Bacteriostatic Effects of Apatite-Covered Ag/AgBr/TiO2 Nanocomposite in the Dark: Anomaly in Bacterial Motility

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Article

ABSTRACT: In this paper, we report a unique property of inactivating Gram-positive/negative bacteria in the dark via apatite-covered Ag/AgBr/TiO2 nanocomposites (AAAT). We demonstrate that the inactivation mechanism is bacteriostatic based on the cellular integrity and motility of bacteria, low toxicity and high durability of AAAT. From straight observations, the catalytic loading affects the bacterial replication and cell envelope as well as inducing an anomaly in bacterial motility (continuous rotation) for both types of bacteria. Both simulation and experimental analyses suggest that the anomaly could be due to posterior intracellular signals rather than purely mechanical effects (e.g., size enlargement and motility retardation). Provoked by chemomechanical stimuli, these signals increase the frequency of flagellar tumbling and eventually entangle the bacteria.

1. INTRODUCTION
Most antibiotics are bactericidal and inactivate bacteria via lysis (e.g., penicillin).1−4 Apart from lysis, chemicals can inactivate bacteria by changing the cellular biochemistry, motility, and reproduction.5−8 When a bacterial cell encounters a chemotactic or a repellent that binds to a chemoreceptor, a ligand-induced conformational change may occur in the chemoreceptor.9−12 The downstream effect is a change in the ratio of the concentration of phosphorylated to unphosphorylated forms of the CheY protein and thereby flagellar motion.13,14 Bacteria tumble less frequently facing an attractant and more frequently facing a repellent, governed by the concentration of phosphorylated CheY.13−18 Other stimuli can also disrupt bacterial motility, such as catalytic loading of bacteria.8

The scope of action, toxicity, and durability are three major concerns in the synthesis of bacteriostatic materials.19−26 Numerous studies have reported bacteriostatic effects of materials, in which silver, titanium, and phosphate components are individually and combinatorially present.27−31 These effects become more prominent when all aforementioned compounds exist simultaneously.32−35 For instance, a bacteriostatic activity has been reported for the porous material, AgTi2(PO4)3 with a very small out-leaching of Ag⁺ ion (<1 μequiv g⁻¹).36 At low concentrations of silver ions, the synthesis of a porous sandwich nanostructure of titanate/silver/titanate with a bacteriostatic rate of 99% has been reported.37 The degree of bacteriostatic activity of these porous material has been attributed to an engineered out-leaching of Ag⁺ ions in the environment but not direct contact of the integrated silver element.38 Silver ions are well known as bactericidal agents, but they have also exhibited bacteriostatic effects at low concentrations, such as inhibition of cell reproduction.39

Photocatalytic bactericidicity of TiO2 and its derivatives have been widely studied in different regions of the electromagnetic spectrum.40−44 Apatite and Ag/AgBr are introduced to TiO2 photocatalytic family as adsorbent and photosensitive agents, respectively.45−47 The antibacterial properties of AAAT are modified compared to those of TiO2, Ag/AgBr/TiO2, and apatite-covered TiO2 in the presence of light.47 In the absence of light, however, the antibacterial functionality of AAAT is yet unresolved. The hydrogen bond between the phosphate group of AAAT and bacteria extracellular polysaccharides has been suggested as the origin of the strong adherence between the two. This adherence has been proposed to obstruct the bacterial outer membrane of Escherichia coli and thus nutrition, while not decomposing the cell wall.46 This proposal raises few
unanswered questions: (1) If the phosphate group is responsible for the adherence on the catalyst end, why is apatite-covered TiO₂ ineffective? (2) What are the possible chemical effects of this adherence? (3) If the cell wall is not damaged, does the catalytic loading of bacteria trigger the inactivation process? (4) Do (chemo)mechanical stimuli influence the bacterial motility and how would this affect the inactivation of bacteria? Herein, we seek possible answers to these questions to provide a better understanding of the nature of the inactivation mechanism.

2. MATERIALS AND METHODS

Apatite-coated Ag/AgBr/TiO₂ was prepared as a sample of synthesized antibacterial group, through deposition of hydroxyapatite, as adsorption bioceramic, and AgBr, as photosensitive material. The preparation procedure has been explained elsewhere (ref 46 of the paper). To measure the catalytic activity under dark media, 24 mg of photocatalyst was added to bacterial suspension. The E. coli (ATCC, 8739) and Bacillus subtilis (ATCC, 6633) were prepared in 1 × 10⁷ colony-forming units (CFU mL⁻¹) bacterial cell concentration. The reaction mixture was stirred with a magnetic stirrer to prevent the precipitation of the photocatalysts, and in certain time intervals, 2 mL of the reaction mixture was diluted with 0.9% saline. Then, 1 mL of diluted solution was incubated at 37 °C for 24 h on soybean casein digest agar, and the colonies were counted. For the transmission electron microscopy (TEM) analysis, the samples were prepared according to the standard procedure. A photocamera coupled with optical microscope was used to follow the motion of bacteria.

3. RESULTS AND DISCUSSION

3.1. Experiment. AAAT exhibits similar inactivation rates for both Gram-negative and Gram-positive bacteria, organisms with different cell envelope structures, in the dark (Figure 1).

![Figure 1. Temporal course of the E. coli and B. subtilis inactivation (1 × 10⁷, 30 mL) in aqueous dispersions containing 24 mg of catalysts: (a) apatite, (b) Ag/TiO₂, (c) Ag/AgBr/TiO₂, (d) apatite-covered TiO₂, (e) TiO₂/P-2, (f) AAAT (acting on B. subtilis), and (g) AAAT (acting on E. coli) in the dark. Catalysts (a)–(e) are inactive on both bacteria types.](image)

Atomic absorbance spectroscopy shows an insignificant increase in the concentration of antibacterial silver ions in the environment (Figure S1). In addition, the increment of CO₂, a byproduct of oxo-degradation, is insignificant in the dark (Figure S2). Finally, the compromised integrity of the cell envelope suggests that the undefined mechanism of inactivation by AAAT has nonlytic nature (Figures 2 and 3). All aforementioned evidences suggest that the cell envelope is not chemically decomposed, and bacterial inactivation may have bacteriostatic origins. Monitoring the bacterial motility under the exposure to AAAT (up to 3 h) confirms that both E. coli and B. subtilis retain mobility, yet cease replication (Movies S1 and S2). Therefore, we propose that AAAT is bacteriostatic rather than bactericidal in the absence of light, contrary to the presence of light.

From the chemical point of view, inefficiency of apatite alone (source of phosphate) and apatite-covered TiO₂ raises doubts about phosphate groups being the only responsible for bacteriostaticity of AAAT (Figure 1). In addition, the neutrality of Ag/AgBr/TiO₂ and TiO₂ suggests that all components participate in the bacteriostatic efficiency of AAAT. Neutral catalysts in Figure 1a–e remain inactive, even at higher dosages (up to 48 mg). Energy-dispersive X-ray analysis indicates that mainly phosphate and Ti (as in TiO₂) interact with the bacterial surface (Table S1). Compared to other apatite containing catalysts, the bacteriostaticity of AAAT can be attributed to its higher (~10 to 15%) Brunauer–Emmett–Teller surface (48 m² g⁻¹). This can increase the AAAT–bacterium interface and maintain the unleaching of silver ions at a proper level for bacteriostatic purposes. However, Ag/AgBr/TiO₂ releases similar amount of silver ions (~1%) in the solution and yet unable to inactivate bacteria within the scope of the experiment. Further assessment on the physico-chemical properties of AAAT is in progress to perceive more on its significant bacteriostaticity and adsorbability against bacteria. A rough estimate of bacterial growth implies that cell replication proceeds up to elongation step prior to arrest (Figure S4). Also, the oscillation in pH of the AAAT/bacteria solution (7.0–7.3), whereas pH of the AAAT bare solution is about 7.5, could be an indicator of disruption of the cellular net proton transfer. Taken together, we speculate that the bacteriostatic mechanism relies on not only direct consequences of the AAAT–bacterium bond (e.g., cell obstruction) but also posterior chemical effects (e.g., endogenous imbalance).

3.2. Simulation. Mechanically speaking, the bacteriostatic mechanism could be explained by an anomaly in the motility of bacteria that is induced by the AAAT surficial fixation. As Figure 3 shows, the heterogeneous AAAT–bacterium adherence makes the bacterium heavier and less flexible, therefore, few flagella may fly apart, and the average forward run velocity drops. This emerges as a random run and tumble motility with a preferable rotation around the more flexible (less loaded) side and intermittent stops (Figure 4). In most cases, a constant tumbling motion is observed without any sensible forward motion (Movies S1 and S2). This leads to a unique rotational motion that is different from the circular trajectory near the solid boundaries and glass surfaces due to the following reasons: (1) the rotational motion occurs in an almost fixed position, (2) tumbling motion dynamically converts clockwise (CW) and counterclockwise (CCW) motions to each other, (3) the frequency of the rotational motion is about 1 order of magnitude (~1 Hz) smaller than that of the circular trajectory (10 Hz). The mechanical origin of this motion is the catalytic loading of bacteria such that the forward motion is hindered. As a result, the average forward run velocity changes as a function of size growth and uniformity (Figures S7 and S8). Even for extreme cases (e.g., 50X growth), the average run velocity is significant; nearly 35 and 15 μm s⁻¹ for run and tumble and conventional tumble (0.1 time scale) motilities, respectively. The increment of tumbling frequency, however, reduces the average run velocity further, independent of the bacterial size and uniformity.
The trajectory of such motility (Figure 4) provides a better explanation for the observed rotational motion. A higher frequency tumbling could reduce the mismatch between simulation and experiment, provoked by endogenous/induced chemotactic signals.

4. CONCLUSIONS

The evaluation of the cell integrity, matrix toxicity, inactivation rate, and bacterial motility demonstrate a bacteriostatic inactivation of *E. coli* and *B. subtilis* bacteria when AAAT is introduced in the dark. Similar to previous studies, structural analyses suggest that bacteriostaticity is interconnected to the porosity of AAAT and its strong adherence to the bacterial surface. We speculate that TiO₂, Ag/AgBr, and apatite serve as the matrix, poring, and adsorbent agents in the structure of AAAT, respectively. The hydroxyl group (as in apatite) could also participate in the reduction of silver ions, cellular proton transfer, and porosity of the nanocomposite.⁴⁶ A purely mechanical effect of catalytic loading is expected to be a retardation of the bacterial motility as a result of its enlargement, evidenced in both simulation and experiment. Nevertheless, modeling this effect fails to capture a quantitative/qualitative feature of the observed rotational motion. On the other hand, increasing the tumbling frequency, induced by intracellular/extracellular stimuli, can better capture this feature. Overall, this study elucidates a few
chemomechanical origins of the AAAT-induced inactivation of bacteria but leaves many questions unanswered. Among nanomaterials, those with silver, titanium, and phosphate in common have exhibited similar properties such as high durability (slow out-leaching of Ag⁺), bacteriostaticity, and porosity, and low toxicity. We strongly suggest that a new class of nanomaterials, in which those properties are modified, is potentially applicable for the in vivo studies and drug delivery.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpcb.8b10710.

REFERENCES

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