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Dynamically Crosslinked Polymer Nanocomposites to Treat Multidrug-Resistant Bacterial Biofilms

Dong Yu Zhu^{#a,b}, Ryan F. Landis^{#b}, Cheng-Hsuan Li^b, Akash Gupta^b, Li-Sheng Wang^b, Yingying Geng^b, Sanjana Gopalakrishnan^b, Jian Wei Guo^a, and Vincent M. Rotello^b

^aSchool of Chemical Engineering and Light industry, Guangdong University of Technology, 100 Wai Huan Xi Road, Guangzhou, Guangdong, 510006, China.

^bDepartment of Chemistry, University of Massachusetts Amherst, 710 North Pleasant Street, Amherst, Massachusetts, 01003, United States. rotello@chem.umass.edu; guojw@gdut.edu.cn

These authors contributed equally to this work.

Abstract

Multidrug-resistant biofilms are highly resistant to current antimicrobial therapies. We have developed an antimicrobial platform that integrates the bacteria-killing phytochemical carvacrol into dynamically crosslinked polymer nanocomposites (DCPNs). Taking advantage of a reversibly crosslinked Schiff-base scaffold throughout the engineered emulsions, DCPNs exhibited long-term shelf-life and good stability in serum, while readily disassembling in acidic microenvironments. Furthermore, we demonstrated that DCPNs efficiently penetrate the biofilm matrix, eradicating both Gram-negative/positive bacteria enclosed within. Moreover, DCPNs showed no observable toxicity to fibroblast mammalian cells within the same antimicrobial concentrations necessary to eradicate MDR biofilms. Given their potent antimicrobial and stimuli-responsive dissociation characteristics in a biofilm setting, DCPNs are a suitable therapeutic platform for combating MDR bacterial infections.

Graphical Abstract

Conflicts of interest

The authors declare no competing financial interest.

Electronic Supplementary Information (ESI) available: Synthesis procedure and characterization spectra of crosslinker ATA, ATR-FTIR spectra of DCPNs and nonPNs, DLS curves for monitoring dynamic Schiff base crosslinking in DCPNs using imine-displacing reagent hydroxylamine, variation of DLS curves for detecting the DCPNs stability in serum with different crosslinker content, DLS curves monitoring the shelf life of DCPN and the nonPN control, SEM micrographs of pathogenic planktonic bacteria. E. coli and S. aureus, antibiofilm and antibacterial effects over time of DCPNs against bacterial biofilms, action mechanism of DCPNs, and MICs of DCPN against different strains of bacteria. See DOI: [10.1039/x0xx00000x](https://doi.org/10.1039/x0xx00000x)

Engineered phytochemical nanocomposites eliminate broad-spectrum biofilms in the presence of mammalian cells and degrade afterwards, reducing vehicle accumulation.

1. Introduction

Multidrug-resistant (MDR) bacterial biofilms are widely found in wounds, indwelling medical devices, and dead tissue fragments.¹ It is reported over 80 % of all human infections are due to biofilms, such as osteomyelitis, chronic bacterial prostatitis, and chronic otitis media.^{2–4} Biofilms are extremely refractory to antimicrobial treatment and easily evades the hosts immune responses.^{5,6} Currently, aggressive antibiotic treatments coupled with debridement (removal of infected tissue and limbs) is the best therapeutic intervention for biofilms. However, this approach fails to fully eradicate biofilms,⁷ and has considerable drawbacks including increased patient suffering, health care costs, and accumulative drug resistance.^{8,9} Hence, it is urgent to develop alternative therapeutic platforms that can eliminate these dangerous infections.

Phytochemical extracts, components isolated from trees and plants, have been previously used as natural antimicrobial agents.^{10,11} However, their lack of solubility and stability in physiological environments limit their practical use.^{12,13} Stabilizing these oils with surfactants or nanoparticles to generate emulsions can improve their solubility in aqueous environments and antimicrobial activity against bacteria.^{14,15} However, long-term mechanical stability and potentially adverse mammalian cell toxicity limit their therapeutic practicality.¹⁶ Covalently crosslinking the emulsions can dramatically improve their stability in serum-containing conditions while maintaining high antimicrobial efficacy.¹⁷ However, this crosslinking strategy does not incorporate degradable linkage sites and may cause accumulation of the antimicrobial system in the body if used long-term.¹⁸

Taking advantage of the acidic microenvironments within biofilms we set out to develop degradable analogues of our previously reported composites. Herein, we describe the design and therapeutic applicability of dynamically crosslinked polymer nanocomposites (DCPNs). DCPNs fabrication is based on spatial-directed crosslinking derived from in situ Schiff-base reactions (Scheme 1).¹⁹ An adamantyl-core tetrakisaldehyde (ATA) was used as the crosslinker. The adamantyl core has excellent solubility in hydrophobic solvents and

contains reactive aldehyde groups to bridge amino-functionalized polymers used to stabilize the oil composite.^{20,21} We hypothesized ATAs would make a suitable polymer crosslinker and produce pH responsive imine bonds throughout and at the oil-water interface of the emulsion. Furthermore, these composites are envisioned to degrade over-time after the antimicrobial payload has been released. We demonstrate DCPNs have good serum stability and shelf-life and easily penetrates the extracellular polymeric substance (EPS) matrix, eliminating enclosed MDR bacteria. Taken together, the therapeutic properties of DCPNs make them promising candidates to treat wound biofilm infections.

2. Experimental

2.1. Chemicals and reagents

Titanium tetrachloride (99.9 %), Dichloromethyl methylether, Hydroxylamine (50 wt% in water) were purchased from Sigma Aldrich, all the other reagents and materials were purchased from Fisher Scientific and used as received. NIH-3T3 cells (ATCC CRL-1658) were purchased from ATCC. Dulbecco's modified Eagle's medium (DMEM; ATCC 30–2002) and fetal bovine serum (Fisher Scientific, SH3007103) were used in cell culture. The Pierce LDH cytotoxicity assay kit was purchased from Fisher Scientific.

2.2. Fabrication of DCPNs

Stock nanocomposite emulsions were prepared in 0.6 mL Eppendorf tubes. To prepare the stock DCPN emulsions, 3 μL of carvacrol oil (containing 5 wt% ATA) was first added to a 0.6 mL Eppendorf tube, then a 20 μL (150 μM) PONI-GAT solution was added into the tube, followed by 477 μL of Milli-Q H_2O (previously adjusted to a pH of 10) and emulsified in an amalgamator for 50 s. The non-crosslinked analogue (Non-PN) was generated in the same fashion, however without ATA dissolved in carvacrol. The emulsions were allowed to rest overnight prior to use.

2.3. MIC Measurements

Bacteria were inoculated in LB broth in a shaker at 275 rpm and 37 °C until they reached stationary phase. The cultures were then harvested by centrifugation and washed with 0.85% sodium chloride solution three times. The concentrations of resuspended bacterial solution were determined by optical density measured at 600 nm. MICs of DCPNs against all bacteria were determined by the microdilution method reported previously with slight modifications.²² Briefly, the DCPNs emulsions were serially diluted with M9 to various concentrations (64–0.5) v/v %. The bacterial suspension was diluted in M9 broth to a final concentration of 10^6 CFU mL^{-1} . Equal volumes (50 μL) of microbial suspension and DCPNs solution with varied concentrations were mixed in each well of a 96-well plate. Thus, the final concentration of bacteria in each well was $5 \times 10^5 \text{ CFU mL}^{-1}$ and those of DCPN emulsions were 32–0.25 v/v%, respectively. M9 broth containing only microbial cells was used as the negative control, and M9 broth only as the growth control. Each test was performed in three replicates. The plates were then incubated in a shaker at 275 rpm and 37 °C overnight and their optical density was measured at 600 nm. The MIC values were reported as the lowest concentration of the antimicrobial to inhibit the visible growth of bacteria.

2.4. Antibiofilm Activity Measurements

Bacteria seeding solutions were made in M9 to reach 0.1 OD (10^8 CFU mL⁻¹). 100 µL portions of the seeding solutions were added to each well of the microplate. M9 medium without bacteria was used as a negative control. The plates were covered and incubated at room temperature under static conditions. The biofilms were used after 1 day. Biofilms were washed with PBS (three times) to remove the planktonic bacteria. Next, varied concentration of DCPNs, made in M9 medium, were added to each well of the microplate. The microplate was then incubated at 37 °C under static conditions. After 3 h, biofilms were washed with PBS three times, and the viabilities were determined using Alamar Blue assay according to the manufacturer's protocol.

2.5. Cytotoxicity Evaluation of DCPNs Against Fibroblast Cells

NIH 3T3 cells (ATCC, Manassas, VA, U.S.A) were cultured in Dulbecco's modified Eagle's medium supplemented with 4.5 g/L glucose, 10 % FBS and 1 % antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin). Cells were maintained at 37 °C in a humidified atmosphere containing 5 % CO₂. Cells were regularly passaged by trypsinization with 0.25 % trypsin with EDTA, (Invitrogen) in PBS (pH 7.4). At ~80 % confluence, cells were trypsinized and seeded in a 96-well plate at a density of 20,000 cells/well. After allowed to attach overnight, cells were washed by PBS and treated with desired concentration of DCPNs for 3 h. At the end of treatment, the cell viability was measured using a Pierce LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific). Procedures were performed according to manufacturer's instruction.

2.6. TEM, Dynamic light scattering (DLS), Confocal scanning laser microscopy (CSLM) and SEM methods

The samples for TEM were prepared by dropping 5 µL of the DCPNs emulsion onto Formvar Film on 300 Square Mesh, Nickel Grids (EMS FF300-Ni). The TEM images were taken on a JOEL 2000FX TEM instrument at an acceleration voltage of 200 kV. DLS measurements were performed on a Malvern NanoZS apparatus operating at a 173-degree scattering angle. The CSLM experiments both for the DCPNs morphology and their penetration into biofilms were performed on a Nikon A1 Resonant scanning confocal microscope. SEM was carried out according to modified procedure.^[23] 2 ml 0.1 OD (10^8 CFU mL⁻¹) bacterial suspension in M9 media was incubated with 4 v/v% DCPNs at 37 °C for 3 h. The bacteria were collected by centrifugation at 1,200 rpm for 10 min and washed twice using PBS (pH=7.4), and then made a thin smear on a silica wafer. Glutaraldehyde solution (2.5 % in PBS) was used to fix the bacteria cells at 4 °C overnight. After rinsing three times using PBS, the samples were dehydrated by ethanol in a gradient alcohol concentration (25 %, 50 %, 75 %, 90 %, and 100 %). Finally, after sputter coating with gold for 1 min, the samples were observed in FEI Magellan 400 field emission scanning electron microscope operated at 1 kV with 13 µA of beam current. Control samples without treatment were also prepared and tested in the same way as mentioned above.

2.7. Evaluation of DCPNs Imine Bond Displacement

The crosslinking of DCPNs through imine bonds was further observed using an imine-oxime displacement reaction. 10 μ L hydroxylamine solution was added to 50 μ L DCPNs stock and mixed. Then the bottles were placed on an orbital shaker (1200 rpm) at room temperature for 1 h and 3 h, respectively. The control samples were also prepared in the same method except using PBS instead of hydroxylamine. The size of both control and treated samples were measured by DLS.

3. Results and Discussion

3.1. Generation and Characterization of DCPNs

As shown in Scheme 1, a poly(oxanorborneneimide) scaffold bearing guanidine, amino, and tetraethylene glycol monomethyl ether groups (PONI-GAT), an adamantyl-based crosslinker ATA, and carvacrol oil are used to generate the antimicrobial nanocomposites. The synthesis and characterization of PONI-GAT has been described previously.¹⁷ The synthesis of ATA was carried out following the modified procedures,^{24,25} and it was characterized using ¹HNMR and ATR-FTIR (Figure S1, Figure S2). The nanocomposites were prepared by emulsifying ATA-containing carvacrol oil (DCPN) or carvacrol oil only (Non-crosslinked control, PN) in a water solution which contains PONI-GAT. The pH of the aqueous solution was pre-adjusted to around 10, so that the amines on PONI-GAT are nucleophilic towards ATA aldehydes. Upon emulsification, PONI-GAT acts as a stabilizer and assembles at the oil-water interface of the oil droplets. In the presence of ATA, PONI-GAT amines crosslink with ATA aldehydes, generating DCPNs. The morphology and size of DCPNs were characterized with transmission electron microscopy (TEM) and dynamic light scattering (DLS), indicating they are spherical-shaped particles with an average size of ~220 nm. ATR-FTIR spectrum of freeze dried DCPNs indicated a complete loss of aldehydes from ATA and formation of imines (Figure S3), demonstrating successful Schiff-base crosslinking. To provide more insight into the Schiff base crosslinking, hydroxylamine (HA, possessing much higher equilibrium constant with benzaldehyde group than that of alkyl amine) was used as a competitive molecule to despoil the benzaldehyde groups reacted with amino groups in DCPNs. DLS results show that the DCPNs becomes unstable after incubating with hydroxylamine for 1 h, with obvious aggregation after 3 h (Figure S4). However, the control samples with only PBS do not show a size change. These results further support that Schiff base crosslinking is responsible for DCPN stability.

We next probed the morphological structure of DCPNs using CSLM. The formulation was modulated to generate micron-sized analogues that are easily seen under CSLM. PONI-GAT was partially labelled with TRITC (red fluorescence) and emulsified with the ATA-containing oil loaded with 3,3-Dioctadecyloxacarbocyanine (DiO, green fluorescence). As shown in Figure 1, the confocal images demonstrate a co-localization of red and green fluorescence, indicating DCPNs adopt a composite morphology as opposed to a core-shell structure. A composite morphology is plausible given PONI-GATs amphiphilic properties and its loss of hydrophilicity as it reacts with hydrophobic ATAs.

3.2. Stability and pH Responsiveness of DCPNs

Nanoemulsion stability in serum media is vital for biomedical applications.^{26,27} An ideal antimicrobial therapeutic platform should be stable enough to maintain its antimicrobial payload both during storage and delivery, while readily dissociating and releasing its payload at the infection site. We further characterized DCPNs and determined its structural integrity in aqueous environments. We hypothesized that DCPNs with imine crosslinking would have suitable stability in the presence of high ionic strength and serum environments, while losing stability in the presence of acidic environments.²⁸ Therefore, we monitored the stability and dissociation of DCPNs using DLS. A series of DCPN samples with increasing amounts of ATA were fabricated and their size monitored after incubation with 10 % fetal bovine serum (FBS) for 6 h (Figure S5). It is observed that the stability of DCPN increases with increasing concentrations of ATA, with a 5 wt% ATA loading showing no evidence of destabilization/aggregation. In stark contrast to non-crosslinked PNs which had no stability in serum (Figure 2a), DCPNs maintained stability in serum within the time duration of our studies. A 5 wt% loading of ATA within the oil core was chosen as the optimized formulation. Afterwards, we monitored the size of optimized DCPNs in PBS media at different pH conditions. As shown in Figure 2b, we observed DCPNs displayed high stability under physiological conditions (pH = 7.4). Furthermore, DCPN samples demonstrated long-term stability in these conditions even after 6 months of storage. (Figure S6). However, the size of DCPN was compromised at pH = 6.5, with further instability observed when the pH was decreased to 5. The DLS results indicate that DCPNs are sufficiently stable in physiological conditions yet become unstable in acidic conditions. According to previous literature, the average biofilm infection pH has been observed to be ~ 5.5.²⁹ At these conditions, DCPNs within biofilms would theoretically dissociate, simultaneously addressing vehicle degradability and payload release (Figure 2c). Assembly size was dictated by the process, and while size may be important, ours system is similar in size to other recent antibiofilm agents¹⁵ showing similar treatment efficacy to DCPN, although with lower antimicrobial activity.

3.3. DCPN Penetration into Biofilm

Antimicrobial penetration into biofilms is essential for biofilm eradication.³⁰ With DCPNs size, morphology, and stability characterized, we next probed DCPNs ability to penetrate biofilms using confocal microscopy. DiO was again loaded into DCPNs oil core and used to track their delivery into biofilms formed by red fluorescent protein (RFP) expressing *Escherichia coli*. DCPNs were incubated with biofilms for 1 h, washed, and their penetration analysed using ImageJ software. As illustrated in Figure 3, the fluorescence overlay shows DCPNs are distributed throughout the entire biofilm, colocalizing with the enclosed bacteria. The results indicate DCPNs are suitable carriers for antimicrobial payloads that can be delivered efficiently to biofilms. We hypothesize DCPN degradability is proportional to crosslinking density, providing a potential tool for modulating response.

3.4. DCPNs Antibiofilm Activity

Having validated DCPNs ability to penetrate a biofilm matrix, we next evaluated their antimicrobial activity against multiple Gram-negative and positive bacteria in their plankton

or biofilm state. First, we determined the minimum inhibition concentrations (MIC) against a library of bacteria. DCPNs demonstrate MIC values ranging from 0.74–1.50 mM (Table S1). Furthermore, we monitored MIC changes to DCPNs when using Cation-adjusted Mueller-Hinton Broth (CAMHB) media (Table S2) against a quality control bacteria strain, *P. aeruginosa* (ATCC-27583). We hypothesized DCPN MICs would not significantly change given that serum proteins did not compromise the vehicles integrity, as shown in Figure 2. As expected, no increase in MIC was observed when using CAMHB. In contrast, a 4 - fold increase in the control colistin MIC was observed when CAMHB media was used in place of M9. In addition to MIC, we investigated the structural integrity of Gram-negative and positive planktonic species using scanning electron microscopy (SEM) after 3 h incubation with DCPNs (Figure S7). In contrast to untreated bacteria, bacteria treated with DCPNs showed obvious cell wall/membrane damage along with a reduction in bacteria populations. Afterwards, four pathogenic bacterial strains of clinical isolates, *Pseudomonas aeruginosa* (*P. aeruginosa*, CD-1006), *Enterobacter cloacae* (*E. cloacae*, CD-1412) complex, *Escherichia coli* (*E. coli*, CD-2), and a methicillin-resistant strain *Staphylococcus aureus* (*S. aureus*, CD-489, MRSA) were chosen to test DCPNs antibiofilm properties. As shown in Figure 4, biofilms treated with DCPNs for three hours were effectively eliminated at a concentration of 4 v/v% (1.50 mM carvacrol oil). Notably, Gram-negative (CD-1006, CD-2, CD-1412) and positive (CD-489) bacterial biofilms can be treated, demonstrating DCPNs have broad-spectrum activity. Going further we investigated antibacterial activity towards biofilms at additional timepoints to better ascertain killing as a function of time (Figure S8) and percentage of biomass remaining after a three-hour treatment with DCPN (Figure S9). A clear trend was observed that as DCPN treatment time increased, the viability and biomass of tested biofilms decreased. As compared to the individual components of DCPN as control samples, DCPN exhibited significantly higher antibiofilm activity than that of pure carvacrol oil or PONI-GAT, supporting our antibiofilm mechanism proposed in Figure 2c. Finally, we investigated the DCPN mechanism of action towards pathogenic bacteria using a propidium iodide (PI) staining assay (Figure S10). Fluorescence from PI occurs upon intercalation with bacteria DNA and is dependant on membrane permeation. PI fluorescence was not observed with negative controls, including ciprofloxacin, a non-membrane disrupting antibiotic. In stark contrast, PI fluorescence was quickly observed when bacteria were subjected to DCPN treatment. Taken together, DCPNs action mechanism is derived from its phytochemical payload that compromises bacterial membranes upon contact.

3.5. Cytotoxicity Assessment of DCPNs Against Fibroblast Cells

Finally, we investigated the cytotoxicity of DCPN to mammalian NIH 3T3 (ATCC CRL-1658) Fibroblast cells. Fibroblasts are critical in rebuilding the structural framework in animal tissues during wound healing processes.³¹ We incubated fibroblast cells with DCPNs using the same experimental conditions as used for biofilms. As shown in Figure 5, no observable cytotoxicity to Fibroblast cells at 4 v/v % of DCPN was observed. Although we envision the degraded products of DCPNs after our *in vitro* experiment would be regenerated ATA and PONI-GAT, the same outcome may not occur *in vivo*. It is plausible that ATA may have off-target effects not demonstrated in simple viability studies, and experiments are on-going to look for and issues.. Given that DCPNs successfully eliminate both Gram-negative and positive biofilms below this concentration, the results suggest that

DCPNs are promising candidates to eliminate pathogenic biofilms in the presence of mammalian cells, a critical need in wound therapy.

4. Conclusions

In conclusion, we have developed a dynamically crosslinked polymeric nanocomposite that demonstrates promise as a therapeutic agent against MDR biofilms. Taking advantage of the dynamic scaffold presented within DCPNs, the nanoemulsions demonstrated good stability in physiological conditions while readily degrading in acidic conditions found commonly in biofilm infections. Furthermore, DCPNs are broad-spectrum antimicrobials that effectively penetrate and eliminate enclosed pathogens within biofilms at concentrations that do not compromise fibroblast cell viability. Given the oil core is a suitable environment to encapsulate a range of other hydrophobic antimicrobials and drugs, we envision future degradable antimicrobial emulsions can build off this strategy, potentially providing potent therapeutic platforms to fight against the rising number of MDR biofilm infections in biomedical settings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Confocal micrographs of the corresponding micron-sized counterparts of DCPNs. PONI-GAT was partially labelled with TRITC (red fluorescence), and the oil core is loaded with DiO (green fluorescence). The results show that red fluorescent PONI-GAT can be seen colocalized with the green fluorescent oil core, indicating a composite (as opposed to core-shell) morphology.

Figure 2.

a) DLS curves of DCPNs, NonPNs and serum media only in 10 % fetal bovine serum (FBS);
 b) DLS curves of DCPN with different pH in PBS; c) Proposed antibiofilm mechanism.

Figure 3. Representative 3D views of confocal images stacks of RFP-expressing DH5- α *E. coli* biofilms, DiO-loaded DCPN and their overlay after treating the biofilm for 1 h with 5 wt% DiO-Loaded DCPN at 5 v/v% concentration in M9 media.

Figure 4.

Viability of 1-day-old (a) *P. aeruginosa* (CD-1006), (b) *E. coli* (CD-2), (c) *En. cloacae* complex (CD-1412) and (d) *S. aureus* (CD-489) biofilms after 3 hour treatment with 5 wt% DCPN, PONI-GAT only, and Carvacrol oil only at different antimicrobial concentrations (mM)/(v/v %). The data are average of triplicates, and the error bars indicate the standard deviations.

Figure 5.

Viability of 3T3 fibroblast cells after treated with 5 wt% DCPN at different emulsion concentrations for three hours. Each result is an average of five experiments, and the error bars designate the standard deviations.

Scheme 1.

a) Schematic depiction of the strategy used to generate DCPNs along with the chemical structures of ATA crosslinker and PONI-GAT; b) TEM micrograph of DCPNs. Scale bar is 100 nm; c) DLS histogram indicating the size distribution of DCPNs in phosphate buffer saline (PBS, 150 mM).