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Phosphorylcholine-Based Polymer Encapsulated Chitosan Nanoparticles Enhance the Penetration of Antimicrobials in a Staphylococcal Biofilm

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Supporting Information

ABSTRACT: Biofilms that contribute to the persistent bacterial infections pose serious threats to human health, due in part to the extracellular polymeric substances (EPS) matrix of biofilm block the diffusion of intact antimicrobials. The poor penetration of antimicrobials into biofilm greatly reduces their bacterial killing efficacy. Here, we have demonstrated a nanocapsule PMPC−CS synthesized by encapsulating a chitosan nanoparticle with poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC). Such PMPC-based surface exhibited low EPS-adsorption, allowing enhanced penetration of PMPC−CS. Additionally, PMPC−CS showed effective targeting toward negatively charged bacterial cell surfaces and pH-responsive drug release mediated by the swelling of chitosan core under the acidic environment of biofilm. These unique features ensured targeted delivery of antimicrobials throughout the depth of a biofilm. Delivery of triclosan with PMPC−CS outperformed direct application of free triclosan in inhibiting the growth of bacteria in biofilm, suggesting the potential of PMPC−CS as an effective delivery system for the treatment of bacterial infections.

Each year, bacterial infections are becoming a more serious threat to human health† and it is anticipated that a continued rise in bacterial resistance by 2050 would lead up to more than 10 million annual deaths worldwide, exceeding the incidence of cancer deaths.‡ More than half of all resistant bacterial infections, ranging from oral to diabetic foot infections, are due to bacteria in their biofilm mode of growth.§ Biofilms are complex bacterial communities embedded in a self-produced matrix of extracellular polymeric substances (EPS), which make biofilm bacteria hard to eradicate.** This EPS matrix that constitute over 90% of the overall biomass consists mostly of polysaccharides, proteins, and DNA.† One of the protective mechanisms operative in infectious biofilms is the poor penetration of antimicrobials through the EPS matrix toward the depth of a biofilm, causing survival of bacteria residing deep inside a biofilm, and therewith, providing a nidus for their regrowth, following arrest of antimicrobial administration.‡‡ Therefore, delivery strategies that enable efficient biofilm penetration of antimicrobials are highly demanded for the effective eradication of biofilm infections. pH-adaptive polymeric nanoparticles have been demonstrated to effectively overcome the biofilm barrier and achieve penetration and accumulation into the biofilm.§§ For example, poly(ethylene)glycol-poly(β-amino esters) micelles demonstrated enhanced biofilm penetration by their stealth nature in combination with a negative surface charge under neutral pH.†† Once penetration, its surface charge becomes positive in response to the acidic environment of the biofilm, allowing their accumulation by the electrostatic double-layer attraction to biofilm components, that is, either targeting bacteria or adhesion to the biofilm EPS matrix. Although it can be advantageous as the nanoparticles will accumulate in the biofilm and form a depot for sustained drug release, it can be counter argued that attraction to EPS-matrix components of biofilm may hamper the distribution of nanoparticles throughout the entire biofilm.‡‡ Hindered diffusion readily leads to gradients in the concentration of antimicrobial-loaded nanoparticles, which potentially reduce the possibility of the nanoparticles to reach the bacteria inside the acidic biofilm and, hence, affect the bactericidal efficacy adversely. To achieve optimal killing efficacy of the bacteria in biofilm, antimicrobial delivery carriers with low adsorption to EPS matrix are required to allow efficient penetration and diffusion in biofilm, as well as efficient attachment to bacterial...
surface to maintain the accumulation of antimicrobials around the bacteria in biofilm.

Nano- and chemical engineering technology provide unparalleled flexibility to control the composition, shape, size, surface chemistry, and functionality of nanostructures, which can be applied to develop a new generation of modified materials or to overcome the biofilm recalcitrance to antimicrobial treatment. Current drug delivery systems applied for biofilm treatment are usually designed to be positively charged surfaces, which can bind to the negatively charged bacterial surfaces via electrostatic double-layer interaction. However, traditional cationic materials also enhance the binding affinity to the EPS matrix of the biofilm, which decreases the efficiency in biofilm penetration. Overwhelming evidence support that phosphorylcholine-based polymers can bind water molecules to yield a unique surface, which can resist nonspecific protein adsorption.\textsuperscript{16,17} Such polymers are considered as potential materials to significantly reduce the adsorption to EPS matrix to allow an efficient biofilm penetration. Herein, we demonstrated a novel antimicrobial delivery nanocapsule that facilitated efficient penetration of antimicrobials into biofilm, allowing the antimicrobials to accumulate around the bacteria and eventually improved their bactericidal effect to biofilm.

Figure 1. Characterization of nanocapsules. (a) Transmission electron micrographs (scale bar, 100 nm), (b) hydrodynamic diameters, and (c) zeta potentials (measured in PBS at pH 5.0 and 7.4) of PMPC–CS, P(MPC\textsubscript{1}-co-APM\textsubscript{1})-CS, and P(MPC\textsubscript{1}-co-APM\textsubscript{4})-CS. Data are presented as mean ± standard error of the mean (s.e.m.) over three independent experiments. Note that the deprotonation of the amino groups in chitosan leads to the aggregation and precipitation of chitosan nanoparticle (CS) at pH 7.4, which made the measurement of particle size and zeta potential impossible.

Scheme 1. Sequential Steps in the Preparation of Triclosan-Loaded Nanocapsules, Including Acrylation, In Situ Polymerization of 2-Methacryloyloxyethyl Phosphorylcholine (MPC) and Glycerol Dimethacrylate (GDMA), Yielding PMPC–CS Nanocapsules, and Their Loading with Triclosan.
nanocapsule (denoted as PMPC−CS) was constructed by encapsulating chitosan nanoparticle (CS) with a thin layer of cross-linked phosphorylcholine-based copolymer (PMPC) synthesized by in situ polymerization of 2-methacryloylox-
ethyl phosphorylcholine (MPC) and glycerol dimethacrylate (GDMA)\textsuperscript{18,19} (Scheme 1 and Table S1 in the Supporting Information (SI)). In this design, CS was employed as the core of the nanocapsule to load hydrophobic antimicrobials for its capability to control the drug release rate under different pH.\textsuperscript{20} Moreover, using CS (pK\textsubscript{a} ~ 6.5) as the core made the nanocapsule exhibiting positively charged, which can readily bind to the bacteria in an acidic biofilm environment.\textsuperscript{21,22} For the better evaluation of the surface effects on the penetration and accumulation, similarly structured nanocapsules with different surface compositions were synthesized by partially replacing MPC with cationic N-(3-aminopropyl) methacrylamide (APM) during the polymerization in molar ratios of APM to MPC of 4 (4:1) and 1 (1:1) (denoted as P(MPC\textsubscript{1-co-APM\textsubscript{4}})-CS and P(MPC\textsubscript{1-co-APM\textsubscript{4}})-CS, respectively). The introduction of APM increased the positive surface charge of nanocapsules, thereby enhancing the electrostatic attraction between nanocapsules and EPS-matrix components. Meanwhile, increasing the portion of APM also weakened the contribution of MPC to the low EPS-adsorption of the nanocapsules surface. In the following study, we explored the penetration, accumulation and killing efficacy of these three types of nanocapsules in a biofilm.

Transmission electron micrographs (TEM) demonstrated that all nanoparticles had a spherical shape (Figure 1a). At pH 5.0, CS exhibited a diameter of 50 ± 22 nm, that increased by maximally 22 nm after encapsulating (Figure 1b). PMPC–CS and P(MPC-co-APM)-CS nanocapsules had a roughly 30% larger diameters at pH 5.0 than at pH 7.4, indicative of swelling in a more acidic environment. This swelling can be explained by the protonation of chitosan core at pH 5.0. At pH 7.4, the chitosan core is deprotonated (pK\textsubscript{a} ~ 6.5) and collapsed, while at pH 5.0, the amino groups in chitosan are protonated, the electrostatic repulsion between protonated amino groups results in swelling of the chitosan core and accordingly to a larger nanoparticle diameter.\textsuperscript{23} Hydrodynamic diameters of the nanoparticles assessed by dynamic light scattering were larger than those measured from TEM (compare Figure 1a,b), as hydrodynamic diameters were measured under fully hydrated conditions, opposite to diameters taken from TEM requiring full dehydration, accompanied by nanoparticle shrinkage. Zeta potential measurements indicated that all three types of the nanocapsules exhibited positive zeta potentials at pH 5.0 and pH 7.4. The zeta potentials of the nanocapsules became more positive as the increase of APM to MPC ratios during the polymerization (Figure 1c).

Staphylococcus aureus (S. aureus) is one of the most common infectious bacterial strains, and can be found on dental implants, orthopedic infections, wound infections and in many other infections.\textsuperscript{24,25} Green-fluorescent S. aureus ATCC12600\textsuperscript{GM} biofilms were grown and exposed to different Cy5-labeled nanocapsules. After 1 h incubation, biofilms were rinsed with PBS and imaged using confocal laser scanning microscopy (CLSM). Figure 2a showed the penetration of different Cy5-labeled nanocapsules into a staphylococcal biofilm (average biofilm thickness: 30 ± 2 μm, see Figure 2a and Figure S1 in the SI). Effective penetration of the nanocapsules evidenced by the Cy5 fluorescence was observed inside biofilms exposed to PMPC–CS. In contrast, penetration decreased with increasing ratio of APM to MPC and was least for P(MPC\textsubscript{1-co-APM\textsubscript{4}})-CS nanocapsules. This observation confirmed that the encapsulation with PMPC could enhance the penetration of the nanocapsules in biofilm. Further evaluation of the penetration was then achieved by

Figure 4. Relative bacterial viability of S. aureus ATCC12600\textsuperscript{GM} biofilm as a function of exposure time to PMPC–CS nanocapsules, free triclosan, T-PMPC–CS, T(P(MPC\textsubscript{1-co-APM\textsubscript{4}})-CS, and T-P((MPM\textsubscript{1-co-APM\textsubscript{4}})-CS nanocapsules in PBS at pH 5.0 with different triclosan or triclosan equivalent concentration: (a) 10, (b) 20, (c) 40, and (d) 80 μg mL\textsuperscript{-1}. Staphylococcal viability was assessed from the green-fluorescence of the biofilm, as S. aureus ATCC12600\textsuperscript{GM} loses its green-fluorescence upon cell death.\textsuperscript{33,34} Accordingly, relative viability was calculated, setting the green-fluorescence of a freshly cultured biofilm in PBS at 100%. Data are presented as mean ± s.e.m. over three independent experiments. Asterisks indicate statistical significance at *p < 0.05 (**), p < 0.01 (***), p < 0.001 (****), and p < 0.0001 (*****). (Student’s t test) between free triclosan and T-PMPC–CS nanocapsules.
quantitatively analyzing the CLSM images with ImageJ and plotting the Cy5 fluorescence intensity as a function of depth in the biofilm (Figure 2b). Integrating the area under the curves indicated the penetration and accumulation ranked as follows PMPC−CS > P(MPC1-co-APM1)-CS > P(MPC1-co-APM4)-CS, suggesting that introduction of APM reduced the penetration of nanocapsules. Since the penetration of the nanocapsules was mainly affected by their interactions with the biofilm components, the adsorption between different nanocapsules and EPS matrix was further investigated using a quartz crystal microbalance with dissipation monitoring (QCM-D). This is achieved by exposing EPS-matrix components coated Au crystals to the solutions of PMPC−CS, P(MPC1-co-APM1)-CS and P(MPC1-co-APM4)-CS at pH 7.4 and pH 5.0. According to the results (Figure 2c), PMPC−CS showed lowest level of adhesion to the EPS-coated Au crystals compared to the other two types of nanocapsules whether at pH 7.4 or pH 5.0, suggesting the effectiveness of PMPC encapsulation in enhancing the penetration of the PMPC−CS by reducing its adsorption. Increase of the APM to MPC ratios during the preparation increased the attachment of the nanocapsules to the EPS matrix. This could be caused by the enhanced electrostatic interactions due to the introduction of APM increased the positive surface charge of the nanocapsules especially under the acidic biofilm environment (Figure 1c). More importantly, the introduction of APM decreased the

Figure 5. (a) Accumulation of triclosan-loaded chitosan nanocapsules in a staphylococcal biofilm as a function of the ratio APM to MPC. (b) Killing efficacy (=100% – relative viability (%)) after 3 h exposure at 20 μg mL−1 as a function of accumulation of triclosan-loaded chitosan nanocapsules in a staphylococcal biofilm. (c) Schematic presentation of the role of electrostatic attraction in the penetration of triclosan-loaded chitosan nanocapsules with different APM to MPC ratios in a staphylococcal biofilm (details are not drawn to scale).
portion of MPC in the polymer surface of the nanocapsules, resulting in a significant decrease in the capability of low EPS adsorption as indicated by the very different EPS matrix adsorption of P(MPC1-co-APM1)-CS and P(MPC1-co-APM4)-CS (Figure 2c) even with similar zeta potentials at pH 7.4. Uniquely, all three types of the nanocapsules could effectively absorb onto the surface of bacterial cells when incubating with staphylococci (zeta potential = −12.6 ± 1.2 mV at pH 5.0) under a weak acidic condition that simulated the pH of biofilm microenvironment (Figure 2d). This feature could ensure the retention of the nanocapsules inside the biofilm and lead to the formation of high local concentration of antimicrobial-loaded nanocapsules in close proximity of the bacterial.21

Triclosan, a hydrophobic antimicrobial,26–29 was loaded into PMPC−CS, P(MPC1-co-APM1)-CS and P(MPC1-co-APM4)-CS nanocapsules (denoted as T-PMPC−CS, T-P(MPC1-co-APM1)-CS, and P(MPC1-co-APM4)-CS, respectively), yielding a high drug loading content (DLC, 46%) and efficiency (DLE, 61%) (Table S2 in the SI). The release of the triclosan from the nanocapsules was assessed using UV−vis spectroscopy, employing a standard curve obtained over a concentration range from 2 to 200 μg mL−1 (Figure S2 in the SI). Release kinetics of triclosan from all three types of nanocapsules were similar (Figure 3), while over the time period of the experiment, all nanocapsules exhibited significantly higher triclosan release rate at pH 5.0 than at pH 7.4 due to the swelling of their chitosan core under acidic conditions (Figure 1b). Importantly, since biofilms have inherently acidic microenvironments (pH 4.5−6.5),17,30,31 this acid-responsive release ensured that most of triclosan would be released after nanocapsule penetration into biofilm. Subsequently, the killing efficacy of free triclosan and triclosan-loaded nanocapsules was first evaluated for their minimal inhibitory and bactericidal concentrations (MIC and MBC, respectively) against planktonic staphylococci. Free triclosan and triclosan-loaded nanocapsules exhibited similar MICs and MBCs of around 1.25 and 10 μg mL−1, respectively (Table S3 in the SI). The killing efficacies were determined against S. aureus ATCC12600 GFP in their biofilm mode of growth. All triclosan-loaded nanocapsules yielded lower staphylococcal viabilities than free triclosan (Figure 4), while unloaded PMPC−CS nanocapsules did not show any reduction in staphylococcal viability. These results indicated that the bactericidal observations were solely caused by the triclosan release from the nanocapsules. Moreover, significant viability differences were observed after 3 h incubation with free triclosan and different triclosan-loaded nanocapsules, indicating that all triclosan-loaded nanocapsules exhibited improved bactericidal effect than free triclosan, and T-PMPC−CS outperformed all the others with the significantly lower bacterial viability after the treatments especially at lower triclosan concentration (20 μg mL−1, Figure 4b). As expected, the bactericidal performance of the nanocapsules was ranked as T-PMPC−CS > T-P(MPC1-co-APM1)-CS > T-P(MPC1-co-APM4)-CS, which was in agreement with their ability to penetrate and accumulate in the staphylococcal biofilm (Figure 2). This result indicated that the bacterial killing efficacy of triclosan-loaded nanocapsules in the biofilm were significantly affected by their surface properties, particularly in the EPS matrix adsorption, which can be tuned by altering the ratio between the two monomers (APM and MPC) during the polymerization of the nanocapsules. As summarized in Figure 5, accumulation of the nanocapsules decreased with increasing ratio of APM to MPC (Figure 5a) due to the strong adhesion of nanocapsules with EPS-matrix components, which hampered their penetration and eventually resulted in a reduced killing efficacy against biofilm (Figure 5b,c).

To conclude, we have demonstrated a rational design of antimicrobial delivery nanocapsules, PMPC−CS, to enhance the penetration of antimicrobials into bacterial biofilm. PMPC−CS was prepared by encapsulating chitosan nanoparticle with PMPC via in situ polymerization. Such PMPC coating exhibited low EPS-adsorption of the bacterial biofilm, leading to significantly enhanced penetration of PMPC−CS into the biofilm. Meanwhile, PMPC−CS could attach to bacterial cell surfaces, allowing them to accumulate in biofilm. Release of triclosan was triggered by the swelling of chitosan core under acidic microenvironment of biofilm. Delivery of triclosan with PMPC−CS effectively eradicated multidrug resistant staphylococcal biofilm at lower triclosan concentrations than that usually required with free triclosan. Moreover, this delivery nanocapsule can be also applied to deliver existing or novel agents to enhance their biofilm penetration and retention, as well as the overall antimicrobial efficacy in the treatment of persistent infectious diseases caused by biofilms.

■ ASSOCIATED CONTENT

# Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmacrolett.9b00142.

Experimental details, standard curve of triclosan, confocal laser scanning micrographs of the penetration and accumulation of nanocapsules into biofilm, minimal inhibitory and minimal bactericidal concentrations of triclosan and triclosan-loaded nanocapsules against planktonic staphylococci (PDF)

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Notes
The authors declare no competing financial interest.

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