Combination therapy of curcumin and silver nanoparticles with enhanced anti-biofilm activity for treatment of endotracheal tube-associated infections

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Summary
Biofilm tolerance has become a serious clinical concern in the treatment of nosocomial pneumonia owing to the resistance to various antibiotics. There is therefore an urgent need to develop alternative antimicrobial agents or combination drug therapies that are effective via different mechanisms. Silver nanoparticles (AgNP) have been developed as anti-biofilm agent for the treatment of infections associated with the use of mechanical ventilations, such as endotracheal intubation. Meanwhile curcumin, a phenolic plant extract, has displayed natural anti-biofilm properties through the inhibition of bacterial quorum sensing systems. The aim of this study was to investigate the possible synergistic/additive interactions of AgNP and curcumin nanoparticles (Cur-NP) against both Gram-negative (*Pseudomonas aeruginosa*) and Gram-positive (*Staphylococcus aureus*) microorganisms. The combination therapy against *P. aeruginosa* showed an additive effect with minimum inhibitory concentration (MIC) of 29.4 µg/mL. Similarly, it was found that the MIC of Cur-NP alone against *S. aureus* was 220 µg/mL, compared to 50 µg/mL for the combination therapy. Combination of AgNP and Cur-NP (termed as Cur-SNP) at ~100 µg/mL disrupted 50% of established bacterial biofilms (formed on microtiter plates). However, further increase in the concentration of Cu-SNP failed to effectively eliminate the biofilms. To achieve the same effect, at least 500 µg/mL of Cur-NP alone was needed. Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) revealed that combination therapy (Cur-SNP) was the most potent to eradicate pre-formed biofilm compared to mono-drug therapy. These agents are also non-toxic to healthy lung cells (BEAS-2B). As a co-therapy, curcumin acts to inhibit biofilm re-assembly and production of virulence factors in biofilm while AgNP inactivates cells activities through binding with sulfur-based compounds.

Introduction
Ventilator-associated pneumonia (VAP) accounts for more than 80% of nosocomial pneumonia cases in hospitals and often manifests in patients after 48 h of receiving mechanical ventilation [1]. A major burden to treat VAP is the rise of antibiotic-resistant bacterial infections and extremely tolerant bacterial biofilm formation [1, 2]. There are currently no definite guidelines to treat VAP but most likely would involve the co-administration of several antimicrobial agents that work synergistically. Silver nanoparticles (AgNP) have been noted for its superior anti-bacterial and anti-biofilm mechanisms against various microorganisms [3, 4]. As AgNP act simultaneously on multiple sites within bacterial cells, it is believed that the probabilities to acquire silver-resistant bacterial genes are quite low [5]. The use of nanoparticulate to treat biofilm is advantageous because, in addition to bactericidal activities conferred by Ag^+, additional killing mechanisms by the nanoparticles themselves exist which includes direct attachment to cell membrane, higher uptake into cells and formation of pits [6, 7]. Curcumin is a natural phenolic compound which acts to interfere with the quorum sensing signaling in biofilms and hinder the production of bacterial virulence factors [8]. Therefore, co-administration of curcumin and AgNP is expected to provide enhanced anti-biofilm efficacy.

In this study, the fabrication of curcumin nanoparticles (Cur-NP) and AgNP were developed using wet chemical synthesis method. The effect of combination therapy on eradication established biofilm was evaluated using static microtiter assay and viable cell counting. Examination of biofilm structure after treatment was performed to further validate the data obtained in static assay. Furthermore, the tolerance of mammalian cells to this combination formulation was also assayed using healthy lung cell lines.

Methods
Preparation of Cur-NP, curcumin silver nanoparticles (Cur-SNP) and AgNP
Colloids of AgNP with average diameter of 7 to 15 nm were prepared according to the method as described previously [3]. Cur-NP were prepared using solvent and anti-solvent precipitation method. In brief, 500 mg of curcumin was dissolved in 150 mL of absolute ethanol and filtered using 0.45 µm membrane filters. The filtered curcumin solution was added immediately into 450 ml chilled pluronic F-127 aqueous solution and homogenized (Silverson L4RT, United States) at 6,000 rpm followed by addition of 100 mL of PVA (0.3% w/v). For the preparation of Cur-SNP, the same procedure was used with a slight modification in which 100 mg of re-dispersed AgNP was added into the solution before the addition of PVA. The resultant Cur-NP and

Cur-AgNP were immediately frozen using liquid nitrogen before being lyophilized at –50 °C (B.Braun, Alpha 1-4).

Physicochemical characterization of NPs

Particle size and polydispersity index (PI) of Cur-NP, Cur-SNP and AgNP were determined using dynamic light scattering (DLS) (Malvern Instruments Nano Series ZS Zetasizer, United Kingdom). The morphology of the particles was observed under transmission electron microscope (TEM) (JEOL 2100).

In vitro biofilm detachment using crystal violet assay

The biofilm detachment was quantified by crystal violet assay in 96-well microtiter plates using two different wild type bacterial strains (*P. aeruginosa* PAO1 and *S. aureus* ATCC 25923) as described previously [3]. The effects of Cur-NP, Cur-SNP and AgNP on the biofilm removal were evaluated at a range of different concentrations containing equivalent amount of curcumin. For AgNP, the concentrations used corresponded to the amount of Ag present in Cur-AgNP.

Qualitative imaging of *P. aeruginosa* PAO1 and *S. aureus* ATCC 25923 biofilms using confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM)

For visual observation of the biofilm after the treatment, samples were analyzed using both CLSM and SEM. For CLSM visualization, both control and treated biofilms were stained with SYTO9 (Invitrogen, Australia) for 1 h followed by 4% paraformaldehyde fixation. The stained and fixed samples were imaged using oil immersion lens (100 x objective lens and numerical aperture of 1.4). Recorded images were reconstructed by Imaris and presented as 3-dimensional structures. For SEM observation, samples were fixed with 4% paraformaldehyde overnight without staining followed by dehydration in a series of graded ethanol baths. Samples were placed onto SEM stubs and dried using a critical-point drier. Dried samples were gold-coated and imaged using SEM (Zeiss Ultra, Germany).

Cytotoxicity of NP on human normal bronchial epithelial cells (BEAS-2B)

The BEAS-2B cells were cultured in Dulbecco's Modified Eagle's medium (DMEM F-12) supplemented with non-essential amino acid, L-glutamic acid and 10% of fetal bovine serum (FBS). BEAS-2B was incubated at 37 °C in CO_2 humidified atmosphere. When the cells had reached about 80% of confluence, cells were trypsinized and seeded into 96-well plates with 50,000 cells per well. The cells were incubated for 24 h to allow cell attachment on the surface. Next, cells were treated with different concentration of NP for 3 days. The cells were then washed with warm PBS (3 times) to remove remaining particles and 200 µL of fresh medium was added to each well. Then, 20 µL of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) reagent was added and incubated for 4 h at 37 °C. The colour intensity was measured at 490 nm with microplate reader (POLAstar). The cytotoxicity was expressed as a percentage of viable cells relative to untreated cells.

Results

TEM and DLS images revealed that AgNP appeared spherical, non-agglomerated and had average size of 20 nm [3]. Cur-NP was seen as clusters of spherical-like particles at approximately 30 nm (Figure 1). Both freeze-dried and re-dispersed particles demonstrated similar morphologies with negligible agglomeration (results not shown). The amount of curcumin and silver present in Cur-SNP was determined prior to antimicrobial susceptibility studies. The concentrations of curcumin and silver in each mg of powdered samples were 31.5 ± 0.80 µg/mg and 4.0 ± 0.5 µg/mg, respectively. It should be noted that the concentrations noted in subsequent experiments were calculated based on equivalent amount of curcumin.

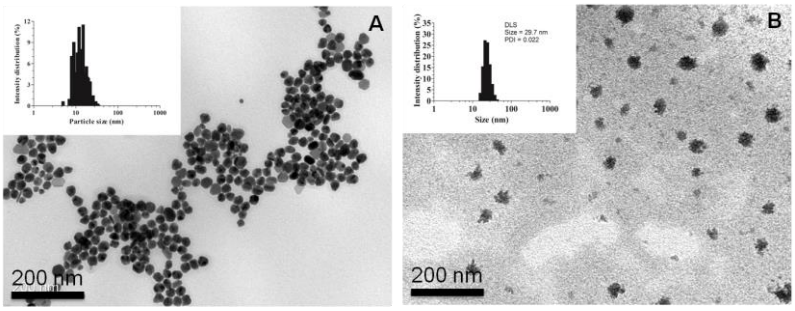

Figure 1. TEM images of (A) AgNPs and (B) Cur-NPs. Insets show the size distributions of these nanoparticles.

Table 1 shows the antimicrobial efficacies of Cur-NP and AgNP against planktonic microorganisms. The MIC of Cur-NP against *P. aeruginosa* and *S. aureus* was 150 and 220 µg/mL, respectively. Meanwhile, the combination therapy (Cur-SNP) demonstrated enhanced antibacterial activity, where the MIC ranged between 29–50 µg/mL. Based on the calculation of synergistic index, this combination demonstrates an additive effect. The MIC of AgNP treatment alone was much higher in comparison to the combination therapy indicating the beneficial effects of combining Cur-NP and AgNP to kill microorganisms. Due to the low solubility of curcumin in aqueous solution, we did not perform the inhibitory studies using raw curcumin (solubilized curcumin in an organic solvent). In addition, the inhibitory experiments using void nanoparticles (without curcumin or silver) demonstrated no antimicrobial effect against both tested microorganisms (results not shown).

Table 1. MIC of planktonic *P. aeruginosa* PAO1 and *S. aureus* ATCC 25923.

	MIC (µg/mL)	
	PAO1	ATCC 25923
Cur-SNP	29.4 (curcumin) or 4.8 (silver)	50 (curcumin) or 8.2 (silver)
Cur-NP	150	220
AgNP	> 4.8	> 8.2

Both SEM and CLSM images of microorganisms provided visual confirmation on the effect of combination therapy (Cur-SNP) on detachment of *S. aureus* and *P. aeruginosa*. Significantly higher cell removal was observed using Cur-SNP treatment in both microorganisms, which correlated to the results obtained from static assay and viable counting (CFU). Only small clusters of cells remained adhered after being treated with Cur-SNP. Although Cur-NP or AgNP alone was less effective compared to Cur-SNP, we also observe significant removal of biofilm when these nanoparticles used separetly. Compared to dense and robust biofilm structure in untreated (control) group, Cur-NP treated samples revealed extensive loss in biofilm density and volume coverage with clusters of small microcolonies evident. Similar finding was observed for AgNP treatment with the exception of lesser biofilm detachment activities (Figure 2).

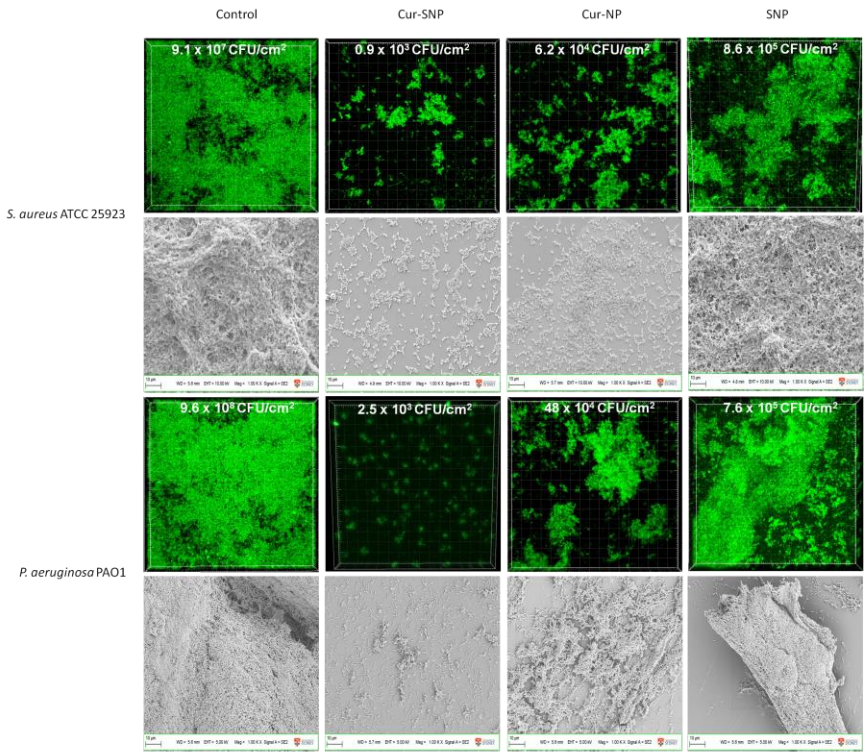

Figure 2. CLSM and SEM images of control pre-formed *P.* aeruginosa PAO1 and S. aureus biofilm colonies grown in their culture media: cation adjusted Mueller Hinton broth (CAMHB) or tryptic soy broth (TSB) and samples treated with Cur-SNP, Cur-NP and AgNP (SNP). Corresponding viable attached cells were expressed in CFU/well.

Curcumin is an established compound with selective target towards cancer cells but benign against healthy cells. To remove lingering doubts regarding the safety of curcumin, the tolerance of healthy cells against curcumin and/or silver was investigated via concentration-dependent killing MTS assay. Figure 3 presents cell viabilities in a few selected representative concentrations. It should be noted here that the concentration of AgNP is equivalent to the amount of Ag present in combination agents (Cur-SNP). In general, the viability of BEAS-2B is concentration-dependent. At low concentration (20 µg/mL), about 90% of cells survived the administration of each antimicrobial formulations. However as the concentration exceeded 200 µg/mL, cell viabilities were decreased to about 60%, for curcumin in particular. In the meantime, CLSM images showed that curcumin was internalized into BEAS-2B cells as depicted in green fluorescent color, thus indirectly correlates to the curcumin's toxicity effect (Figure 3). For AgNP treated biofilm samples, only DAPI-stained nucleus was visible.

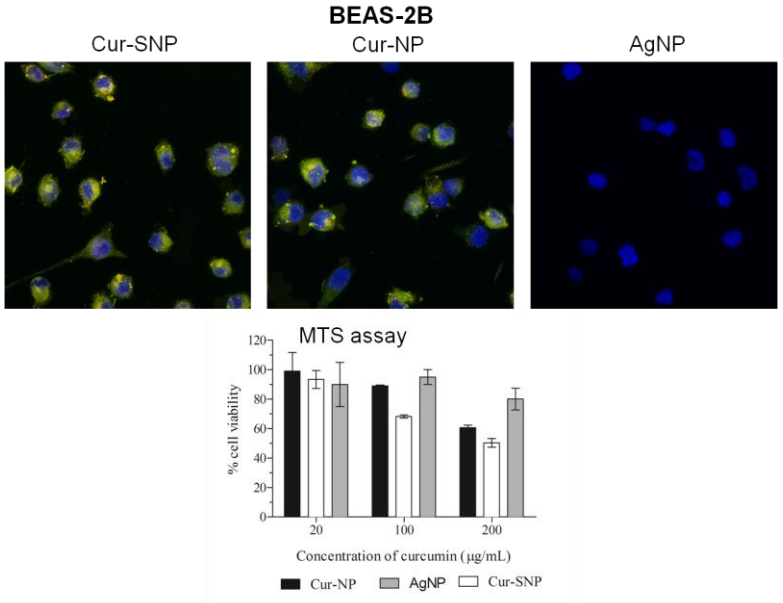

Figure 3. CLSM images showing the internalization of curcumin into BEAS-2B cultured at high curcumin concentration. Cytotoxicity MTS assay demonstrating the viabilities of BEAS-2B treatment with Cur-NP, AgNP or Cur-SNP (combination) in comparison against control.

Conclusions

This study showed that combination therapy of curcumin and silver nanoparticles is an effective tool to eradicate established mature biofilm. These formulations could be administered either directly as 'free' solution to produce rapid alleviation in bacterial infections or as a coating on endotracheal tubes to achieve prolonged, sustained antibacterial effect. Hydrogels of Cur-SNP for coatings on endotracheal tubes are currently being developed and their performance and bio-compatibility are being investigated.

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