concentrations ranging from 0.3 to 20 $\mu\text{g/mL}$. Bacterial culture was supplemented with TCEP and Triton X-100 to mimic reducing environment inside the mammalian cells, and cultured for 24 h at 37°C [28]. Following the incubation, vancomycin-loaded RBC-nanogels exhibited an anti-MRSA efficacy comparable to that of free vancomycin, with a minimum inhibition concentration (MIC) of 2.5 $\mu\text{g/mL}$ for both groups (Fig. 6A). In contrast, vancomycin-loaded control nanogels showed a reduced anti-MRSA efficacy with an MIC value of 5 $\mu\text{g/mL}$. Since Triton and TCEP were added to all samples, a higher MIC value of control nanogels compared to RBC-nanogels was attributable to the slow release of vancomycin from non-responsive nanogel formulation. These results imply that rapid release of vancomycin after RBC-nanogels are taken up by macrophages will be beneficial for treating intracellular bacterial infection.

Lastly, we tested the anti-MRSA efficacy of vancomycin-loaded RBC-nanogels on an in vitro intracellular infection model by using THP-1 derived macrophages as the cellular reservoir for MRSA USA300. In the study, we incubated macrophages with MRSA bacteria at a ratio of 1:10 and used gentamicin to eradicate extracellular bacteria. Under the condition, the ratio of macrophages to the intracellular bacteria was determined to be ~20:1. Next, the infected macrophages were treated with PBS, empty RBC-nanogels, empty control nanogels, vancomycin-loaded RBC-nanogels, vancomycin-loaded control nanogels and free vancomycin. After culturing the cells for 6 h, macrophages were lysed and the intracellular MRSA bacteria were quantified. Cells treated with PBS and vehicle controls (empty RBC-nanogels and empty control nanogels) showed a significant increase of intracellular bacterial burden to more than 15 CFU/100 macrophages. In comparison, cells treated with free vancomycin and vancomycin-loaded control nanogels only showed a moderate increase of bacterial burdens to 6 and 7 CFU/100 macrophages, respectively. Among all the groups,

only vancomycin-loaded RBC-nanogels demonstrated an inhibition of intracellular MRSA bacteria (Fig. 6B).

4. Conclusions

In summary, we synthesized a cell membrane-coated nanogel system consisting of a redox-responsive hydrogel core and an RBC membrane shell. The RBC membrane coating is for spontaneously absorbing and neutralizing virulence factors secreted by MRSA bacteria. The antibiotics-loaded hydrogel core is crosslinked with disulfide bonds responsive to intracellular reducing environment for ‘on-demand’ drug release specifically targeting intracellular MRSA bacteria. We demonstrated that in extracellular environment the RBC-nanogels effectively neutralized MRSA-associated toxins, which promoted bacterial uptake by macrophages. Once inside the cells, the RBC-nanogels rapidly released their carried antibiotics and showed remarkable antibacterial activity as compared to free antibiotics and non-responsive control nanogels. It is well documented that bacteria-secreting toxins play a critical role in bacterial pathogenesis [34]. Therefore antivirulence treatment by the RBC-nanogels can be potentially applied to a variety of different bacteria that are associated with hemolytic toxins. Meanwhile, although we chose vancomycin as a model anti-MRSA antibiotic in the study, cell membrane-templated nanogel synthesis can be applied to load other water-soluble antibiotics; membranes of other cells, especially those that target MRSA bacteria such as platelets, can be also used to template nanogel synthesis [35, 36]. Moreover, with the advancement of hydrogel technology, other responsive mechanisms, especially those responsive to bacterium-specific enzymes, can be incorporated into the hydrogel cores to further improve the controlled release and tempopsatial resolution during the treatment [37]. These features together make the RBC-nanogels a promising system for the treatment of various types of bacteria.

Acknowledgments

This work is supported by the National Institutes of Health under Award Numbers R01CA200574 and R01EY025947.

References

1. Foster TJ, Geoghegan JA, Ganesh VK, Hook M. Adhesion, invasion and evasion: The many functions of the surface proteins of *Staphylococcus aureus*. Nat. Rev. Microbiol. 2014; 12:49–62. [PubMed: 24336184]
2. Pantosti A. Methicillin-resistant *Staphylococcus aureus* associated with animals and its relevance to human health. Front. Microbiol. 2012; 3 article number 127.
3. Chambers HF, Deleo FR. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. Nat. Rev. Microbiol. 2009; 7:629–641. [PubMed: 19680247]
4. Yoong P, Pier GB. Antibody-mediated enhancement of community-acquired methicillin-resistant *Staphylococcus aureus* infection. Proc. Natl. Acad. Sci. U. S. A. 2010; 107:2241–2246. [PubMed: 20133867]
5. Edwards B, Andini R, Esposito S, Grossi P, Lew D, Mazzei T, Novelli A, Soriano A, Gould IM. Treatment options for methicillin-resistant *Staphylococcus aureus* (MRSA) infection: Where are we now? J. Glob. Antimicrob. Resist. 2014; 2:133–140. [PubMed: 27873719]
6. Vuong C, Yeh AJ, Cheung GYC, Otto M. Investigational drugs to treat methicillin-resistant *Staphylococcus aureus*. Expert Opin. Investig. Drugs. 2016; 25:73–93.

7. Tirilomis T. Daptomycin and its immunomodulatory effect consequences for antibiotic treatment of methicillin-resistant *Staphylococcus aureus* wound infections after heart surgery. *Front. Immunol.* 2014; 5 article number 97.
8. Burke SL, Rose WE. New pharmacological treatments for methicillin-resistant *Staphylococcus aureus* infections. *Expert Opin. Pharmacother.* 2014; 15:483–491. [PubMed: 24437531]
9. Kumar K, Chopra S. New drugs for methicillin-resistant *Staphylococcus aureus*: An update. *J. Antimicrob. Chemother.* 2013; 68:1465–1470. [PubMed: 23429643]
10. Gardete S, Tomasz A. Mechanisms of vancomycin resistance in *Staphylococcus aureus*. *J. Clin. Invest.* 2014; 124:2836–2840. [PubMed: 24983424]
11. Peacock SJ, Paterson GK. Mechanisms of methicillin resistance in *Staphylococcus aureus*. *Annu. Rev. Biochem.* 2015; 84:577–601. [PubMed: 26034890]
12. Singer AJ, Talan DA. Management of skin abscesses in the era of methicillin-resistant *Staphylococcus aureus*. *New Engl. J. Med.* 2014; 370:1039–1047. [PubMed: 24620867]
13. Brauner A, Fridman O, Gefen O, Balaban NQ. Distinguishing between resistance, tolerance and persistence to antibiotic treatment. *Nat. Rev. Microbiol.* 2016; 14:320–330. [PubMed: 27080241]
14. Hu CMJ, Zhang L. Nanotoxoid vaccines. *Nano Today.* 2014; 9:401–404. [PubMed: 25285152]
15. Greenlee-Wacker M, DeLeo FR, Nauseef WM. How methicillin-resistant *Staphylococcus aureus* evade neutrophil killing. *Curr. Opin. Hematol.* 2015; 22:30–35. [PubMed: 25394313]
16. Hu CMJ, Fang RH, Copp J, Luk BT, Zhang L. A biomimetic nanosponge that absorbs pore-forming toxins. *Nat. Nanotechnol.* 2013; 8:336–340. [PubMed: 23584215]
17. Hu CMJ, Fang RH, Luk BT, Zhang L. Nanoparticle-detained toxins for safe and effective vaccination. *Nat. Nanotechnol.* 2013; 8:933–938. [PubMed: 24292514]
18. Wang F, Gao W, Thamphiwatana S, Luk BT, Angsantikul P, Zhang QZ, Hu CMJ, Fang RH, Copp JA, Pornpattananangkul D, Lu W, Zhang L. Hydrogel retaining toxin-absorbing nanosponges for local treatment of methicillin-resistant *Staphylococcus aureus* infection. *Adv. Mater.* 2015; 27:3437–3443. [PubMed: 25931231]
19. Foster TJ. Immune evasion by staphylococci. *Nat. Rev. Microbiol.* 2005; 3:948–958. [PubMed: 16322743]
20. Lehar SM, Pillow T, Xu M, Staben L, Kajihara KK, Andlen RV, DePalatis L, Raab H, Hazenbos WL, Morisaki JH, Kim J, Park S, Darwish M, Lee BC, Hernandez H, Loyet KM, Lupardus P, Fong RN, Yan DH, Halouni CC, Luis E, Khalfin Y, Plise E, Heong JC, Lyssikatos JP, Strandh M, Koefoed K, Andersen PS, Flygare JA, Tan MW, Brown EJ, Ariathasan SM. Novel antibody-antibiotic conjugate eliminates intracellular *S. Aureus*. *Nature.* 2015; 527:323–328. [PubMed: 26536114]
21. Kubica M, Guzik K, Koziel J, Zarebski M, Richter W, Gajkowska B, Golda A, Maciag-Gudowska A, Brix K, Shaw L, Foster T, Potempa J. A potential new pathway for staphylococcus aureus dissemination: The silent survival of s. Aureus phagocytosed by human monocyte-derived macrophages. *PLoS One.* 2008; 3 article number e1409.
22. Kim HK, Thammavongsa V, Schneewind O, Missiakas D. Recurrent infections and immune evasion strategies of staphylococcus aureus. *Curr. Opin. Microbiol.* 2012; 15:92–99. [PubMed: 22088393]
23. Maya S, Indulekha S, Sukhithasri V, Smitha KT, Nair SV, Jayakumar R, Biswas R. Efficacy of tetracycline encapsulated o-carboxymethyl chitosan nanoparticles against intracellular infections of staphylococcus aureus. *Int. J. Biol. Macromol.* 2012; 51:392–399. [PubMed: 22705573]
24. Kamaruzzaman NF, Firdessa R, Good L. Bactericidal effects of polyhexamethylene biguanide against intracellular *staphylococcus aureus* EMRSA-15 and USA 300. *J. Antimicrob. Chemother.* 2016; 71:1252–1259. [PubMed: 26825118]
25. Xiong MH, Li YJ, Bao Y, Yang XZ, Hu B, Wang J. Bacteria-responsive multifunctional nanogel for targeted antibiotic delivery. *Adv. Mater.* 2012; 24:6175–6180. [PubMed: 22961974]
26. Meng YS, Hou XC, Lei JX, Chen MM, Cong SC, Zhang YY, Ding WM, Li GL, Li XR. Multi-functional liposomes enhancing target and antibacterial immunity for antimicrobial and anti-biofilm against methicillin-resistant *Staphylococcus aureus*. *Pharm. Res.* 2016; 33:763–775. [PubMed: 26666773]

27. Zhang J, Gao W, Fang RH, Dong A, Zhang L. Synthesis of nanogels via cell membrane-templated polymerization. *Small*. 2015; 11:4309–4313. [PubMed: 26044721]
28. Gao W, Langer R, Farokhzad OC. Poly(ethylene glycol) with observable shedding. *Angew. Chem. Int. Ed*. 2010; 49:6567–6571.
29. Fairbanks BD, Schwartz MP, Bowman CN, Anseth KS. Photoinitiated polymerization of PEG-diacrylate with lithium phenyl-2,4,6-trimethylbenzoylphosphinate: Polymerization rate and cytocompatibility. *Biomaterials*. 2009; 30:6702–6707. [PubMed: 19783300]
30. Ma YJ, Thiele J, Abdelmohsen L, Xu JG, Huck WTS. Biocompatible macro-initiators controlling radical retention in microfluidic on-chip photopolymerization of water-in-oil emulsions. *Chem. Commun*. 2014; 50:112–114.
31. Hu CMJ, Zhang L, Aryal S, Cheung C, Fang RH, Zhang L. Erythrocyte membrane-camouflaged polymeric nanoparticles as a biomimetic delivery platform. *Proc. Natl. Acad. Sci. U. S. A*. 2011; 108:10980–10985. [PubMed: 21690347]
32. McGee MP, Kreger A, Leake ES, Harshman S. Toxicity of staphylococcal alpha-toxin for rabbit alveolar macrophages. *Infect. Immun*. 1983; 39:439–444. [PubMed: 6822424]
33. Berube BJ, Wardenburg JB. *Staphylococcus aureus* alpha-toxin: Nearly a century of intrigue. *Toxins (Basel)*. 2013; 5:1140–1166. [PubMed: 23888516]
34. Ray K, Marteyn B, Sansonetti PJ, Tang CM. Life on the inside: The intracellular lifestyle of cytosolic bacteria. *Nat. Rev. Microbiol*. 2009; 7:333–340. [PubMed: 19369949]
35. Hu CMJ, Fang RH, Wang KC, Luk BT, Thamphiwatana S, Dehaini D, Nguyen P, Angsantikul P, Wen CH, Kroll AV, Carpenter C, Ramesh M, Qu V, Patel SH, Zhu J, Shi W, Hofman FM, Chen TC, Gao WW, Zhang K, Chien S, Zhang L. Nanoparticle biointerfacing by platelet membrane cloaking. *Nature*. 2015; 526:118–121. [PubMed: 26374997]
36. Hu QY, Sun WJ, Qian CG, Wang C, Bomba HN, Gu Z. Anticancer platelet-mimicking nanovehicles. *Adv. Mater*. 2015; 27:7043–7050. [PubMed: 26416431]
37. Li YM, Liu GH, Wang XR, Hu JM, Liu SY. Enzyme-responsive polymeric vesicles for bacterial-strain-selective delivery of antimicrobial agents. *Angew. Chem. Int. Ed*. 2016; 55:1760–1764.

Fig. 1.

Schematic illustrations of a red blood cell (RBC) membrane-coated nanogel (RBC-nanogel) system for combinatorial extracellular toxin neutralization and intracellular redox-responsive antibiotic release. (A) The RBC-nanogel is constructed with a natural RBC membrane shell and a hydrogel core containing antibiotic payload. The hydrogel core is crosslinked with disulfide bonds, cleavable in reducing environment. (B) In extracellular environment, the RBC-nanogel absorbs and neutralizes pore-forming toxins (PFT) secreted by bacteria. Such toxin sequestration facilitates the intracellular uptake of the toxin-secreting bacteria and the RBC-nanogel. Once inside the cell, the RBC-nanogel rapidly releases its encapsulated antibiotic payload triggered by a redox reaction.

Fig. 2.
Characterization of cystine dimethacrylate (CDA), a redox-responsive crosslinker. (A) ^1H -NMR spectrum and (B) MALDI-TOF spectrum of CDA.

Fig. 3.

The formulation and characterization of RBC-nanogels. (A) RBC-nanogels loaded with rhodamine B were formulated and subjected to (i) no treatment, (ii) treated with Triton X-100 and proteinase K, or (iii) Triton X-100 and proteinase K followed by tris(2-carboxyethyl) phosphine (TCEP). The RBC-nanogels were then filtered to collect the released dye, which was further measured by a UV-vis spectrophotometer. (B) A representative TEM image of RBC-nanogels (scale bar = 100 nm). (C) Dynamic light scattering (DLS) measurements of the size and size distribution of RBC-vesicles, RBC-

nanogels, and non-responsive RBC nanogels (Control nanogels) subjected to the same treatment as in (A).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 4.

Toxin neutralization by RBC-nanogels promotes intracellular uptake of MRSA USA300 bacteria by THP-1 derived macrophages. (A) The relative hemolysis activity of α -toxin in the presence or absence of 2.4 μ g (protein amount) of RBC-nanogels is quantified by measuring the absorption of released hemoglobin in the supernatant. (B) Representative flow cytometry analyses of bacterial uptake by macrophages. The bacteria were labeled with calcein-AM and exposed to macrophages after treated with 20 μ g free α -toxin or 20 μ g α -toxin mixed with 50 μ g RBC-nanogels (protein amount). Untreated sample served as a control group. (C) Quantification of the mean fluorescence intensity values based on the histograms in (B). Data are shown in mean $\pm$ SEM (* p < 0.5). Three independent experiments were carried out.

Fig. 5.

The cumulative release profiles of vancomycin from (A) RBC-nanogels and (B) non-responsive RBC-nanogels (Control nanogels). The nanogels were treated with Triton X-100, treated with Triton X-100 followed by TCEP, or not treated by anything.

Fig. 6. Antimicrobial efficacy of vancomycin-loaded RBC-nanogels against intracellular MRSA USA300 bacteria. (A) In vitro antibacterial activity of free vancomycin (Free Vanc), vancomycin-loaded RBC-nanogels (RBC-nanogels(Vanc)), and vancomycin-loaded control nanogels (control nanogel(Vanc)) at different vancomycin concentrations. (B) Quantification of bacterial burden inside macrophages treated with PBS, empty RBC-nanogels, empty control nanogels, RBC-nanogels(Vanc), control nanogel(Vanc) and free Vanc, respectively. For treatment groups, vancomycin concentration was 5 μg/mL. The dash line marks initial intracellular bacterial burden. The viable bacteria of each group were enumerated 6 h after the treatment. The statistical significance was assessed with student *t* test: *ns*, no significant difference, **p* < 0.05, ***p* < 0.01, ****p* < 0.001.