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Shell crosslinked nanoparticles carrying silver antimicrobials as therapeutics†

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Abstract

Amphiphilic polymer nanoparticles loaded with silver cations or/and *N*-heterocyclic carbene–silver complexes were assessed as antimicrobial agents against Gram-negative pathogens *Escherichia coli* and *Pseudomonas aeruginosa*.

Silver has long been prized as an antimicrobial; ancient Egyptians used silver in food storage. In the present day, silver compounds are widely used as antimicrobial agents, especially in the treatment of wounds and burns.[1,2] Silver cation (Ag^+) is highly toxic, or described as "oligodynamic," against a broad spectrum of microorganisms, probably because of its inhibition of certain oxidative enzymes, protein denaturation, or interference with DNA replication.[3] Unlike traditional antibiotics, Ag^+ is of low toxicity to human tissues and has elicited only rare instances of bacterial silver resistance.[4–6] A variety of silver-based antimicrobials, therefore, has been synthesized and evaluated. Fox introduced silver sulfadiazine (SSD) in the 1960s;[7] SSD remains routinely used as a topical treatment of burns. Although it has been recognized as an antimicrobial since at least the 1800s, Moyer repopularized $AgNO_3$ for treatment of burns, which prompted development of SSD.[2] Unfortunately, $AgNO_3$ is not practical *in vivo*, because Ag^+ complexes with salts and other biological agents in the bloodstream.[7] Over the past decade, an array of silver *N*-heterocyclic carbene (NHC) complexes, which exhibit improved stability to light and aqueous solution, have been synthesized and investigated by Youngs and Cannon as potential antimicrobial agents and have shown very promising results with both *in vitro* and *in vivo* studies in a variety of bacteria including BSL3 organisms.[8–11] Small molecule antibiotics also have a major problem, however, that of rapid clearance from the human body and, in the case of silver, reaction with sulfur-containing proteins and chloride in the bloodstream.[12] Therefore, there

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remains a need for packaging and protection for therapeutic delivery of Ag^+ or silver–carbene complexes (SCCs).

Silver-containing (mostly silver(0) nanoparticles) polymers,[13] hyperbranched polymers,[14] and dendrimers[15] have been investigated for improved solubility and processability to form antimicrobial surfaces for biomaterial-related infections. Youngs and Cannon *et al.* had previously loaded SCCs into L-tyrosine polyphosphate nanoparticles and demonstrated potent antimicrobial efficacy in *in vitro* and *in vivo* studies against *Pseudomonas aeruginosa*.[16] In this study, we developed shell crosslinked knedel-like (SCK) nanoparticles[17–20] as an antimicrobial device, designed to encapsulate and protect Ag^+, SCCs, or the two agents coincidentally, and evaluate the relative efficacy of each system. The SCKs were constructed by the supramolecular assembly of amphiphilic block copolymers, poly(acrylic acid)-*b*-polystyrene (PAA-*b*-PS), into micelles, followed by covalent crosslinking throughout the shell layer to afford discrete nanostructures having a hydrophobic core domain and a hydrophilic shell region. Four procedures were then followed for loading of the SCKs with silver: (1) Ag^+ was incorporated from $AgNO_3$ into the hydrophilic PAA shell region ($AgNO_3$–SCK); (2) 1-hexyl-3-methyl-4,5-dichloro-imidazole-2-ylidene silver(I) acetate (SCC10, which undergoes decomposition in the presence of saline solution to release active Ag^+)[21] was loaded into the hydrophobic PS core domain and/or the core–shell interface (SCC10–SCK);[22,23] (3) and (4) both methods were applied in opposite order of addition ($AgNO_3$–SCC10–SCK or SCC10–$AgNO_3$–SCK) (Fig. 1). In all cases, free silver was eliminated using a centrifugal filter device (100 kDa MWCO), and the filtrates were examined by UV-visible spectroscopy to confirm removal of free silver. The resulting silver-bearing nanoparticles were characterized and their antimicrobial activities against common Gram-negative pathogenic bacteria were evaluated *in vitro*.

The silver-loading capacities of the nanoparticles were measured by inductively coupled plasma-mass spectrometry (ICP-MS) using Tl as an internal standard (see ESI†). The amount of Ag loaded increased with increasing feed amounts. $AgNO_3$–SCK reached a [Ag] loading capacity of *ca.* 370 μg mL^{-1} at the highest $AgNO_3$ feed of 200 mol% with respect to the combined total theoretical moles of acrylic acid and amide residues in the SCK shell, whereas SCC10–SCK had a capacity of *ca.* 75 μg mL^{-1} at 200 wt% feed of SCC10 with respect to the polymer weight of the SCK solution. The efficiencies for loading, measured as the percentage of silver loaded into the SCKs *vs.* the amount of silver in the feed, were constant across the feed ratios, and were consistently higher for the $AgNO_3$ loading method. Sequential silver loading by both methods (performed in either order) did not improve silver capacity over Ag^+-loading only, reaching a total [Ag] of *ca.* 220 μg mL^{-1} at 150% feed. Higher feeds of silver caused precipitation. The silver-bearing nanoparticles were examined by transmission electron microscopy (TEM), and were observed to be uniform nanostructures of sizes that agreed with the non-Ag-loaded SCKs (Fig. 2). Some elemental silver nanoparticles were observed in the $AgNO_3$–SCK sample (see ESI†), which might be due to the reduction of Ag^+ to Ag(0) in the amine-containing polymer matrix.[9,15,24]

Release of silver from the SCK nanoparticles was assessed by monitoring the decrease over time of the concentration of silver in dialysis cassettes, performed at 37 °C in 5 mM PBS at pH 7.4 and analyzed by ICP-MS (Fig. 3). Each loading protocol gave *ca.* 50% release of silver within *ca.* 1 day and *ca.* 80% release within 2 days, obtaining a plateau with full silver release by *ca.* 4 days, a time period that would provide a desired depot effect for therapeutic delivery. Moreover, the stability of these Ag–SCK complexes over many hours in PBS is a distinct advantage, relative to simple silver salt solutions, for future *in vivo* studies.

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The antimicrobial activities of the silver-loaded nano-constructs against common Gram-negative pathogenic bacteria were measured. We first tested the antimicrobial activity of SCC10 (in aqueous solution with 1% dimethyl sulfoxide) by determining the minimal inhibitory concentration (MIC) in Mueller–Hinton (MH) broth against urinary isolates of *Escherichia coli* and respiratory isolates of *P. aeruginosa* from patients with cystic fibrosis. These MICs were physiologically relevant, ranging from 1 to 6 μg mL^{-1} (see ESI†). As positive and negative controls, the MICs of SCC10 against *E. coli* strain J53 with and without the silver resistance plasmid pMG101[5,6] were tested. The MIC of SCC10 was 1 μg mL^{-1} for J53 but > 10 μg mL^{-1} for J53/pMG101, demonstrating that the antimicrobial activity of SCC10 is conferred by the silver moiety.

Next, we tested the activity of our silver-bearing SCK constructs against representative strains of *E. coli* (strain UTI89; MIC [SCC10] = 2 μg mL^{-1}) and *P. aeruginosa* (strain PAM57-15; MIC [SCC10]=1 μg mL^{-1}). Defined suspensions of these strains in MH broth were treated in 96-well plates with the silver-bearing SCKs, equalized for [Ag] by the ICP-MS data. Bacterial growth was measured by optical density (650 nm) in a microplate spectrophotometer 6 h after treatment. SCKs without loaded silver had no antimicrobial activity (data not shown). Independent of the silver-loading method, decrements in growth of *E. coli* UTI89 were observed at [Ag] of 1 μg mL^{-1}, and growth was completely inhibited at [Ag] of 2 μg mL^{-1} (Fig. 4a). For *P. aeruginosa* PAM57-15, decrements in growth were observed at [Ag] of 2–4 μg mL^{-1} and growth was completely inhibited at [Ag] of 8 μg mL^{-1} (Fig. 4b). Activity of the silver-bearing SCKs was generally inferior to that of naked $AgNO_3$ by ≤1 two-fold dilution in inhibition of bacterial growth, suggesting that the SCKs provide availability of silver for antimicrobial action.

These silver-loaded SCK nanoparticle delivery systems exhibited antimicrobial activities, which were nearly comparable to $AgNO_3$. There appeared to be no advantage to the use of the silver–carbene compounds *vs.* loading with silver cations directly. The sustained release over a period of hours suggests that these nanoparticle delivery systems may be beneficial in the treatment of microbial infections *in vivo*. Packaging in the nanoparticle framework is expected to provide for *in vivo* stability. Furthermore, they can be functionalized, which may permit control over biodistribution,[25] tissue-selective targeting[26] and *in vivo* clearance.[27,28] We are currently investigating their potential in the treatment of pulmonary and urinary tract infections.

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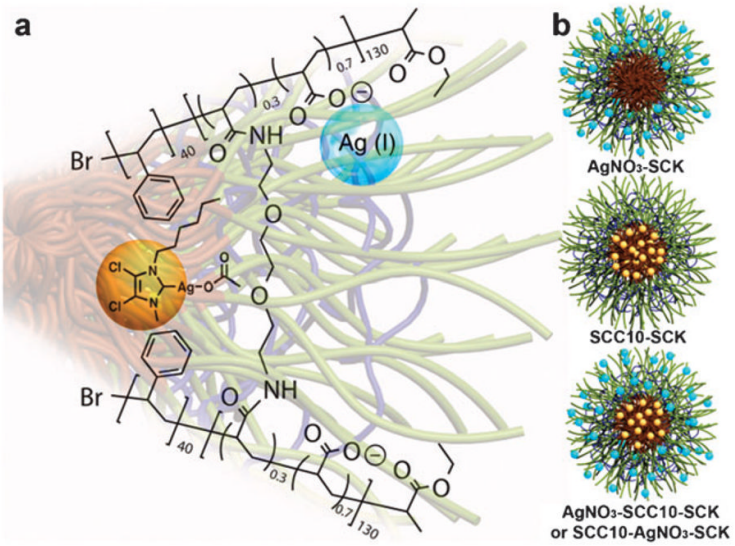

Fig. 1.

Schematic representations of (a) SCC10 (yellow ball) incorporated into the core and Ag^+ (blue ball) from $AgNO_3$ chelated into the shell of an SCK prepared from PAA_{130}-*b*-PS_{40}; and (b) $AgNO_3$–SCK, SCC10–SCK, and $AgNO_3$–SCC10–SCK or SCC10–$AgNO_3$–SCK. Note: the placements of the silver species within the SCK framework are hypothetical locations, which have not been confirmed experimentally.

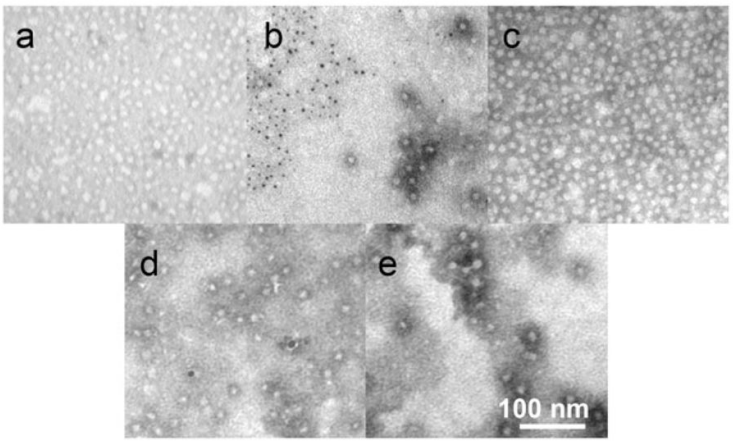

Fig. 2.

TEM images of SCKs and silver-loaded SCKs, each with negative staining by 1% phosphotungstic acid, (a) SCK, (b) $AgNO_3$–SCK, (c) SCC10–SCK, (d) $AgNO_3$–SCC10–SCK, and (e) SCC10–$AgNO_3$–SCK. The scales are consistent.

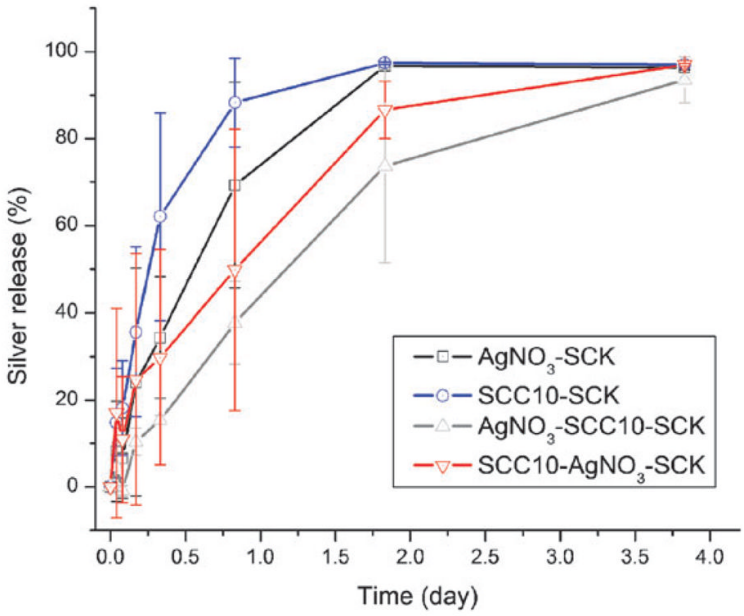

Fig. 3.
Release profiles of silver from silver-bearing nanoparticles at 37 °C in 5 mM PBS at pH 7.4 (duplicate).

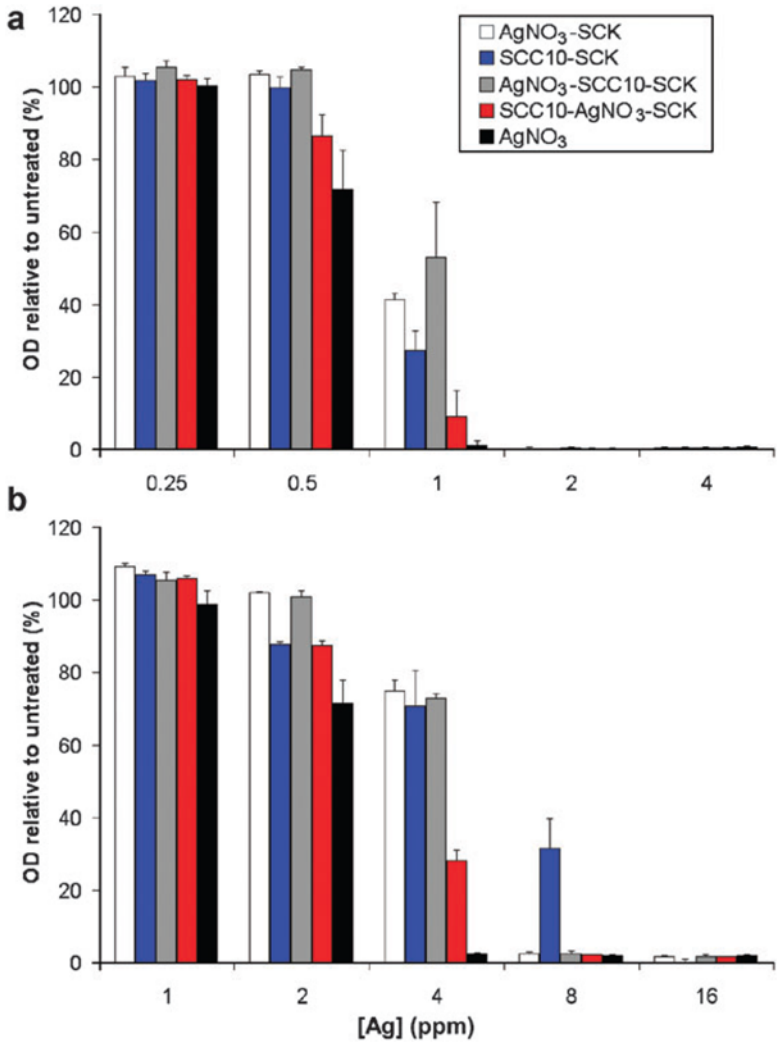

Fig. 4.

Inhibition of growth of *E. coli* strain UTI89 (a) and *P. aeruginosa* strain PAM57-15 (b) by silver-bearing nanoparticles and naked $AgNO_3$. Relative optical density (650 nm) after 6 h is shown for each construct at the indicated silver concentrations.