Encapsulation of collagen mimetic peptide-tethered vancomycin liposomes in collagen-based scaffolds for infection control in wounds

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Abstract

Wound infections are a significant clinical problem affecting millions of people worldwide. Topically applied antibacterial formulations with longer residence time and controlled antimicrobial release would offer significant benefits for improved prevention and treatment of infected wounds. In this study, we developed collagen mimetic peptide (CMP) tethered vancomycin (Van)-containing liposomes (Lipo) (CMP-Van-Lipo) hybridized to collagen-based hydrogels ('co-gels,' e.g., collagen/fibrin combination hydrogels) for the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infections in vitro and in vivo. Tethering CMP-Van-Lipo nanostructures to co-gels enabled sustained Van release and enhanced in vitro antibacterial effects against MRSA as compared to Van loaded co-gels or Van-Lipo loaded co-gels following multiple fresh bacterial inoculations over a period of 48 h. These results were successfully translated in vivo wherein MRSA infected wounds were effectively treated with CMP-Van-Lipo loaded co-gels up to 9 days, whereas the activity of Van loaded co-gels and Van-Lipo loaded co-gels were limited to < 2 days. Moreover, CMP-Van-Lipo retained in vivo antibacterial activity even after re-inoculation with bacteria; however, Van loaded co-gels and Van-Lipo loaded co-gels allowed significant bacterial growth resonating their limited efficacy. Altogether, these results provide proof-of-concept that CMP-Van-Lipo loaded co-gels can be effective topical formulations for preventative treatment of MRSA wound infections.

Keywords: Co-gel; Collagen mimetic peptide; Liposomes; MRSA; Vancomycin; Wound infection

1. Introduction

Superficial infections pose a serious problem in the healing of wounds leading to the development of a chronic, non-healing condition [1, 2]. One of the common examples of such wounds is the diabetic foot ulcer, which affects 9.1 - 26.1 million people worldwide [3]. The most prevalent genera that constitute microbiota of chronic wounds include *Staphylococcus* and *Pseudomonas* [4]. Bacterial infection of wounds can have several adverse consequences, including excessive inflammation and prevention of wound healing [5, 6]. Furthermore, biofilms produced by microbial colonies (prevalent in 60% of chronic wounds and 6% of acute wounds) interact with host tissue in a parasitic manner [5, 7]. For example, the extracellular adherence protein (Eap) of *Staphylococcus aureus* has potent anti-inflammatory and anti-angiogenic properties that interfere with the host defense and repair mechanisms and thereby inhibit wound healing [8]. *Staphylococcus aureus* biofilms deplete oxygen, increase pH, and induce cell death [9]. Meanwhile, infections caused by *Pseudomonas aeruginosa* or a combination of methicillin resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* contribute to the enhanced expression of pro-inflammatory cytokines, which also results in an inhibited wound healing process [10].

Given these adverse effects, efforts to treat infections through antimicrobial delivery are of great interest due to their potential to aid in the healing process [11]. Oral or parenteral antimicrobial delivery is used most commonly, due to rapid absorption and high bioavailability, but oral delivery approaches require higher doses to produce therapeutically-relevant concentrations at the infection site. Topical application would be a viable approach to reduce the dose and maintain controlled levels of the active drug locally [12], enhancing antibacterial effects and resisting reinfection within wounds that exhibit delayed healing.

Hydrogels are appealing scaffolds for topical application and controlled release of biomolecules or small drug molecules [13]. For example, engineered collagen hydrogels were designed for the sustained release of biomolecules (e.g., TGF- β1) for > 24 h to promote the growth of human gingival cells [14]. Heparin-modified collagen gels were developed for controlled release of pleiotrophin over a > 10 day period, to potentially improve the biological performance and integration of vascular grafts [15]. Hydrogels also offer capacity for sustained release of small molecule agents such as antimicrobials for infection control; however, small drug molecules migrate freely and hence hydrogels cannot sustain their release profile over the requisite multi-day periods for chronic wound treatment [16]. Therefore, appropriate modifications either to reduce the mesh size of the hydrogel or to incorporate nano- or microparticles for controlling drug diffusion are potentially viable approaches [16, 17].

A variety of nano- and microparticles offer capacity to control the release of small molecules over extended time periods. For example, polyvinylpyrrolidone-capped zinc oxide nanoparticle-cross-linked collagen shields were prepared for sustained delivery (over > 14 days) of pilocarpine hydrochloride in the treatment of glaucoma [18]. Liposomes may offer additional advantages in the context of antimicrobial delivery due to their biocompatibility, biodegradability, ease in formulation, high drug loading capacity, and controlled drug release properties [19-21]. A series of in vitro studies report the preparation of vancomycin-loaded liposomes for the induction of biofilm-inhibition effects in infections of *Staphylococcus epidermidis* (cationic liposomes) [22], *Staphylococcus aureus* (cationic liposomes) [23], or *E. coli* and *Pseudomonas aeruginosa* (fusogenic liposomes) [24]. In other examples, liposome-encapsulating biomaterials have been designed to increase the residence time of liposomes at the application site, resulting in localized antimicrobial release [25]. A nano-hydroxyapatite/chitosan/konjac glucomannan scaffold loaded

with cationic liposomal Van effectively inhibited the in vitro formation of *Staphylococcus aureus* biofilms [26], and similarly, 3D-printed poly(ε-caprolactone) composite scaffolds incorporating Van loaded polylactic acid-glycolic acid microspheres exhibited good biocompatibility and sustained antibacterial effects in vitro [27]. Drug release from nano-hydroxyapatite/chitosan/konjac glucomannan scaffold loaded with cationic liposomal Van was sustained for > 48 h, however, initial burst release ~50-70% of Van was observed that could limit the antibacterial activity at > 8 h time points [26]. 3D-printed poly(ε-caprolactone) composite scaffolds incorporating Van loaded polylactic acid-glycolic acid microspheres sustained Van release for 28 days, however, controlled (~30%) drug was released after 24 h which might not be enough to effectively control the log phase growth of bacteria and might lead to the development of resistance [27]. Furthermore, poly(ε-caprolactone) is non-biodegradable and hence would affect the wound integrity during the wound healing phase. These studies support the promise of particle/hydrogel formulations for localized and sustained antimicrobial delivery, yet control over particle retention was limited, and in vivo experiments were lacking to support the effectiveness of the systems in promoting healing. Creating extracellular matrix (ECM)-inspired biomaterials that can control drug delivery via binding/sequestration in the matrix, while simultaneously promoting cellular healing responses via the bioactivity of the scaffold, offer a compelling alternative that may produce significant improvements in overall wound repair activity.

Collagen is involved in all three phases of wound healing - inflammation, proliferation, and maturation - and it stimulates cell migration and contributes to new tissue development [28]. Collagen dressings promote the deposition and organization of newly formed collagen, developing an environment that fosters wound healing because of the dressing's chemotactic properties on wound fibroblasts. These biomaterial scaffolds can provide moisture or absorption and are easy to

apply/remove and are conformable [28]. Moreover, the versatile triple helical structure of collagen can be harnessed to incorporate nanocarriers and other therapeutic agents as pendant groups on the collagen fibrils [29-31]. For this purpose, collagen mimetic peptides (CMPs) offer an especially appealing modification approach. CMPs are comprised of collagen-like $(GXY)_n$ motifs that can spontaneously fold into the hallmark collagen triple helix, and CMPs also can be incorporated into native collagen triple helices via strand invasion and exchange [32]. Herein, CMPs were conjugated to the surface of Van-loaded liposomal agents via maleimide-thiol click chemistry. These CMP-tethered liposomal particles (CMP-Van-Lipo) were incorporated within collagen scaffolds to retain the Lipo for sustained Van delivery (**Scheme 1**). Tethering CMP-Van-Lipo to co-gels sustained Van delivery as compared to Van-Lipo or Van-Lipo loaded co-gels for up to 48 h. The in vitro antibacterial effects of CMP-Van-Lipo loaded co-gels on MRSA were superior to Van loaded co-gels and Van-Lipo loaded co-gels even after multiple fresh bacterial inoculations over a total period of 48 h. These antibacterial effects of CMP-Van-Lipo loaded co-gels were successfully translated in vivo in a MRSA-inoculated murine excisional wound model. More importantly, the in vivo antibacterial effects of CMP-Van-Lipo, but not of Van solution, Van loaded co-gels, or Van-Lipo loaded co-gels, were retained even following a second fresh bacterial inoculation on the wound. This study is a proof-of-concept for the potential application of CMP-Van-Lipo loaded co-gel for sustained antimicrobial release and potential infection control in wounds.

2. Materials and methods

2.1. Materials

Fmoc-protected amino acids, H-Rink amide ChemMatrix® resin, and O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) were purchased from Anaspec (Fremont, CA, USA), PCAS Biomatrix (Quebec, Canada), and Novabiochem (San Diego, CA, USA), respectively. High performance liquid chromatography (HPLC)-grade acetonitrile, N,N dimethyl formamide (DMF), trifluoroacetic acid (TFA), and cell culture reagents, including Opti-MEM® I Reduced Serum Media (Opti-MEM), Dulbecco's phosphate buffered saline (DPBS), Dulbecco's modified Eagle's medium (DMEM), and trypsin were purchased from Fisher Scientific (Fairlawn, NJ, USA). Fetal bovine serum (FBS) and Type I bovine collagen were purchased from Corning (Manassas, MA, USA) and Advanced BioMatrix (San Diego, CA, USA), respectively. CellTiter 96® AQ$_{ueous}$ One Solution [contains 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine ethosulfate (PES)] was procured from Promega (Madison, WI, USA). All cleavage cocktail components, piperidine, 4-methylmorpholine, and syringes were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000-maleimide] (DSPE-PEG-Mal) and cholesterol were procured from Avanti Polar Lipids, Inc. (Alabama, USA), Sigma Aldrich (St. Louis, MO, USA), and Nanocs Inc. (New York, USA), respectively. A luminescent strain of *Staphylococcus aureus* (SAP231, luminescent version of USA300 MRSA strain NRS384) was kindly gifted by Dr. Roger Plaut [33]. Van, tryptic soy broth, tryptic soy agar, and chloramphenicol were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Animals

BALB/c mice (8 week-old, male) were procured from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA). All experiments were performed in accordance with the guidelines from Institutional Animal Care and Use Committee-approved protocol.

2.3. Collagen-mimetic peptide (CMP) synthesis

An automated Fmoc solid-phase peptide synthesis method was used to synthesize the CMP sequence [$(GPP)_3$GPRGEKGERGPR$(GPP)_3$GPCCG] containing 35 amino-acids using a Tribute peptide synthesizer (Protein Technologies Inc., Tucson, AZ) as explained previously [32]. The Rink amide MBHA resin was used for peptide synthesis. Specifically, the amino acid residues were activated for coupling with HBTU in 0.4 M methylmorpholine in DMF and de-protected using 20% piperidine in DMF for 10 min. A coupling time of 1 h was used, and all amino acid residues after the 15_{th} residue were double coupled. The CMP sequence was cleaved from the resin using a cocktail of 94:1:2.5:2.5 TFA/triisopropylsilane (TIS)/water/1,2-ethanedithiol (EDT) for 6 h. The cocktail was then evaporated and the cleaved peptides were precipitated in ethyl ether, dissolved in water, and lyophilized.

Crude peptides were purified using a reverse phase-high performance liquid chromatography (HPLC) on a Prominence Chromatography Instrument (Shimadzu, Inc., Columbia, MD) equipped with a Viva C18 (4.2 mm x 50 mm, 5 μm particle diameter) column from Restek (Lancaster, PA). Water with 0.1% TFA (Solvent A) and acetonitrile with 0.1% TFA (Solvent B) were employed as HPLC solvents with a gradient of solvent B from 25-35% over 30 mins. The eluent absorbance was monitored at λ = 210 nm and purity was determined to be ~70% (**Figure S1A**). The purity of crude peptides was calculated based on the ratio of the area for all

peaks observed under HPLC to that of the pure peptide peak. During HPLC, the pure peptides were collected separately from within the pure peptide peak area observed and were lyophilized for further studies. Such collected peptides exhibited purity of > 95% (insets in Figure S1A) and were used for further studies. Matrix-assisted laser desorption/ionization-mass spectroscopy (MALDI-MS) analysis was performed to confirm the product based on observed m/z (≈ 1610, 2+ charged species); the observed molecular mass was ≈ 3220 (**Figure S1B**). Circular dichroism studies confirmed the triple helical structure and melting temperature (≈ 41.04 °C) of the synthesized CMPs (**Figure S1C and S1D**), using previously described methods [34].

2.4. Preparation and characterization of liposomes

2.4.1. Preparation of CMP-Van-Lipo

Liposomes were prepared using a thin film hydration technique (**Figure S2**). Lipids at a molar ratio of 73:24:3 (DPPC:Cholesterol:DSPE-PEG-Mal) were added to a round-bottom flask containing a mixture of chloroform and methanol (4:1 volume ratio). The round-bottom flask was then connected to a rotary evaporator and a thin film was developed by overnight vacuum drying the solvents in a water bath set at 40 °C. Afterwards, the temperature of the water bath was increased to ≈ 60 °C. Separately, an aqueous solution of Van was prepared by dissolving Van in PBS and heating to 60 °C for 10 min. The heated Van solution was added to the thin film and the mixture was rotated in the round bottomed flask to develop unilamellar vesicles. After 10 mins of rotation, the round bottomed flask was removed from the rotary evaporator and the contents sonicated for 30 s. Immediately after sonication, the dispersion was extruded using a mini-extruder (Avanti Polar Lipids Inc., Alabaster, AL) to obtain a monodisperse Van-Lipo formulation. The

prepared Van-Lipos were then flushed with nitrogen gas, and CMP solution was subsequently added at a molar ratio of 1:1 (CMP:DSPE-PEG-Mal). The thiol-maleimide click chemistry reaction was allowed to proceed overnight to produce CMP-Van-Lipo formulations that were then dialyzed to remove excess Van and CMP. Finally, the dialyzed CMP-Van-Lipo was lyophilized in the presence of 20 mM sucrose.

2.4.2. Hydrodynamic particle diameter, polydispersity, and zeta potential analysis

Particle diameter, polydispersity index (PDI), and zeta potential measurements were carried out using dynamic light scattering (DLS) with a Nano-S90 ZetaSizer (Malvern Instruments, Worcestershire, U.K.). Following measurements, Stokes-Einstein equation was used to determine the final particle diameter while PDI and zeta potential were evaluated using Nano DTS software (version 6.34). All measurements were performed at 25 °C with at least three sets of 10 runs.

2.4.3. Transmission electron microscopy

The cross-sectional morphology of the CMP-Van-Lipo nanoparticles were examined using transmission electron microscopy (TEM, H7600, Hitachi, Tokyo, Japan). The CMP-Van-Lipo solutions were mixed with 2% phosphotungstic acid solution, added to a carbon-coated copper grid, and air dried. The grid was then viewed under the accelerating voltage using a microscope.

2.4.4. Determination of CMP conjugation of Van-Lipo

CMP conjugation to the Van-Lipo was evaluated by using a 0.2 mg/mL collagen coated Nunc Maxisorp black plate. Neutralized collagen (50 μL) was added and plate was shaken for 1 h. Afterwards, the solution from the wells was removed and 0.1% BSA in PBS was used for washing the plate three times. A solution containing 5% BSA in PBS was used for blocking by incubation at room temperature for 1 h followed by two washes with 0.1% BSA. For the development of a calibration curve, Carboxyfluorescein-CMP (CF-CMP) was diluted in DI water at a concentration range from 1 to 120 μM. CF-CMP conjugated Van-Lipo were separately prepared by adding CF-CMP to give a final concentration of 102 μM. Samples were then heated at 60 °C for 5 min, added to the wells, incubated at room temperature with shaking for 2 h, and washed with 0.1% BSA three times. Fluorescence measurements on the plate were taken using a plate reader (ex. λ = 489 nm; em. λ = 533 nm). CF-CMP/Lipo conjugation was calculated based on the fluorescence measurements. Furthermore, zeta potential measurements for the Van-Lipo and CMP-Van-Lipo samples were evaluated.

2.4.5. Determination of encapsulation efficiency (EE)

The entrapment of Van within the liposomes was calculated by determining the free and total drug concentrations in the formulation. Briefly, the CMP-Van-Lipo formulation was filtered using an Amicon centrifugal ultrafiltration device (MWCO 10,000 Da; Millipore, Billerica, MA, USA). The concentration of free Van was determined using UV-Vis spectroscopy at a wavelength of 280 nm. For determination of the total Van concentration, liposomes were disrupted using Triton X-100 solution (empty liposomes were used as blank), and the resulting absorbance in the

solution was analyzed with UV-Vis spectroscopy. The encapsulation efficiency (EE) (%) was calculated using the following formula:

$$EE = \frac{W_{CMP-Van-Lipo}}{W_T} \times 100$$

where $W_{CMP\text{-}Van\text{-}Lipo}$ = weight of Van entrapped in CMP-Van-Lipo, and W_T = total weight of Van added to the formulation.

2.4.6. In vitro drug release study

The in vitro Van release from the CMP-Van-Lipo structures was assessed using dialysis and UV-Vis spectroscopic detection of Van. An aliquot of each liposomal formulation was placed in dialysis membrane tubing (Spectra/Por, MWCO 3500 Da; Spectrum Labs, Rancho Dominguez, CA, USA) and immersed in 10 mL of phosphate buffered saline (PBS, pH 7.4) on a shaker. The shaker was maintained at 100 rpm at 37 °C, and samples were withdrawn and replaced with fresh medium at predetermined time intervals. The concentration of Van released into the medium was quantified using UV-Vis spectroscopy as described previously.

2.5. Preparation and characterization of co-gels

2.5.1. Preparation of liposome loaded co-gels

Modified collagen gels, composed of collagen, fibrinogen and thrombin, were prepared and are referred to as “co-gels”. First, neutralized collagen was prepared by mixing collagen type I (Firbricol®, Advanced BioMatrix, San Diego, CA, USA) with 10X PBS and 0.1 N NaOH.

Various concentrations of collagen (2, 3, or 4 mg/ml) along with fibrinogen (1.25 mg/ml) and thrombin (0.156 IU/ml) were uniformly mixed in the given order and incubated at 37 °C to form co-gels. The polyplex-loaded co-gels were prepared by mixing the lyophilized polyplexes in neutralized collagen followed by incubation at 4 °C for 1 h before the addition of fibrinogen and thrombin to form the co-gel via incubation at 37 °C for at least 6 h. The CMP-Van-Lipo loaded co-gels were prepared by mixing the lyophilized CMP-Van-Lipo in neutralized collagen and storing the resulting solution at 4 °C for 1 h before the addition of fibrinogen and thrombin followed by incubation at 37 °C.

2.5.2. Rheology studies

The oscillatory rheology measurements were performed on an DHR-3 rheometer (TA Instruments, New Castle, DE) with a 20 mm diameter stainless steel parallel-plate geometry. The components of the co-gels mentioned above were mixed immediately prior to the addition to rheometer plate. A gap distance of 500 μm and temperature of 37 °C was used for the study. The viscoelastic properties of the co-gels were assessed via frequency sweep (0.1 to 10 rad s^{-1}) and strain sweep (0.1-10%) experiments. The gelation of co-gels was monitored using a time sweep measurements conducted in the linear viscoelastic regime at 1% strain and an angular frequency of 10 rad s^{-1}. Shear storage moduli (G') and loss moduli (G") for the co-gels were recorded. Experiments were repeated at least on three individual, separate samples for co-gels. Among the different co-gel concentrations tested, co-gels with 4 mg/ml collagen with fibrinogen (1.25 mg/mL) and thrombin (0.156 IU/mL) presented the best integrity based on storage (G') and loss (G") modulus analysis (**Figure S3**), and this composition was used for further studies. The

recorded storage modulus (G') and loss modulus (G") of the prepared co-gels were 149 Pa and 27 Pa, respectively.

2.5.3. Liposome release study from co-gels

Determination of Lipo release from the co-gels was evaluated by using DiR' dye-labeled Lipo. Firstly, blank Lipo were prepared with or without CMP modification. DiR' dye was then added to the Lipo at a molar ratio of 100:1 (lipid:DiR' dye), and the labeled Lipo were centrifuged to remove excess dye and subsequently lyophilized. The DiR' dye absorbs strongly only when incorporated into the membrane, so provides a faithful measurement of liposome release. Co-gel solutions were prepared with a composition of 4 mg/mL collagen, 1.25 mg/mL fibrinogen, and 0.156 IU/mL thrombin. Lyophilized DiR' Lipo or DiR' CMP-Lipo (30 μg/gel DiR') were incorporated directly into the co-gel solutions prior to gelation. Five hundred microliters of the co-gel solution was placed onto a petri dish and allowed to gel at 37 °C for 6 h. Following gelation, 5 mL PBS was added to each petri dish to facilitate DiR' Lipo release. Samples were collected at 1, 3, 6, 12, 24, 36, 48, 60, and 72 h, and cumulative Lipo release was determined for each sample using absorbance measurements (λ = 750 nm) in a Nanodrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.5.4. Drug release study from co-gels

In vitro drug release of Van from the co-gels was evaluated by comparing the drug release between free drug, Van-Lipo, and CMP-Van-Lipo loaded co-gels. Co-gels were prepared with a

composition of 4 mg/mL collagen, 1.25 mg/mL fibrinogen, and 0.156 IU/mL thrombin. Free drug, Van-Lipo, and CMP-Van-Lipo (equivalent to 1.25 mg/gel Van) were incorporated into the co-gels. Five hundred microliters of each co-gel solution was placed onto a petri dish and allowed to gel at 37 °C for 6 h. Following gelation, 5 mL PBS was added to each petri dish to facilitate Van release. Samples were collected at 1, 3, 6, 12, 24, 36, and 48 h, and cumulative Van release was determined for each sample using absorbance measurements (λ = 280 nm) in the Nanodrop Spectrophotometer.

2.6. Determination of Van minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of Van for luminescent methicillin-resistant *Staphylococcus aureus* (MRSA) was determined by using the broth dilution method. One hundred fifty microliters of tryptic soy broth containing $5x10_5$ cfu/mL of MRSA was added to each well of a 96-well plate. Separately, 150 µL of Van diluted at concentrations of 128, 64, 32, 16, 8, 4, 2, 1, 0.50, 0.25, 0.125, and 0.06 µg/mL was added to the wells. The plate was incubated at 37 °C for 16 h and subsequently the O.D. was measured at 600 nm to determine the MIC. A total of three groups were included for the study: negative control (tryptic soy agar only), positive control (tryptic soy agar with bacteria), and test group (tryptic soy agar with bacteria and Van).

2.7. Determination of in vitro antibacterial activity of co-gels loaded with Van loaded formulations

Co-gels (100 μL) loaded with free Van, Van-Lipo, or CMP-Van-Lipo at concentrations of 4, 7, and 10 μg/mL Van per gel were added to the wells of a 96-well plate, and the plate was incubated at 37 °C overnight for gelation. The MRSA stock was diluted in tryptic soy broth to obtain the desired O.D. (≈ $5x10^5$ cfu/mL). Aliquots of this bacterial culture (200 μL) were added to each well in the 96 well plate after the co-gels were washed with broth. The plate was incubated at 37 °C for 16 h and O.D. was checked every hour. Sixteen hours after the first inoculation, the bacterial cultures were removed from the wells and the wells were washed once with broth. The wells were re-inoculated with the bacterial culture and incubated at 37 °C for an additional 16 h to evaluate bacterial growth subsequent to repeated inoculation. The process was repeated for the third time and O.D. measurements were taken for a total of 48 h and were compared to untreated control samples.

In vivo infected wound healing follows complex mechanisms, and an in vitro experiment cannot completely simulate these mechanisms. The major considerations are: wound fluids containing bacteria, re-infection caused by infesting bacteria, and antibiotic delivery to control bacterial growth. Thus, the main goal of our in vitro experiments was to capture these key factors defining infected wound healing. We specifically sought to maintain the co-gel in direct contact with the wound fluid containing bacteria. However, we note that the experimental set-up was inverted as compared with in vivo wound co-gel application because we used co-gel loaded 96 well plates and added bacterial broth to the wells of these plates (vs. adding a broth and applying co-gel to the broth); this approached enabled direct analysis of Van release from the co-gels based on the observed antibacterial activity elicited by the released Van. Moreover, chronic wounds often remain open over prolonged time periods, providing repeated opportunities for bacterial exposure/infection. Therefore, we designed the experiment to simulate the potential for serial re-

exposure to bacteria within the chronic wound bed, with the specific goal of testing the antibacterial activity of the co-gels for up to 16 h following each fresh inoculation to resemble the potential re-infection in a chronic wound scenario. Furthermore, this study design gives information on the real-time antibacterial effects of the released Van localized to the surface of the co-gels. This study is a first step toward new ways of managing antibiotic delivery to wounds. Future additional modifications in the antibiotic delivery methods and experiments can further illuminate the beneficial effects of this system in effectively treating chronic wound infections.

An In Vivo Imaging System Lumina (IVIS®) (Perkin Elmer, Waltham, MA, USA) was used for imaging and quantitative evaluation of the luminescent bacteria numbers following the 16 h serial incubations. The settings for IVIS imaging included: F/stop: 1, exposure: 1 min, binning: small. Photographic images were taken and luminescence was calculated using the ROI tool from the IVIS software.

2.8. Cell viability assay of fibroblasts

Cell viability in the presence of various liposome and co-gel formulations was evaluated by treating fibroblasts (NIH-3T3 cells) grown in 96-well plates with free Van, Van-Lipo, or CMP-Van-Lipo at Van concentrations of 0.1, 1, 10, 25, 50, and 100 µg/mL. Separately, co-gels (100 µL) encapsulating free Van, Van-Lipo, or CMP-Van-Lipo were added to 96 well-plates at concentrations of 0.1, 1.0, 10.0, 25.0, 50.0, and 100.0 µg/mL, as explained previously. The plates were incubated at 37 °C overnight for gelation. Blank co-gel was used as control. The next day, the co-gels were washed with PBS and incubated with complete DMEM [DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S)] for 1 h. The full media

was removed after 1 h and 10,000 NIH-3T3 cells/cm_2 were added to each well. The cells were incubated for an additional 24 or 48 h, and the cells were subsequently treated with CellTiter 96® AQ_{ueous} One Solution for determination of cell viabilities by measuring O.D. at 490 nm.

2.9. Determination of in vivo antibacterial activity in wound infection model

The in vivo wound infection model was developed in BALB/c mice. First, mice were anesthetized and the hair on their dorsa was removed by using depilatory cream. Punch biopsy wounds (one per mouse) were subsequently created using a 5 mm diameter punch. Immediately after wound development, 5 µL ($1x10_8$ cfu) of luminescent *Staphylococcus aureus* was applied on the wounds. Following the inoculation of bacteria in the wounds, co-gels (encapsulating Van, Van-Lipo, or CMP-Van-Lipo) were applied each corresponding to a Van dose of 100 µg per gel. PBS and blank co-gel treatments were used as controls. The in vivo wounds were then covered with Opsite wound dressing and imaged using IVIS Lumina imaging for 9 days. ROI measurements were used to evaluate the number of bacteria present in the treated wounds. At the end of day 9, mice were sacrificed and skin samples from the wound area were collected. The obtained biopsies were stained with hematoxylin and eosin (H&E) as well as gram staining.

The current treatment procedure was designed to retain the bacteria as well as the co-gel formulation within the punch biopsy wound. From our previous experience with in vivo wound infection models, the applied bacterial suspension can drip off the wounds allowing minimal or no infection. Therefore, in our study, we applied Opsite wound dressing after adding the bacteria to the wounds. The wound dressing is sticky and cannot be easily removed a day after the bacterial inoculation for co-gel application. Furthermore, the co-gel forms instantly after mixing the

components and hence is straightforward to apply while the wounds are still open and not covered with wound dressing. Our results supported the fact that bacterial infections developed relatively quickly after bacterial inoculation and application of Opsite wound dressing, and the infection was sustained without treatment for several days. Therefore, this method was used for the development of wound infection and simultaneous treatment using CMP-Van-Lipo loaded co-gel formulations.

2.10. Statistical analysis

Results are expressed as mean ± standard deviation (SD). One-way ANOVA and Student's *t* tests were used to assess differences between the various control and experimental groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Preparation and characterization of CMP-Van-Lipo

CMP-Van-Lipo nanostructures were prepared using a step-wise process that included moderate heat treatment (50 °C) to produce monomeric CMPs as well as lyophilization to enable co-gel incorporation (**Figure S2**). Accordingly, liposomes were serially characterized at each step of the process. Liposomes were formulated using a thin film hydration technique with a lipid composition of DPPC:Cholesterol:DSPE-PEG-Mal. First, blank Lipo samples were prepared to evaluate the baseline nanoparticle characteristics (**Figure S4a**). Freshly prepared blank Lipo were ≈ 165 nm in diameter with a low PDI (≈ 0.05) and a negative zeta potential (≈ -5.0 mV). Storage at room temperature for up to 48 h led to a moderate increase in liposomal particle diameter (≈ 180

nm) and PDI (≈ 0.15) and a decrease in zeta potential (≈ -7.0 mV). Van-Lipo samples were prepared using the same method as blank Lipo except for the addition of Van to the hydrating buffer. Van-Lipo were ≈ 150 nm in diameter with a low PDI (≈ 0.05) and a modestly negative zeta potential (≈ -3.0 mV); these samples also maintained their particle characteristics for up to 48 h with only slight to moderate changes in particle diameter, PDI, and zeta potential (**Figure S4b**).

The temperature stability of the Van-Lipo samples was assessed following heating to 50 °C for 24 – 48 h (**Figure S4c**). After a 24 h incubation, the particle diameter increased to ≈ 225 nm with higher PDI and zeta potential. By the end of a 48 h incubation, a significant increase in particle diameter (≈ 325 nm) and PDI (≈ 0.4) were evident suggesting that prolonged heating led to liposome instability. As heat treatment was designed to produce monomeric CMPs prior to lyophilization, a process that typically requires only ≈ 1 h at elevated temperatures [32], these results indicated that brief heat treatment of Van-Lipo at 50 °C would allow CMP melting without altering the diameter and surface charge of the liposomes.

Lyophilized Lipo, prepared using sucrose as a cryoprotectant, were employed to enable incorporation of liposomes into co-gels that were used for in vitro and in vivo studies. Accordingly, changes in the liposomes following lyophilization were evaluated. A comparison of the particle properties for non-lyophilized and lyophilized Lipo is presented in **Figure S5a**. A slight increase in particle diameter, PDI, and zeta potential was observed, but the changes were not significant. The lyophilized Lipo were resuspended in PBS and incubated at 25 °C or 37 °C to evaluate any changes in particle stability evident upon prolonged incubation (**Figure S5b and S5c**). At both temperatures, Lipo samples maintained the same particle diameters with only modest changes in PDI and zeta potential, indicating their stability under the conditions employed for their formulation.

The Lipo is composed of DSPE-PEG-Mal and CMPs possess thiol groups. Therefore, thiol-maleimide click chemistry is possible to produce DSPE-PEG-CMP and enable CMP linkage to liposomes. CMP/Lipo conjugation was confirmed by measuring the zeta potential values for the liposomes before and after reaction. Blank Lipo nanostructures possessed negative zeta potentials (-8.10±0.36 mV). Following CMP conjugation, the zeta potential value was slightly positive (0.26±0.11 mV) resulting from the overall positive charge from the amino acid sequence of the CMP. The CMP conjugation percentage was further determined by conjugating CF-CMP to the Lipo structures and performing a collagen film binding assay. A 1:1 molar ratio of DSPE-PEG-Mal to CF-CMP was used for conjugation. Based on the fluorescence measurements for CF, the percentage CMP/Lipo conjugation was 50.3±7.0%. During the formation of Lipo, DSPE-PEG-Mal groups can face either to the outer hydrophilic surface or inner hydrophilic core and hence not all maleimide groups are available for conjugation, which is consistent with the observed conjugation efficiency.

Particle characteristics of the CMP-Van-Lipo were evaluated both before and after lyophilization (**Figure 1**). A slight increase in particle diameter, PDI, and zeta potential were evident for lyophilized CMP-Van-Lipo as compared to non-lyophilized CMP-Van-Lipo (**Figure 1a and 1b**). The EE of CMP-Van-Lipo was nominally reduced following lyophilization and resuspension in PBS (**Figure 1b**). The TEM images for non-lyophilized CMP-Van-Lipo demonstrate the presence of circular liposomal nanoparticles and that particle integrity was maintained even after resuspension of the lyophilized CMP-Van-Lipo (**Figure 1c**). In vitro Van release studies were performed using the dialysis method to compare drug release from non-lyophilized and lyophilized CMP-Van-Lipo (**Figure S6**). Non-lyophilized CMP-Van-Lipo presented a controlled drug release profile for up to 48 h. The lyophilized CMP-Van-Lipo,

following resuspension in PBS, maintained drug release profiles similar to those of non-lyophilized CMP-Van-Lipo.

3.2. Liposome release study from co-gels

The release profiles of DiR' dye conjugated Lipo and CMP-Lipo from co-gels were evaluated to determine the capacity of the CMP to retain the liposomes within the co-gel (**Figure 2**). DiR' dye (blue colored) only stains the lipid components and can be used to evaluate the amount of Lipo released from the co-gels. The dye is weakly fluorescent in water but highly fluorescent and quite photostable when incorporated within the lipid membranes [35]. The photographs for DiR' Lipo loaded co-gels demonstrated a visible reduction in the blue color over a 0 h to 72 h time period. These results were replicated in the quantitative evaluations of the % Lipo released, with a cumulative ≈ 50% Lipo release in 72 h. However, there were no visible changes in the color intensity for DiR' CMP-Lipo loaded co-gels even at 72 h. Quantitative evaluations of the % CMP-Lipo release indicated that ≈ 10% CMP-Lipo was released in 72 h. These results clearly support the fact that CMP conjugation to the Lipo nanostructures was beneficial in hybridizing them with the collagen in the co-gels, and thus increase their retention.

3.3. Drug release study from co-gels

A comparative drug release study was performed to determine Van release from free Van, Van-Lipo, and CMP-Van-Lipo loaded co-gels (**Figure 3**). Uniformity in the gel appearance was observed based upon the photographs taken at 0 h for different Van formulations loaded into the co-gels; moreover, the integrity of the co-gels was maintained even after 72 h. Drug release from

the Van loaded co-gels was abrupt, and complete Van release was achieved within 12 h. Van-Lipo loaded co-gels controlled the drug release for ≈ 32 h whereas CMP-Van-Lipo loaded co-gels controlled the drug release for ≈ 48 h. Drug molecules for Van are small enough to diffuse easily through the co-gel pores and hence were able to be released within a shorter period of time. Liposomes, on the other hand, provide sustained release both based upon their retention within the co-gels and based upon the reduction in mass transfer of the Van afforded by the lipid bilayer. Therefore, Van-Lipo formulations were able to prolong the Van release time from the co-gel, and CMP-Van-Lipo formulations further extended the drug release time. Our Lipo release studies from the co-gels (**Figure 2**) demonstrated that CMP/Lipo conjugation significantly reduced Lipo release from the co-gels as compared to CMP-free Lipo. Thus, our results indicated that these differences in Lipo retention resulted in improved control over Van release from the CMP-Van-Lipo loaded co-gels, with Van release primarily dependent on drug diffusion from the Lipo that are retained within the co-gel.

3.4. In vitro antibacterial activity of co-gels loaded with Van formulations

In vitro antibacterial activity of the co-gels loaded with Van formulations was evaluated using luminescent MRSA (**Figure 4a**). The MIC value of free Van was calculated to determine the concentration ranges to be used for the bacterial studies. However, the MIC of CMP-Van-Lipo loaded co-gels was not calculated because the antibacterial effect is only elicited by the released Van. Based on our experiments, the MIC of Van for luminescent MRSA was determined to be 2 μg/mL. Co-gels loaded with free Van, Van-Lipo, or CMP-Van-Lipo encapsulating 4, 7, or 10 μg/mL Van per co-gel were prepared. Luminescent MRSA were subsequently added to the co-

gels. A total of three serial fresh bacterial inoculations (each for 16 h) were carried out to determine antibacterial activity by measuring the O.D. Following the first inoculation, the Van loaded co-gels exhibited potent antibacterial activity for all tested concentrations of Van. However, with Van-Lipo and CMP-Van-Lipo loaded co-gel samples, there was bacterial growth for the earlier time points (up to 7 h), similar to the control group. After 7 h, the antibacterial activity became evident in these samples. Free Van can easily diffuse through the co-gels, and hence potent antibacterial activity was observed from the earlier time points for Van loaded co-gels. In contrast, for Van-Lipo and CMP-Van-Lipo loaded co-gels, the drug diffusion from the liposomes, followed by diffusion from the co-gels, delay the initiation of antibacterial activity, resulting in the longer observed time required to observe the antibacterial effect.

Following the second inoculation, exponential bacterial growth was observed for both the control and Van loaded co-gel groups. A lag time in bacterial growth was observed for both Van-Lipo and CMP-Van-Lipo loaded co-gels. With an increase in the amount of loaded Van from 4 μg/mL to 7 μg/mL, the lag time for bacterial growth was increased for Van-Lipo and CMP-Van-Lipo loaded co-gels. CMP-Van-Lipo loaded co-gels encapsulating 10 μg/mL Van were able to effectively control bacterial growth even after second inoculation. Free Van was completely released within the first 16 h inoculation; therefore, with a second inoculation there was minimal Van concentration present within the Van co-gels, and hence these co-gels did not limit bacterial growth. For Van-Lipo formulations, the drug release was prolonged and hence antibacterial effects were evident for longer periods of time. However, even Van-Lipo loaded co-gels with 10 μg/mL Van were not able to inhibit the bacterial growth at later time points (> 26 h) suggesting that the drug diffusion, along with Lipo release, liberated Van from the co-gels at times earlier than 26 h. These results are in perfect correlation with the in vitro Van release profiles wherein > 70% drug

released by 24 h and < 10% drug was released between 24-36 h time points from Van-Lipo loaded co-gels. CMP-Van-Lipo loaded co-gels sequestered Lipo via hybridization within the co-gels, and hence these co-gel samples were able to release drug for up to 32 h and control bacterial growth. In vitro Van release profiles support these results wherein ~ 60% drug released by 24 h and > 15% drug was released between 24-36 h time points from CMP-Van-Lipo loaded co-gels.

Following a third inoculation, the bacterial growth for all the treatment groups was similar to the control group suggesting exponential growth of bacteria for Van concentrations of 4, 7, and 10 μg/mL. However, at the 10 μg/mL concentration, a lag time in bacterial growth was observed for CMP-Van-Lipo loaded co-gels but not for other treatment groups, suggesting that CMP-Van-Lipo can control Van release for even longer periods (> 36 h). The ability to control bacterial growth following multiple fresh bacterial inoculations makes the CMP-Van-Lipo loaded co-gels a promising formulation for effective treatment of bacteria-infected wounds.

Bacterial counts also were evaluated for bacterial cultures collected at the end of each inoculation time (every 16 h). An agar plate dilution method was used to calculate the number of bacterial colonies present following each treatment (**Figure 4b**). The results were in agreement with the O.D. measurements, wherein low colony forming unit (cfu) counts were observed for free Van loaded co-gels as compared to Van-Lipo loaded co-gels and CMP-Van-Lipo loaded co-gels for the first 16 h incubation. Overall, low cfu counts were observed for all treatment groups and at all Van concentrations subsequent to the first inoculation. With the second inoculation, cfu counts were drastically increased for all the treatment groups at all given Van concentrations except for the CMP-Van-Lipo loaded co-gel containing 10 μg/mL Van. The cfu counts following the third inoculation were similar for all the treatment groups. These results are perfectly consistent with

and confirm the results obtained via IVIS Lumina imaging of the bacterial cultures at the end of every inoculation (**Figure S7**).

3.5. Cell viability assay of fibroblasts

The effects of Van, Van-Lipo, and CMP-Van-Lipo on NIH-3T3 cell viability were determined (**Figure S8**), as free Van and lipid components from the liposomal formulation might affect viability of fibroblastic cells. Free Van can induce toxic effects in fibroblasts by lysosomal lipid-antibiotic interference [36, 37]. Liposomes are biocompatible and nominally toxic to the cells [38, 39]; however, even a minimal reduction in cell viability in the wound site might affect the overall healing time, especially under infected conditions [40]. Given that lyophilized liposomal formulations were used in the co-gels, lyophilized blank Lipo, lyophilized Van-Lipo, and lyophilized CMP-Van-Lipo at Van concentrations ranging from 0.1 – 100 μg/mL Van were used for viability assays. Free Van minimally reduced cell viability even at a high concentration of 100 μg/mL following up to 48 h incubation. However, blank Lipo resulted in a concentration-dependent reduction in fibroblast viability. The cytotoxicity of the lipid components of the Lipo [41, 42] resulted in the approximate 30 % reduction in cell viability (at 100 μg/mL Van) following incubation for up to 48 h. Van-Lipo and CMP-Van-Lipo treatments resulted in comparable cell viability reductions to that of blank Lipo indicating minimal toxic effects of Van on the fibroblasts. With the conjugation of CMP to Van-Lipo, as presented in **Figure S8a**, there are non-significant changes in the cell viability as compared to the Van-Lipo lacking the CMP, thus confirming the minimal cytotoxicity of the CMP itself.

Fibroblast viability was further evaluated in the presence of co-gel formulations loaded with free Van, Van-Lipo, or CMP-Van-Lipo. Cell viability data for free Van loaded co-gels were in agreement with free Van treatments, demonstrating minimal cell viability reductions. Van-Lipo loaded co-gels also reduced the cell viability at a similar extent to Van-Lipo treatments following 48 h incubation. However, CMP-Van-Lipo loaded co-gels enhanced the fibroblast cell viability by 20% as compared to CMP-Van-Lipo treatments alone. As determined from Lipo release studies, Van-Lipo can be released from the co-gels during the incubation period, resulting in cell death caused by the cytotoxicity of the lipids. By contrast, CMP-Van-Lipo is mostly (≈ 90 %) retained within the co-gels for 72 h, and hence the liposomal components of the CMP-Van-Lipo co-gel samples were not released and did not significantly affect cell viability.

3.6. Determination of antibacterial activity on in vivo wound infection model

The in vivo antibacterial activity of the Lipo-loaded co-gels was evaluated using a punch biopsy wound model inoculated with luminescent MRSA, with IVIS Lumina imaging employed to detect and quantify bacteria (**Figure 5**). Re-inoculations were performed at day 1 to enable comparison of the in vitro and in vivo antibacterial effects of co-gel formulations upon repeated bacterial challenge. IVIS Lumina images were used to visually compare the luminescence of the inoculated bacteria following treatment with different co-gel formulations (**Figure 5a**). Quantitative evaluations of the luminescence observed by IVIS Lumina imaging and their respective bacterial counts are presented in **Figures 5b and 5c**. No bacterial luminescence was observed for uninoculated wounds (G1). High bacterial luminescence was observed even at day 9, with high bacterial counts (≈ $5x10^7$ cfu per wound), in G2 samples that were inoculated with

bacteria but not treated. Blank co-gel treatments (G3) also exhibited high bacterial luminescence and bacterial counts (≈ $2x10^7$ cfu per wound) by the end of day 9, confirming minimal/no antibacterial activity of the co-gel itself. Free Van solutions (G4) also were not able to reduce the bacterial luminescence and bacterial count (≈ $7x10^7$ cfu per wound) at day 1, and higher overall bacterial counts were observed during the treatment period. Van solutions can easily leak out of the wounds resulting in minimal Van retained in the wound site, and subsequent minimal antibacterial effects. However, there was a significant reduction in bacterial luminescence and cfu for G4 at day 9. At this time point, the G4 samples exhibited weakened Opsite dressing adhesive forces along the wound edges that may have disrupted the bacterial layer, contributing to lower luminescence and cfu. However, the wound size was much larger as compared to co-gel formulations treated groups confirming deleterious effects of bacteria at least for > 6 days (or until the wound dressing was removed). Van loaded co-gels (G5) enhanced the antibacterial effects resulting from prolonged contact time, with Van accumulated in the wound site following diffusion through the co-gels. At day 1, luminescence and bacterial counts (≈ $1x10^5$ cfu per wound) were significantly reduced; however, bacterial luminescence increased from day 2 suggesting minimal control in drug diffusion that resulted in complete drug release within a short period of time (< 1 day), allowing re-growth of surviving bacteria. Re-inoculation of bacteria on day 1 was performed for Van loaded co-gel-treated wounds (G5R) to reinstate the bacterial luminescence. High bacterial luminescence and bacterial counts (≈ $2x10^7$ cfu per wound) were exhibited by the end of day 9, indicating that these co-gel formulations were absent of Van and thereby could no longer control bacterial growth. Van-Lipo loaded co-gels (G6) and Van-Lipo loaded co-gels with bacterial re-inoculation (G6R) showed similar bacterial control and growth patterns to G5 and G5R, respectively. However, better bacterial growth control was evident in the G6 and G6R groups at

day 9, resulting from control of Van diffusion from the Lipo and co-gel components in the Van-Lipo loaded co-gels. CMP-Van-Lipo loaded co-gels (G7) presented the best antibacterial activity, with minimal bacterial luminescence and bacterial counts ($<1x10^4$ cfu per wound) observed up to day 9. Following re-inoculation (G7R), the bacterial luminescence and bacterial counts were better controlled from day 2 to day 9 as compared to free Van or Van-Lipo loaded co-gels. CMP-mediated Lipo retention within the co-gel matrix resulted in controlled Van release for long time periods with effective antibacterial activity. There were no significant changes in the body weights of mice treated with free drug or co-gel formulations suggesting no adverse effects (**Figure 5d**).

Histological evaluations of wound skin samples at day 9 following treatment with different formulations are presented in **Figure 6**. H&E and gram staining of the skin sections revealed the wound healing patterns for different treatment groups based on bacterial prevalence. For uninoculated wounds (G1), normally healing wounds with epidermal layers covered by a clot were evident. No bacterial colonies were inoculated within the wounds and hence the normal wound healing patterns were visible. The tissue morphology was distorted for tissue sections from wounds inoculated with bacteria without any treatment (G2), with no clear epidermal layer formation. Large numbers of bacterial colonies were observed within the skin sections. Furthermore, larger void gaps were observed within the tissue sections from dermal layers possibly resulting from the cell apoptosis and toxicity caused by the endotoxins produced by the bacteria in the wound site. A similar pattern of tissue sections were obtained for blank co-gel treatments (G3), where no clear epidermal layer formations were visible and a large number of bacterial colonies were present, confirming the lack of antibacterial effects of the blank co-gels. A thicker epidermal layer with substantial bacterial colonies was observed in skin sections from groups treated with free Van solution (G4), resulting from the limited antibacterial effect of free Van due to its leakage out of

the wound site. An enhanced tissue integrity and a substantial reduction in the bacterial count were evident for skin sections from Van loaded co-gel treatments (G5), wherein Van release as well as Van residence times were prolonged. The reinoculation of bacteria in the Van loaded co-gel treatment samples (G5R) resulted in tissue patterns similar to those in the inoculated untreated wounds (G2), along with a high number of bacterial colonies, confirming the inability of the co-gels to control free Van release for longer periods of time, with correspondingly limited antibacterial effects. A similar pattern in the histological sections was observed for Van-Lipo loaded co-gels (G6) and Van-Lipo loaded co-gels with bacterial re-inoculation (G6R) relative to G5 and G5R. However, the presence of bacterial colonies in the G6R samples was lower as compared to G5R attributable to better control over Van release from the Lipo nanostructures. The skin sections for the CMP-Van-Lipo loaded co-gel treated group (G7) presented better epidermal layer formations that were comparable to the non-infected wound models with minimal/no bacterial colonies visible. Reinoculation of the CMP-Van-Lipo loaded co-gel treated wounds (G7R) resulted in epidermal layer formations with minimal bacterial counts. Co-gels loaded with CMP-Van-Lipo can retain Lipo for longer periods of time, thus controlling the Van release for induction of effective antibacterial activity even with repeated bacterial inoculations/infections.

4. Discussion

Wound infections (defined by a microbial count of $> 10^5$ cfu/g) impede the wound healing process based upon the virulence (e.g. bacterial proteases) of the invading microorganisms [43]. Furthermore, bacterial biofilm formation and subsequent endotoxin production adversely affect the normal wound healing process due to potent inflammatory and anti-angiogenic effects [6].

Staphylococcus, being the most prevalent genera, causes wound infections in a substantial fraction of the chronic (e.g diabetic) wound population [44]. Therefore, wound treatments with effective antibacterial activity are required for controlling infections occurring in wounds.

Several clinical studies report the use of Van powder application onto wounds, with some studies reporting beneficial effects for wound healing [45, 46], and other studies indicating that Van powder application further complicates the wound condition by skin erosion, wound dehiscence, and prolonged wound healing [47, 48]. In critically colonized wound infections, serous exudates are increased, resulting in a higher probability that the applied Van powder leaks out of the wound with resulting limitations in efficacy of the high dose applied at the wound site [49]. Several studies have established the ability of liposomes to sustain the release of drug molecules (e.g., Van) for longer periods of time (few hours to several days) [24, 50]. However, when Van-Lipo formulations are applied topically, rapid Van-Lipo clearance results in minimal Van remaining in the wound site. Therefore, combination approaches to control Van release kinetics and maintain Van locally would have significant benefits for improving antibacterial and wound healing efficacy.

Pharmacokinetically, the efficacy of free Van is inherently limited due to its high aqueous solubility and thus poor absorption and quick elimination [51]. Efforts to address this problem have typically employed Van loaded nano-/micro-particles combined with hydrogels to provide a localized depot of Van [52-54]. However, minimal efforts have been applied for retaining the nanoparticles within the hydrogel to better control Van delivery once applied in vivo. Additionally, the polymers used in these studies [e.g., chitosan, Spanish Broom fibers, polycaprolactone-poly(ethylene glycol)-polycaprolactone] do not aid in the wound healing process. To address these issues, in our study, we have developed a CMP conjugated Van-Lipo formulation and incorporated

it into a collagen-based scaffold for potential wound management applications. A collagen-based scaffold precursor solution composed of collagen, fibrinogen, and thrombin was developed to incorporate CMP-Van-Lipo. Collagen is a native protein in mammals that is commonly used as a wound healing material [32, 55], and fibrinogen/thrombin are usually present in wound sites following injury to promote cell recruitment and enhance the wound healing process [56]. Accordingly, the combination of collagen, fibrinogen, and thrombin was designed to provide a suitable mimic of the natural healing environment [57]. The ability of CMPs to form triple helices with collagen was used to enable improved retention of the Van-Lipo within these collagen-based scaffolds for prolonged release of Van in wound sites [32].

There were no significant changes in the particle characteristics of liposomes with or without Van loading suggesting the successful incorporation of Van (a hydrophilic drug) within the hydrophilic core of the liposomes [58]. The particle characteristics and stability were maintained at different temperatures (25 °C and 37 °C) relevant to in vivo wound application. The Lipo/CMP conjugation did not alter the particle characteristics even after the lyophilization of the CMP-Van-Lipo nanostructures, providing a great advantage for long-term storage of the formulation prior to incorporation into the scaffold. Lyophilized CMP-Van-Lipo were incorporated into the co-gels via CMP/collagen hybridization, with the CMPs forming triple helices with the collagen to enable retention within the co-gel and controlled Van release for longer durations of time. Accordingly, a substantial reduction in Lipo release from the co-gels was achieved simply by conjugating CMP to the Lipo. CMP-Van-Lipo loaded co-gels presented more controlled Van release kinetics in vitro as compared to free Van or Van-Lipo loaded co-gels, making the CMP-Van-Lipo formulations suitable candidates for topical application to elicit prolonged localized antibacterial effects.

Fibroblast cell proliferation and migration during wound healing play a significant role in timely healing of wounds, and fibroblasts drive granulation tissue formation as well as wound bed contraction [59, 60]. Therefore, reductions in cell viability following application of liposomal formulations would be detrimental for wound healing. As expected based upon literature [41, 42], the lipid composition (DPPC:Cholesterol:DSPE-PEG-Mal) in the liposomes was determined to reduce fibroblast cell viability in a concentration dependent manner at a range of therapeutically-relevant Van concentrations (0.1 to 100 μg/mL). The release of Van and/or liposome components could also trigger substantial reductions in fibroblast viability at the wound site. This detrimental effect was minimized by the addition of the CMPs to the Van-Lipo agents, such that the Van-Lipo was hybridized within the co-gels for long periods, reducing the local concentrations of Van and Lipo components at any given time, and correspondingly, improving fibroblast viability [32].

The in vitro antibacterial activity of CMP-Van-Lipo loaded co-gels was evaluated using luminescent MRSA. *Staphylococcus* is the most prevalent genera of bacteria present in the skin and has easy access to wounds [61, 62]. Van is the first line drug of choice for treating infections caused by MRSA [63]. A dose-dependent antibacterial effect was observed depending on the Van loading (4, 7, or 10 μg/mL), and the antibacterial effect also changed as a function of the formulation type (free Van, Van-Lipo, or CMP-Van-Lipo loaded co-gels). As expected, the antibacterial effects of free Van loaded co-gels showed the best antibacterial effects for the first inoculation at all given Van loading concentrations (≥ MIC) due to the abrupt release of most of the encapsulated Van. An initial bacterial growth was observed for Van-Lipo and CMP-Van-Lipo loaded co-gels resulting from the lag time required for Van diffusion through the liposomes and through the gel, commensurate with the Van release profiles observed from the in vitro release studies. However, after 16 h, a comparable antibacterial effect between the groups was evident.

Our results are in close agreement with a previous study using oligo(poly(ethylene glycol)fumarate/sodium methacrylate (OPF/SMA) co-polymer hydrogels for controlled Van release that presented significant reductions in the cfu counts of MRSA (after a 12 h treatment) following the release of drug concentrations close to the MIC [51]. In another study, similar results were obtained by using chitosan/hyaluronic acid hydrogels for controlled Van release [54]. The antimicrobial activity of 3D-printed polycaprolactone composite scaffolds loaded with Van-PLGA microspheres presented in vitro antibacterial activity up to 28 days [27]. However, these studies lacked in vivo results wherein the formulation might behave differently in regard to both antibacterial efficacy as well as wound healing.

Wound infections, especially in chronic wounds, are recurring [64, 65], and hence formulations capable of inhibiting recurring infections are essential. Therefore, in vitro antibacterial studies incorporating multiple fresh bacterial inoculations were performed. This study, to the best of our knowledge, is the first report of its kind using repeated bacterial challenges to test the in vitro and in vivo wound healing efficacy in Van formulations. Following a second inoculation, the free Van loaded co-gels did not control the exponential growth of bacteria because of the complete loss of Van from the co-gel during the time period following the first inoculation. Van-Lipo showed a lag time in bacterial growth for the initial hours of the second inoculation attributed to the Van release from liposomes that were still retained within the co-gels. CMP-Van-Lipo loaded co-gels were able to control bacterial growth even after the second inoculation attributable to the higher number of liposomes that were still retained within the co-gel, that could release enough Van to elicit the antibacterial effects. Following a third inoculation, only CMP-Van-Lipo showed a lag time before the exponential growth of the bacteria, demonstrating the capacity of these formulations to continue to hinder bacterial growth. These results clearly indicate

that CMP conjugation to Van-Lipo provides retention of the liposomes for longer times that are sufficient to control bacterial growth following multiple infections during the course of wound healing. The effective antibacterial activity, following at least two fresh bacterial inoculations, is a step toward an important potential clinical tool for infection control in wounds that could enhance patient compliance by reducing the frequency of formulation application to once in every two days.

The in vivo antibacterial activity of the Van loaded co-gels was evaluated in punch biopsy wound models of mice inoculated with luminescent MRSA. High bacterial luminescence (similar to previously published reports [66, 67]) was maintained in the wounds even at the end of day 9 for either saline or blank co-gel treatments. Van solution also was not effective in reducing the bacterial luminescence, presumably due to Van solution loss from the wound site following application. Van loaded co-gels were effective in reducing the bacterial luminescence at day 1; however, the bacterial growth re-started after day 2. Re-inoculation of bacteria in wounds treated with Van loaded co-gels at day 1 showed no control in the bacterial luminescence as a consequence of the abrupt release of free Van within short time periods (< 1 day), resulting in minimal residual drug to address the second bacterial inoculation. The Van-Lipo loaded co-gels showed similar patterns of reduction in bacterial luminescence to that of the free Van loaded co-gels due to the combination of drug and Lipo release from the co-gels that enhanced the cumulative Van release. CMP-Van-Lipo loaded co-gels were able to completely inhibit bacterial growth during the study period. Furthermore, following re-inoculation with bacteria, the luminescence was reduced as compared to Van-Lipo loaded co-gels. H&E and gram staining of the treated wounds showed comparable bacterial colonies to the observed in vivo luminescence. These results clearly exemplified the advantage of using CMP-Van-Lipo loaded co-gels for the effective treatment of wound infections by their capacity to control Van release for prolonged periods of time. Suitable

optimization of the Van concentration to be loaded within the co-gels could further enhance the antibacterial efficacy of the formulation by maintaining effective drug concentrations for even longer time periods.

5. Conclusions

A CMP-Van-Lipo loaded collagen-based formulation was successfully developed for the potentail treatment of MRSA-infected wounds. Effective CMP-based liposomal hybridization within the scaffold and controlled Van release for prolonged periods of time synergistically enhanced antibacterial effects of the formulations both in vitro as well as in vivo. Furthermore, enhanced antibacterial efficacy was evident even after multiple bacterial inoculations, demonstrating the potential of the CMP-Van-Lipo co-gel as a topical formulation for effective treatment of persistent wound infections.

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Conflict of interest

The authors declare no conflict of interest.

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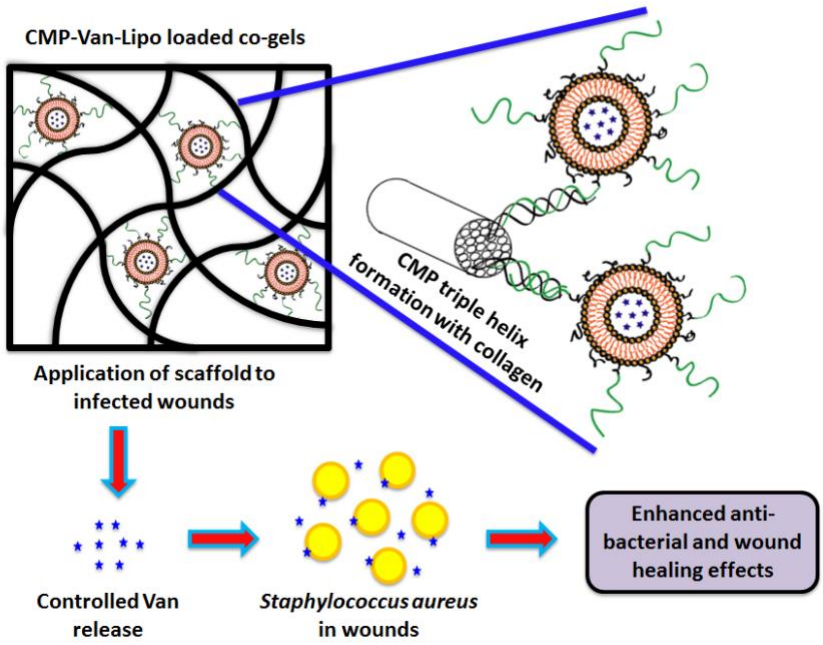
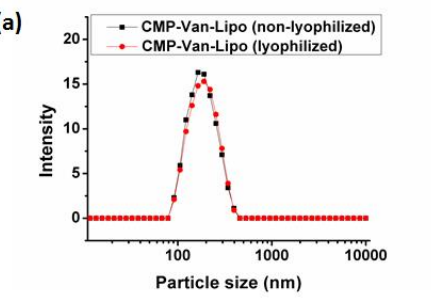

CMP-Van-Lipo loaded co-gels
CMP triple helix formation with collagen
Application of scaffold to infected wounds
Controlled Van release
Staphylococcus aureus in wounds
Enhanced anti-bacterial and wound healing effects

Scheme 1

(a)



(b)

	Non-lyophilized CMP-Van-Lipo	Lyophilized CMP-Van-Lipo
Particle size (nm)	182.2±8.1	196.7±11.5
PDI	0.24±0.02	0.26±0.01
Zeta potential (mV)	-5.96±0.74	-8.95±0.28
Van encapsulation efficiency (%)	7.94±1.14	7.69±1.15

(c)

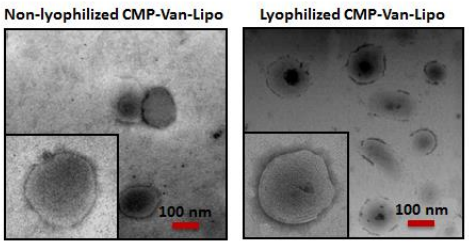


Figure 1

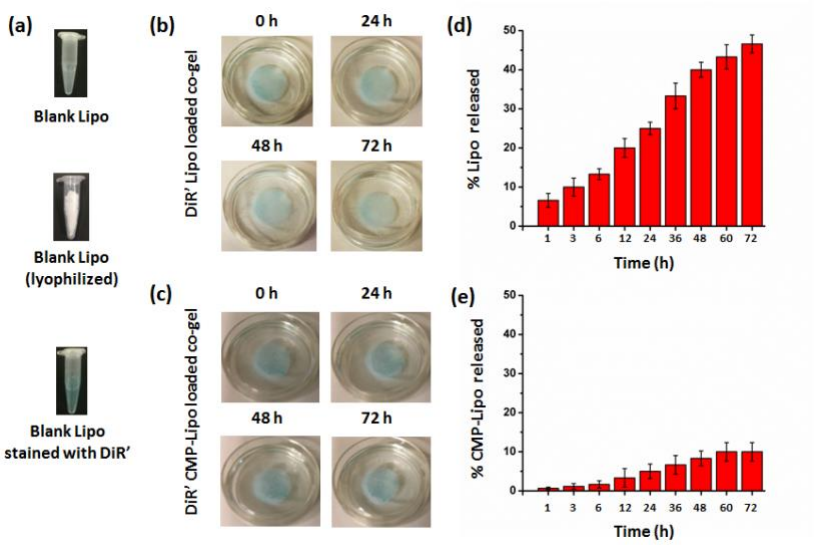

(a)
Blank Lipo
Blank Lipo
(lyophilized)
Blank Lipo
stained with DiR'
(b)
0 h
24 h
48 h
72 h
DiR' Lipo loaded co-gel
(c)
0 h
24 h
48 h
72 h
DiR' CMP-Lipo loaded co-gel
(d)
% Lipo released
Time (h)
(e)
% CMP-Lipo released
Time (h)

Figure 2

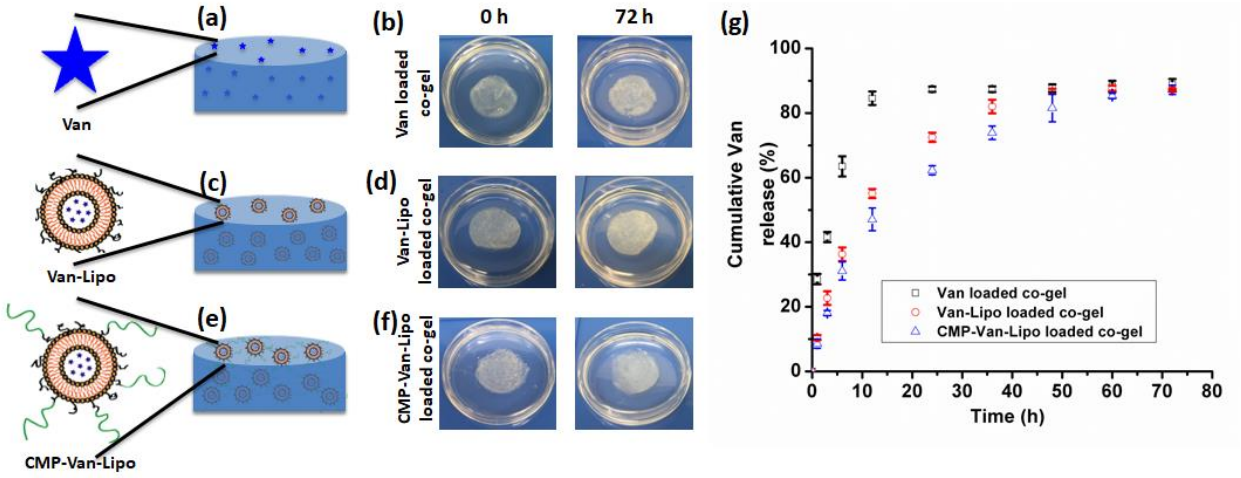

(a)
Van
(b)
0 h
72 h
Van loaded co-gel
(c)
Van-Lipo
(d)
Van-Lipo loaded co-gel
(e)
CMP-Van-Lipo
(f)
CMP-Van-Lipo loaded co-gel
(g)
Cumulative Van release (%)
Time (h)
Van loaded co-gel
Van-Lipo loaded co-gel
CMP-Van-Lipo loaded co-gel

Figure 3

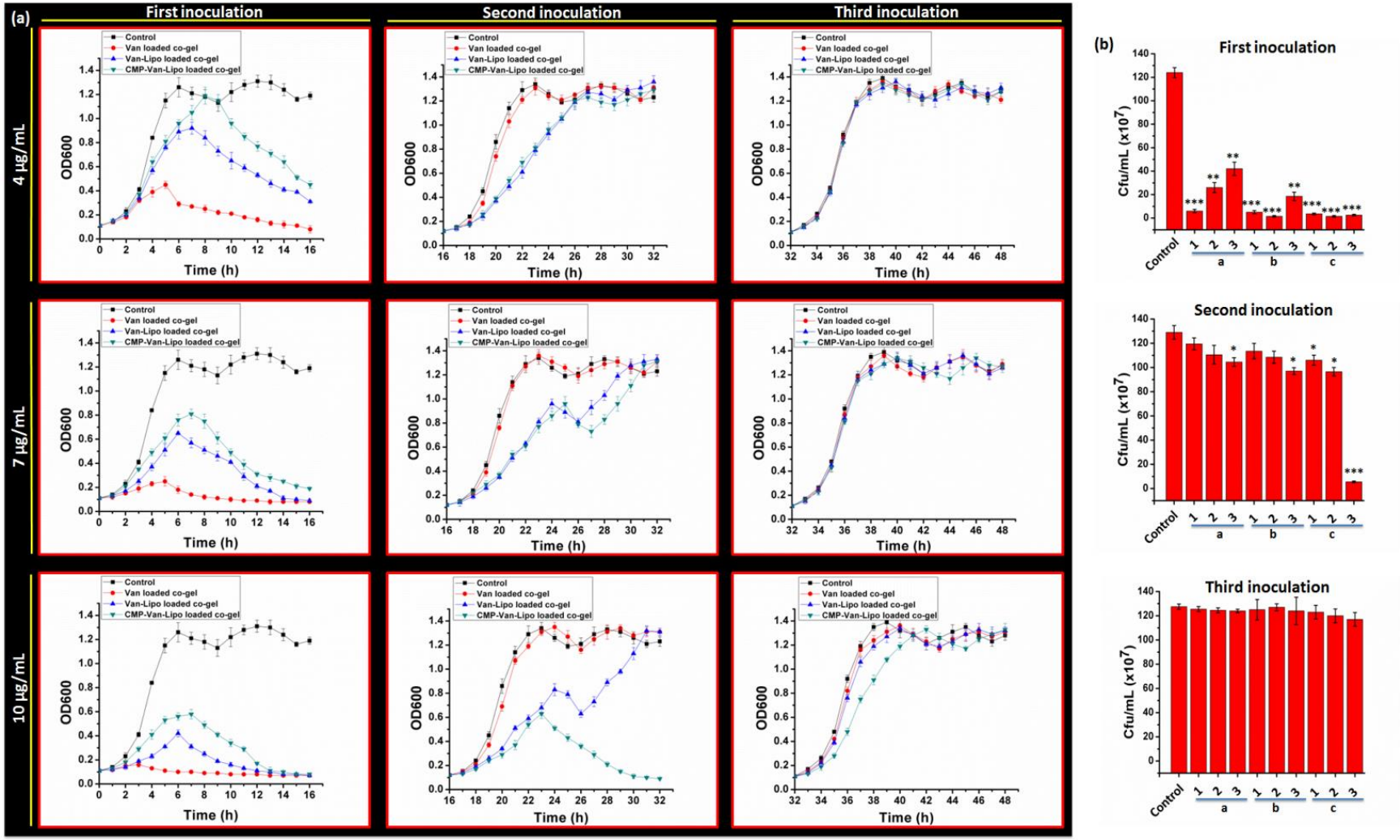

(a)
First inoculation
Second inoculation
Third inoculation
4 µg/mL
7 µg/mL
10 µg/mL
Control
Van loaded co-gel
Van-Lipo loaded co-gel
CMP-Van-Lipo loaded co-gel
OD600
Time (h)
(b)
First inoculation
Second inoculation
Third inoculation
Cfu/mL (x10^7)
Control
a
b
c

Figure 4

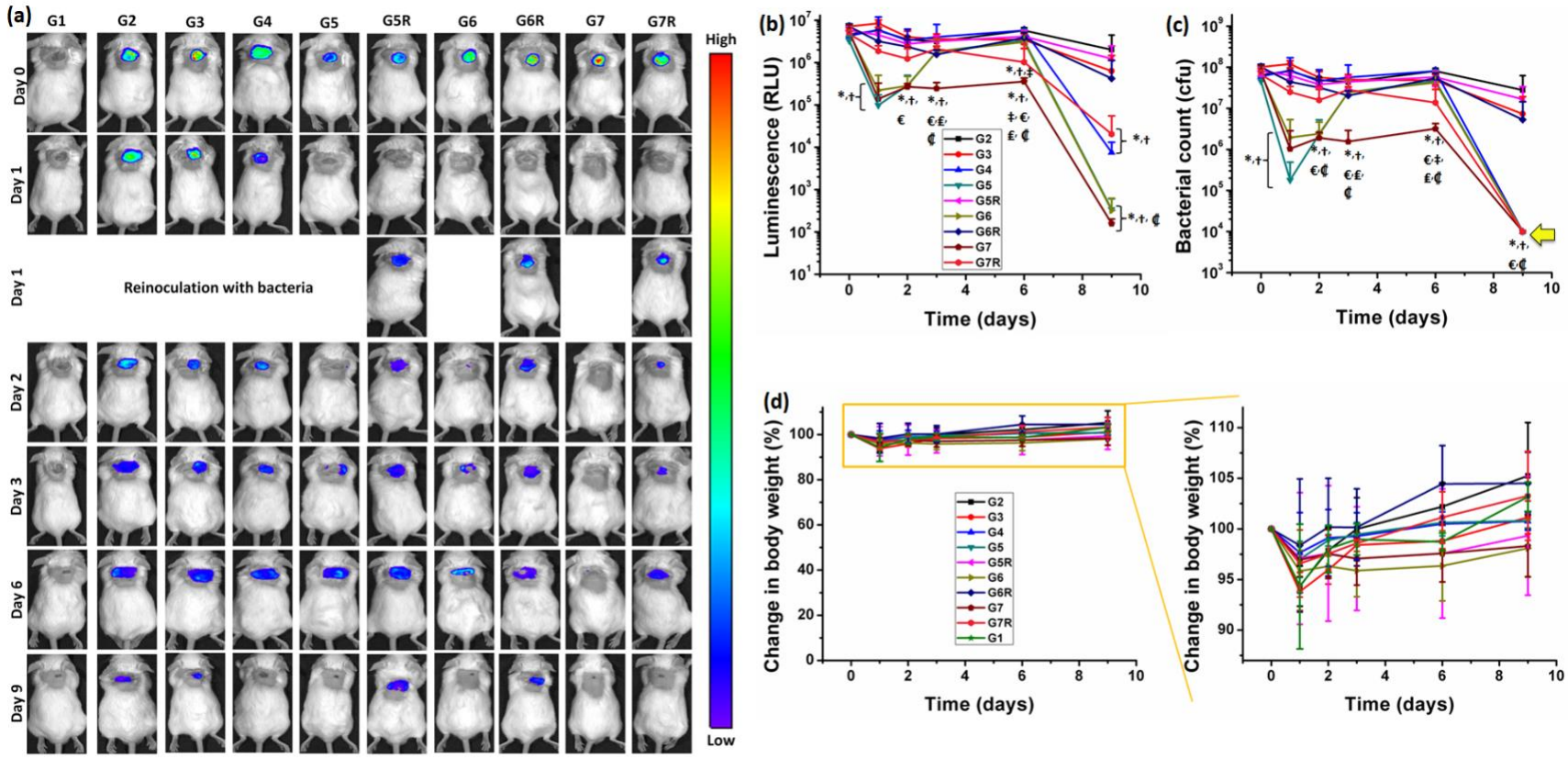

(a)
G1
G2
G3
G4
G5
G5R
G6
G6R
G7
G7R
High
Low
Day 0
Day 1
Day 1
Reinoculation with bacteria
Day 2
Day 3
Day 6
Day 9
(b)
Luminescence (RLU)
Time (days)
G2
G3
G4
G5
G5R
G6
G6R
G7
G7R
(c)
Bacterial count (cfu)
Time (days)
(d)
Change in body weight (%)
Time (days)
G1

Figure 5

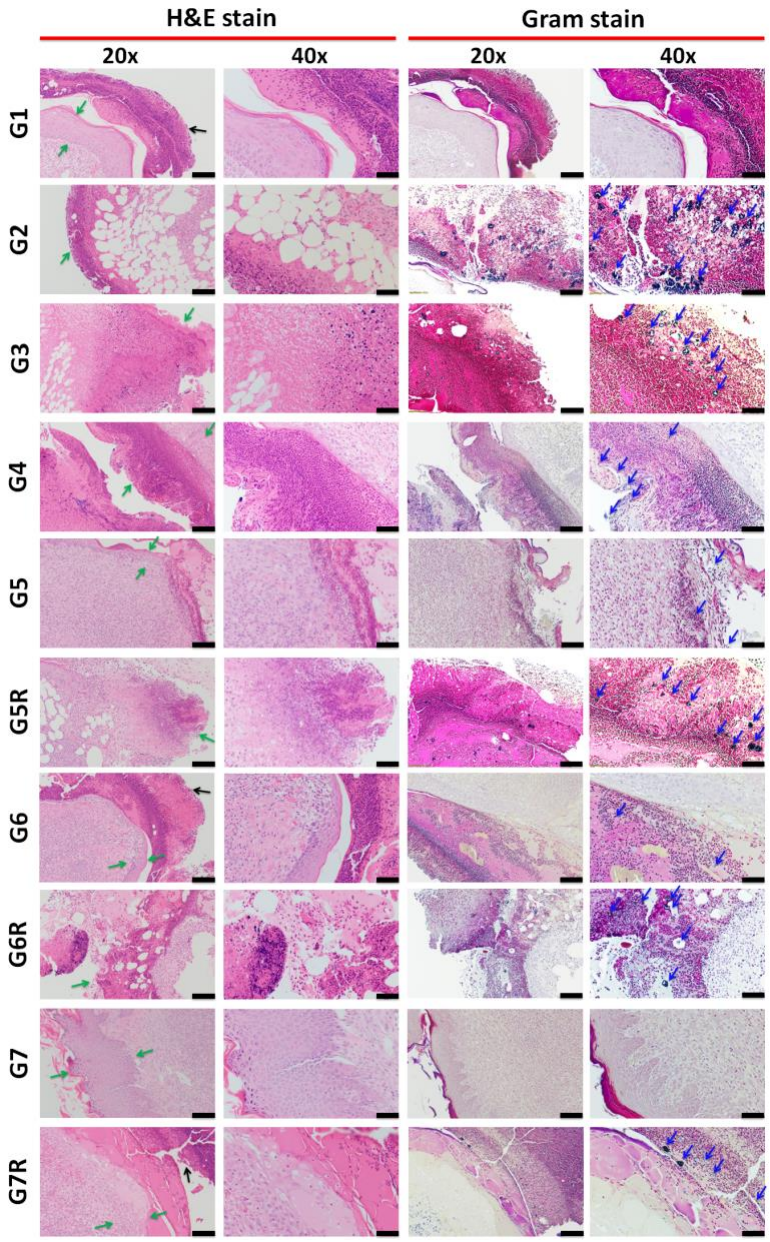

H&E stain
Gram stain
20x
40x
20x
40x
G1
G2
G3
G4
G5
G5R
G6
G6R
G7
G7R

Figure 6