Published in final edited form as:

Biomaterials. 2009 August ; 30(22): 3771–3779. doi:10.1016/j.biomaterials.2009.03.044.

The antimicrobial efficacy of sustained release silver–carbene complex-loaded L-tyrosine polyphosphate nanoparticles: Characterization, *in vitro* and *in vivo* studies

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Abstract

The pressing need to treat multi-drug resistant bacteria in the chronically infected lungs of cystic fibrosis (CF) patients has given rise to novel nebulized antimicrobials. We have synthesized a silver–carbene complex (SCC10) active against a variety of bacterial strains associated with CF and chronic lung infections. Our studies have demonstrated that SCC10-loaded into L-tyrosine polyphosphate nanoparticles (LTP NPs) exhibits excellent antimicrobial activity *in vitro* and *in vivo* against the CF relevant bacteria *Pseudomonas aeruginosa*. Encapsulation of SCC10 in LTP NPs provides sustained release of the antimicrobial over the course of several days translating into efficacious results *in vivo* with only two administered doses over a 72 h period.

Keywords

Antimicrobial; Silver; Controlled drug release; Nanoparticle; Cystic fibrosis

1. Introduction

Cystic fibrosis (CF) is a life-threatening autosomal recessive disorder caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator [1]. CF is a multisystem disease that affects the lungs and upper respiratory tract, the gastrointestinal tract, pancreas, liver, sweat glands and genitourinary tract. Most CF patients experience polymicrobial airway colonization with pathogens including *Staphylococcus aureus*, *Burkholderia cepacia* complex organisms, and in particular, *Pseudomonas aeruginosa* followed by intense inflammation in the lungs. A progressive decline in lung function usually occurs, which can lead to respiratory failure and death. The present treatment for chronically

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Appendix. Supplementary data: Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.biomaterials.2009.03.044.

infected CF patients consists of frequent high-dose administration of intravenous antibiotics including aminoglycosides and β -lactams active against *P. aeruginosa*, such as tobramycin and ticarcillin plus clavulanic acid, respectively. Even with this type of aggressive therapy, complete eradication of the bacteria is difficult, due to the ability of the organisms to form biofilms [2]. The persistence of bacteria has led to the rise of resistant bacterial strains with enhanced tolerance to current antibiotics, leading to chronic infection in the lungs. In addition, the high and frequent dosing of the intravenous antibiotics necessary to achieve significant serum concentration of the drugs can cause serious side effects including, in the case of aminoglycosides, nephrotoxicity and ototoxicity [3].

In order to address these two major challenges, resistance and toxicity, a new form of therapy is necessary. Silver based antimicrobials have become an attractive alternative to address the resistance problem. Silver has been used since ancient times to treat infections and is still widely used in a variety of medical applications such as the treatment of burns, coatings on catheters and endotracheal tubes, disinfectants and wound dressings [4,5]. Of major importance are the relatively few reported accounts of silver resistance despite its wide and continuous use [6–10]. The rarity of silver resistance may be attributed to the multiple suggested mechanisms of action of silver. One theory suggests that Ag(I) ions cause structural changes to the bacterial cell wall by reacting with surface proteins [11,12]. Furthermore, silver ions have been thought to bind and inactivate bacterial DNA and RNA [11,12]. Likely due to the multiple mechanisms of cellular killing through fundamental cell processes, silver has broad spectrum activity. Thus, silver is very effective against a wide variety of Gram-positive and Gram-negative bacteria including *S. aureus*, *P. aeruginosa* and *Escherichia coli* [12]. Our group has reported the antimicrobial activity of several silver *N*-heterocyclic carbene complexes (SCCs) against *P. aeruginosa* and *B. cepacia* complex species [13–15]. In addition, recent studies have reported the *in vitro* antimicrobial activity of silver, in the form of silver sulfadiazine, on mature biofilms of *P. aeruginosa* [11].

To combat the second major problem, antibiotic toxicity, direct administration of antimicrobials to the lungs via inhalation has been explored [16,17]. Aerosolized antibiotics have proved effective due to localized delivery at the site of infection. Direct delivery to the organs of interest has other favorable outcomes such as lower necessary dosages for therapeutic outcomes, decreased systemic toxicity, and diminished systemic absorption. However, the challenge of rapid clearance from the lungs as a result of the relatively small size of the antibiotics has been a major problem [17,18]. In order to maintain a drug concentration in the lungs above the minimum inhibitory concentration (MIC) effective against pulmonary pathogens, several inhaled treatments must be administered throughout the day. To avoid this inconvenience, we have explored the use of inhaled nanoparticles (NPs) to deliver, directly to the site of infection, a novel silver *N*-heterocyclic carbene complex, SCC10, which possesses potent antimicrobial properties. This unique formulation will allow the sustained release of SCC10, thereby creating a depot effect in chronically infected lungs. Sustained release of the active drug also has the advantage of decreased administration frequency, which should lead to improved patient compliance.

2. Materials and methods

2.1. Materials

Unless otherwise specified, all manipulations were carried out under aerobic conditions. 4,5-Dichloroimidazole (Alfa Aesar), iodomethane (Alfa Aesar), sodium tetraphenylborate (Acros Organics), silver acetate (Acros Organics) and bromohexane (TCI) were all used as received. The ι -tyrosine polyphosphate (LTP) polymer, with a molecular weight of 8–10 kDa, was prepared using established methods [19,20]. The molecular structure of LTP used for this study has been previously published [21]. Linear polyethylenimine (LPEI) with a molecular weight

of 25 kDa was purchased from PolyScience Inc. Polyethylene glycol grafted to chitosan (PEG-g-CHN) (80% acetylation) was purchased from CarboMer Inc. The surfactant, 10% polyvinylpyrrolidone (PVP) was purchased from Sigma–Aldrich. Dry solvents were used, which were obtained from a PureSolv™ solvent purification system. Water was distilled and deionized with Barnstead NanoPure II System. ^1H and ^{13}C NMR data were collected on a Varian Gemini 300 MHz instrument. The ^1H and ^{13}C NMR spectra obtained were referenced to the residual protons of the deuterated solvents and the solvent resonances, respectively. Mass spectrometry data were collected on a Bruker Daltons (Billerica, MA) Esquire-LC mass spectrometer equipped with ESI. Elemental analyses were performed at the Microanalyses Laboratory at The University of Illinois and Galbraith Laboratories.

2.2. Synthesis of precursor 1-hexyl-3-methyl-4,5-dichloroimidazolium iodide (1)

4,5-Dichloroimidazole (1.23 g, 9.00 mmol) and potassium hydroxide (2.24 g, 40.0 mmol) were stirred in acetonitrile (50 mL) for 2 h at room temperature (Scheme 1). The unreacted KOH was filtered from the solution and bromohexane (1.26 mL, 9.00 mmol) was added (Eq. 1). The reaction mixture was refluxed for 24 h. Excess iodomethane (2.5 mL, 40.2 mmol) was added. The mixture was refluxed at 85 °C for 24 h. The volatile components were removed and the crude product was redissolved in dichloromethane. The solid, presumably KBr, was filtered and discarded and the volatile components were removed in vacuo to yield a yellow oily product. The product was stirred in diethyl ether and filtered to yield a yellow powder. Characterization results including the ^1H and ^{13}C NMR spectra of **1** can be found in the Supplementary data.

For recrystallization purposes, **1** (0.5 mmol, 0.182 g) was dissolved in water (20 mL) and sodium tetraphenylborate (1 mmol, 0.342 g) was added. A white solid of the tetraphenylborate (BPh₄) adduct, **1a**, precipitated from the solution and was filtered and washed with 10 mL of water. Crystals suitable for single X-ray diffraction studies were grown from acetone. The X-ray crystal structure determination detail including the crystal structure analysis of **1a** can be found in the Supplementary data.

2.3. Synthesis of (1-methyl-3-hexyl-4,5-dichloroimidazole-2-ylidene) silver (I) acetate (SCC10)

Compound **1** (2.20 g, 6.07 mmol) was dissolved in dichloromethane (50 mL) and silver acetate (2.03 g, 12.1 mmol) was added. The mixture was stirred at room temperature for 3 h. The yellow precipitate, presumably AgI, was filtered and discarded. The volume of the reaction mixture was reduced under pressure to 5 mL. Hexane (300 mL) was added and the mixture was cooled in an ice bath. A fine off-white solid precipitated from the solution. It was filtered and washed with 20 mL of hexane. The powder was placed under a vacuum overnight to ensure all traces of volatiles had been removed. Characterization results including the ^1H and ^{13}C NMR spectra of SCC10 can be found in the Supplementary data.

2.4. Preparation of SCC10–LTP NPs

NPs loaded with SCC10 were prepared using water-in-oil-in-water emulsion method [21] and solvent evaporation. For the blank LTP nanoparticle formulation, the initial water-in-oil emulsion consisted of 300 mg of LTP dissolved in 3.0 mL of chloroform, 3.0 mg of PEG-g-CHN dissolved in 0.9 mL of 0.1 N acetic acid (for 48 h at 37 °C under rotation), 3 mg of LPEI in 1 mL of distilled and deionized water (DH₂O), and 1 mL of DH₂O in a double-neck round-bottom flask. The initial water-in-oil emulsion was vortexed for 2 min at 3000 rpm by an impeller. The second water-in-oil-in water emulsion had 100 mL of 10% PVP containing 10 mg/mL NaNO₃ added to the initial emulsion and vortexed for 3 min at 1600 rpm. Chloroform was allowed to evaporate for 5 h while being stirred and vented. NPs were collected and washed with DH₂O by centrifugation at 6000 × g for 10 min. Finally, the NPs were shell frozen in 10

mL of DH₂O, and placed in a lyophilizer for 72 h. The lyophilized NPs were stored in a desiccator. To prepare SCC10–LTP NPs, the only deviation from the previously described method is dissolving both SCC10 and LTP into 3 mL of chloroform. The resulting NPs were aliquoted and stored in desiccated containers until the day of the experiment.

2.5. Characterization of NP morphology

Scanning electron microscopy (SEM, Hitachi S2150) was used to measure the morphology of the NPs. The SEM samples were prepared by suspending 1 mg of NPs in 1 mL of DH₂O. Then, 200 μ L of the suspended NPs was transferred onto a stub, dehydrated, sputter coated with silver/palladium, and examined.

2.6. Quantification of NP size

Dynamic laser light scattering was used to further quantify the size of the LTP NPs. The nanoparticle sample was prepared by suspending 1 mg of NPs in 10 mL of 1 $\times$ phosphate buffered saline (PBS) that was previously passed through a 0.2- μ m filter. The suspended NPs were centrifuged at 1000 $\times$ g for 10 s to remove any large aggregates. Then, samples were decanted into a glass scintillation vial. A dynamic laser light scattering system (Brookhaven Instruments BI-200SM) calculated the nanoparticle diameter by the Regularized Non-negatively Constrained Least Squares (CONTIN) method. The range of nanoparticle size was reported as differential distribution values.

2.7. Loading of SCC10 into LTP NPs

The loading of SCC10 was determined by measuring the amount of SCC10 extracted from the NPs using an absorbance plate reader (Molecular Devices). Blank LTP (1 mg) or SCC10–LTP NPs (1 mg) were dissolved in 600 μ L of chloroform for 15 min in 1.5 mL centrifuge tubes. HCl (400 μ L, 0.1 N) was added to the dissolved NPs, emulsified for 2 min by shaking, and allowed to phase separate for 10 min. A sample of the HCl phase (200 μ L) was diluted to 1 mL, and the absorbance of the precipitated silver was measured from 450 to 510 nm using an absorbance plate reader (Molecular Devices). The concentration of the SCC10 was calculated according to a standard curve.

2.8. Release of SCC10 from LTP NPs

Release profiles were determined by measuring the absorbance of the supernatant acquired from SCC10–LTP NPs incubated in PBS at physiological temperature. Release samples were prepared by suspending 1 mg of NPs in 1 mL of PBS and incubated at 37 °C under rotation. At pre-determined intervals, the samples were centrifuged at 6000 $\times$ g and 900 μ L of buffer was removed and exchanged with fresh buffer. The samples were lyophilized and suspended in 1 mL of chloroform. The absorbance was measured at a pre-determined wavelength using an absorbance plate reader and the SCC concentration was calculated according to a standard curve.

2.9. Drugs and delivery

Both SCC10–LTP NPs and the blank LTP NPs were reconstituted in 5 mL of a phosphate buffer (18 mg/mL Na₂HPO₄ and 3 mg/mL KH₂PO₄) to a concentration of 7.2 and 6.8 mg of NPs/mL, respectively. The solution contained no Cl[−] to avoid precipitation of AgCl. The reconstituted loaded and blank LTP NPs were delivered via an Aeroneb Lab apparatus (Aerogen Inc., Galway, Ireland) connected to a multi-dosing animal chamber. The Aerogen nebulizer is based on micropump technology that produces fine particles (1–5 μ m) in a low velocity aerosol [22]. The multi-dosing chamber is a square plexiglass box with inner dimensions of 8 $\times$ 8 $\times$ 4.5 inches height. The nebulizer is mounted in the center of the lid. To test whether the LTP NPs could be readily nebulized, approximately 5 mg of SCC10–LTP NPs

was suspended in 1 mL of phosphate buffer. The NPs were placed into the nebulizer, aerosolized, and the vapor was collected directly into a 50 mL conical tube. The condensed mist was placed on a glass slide and examined under a Zeiss microscope. Microscopy images were acquired using Axiovision 3.1 software (Zeiss).

2.10. Bacteria

The *P. aeruginosa* isolate designed PAO1 is a standard laboratory strain. A clinical isolate of *P. aeruginosa* designed PA M57-15 was provided by Dr. Thomas Ferkol (Washington University, St. Louis, MO). This isolate is a mucoid strain obtained from a patient with cystic fibrosis and has been extensively studied in animal models [23]. The *E. coli* strains were provided by Dr. Simon Silver (University Of Chicago, IL). The J53 strain is known to be sensitive to killing by silver cations and serves as a positive control. In contrast, the J53 + pGM101 is a J53 derivative that harbors the pGM101 plasmid originally conferring silver resistance to a burn ward isolate of *Salmonella* and serves as a negative control. *B. cepacia* complex species either were obtained from the Clinical Microbiology Laboratory at St. Louis Children's Hospital (CF clinical isolates of *Burkholderia multivorans*) or were provided by Dr. John LiPuma of Ann Arbor, MI, Dr. Johannes Huebner of Boston, MA (CF clinical isolates of *Burkholderia dolosa*), or the American Type Culture Collection.

2.11. Mice

Male C57BL/6J mice (Jackson Laboratories, Bar Harbor, Maine) at 7–10 weeks of age were used for these studies that were approved by the Washington University School of Medicine Animal Studies Committee. Animals were housed in a barrier facility under pathogen-free conditions until they were inoculated with bacteria.

2.12. MIC and MBC determinations

Minimal inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were determined by a standard Clinical and Laboratory Standards Institute (CLSI) micro-dilution method. Bacteria were streaked from glycerol-frozen stocks onto blood agar plates and incubated overnight at 37 °C. Cells from the fresh plates were suspended in the CLSI standard Mueller–Hinton broth (M–H) to an optical density at 650 nm (OD₆₅₀) of 0.25 and grown at 37 °C in a shaking incubator at 200 rpm to an OD₆₅₀ of 0.4, which corresponds to $\sim 2 \times 10^8$ colony forming units (CFU)/mL, confirmed by plating serial dilutions. The bacteria were diluted in broth to a concentration of 10^5 CFU in 100 μ L, which was added to triplicate wells of a 96-well plate containing 100 μ L of SCC10 stock diluted in sterile water. The SCC10 stock solution was made by dissolving 10 mg of SCC10 in 1 mL of DMSO. The final SCC10 concentrations tested were, 1, 2, 4, 6, 8, and 10 μ g/mL. The plate was incubated for 18–20 h at 37 °C and the MIC determined as the lowest concentration with clear wells. The MBC of SCC10 was determined by plating the wells with growth inhibition (clear) on blood agar plates and noting the lowest concentration that resulted in no growth.

2.13. Bacterial growth assay

A clinical isolate of *P. aeruginosa*, PA M57-15, was streaked from glycerol-frozen stocks onto tryptic soy agar (TSA) plates and incubated overnight at 37 °C. Cells from the fresh plates were suspended in M–H (5 mL) to an OD₆₅₀ of 0.2 and grown at 37 °C in a shaking incubator at 200 rpm to an OD₆₅₀ of 0.4 ($\sim 2 \times 10^8$ CFU/mL). Serial dilutions of SCC10 and SCC10–LTP NPs were made from stock solutions of 10 mg/mL and 64 μ g (SCC10 component)/mL, respectively. Aliquots of 200 μ L of the bacterial suspension were loaded in triplicate into wells of a 96-well plate containing 50 μ L of SCC10 or SCC10–LTP NPs diluted in sterile water to give final concentrations of SCC10 or SCC10–LTP NPs of 4, 8, 16 and 32 μ g/mL. The 96-well plate was placed in a shaking microplate reader (Synergy 2 Plate reader, BioTek) overnight

at 37 °C with OD₆₅₀ readings every 15 min. Data were graphed using Prism 4 (GraphPad Software, Inc., San Diego, CA).

2.14. Infection model and treatment protocol

Mice were anesthetized using brief exposure to isoflurane. PA M57-15 in Luria broth (LB) was delivered to each mouse intranasally. The dose ranged from 1.1×10^7 to 4.3×10^7 CFU per mouse in each of 4 separate experiments with 8 mice per group. Mice were weighed daily and observed for a total of 72 h after inoculation for survival. To allow nose-only delivery of drug, animals were placed in CH-247 tubes (CH Technologies, Westwood, NJ). Four animals at a time, each housed in individual tubes, were placed into a multi-dosing chamber. One hour after inoculation with *P. aeruginosa*, the animals were exposed for 15 min to either nebulized blank LTP NPs (34 mg) or SCC10–LTP NPs (36 mg) suspended in 5 mL each of phosphate buffer. The animals received a second nebulized dose of NPs 24 h after the first. Once the concentration throughout the dosing chamber equilibrated as indicated by diffusion of the mist cloud, the tail movements of the animals were used as an indicator of their status. Mice were then euthanized 72 h after inoculation by carbon dioxide inhalation. Lungs and spleens were harvested for quantitative bacteriology within 1 h after death for animals that did not survive to 72 h and immediately after euthanasia for the remaining animals as previously described [23]. The organs were homogenized in 1 mL of LB broth (PowerGen 125 homogenizer, Fischer Scientific) and after performing a 10-fold serial dilution, 10 μ L of each of the dilutions was placed in duplicate on TSA plates overnight at 37 °C. Presence or absence of bacterial colonies was noted to determine bacteremia. The results are pooled from 4 separate experiments for a total of 16 animals in each treatment group.

2.15. Statistical analysis

All analyses were performed using Prism 4. The *in vivo* survival curves in the infection model were compared using a log-rank test. Changes in animal weights were compared by ANOVA. The lung and spleen bacteriology of surviving animals were compared by *t*-test. Data are given as mean $\pm$ standard deviation. The risk of bacteremia between the two groups, animals receiving blank LTP NPs versus SCC10–LTP NPs, was compared by Fisher's exact test of a contingency table (number of animals with bacteria in the spleen versus number of animals cleared).

3. Results and discussion

3.1. Characterization of precursors and SCC10

The ¹H NMR spectrum of the imidazolium salt precursor **1** shows a resonance at 9.51 ppm which is indicative of the formation of an imidazolium salt and is consistent with the general C₂–H acidic proton shift of other imidazolium salts (δ 8–10 ppm) [24]. The ¹³C NMR shift of the N–C–N sp² carbon, which later becomes the carbene center was observed at 136.3 ppm.

The molecular structure of the cationic portion of **1** obtained by X-ray crystallography is depicted in Fig. 1. Following metallation of **1** with silver acetate, the ¹H NMR spectrum of SCC10 showed a loss of the imidazolium proton resonance at 9.51 ppm indicating carbene formation. The most definitive evidence for the formation of the silver–carbene was observed in the ¹³C NMR spectrum of SCC10. A shift of the resonance corresponding to the N–C–N sp² carbon from 136.3 ppm to 179.6 ppm was observed confirming formation of a carbene center. This shift is consistent with those typically observed for *N*-heterocyclic carbene complexes of Ag(I) [25,26].

3.2. Characterization of SCC10–LTP NPs

SCC10 was encapsulated into NPs formulated with LTP using a water-in-oil-in-water emulsion technique. The resulting NPs are spherical with smooth surface morphology for both SCC10–LTP NPs and blank LTP NPs (Fig. 2A and B, respectively) as determined by scanning electron microscopy (SEM). Using emulsion techniques, the encapsulation of low molecular weight drugs can be difficult since the encapsulated drugs can diffuse into the external water phase during the solvent evaporation [27,28]. Indeed, previous attempts to encapsulate silver–carbene complexes without a hydrophobic group resulted in undetectable amounts of the drug as determined by loading studies. Thus, the conjugation of the hexyl side chain and the ionic charge gradient created by the addition of the NaNO_3 in the external aqueous phase are carefully designed to confine SCC10 in the polymeric phase. Furthermore, incorporation of PEG-*g*-CHN and LPEI during nanoparticle formulation stabilizes the emulsion and prevents aggregations [21]. Due to the amphiphilic nature of these polymers, they enriched at the water–oil interface and prevented coalescence of the LTP droplets by steric repulsion [29,30].

This method for encapsulating SCC10 has yielded NPs with properties that may alleviate rapid clearance of SCC10 from the lungs and allow delivery to biofilms in the airways. Encapsulating low molecular weight drugs into NPs has been shown to retard drug clearance from tissues [31,32]. Furthermore, studies by Owusu-Ababio et al. have shown that NPs are effective for delivering antimicrobial drugs into biofilm infections [33]. To effectively treat biofilms in small airways, NPs must be small enough to navigate through the respiratory tract without impaction in the nasopharynx, while large enough to deposit onto the matrix of the biofilms [34–36]. The sizes of the blank LTP and SCC10–LTP NPs quantified using a dynamic laser light scattering system, ranged between 471 and 2891 nm with median diameter of 922 nm and between 555 and 1519 nm with median diameter of 1172 nm, respectively (Fig. 3). Hence, these NPs are the proper size to navigate through the respiratory tract and deposit onto biofilms. Furthermore, both SCC10–LTP NPs and LTP NPs were delivered in a suspension form by a nebulizer that produces a narrow range of droplet size, between 4 and 6 μm , optimized for delivery to human airways.

3.3. Measurement of SCC10 release from LTP NPs

The encapsulation techniques for preparing SCC10–LTP NPs resulted in satisfactory loading efficiency. Our minimum target for SCC10 loading was 2.5% of the total polymer weight to ensure optimal dosing while minimizing the delivery of polymer. The loading has been determined by quantifying the amount of silver extracted from LTP NPs via absorbance spectrophotography. The LTP NPs were formulated to be 10% (w/w) of SCC10; however, the actual loading was determined to be $7.1 \pm 1\%$. The encapsulation efficiency translates to $71 \pm 10\%$.

Release of the encapsulated SCC10 from the LTP NPs was characterized to determine whether this formulation would provide the desired depot delivery of the drug. The LTP NPs release the entire encapsulated drug in 7 days under *in vitro* incubation in PBS (Fig. 4). The cumulative release after 7 days from 1 mg of LTP NPs was $74 \pm 10 \mu\text{g}$ (Fig. 4), which is comparable to the loading data of $71 \pm 1 \mu\text{g}$. Approximately 80% of the SCC10 is released within the first 2 days from the LTP NPs, which corresponds to $40 \mu\text{g}$ (54%) and $20 \mu\text{g}$ (27%) SCC10 per 1 mg of NPs on days 1 and 2, respectively. For the next 5 days, the release amounts of SCC10 are minimal. This release rate is consistent with previous studies that attribute the rapid degradation rates to the presence of hydrolytically unstable phosphoester linkages in LTP's polymeric backbone [19,21].

The rapid degradation rate makes LTP NPs ideal candidates for drug delivery into the lungs, because they do not accumulate in the airways. Furthermore, the degradation products of the

LTP polymer have been shown to result in an insignificant decrease in the local pH, unlike other biomaterials such as poly[lactic-co-glycolic acid] [19,20]. The probable nontoxic degradation products of LTP include L-tyrosine based derivatives, phosphate ions, and alcohols [37]. These degradation products are easily metabolized or excreted.

3.4. In vitro MIC, MBC and bacterial growth determinations for SCC10 and SCC10–LTP NPs

The *in vitro* antimicrobial properties of the SCC10–LTP NPs were characterized through comparison with the well-studied SCC10 parent compound, SCC5 [13]. Using a standard micro-dilution assay, the MIC of SCC5, when assayed in the presence of phosphate buffer (18 mg/mL Na₂HPO₄ and 3 mg/mL KH₂PO₄ without NaCl), was uniformly 1 µg/mL for all organisms tested excluding the *E. coli* J53 strain with the silver resistant plasmid pMG101 (Table 1). Both these MICs and the corresponding MBCs were lower than those reported previously for SCC5 against these organisms [13]. The antimicrobial activity of SCC10 is likely linked to the release of the Ag(I) from the carbene, and thus, the free-Ag(I) concentration in the media determines the efficacy. The phosphate buffer in these assays may have driven a more rapid dissociation of the active Ag(I) from the carbene providing a higher free-Ag(I) concentration. The effects of DMSO on the MIC of SCC5 were evaluated in order to insure that the apparent MICs and MBCs of SCC10, which must be solubilized with DMSO due to the hydrophobic nature of the molecule, would not be falsely lowered due to DMSO toxicity. The DMSO changed the MIC and MBC of SCC5 against only one organism, *P. aeruginosa* strain PA M57-15, and rather than lowering the MIC, DMSO raised it. While no explanation for this observation is available yet, it is likely that the efficacy of SCC10 was not falsely overestimated due to the presence of DMSO in the assay.

SCC10 appeared equally efficacious *in vitro* compared with SCC5. The MICs of SCC10 were again, uniformly 1 µg/mL for non-silver resistant organisms. The MBCs of SCC10 were lower than those of SCC5. Encapsulating SCC10 in the LTPNPs resulted in an increase in the apparent MIC of the SCC10 component as one would expect due to the slow release and hence, lower free-Ag(I) concentration at any given SCC10 concentration. As mentioned, SCC5 cannot be readily encapsulated into LTPNPs using current techniques. Consequently, no SCC5–LTP NPs were tested. The LTP NPs themselves had no antimicrobial activity in these assays. Additional MIC and MBC studies of SCC10 on other CF relevant bacteria can be found in the Supplementary data.

To further explore the impact of SCC10 and SCC10–LTP NPs on the growth properties of the *P. aeruginosa* strain PA M57-15, the bacterial growth was measured at OD₆₅₀ in a microplate spectrophotometer in the presence of free SCC10 and SCC10–LTP NPs (Fig. 5). The control PA M57-15 growth curve exhibited log growth between 1 and 5 h. SCC10 at 4 µg/mL shifted the onset of log growth to between 3 and 8 h, while SCC10 concentrations of 8 µg/mL and above inhibited bacterial growth. In this assay, the apparent MIC of SCC10 against PA M57-15 is between 4 and 8 µg/mL, which correlates well with the MIC of 4 µg/mL reported previously for SCC5 against PA M57-15 assayed using standard microtiter dilution methods, also in the absence of the phosphate buffer [13].

In contrast, incubation of PA M57-15 with SCC10–LTP NPs to give final SCC10 concentrations in the microtiter plate well of up to 8 µg/mL resulted in no obvious growth inhibition, as the growth curves appeared similar to the control. At concentrations of SCC10 in LTP NPs of 16 and 32 µg/mL, the lag before onset of log phase growth increased in a dose-dependent manner from 2 to 5 h indicating growth inhibition (Fig. 5B). Once again, the free-Ag(I) concentration in the media containing SCC10–LTP NPs is lower than that of media containing the equivalent concentration of SCC10 due to the slow release of the intact SCC10 prior to subsequent free-Ag(I) release. Thus, we have illustrated the depot delivery properties of the SCC10–LTP NPs in a biologically relevant assay.

3.5. Evaluation of the nebulization efficiency of SCC10–LTP NPs

Different solutions are nebulized with different efficiencies via the micropump technology [38]. Similar to the findings of others [38], the efficiency appears in our hands to correlate with the ionic strength of the liquid, in that solutions with higher ionic strength nebulize more readily. Because the outer shell of LTP NPs is predicted to be non-polar, they were not predicted to nebulize efficiently when suspended in water without the addition of salts. This hypothesis was tested directly by suspending LTP NPs in water, placing an aliquot into the nebulizer and attempting to collect the aerosolized suspension. As expected, the nebulization of phosphate buffer alone was free of NPs. In contrast, condensate from nebulized SCC10–LTP NPs had a concentration equal to that of the original suspension demonstrating that NPs readily passed through the nebulizer (Fig. 6). These results indicate that the nebulization of the LTP NPs is a viable method for localized delivery of SCC10 into the lungs.

Encapsulating SCC10 into degradable NPs provides an avenue for targeting bacteria in biofilms. Targeting moieties such as peptides specific to the *P. aeruginosa* bacteria can be easily incorporated into the nanoparticle formulations. For example, toll-like receptors that recognize the lipopolysaccharide on the membranes of Gram-negative bacteria including *P. aeruginosa* could be bound to the surfaces of NPs [39,40]. Currently, polysaccharides and antibodies have been used in nanoparticle formulations for targeting to specific cells and bacteria [41,42].

3.6. In vivo evaluation of the antimicrobial efficacy of SCC10–LTP NPs

As an initial evaluation of the *in vivo* antimicrobial effects of LTP NPs, two doses of aerosolized blank LTP and SCC10–LTP NPs were delivered 24 h apart in a nose-only fashion to *P. aeruginosa*-infected mice in a multi-dosing chamber (Fig. 7A). Treatment with SCC10–LTP NPs resulted in an almost 20% survival advantage (Fig. 7B: 12/16, 75% SCC10–LTP NPs versus 9/16, 56% blank LTP NPs; $p = 0.23$) and the surviving animals demonstrated less weight loss compared with surviving animals treated with blank NPs (Fig. 7C: $p = 0.009$ by ANOVA). In other studies, mice were pretreated with a 5 mg nebulized dose of free SCC1, a silver complex with a methylated caffeine carrier, prior to infection with *P. aeruginosa* (unpublished results). The mice then received 8 more doses of 5 mg each over a 72-hour study period to achieve a survival advantage of approximately 26%. Thus, treatment with SCC10–LTP NPs using less than one tenth of the silver–carbene dose and only 2 doses in 72 h achieved a similar survival advantage.

To better quantify the antimicrobial effects of the NPs, the lungs and spleens of each animal were harvested for gross examination and quantitative bacteriology. The lungs from the majority of mice treated with SCC10–LTP NPs appeared grossly normal (bright pink with a smooth surface). In contrast, the lungs from most of the mice that received blank LTP NPs appeared inflamed (red or dark pink with an enlarged volume). The spleens from both groups showed no significant morphological disparity. Treatment with SCC10–LTP NPs resulted in a significant decrease in the bacterial burden in the lungs of surviving animals (Fig. 8A: $3.6 \times 10^4 \pm 2.8 \times 10^4$ SCC10–LTP NPs versus $5.3 \times 10^5 \pm 2.6 \times 10^5$ blank LTP NPs; $p = 0.026$, data from one animal is missing due to contamination of the plates). The bacterial counts in the lungs of animals that died were significantly higher than these in both groups and may represent post-mortem replication of bacteria (data not shown). The bacterial burden in the spleens of the surviving animals treated with SCC10–LTP NPs was less than that of the sham-treated animals (Fig. 8B: $1.3 \times 10^2 \pm 8.9 \times 10^1$ for SCC10–LTP NPs versus $5.7 \times 10^2 \pm 3.2 \times 10^2$ for blank LTP NPs), although the difference was not statistically significant ($p = 0.074$). Survival is likely related to bacteremia as demonstrated by dissemination of bacteria to the spleen. Indeed, all of the animals that died had bacteria recovered from their spleens. The animals treated with SCC10–LTP NPs had a significantly lower probability of dissemination of bacteria

to the spleen (Fig. 8C; $p = 0.029$, contingency table), perhaps explaining the survival advantage. These findings indicate that treatment with SCC10–LTP NPs can effectively decrease the burden of bacteria in the lung, bacteremia, and likelihood of death in a *P. aeruginosa* pneumonia model.

4. Conclusions

We have illustrated through *in vitro* and *in vivo* studies that SCC10–LTP NPs are effective against the CF relevant bacteria *P. aeruginosa*. SCC10–LTP NPs provide sustained release of the active drug species over the course of several days providing a significant survival advantage in mouse infection models with only two doses. These results are promising for translating clinically into less frequent dosing of the antibiotic and the added advantage of improved patient compliance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the University of Akron (Firestone Fellowship), Washington University School of Medicine, the National Institute of Allergies and Infectious Diseases (1 R01 A106785601), and the National Institute of General Medical Sciences (5 R01 GM086895-02). We thank the National Science Foundation (CHE-8808587 and CHE-9977144) for funds used to purchase the Varian Gemini 300 MHz and the Varian INOVA 400 MHz NMR instruments used in this work. We would like to acknowledge the National Science Foundation (CHE-0116041) and the Ohio Board of Regents for funds used to purchase the Bruker-Nonius Apex CCD X-ray diffractometer used in this research.

References

1. Welsh, MJ.; Ramsey, BW.; Accurso, F.; Cutting, GR. Cystic fibrosis. In: Scriver, CR.; Beaudet, AL.; Sly, WS.; Valle, D.; Childs, B.; Vogelstein, B., editors. The metabolic and molecular basis of inherited disease. Vol. 8th. Vol. 3. New York: McGraw Hill; 2001. p. 5121-89.
2. Beaulac C, Clement-Major S, Hawari J, Lagace J. Eradication of mucoid *Pseudomonas aeruginosa* with fluid liposome-encapsulated tobramycin in an animal model of chronic pulmonary infection. *Antimicrob Agents Chemother* 1996;40:665–9. [PubMed: 8851590]
3. Marier J, Brazier J, Lavigne J, Ducharme M. Liposomal tobramycin against pulmonary infections of *Pseudomonas aeruginosa*: a pharmacokinetic and efficacy study following single and multiple intratracheal administrations in rats. *J Antimicrob Chemother* 2003;52:247–52. [PubMed: 12837733]
4. Melaiye A, Youngs WJ. Silver and its application as an antimicrobial agent. *Expert Opin Ther Pat* 2005;15:125–30.
5. Kollef MH, Afessa B, Anzueto A, Veremakis C, Kerr KM, Margolis BD, et al. NASCENT Investigation Group. Silver-coated endotracheal tubes and incidence of ventilator-associated pneumonia. The NASCENT randomized trial. *JAMA* 2008;300:805–13. [PubMed: 18714060]
6. Gupta A, Matsui K, Lo JF, Silver S. Molecular basis for resistance to silver cations in *Salmonella*. *Nat Med* 1999;5:183–8. [PubMed: 9930866]
7. Li XZ, Nikaido H, Williams KE. Silver-resistant mutants of *Escherichia coli* display active efflux of Ag^+ and are deficient in porins. *J Bacteriol* 1997;179:6127–32. [PubMed: 9324262]
8. Silver S. Bacterial silver resistance: molecular biology and uses and misuses of silver compounds. *FEMS Microbiol Rev* 2003;27:341–53. [PubMed: 12829274]
9. Modak SM, Stanford JW, Bradshaw W, Fox CL Jr. Silver sulfadiazine (AgSD) resistant *Pseudomonas* infection in experimental burn wounds. *Panminerva Med* 1983;25:181–8. [PubMed: 6669393]

10. Pimay JP, De Vos D, Cochez C, Biloq F, Pirson J, Struelens M, et al. Molecular epidemiology of *Pseudomonas aeruginosa* colonization in a burn unit: persistence of a multidrug-resistant clone and a silver sulfadiazine-resistant clone. *J Clin Microbiol* 2003;41:1192–202. [PubMed: 12624051]
11. Bjarnsholt T, Kirketerp-Moller K, Kristiansen S, Phipps R, Nielsen A, Jensen P, et al. Silver against *Pseudomonas aeruginosa* biofilms. *APMIS* 2007;115:921–8. [PubMed: 17696948]
12. Lansdown AB. Silver. I: its antibacterial properties and mechanism of action. *J Wound Care* 2002;11:125–30. [PubMed: 11998592]
13. Hindi K, Siciliano T, Durmus S, Panzner MJ, Medvetz D, Reddy V, et al. Synthesis, stability, and antimicrobial studies of electronically tuned silver acetate *N*-heterocyclic carbenes. *J Med Chem* 2008;51:1577–83. [PubMed: 18288795]
14. Kascatan-Nebioglu A, Melaiye A, Hindi K, Durmus S, Panzner MJ, Hogue L, et al. Synthesis from caffeine of a mixed *N*-heterocyclic carbene–silver acetate complex active against resistant respiratory pathogens. *J Med Chem* 2006;49:6811–8. [PubMed: 17154511]
15. Kascatan-Nebioglu A, Panzner MJ, Tessier CA, Cannon CL, Youngs WJ. *N*-Heterocyclic carbene–silver complexes: a new class of antibiotics. *Coord Chem Rev* 2007;251(5–6):884–95.
16. Pai V, Nahata M. Efficacy and safety of aerosolized tobramycin in cystic fibrosis. *Pediatr Pulmonol* 2001;32:314–27. [PubMed: 11568993]
17. Patton J, Fishburn S, Weers J. The lungs as a portal of entry for systemic drug delivery. *Proc Am Thorac Soc* 2004;1:338–44. [PubMed: 16113455]
18. Meers P, Neville M, Malinin V, Scotto A, Sardaryan G, Kurumunda R, et al. Biofilm penetration, triggered release and in vivo activity of inhaled liposomal amikacin in chronic *Pseudomonas aeruginosa* lung infections. *J Antimicrob Chemother* 2008;61:859–68. [PubMed: 18305202]
19. Sen Gupta A, Lopina ST. Synthesis and characterization of l-tyrosine based novel polyphosphates for potential biomaterial applications. *Polymer* 2004;45:4653–62.
20. Sen Gupta A, Lopina ST. Properties of l-tyrosine based polyphosphates pertinent to potential biomaterial applications. *Polymer* 2005;46:2133–40.
21. Ditto AJ, Shah PN, Lopina ST, Yun YH. Nanospheres formulated from l-tyrosine polyphosphate as a potential intracellular delivery device. *Int J Pharm* 2009;368:199–206. [PubMed: 19026734]
22. Geller DE, Rosenfeld M, Waltz DA, Wilmott RW. Efficiency of pulmonary administration of tobramycin solution for inhalation in cystic fibrosis using an improved drug delivery system. *Chest* 2003;123:28–36. [PubMed: 12527599]
23. van Heeckeren AM, Tscheikuna J, Walenga RW, Konstan MW, Davis PB, Erokwu B, et al. Effect of *Pseudomonas* infection on weight loss, lung mechanics, and cytokines in mice. *Am J Respir Crit Care Med* 2000;161:271–9. [PubMed: 10619831]
24. Herrmann WA, Köcher C. Essays on organometallic chemistry. 9. *N*-Heterocyclic carbenes. *Angew Chem Int Ed Engl* 1997;36:2162–87.
25. Garrison JC, Youngs WJ. Ag(I) *N*-heterocyclic carbene complexes: synthesis, structure, and application. *Chem Rev* 2005;105(11):3978–4008. [PubMed: 16277368]
26. Lin IJB, Vasam CS. Preparation and application of *N*-heterocyclic carbene complexes of Ag(I). *Coord Chem Rev* 2007;251(5–6):642–70.
27. Stringer JL, Peppas NA. Diffusion of small molecular weight drugs in radiation-crosslinked poly (ethylene oxide) hydrogels. *J Control Release* 1996;42:195–202.
28. Sandor M, Ensore D, Weston P, Mathiowitz E. Effect of protein molecular weight on release from micron-sized PLGA microspheres. *J Control Release* 2001;76:297–311. [PubMed: 11578744]
29. Chern CS, Lin SY. Emulsion polymerization of styrene stabilized by mixed anionic and nonionic surfactants. *Polymer* 1997;38:1977–84.
30. Ouchi T, Nishizawa H, Ohya Y. Aggregation phenomenon of PEG-grafted chitosan in aqueous solution. *Polymer* 1998;39:5171–5.
31. Lee ES, Park KH, Kang D, Park IS, Min HY, Lee DH, et al. Protein complexed with chondroitin sulfate in poly(lactide-*co*-glycolide) microspheres. *Biomaterials* 2007;28:2754–62. [PubMed: 17337049]

32. Sarkar S, Lee GY, Wong JY, Desai TA. Development and characterization of a porous micro-patterned scaffold for vascular tissue engineering applications. *Biomaterials* 2006;27:4775–82. [PubMed: 16725195]
33. Owusu-Ababio G, Rogers J, Anwar H. Effectiveness of ciprofloxacin microspheres in eradicating bacterial biofilm. *J Control Release* 1999;57:151–9. [PubMed: 9971894]
34. Martin AR, Finlay WH. A general, algebraic equation for predicting total respiratory tract deposition of micrometer-sized aerosol particles in humans. *J Aerosol Sci* 2007;38:246–53.
35. Sakagami M, Sakon K, Kinoshita W, Makino Y. Enhanced pulmonary absorption following aerosol administration of mucoadhesive powder microspheres. *J Control Release* 2001;77:117–29. [PubMed: 11689265]
36. Tamilvanan S, Venkateshan N, Ludwig A. The potential of lipid- and polymer-based drug delivery carriers for eradicating biofilm consortia on device-related nosocomial infections. *J Control Release* 2008;128:2–22. [PubMed: 18342974]
37. Sen Gupta, A. PhD thesis. Akron, OH: The University of Akron; 2003. Synthesis and characterization of L-tyrosine based novel biodegradable polyphosphates and polyurethanes for biomaterial applications.
38. Zhang G, David A, Wiedmann TS. Performance of the vibrating membrane aerosol generation device: aeroneb micropump nebulizer. *J Aerosol Med* 2007;20:408–16. [PubMed: 18158713]
39. Pier GB. *Pseudomonas aeruginosa* lipopolysaccharide: a major virulence factor, initiator of inflammation and target for effective immunity. *J Med Microbiol* 2007;297:277–95.
40. Medshitev R, Preston-Hurlburt P, Janeway CA Jr. A human homolog of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* 1997;388:394–7. [PubMed: 9237759]
41. Keegan ME, Royce SM, Fahmy T, Saltzman WM. In vitro evaluation of biodegradable microspheres with surface-bound ligands. *J Control Release* 2006;110:574–80. [PubMed: 16386325]
42. Schlottmann SA, Jain N, Chirmule N, Esser MT. A novel chemistry for conjugating pneumococcal polysaccharides to Luminex microspheres. *J Immunol Methods* 2006;309:75–85. [PubMed: 16448665]

Fig. 1. Crystal structure of the cationic portion of **1** with thermal ellipsoids shown at 50% probability. Hydrogen atoms and anions are removed for clarity.

Fig. 2.
2000× SEM image of (A) SCC10-LTP NPs and (B) blank LTP NPs.

Fig. 3. Diameter distribution from dynamic laser light scattering of SCC10-LTP NPs and blank LTP NPs. Values reported as differential distribution values.

Fig. 4.
Cumulative release of SCC10 from 1 mg of NPs after incubation in PBS at 37 °C for 7 days.

Fig. 5. Effect of increasing concentrations of SCC10 on the growth of *P. aeruginosa*. (A) Free SCC10. (B) SCC10-LTP NPs. The key refers to the SCC10 concentration in µg/mL.

Fig. 6.

Light microscope images of SCC10–LTP NPs after nebulization. A) Gross image of nebulized nanoparticles using 10× objective. B) Magnified image LTP NPs with 63× objective after nebulization. C) Magnified image phosphate buffer with 63× objective after nebulization. Nanoparticles were not observed in this solution. The air/glass interface can be observed in the lower right corner.

Fig. 7. Effect of nebulized SCC10-LTP NPs on survival and weight loss in a mouse model of *Pseudomonas pneumonia*. (A) Treatment protocol. Mice received treatment with nebulized blank LTP or SCC10-LTP NPs 1 h after infection with *P. aeruginosa* PA M57-15, and again 24 h after the first treatment, until mice were evaluated on day 3 (72 h). (B) Kaplan-Meier survival curves. The survival of mice treated with SCC10-LTP NPs was greater than that of those treated with blank LTP NPs (75% survival in 12/16 SCC10-treated mice versus 56% in 9/16 blank-treated mice). (C) Weight loss. Mice treated with SCC10-LTP NPs lost less weight than animals treated with blank nanoparticles (displayed mean % weight change from inoculation weight $\pm$ SEM, no statistical difference between groups at each time point).

Fig. 8.

Effects of nebulized SCC10-LTP NPs on lung and spleen bacterial burden and likelihood of dissemination in a mouse model of *Pseudomonas* pneumonia. (A) Total number of bacteria recovered from the lungs of mice surviving at 72 h. Bar represents the mean bacterial count and was significantly different between samples ($*p < 0.03$). (B) Bacteremia as indicated by recovery of bacteria from the spleen after the indicated treatment in surviving mice analyzed at 72 h. Bar represents the mean bacterial count. (C) Bacteremia in all of the mice plotted as number of spleens in each treatment group with (black bars) or without (yellow bars) bacteria. Mice receiving SCC10-LTP NPs had a significantly reduced likelihood of bacteremia ($*p <$

0.03). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Scheme 1.
Synthesis of **1**.

Equation 1.
Synthesis of SCC10.

Table 1

MIC and MBC comparison of SCC5, SCC10 and SCC10-LTP NPs.

Species	SCC5			SCC5 w/DMSO			SCC10 w/DMSO			SCC10-LTP NPs			LTP NPs		
Strain	MIC ^a	MBC ^a		MIC	MBC		MIC	MBC		MIC	MBC		MIC	MBC	
<i>Pseudomonas aeruginosa</i>															
PAO1	1	4		1	2		1	4		6	10		>10	na	
PA M57-15	1	6		8	>10		1	2		4	>10		>10	na	
<i>Escherichia coli</i>															
J53	1	8		1	8		1	6		6	6		>10	na	
J53 + pMG101	>10	na ^b		>10	na		>10	na		>10	na		>10	na	

^a All concentrations are in mg/mL.

^b Not applicable.