Antibacterial Activity of Ti$_3$C$_2$T$_x$ MXene

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Supporting Information

ABSTRACT: MXenes are a family of atomically thin, two-dimensional (2D) transition metal carbides and carbonitrides with many attractive properties. Two-dimensional Ti$_3$C$_2$T$_x$ (MXene) has been recently explored for applications in water desalination/purification membranes. A major success indicator for any water treatment membrane is the resistance to biofouling. To validate this and to understand better the health and environmental impacts of the new 2D carbides, we investigated the antibacterial properties of single- and few-layer Ti$_3$C$_2$T$_x$ MXene flakes in colloidal solution. The antibacterial properties of Ti$_3$C$_2$T$_x$ were tested against *Escherichia coli* (*E. coli*) and *Bacillus subtilis* (*B. subtilis*) by using bacterial growth curves based on optical densities (OD) and colonies growth on agar nutritive plates. Ti$_3$C$_2$T$_x$ shows a higher antibacterial efficiency toward both Gram-negative *E. coli* and Gram-positive *B. subtilis* compared with graphene oxide (GO), which has been widely reported as an antibacterial agent. Concentration dependent antibacterial activity was observed and more than 98% bacterial cell viability loss was found at 200 μg/mL Ti$_3$C$_2$T$_x$ for both bacterial cells within 4 h of exposure, as confirmed by colony forming unit (CFU) and regrowth curve. Antibacterial mechanism investigation by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) coupled with lactate dehydrogenase (LDH) release assay indicated the damage to the cell membrane, which resulted in release of cytoplasmic materials from the bacterial cells. Reactive oxygen species (ROS) dependent and independent stress induction by Ti$_3$C$_2$T$_x$ was investigated in two separate abiotic assays. MXenes are expected to be resistant to biofouling and offer bactericidal properties.

KEYWORDS: MXene, Ti$_3$C$_2$T$_x$, antibacterial, B. subtilis, E. coli, membrane, oxidative stress

Recently, the family of 2D materials has been augmented by a large group of early transition metal carbides.\textsuperscript{1,2} This new family of 2D materials has been labeled "MXenes", where M is an early transition metal and X is carbon and/or nitrogen. Ti$_3$C$_2$T$_x$ (T is standing for the surface termination, such as −O, −OH, or −F) is the most studied MXene, and recently, we reported the selective ion sieving through micrometer-thick Ti$_3$C$_2$T$_x$ membranes.\textsuperscript{7} The hydrophilic nature of Ti$_3$C$_2$T$_x$$_{sp}$ together with the hydrated interlayer spacing, promotes ultrafast water flux and differential sieving toward single-, double-, and triple-charged metal cations of different sizes. Ti$_3$C$_2$T$_x$ outperformed graphene oxide (GO) membranes in the separation of higher charge cations. However, antibacterial characteristics of MXenes have never been studied. It is important to investigate the antibacterial properties of MXenes for their potential use as a biocide in water treatment and biomedical applications.

Several studies have compared the antibacterial activity of 2D graphene-based materials (graphite oxide, GO, and reduced GO (rGO)) against Gram-negative (Gram (−)) and Gram-positive (Gram (+)) bacteria through direct contact.\textsuperscript{8−14} The antibacterial activity of metal and metal oxide nanoparticles (e.g., Ag, ZnO, and TiO$_2$) have also been well documented by a sizable number of studies.\textsuperscript{15,16} Antibacterial activity of these nanoparticles has been associated with production of reactive oxygen species (ROS) and direct contact with bacteria membrane, penetrating into the bacteria and interacting with sulfur-containing proteins as well as phosphorus-containing DNA, leading to bacterial cell death.\textsuperscript{17−21} Similarly, the antimicrobial activities of graphene have been found to be the synergy of both “chemical” and “physical” effects.\textsuperscript{16,19} Most of the studies have attributed the antibacterial activity of GO and rGO to oxidative and physical stress induced by sharp edges of graphene nanosheets, which may result in mechanical damage of cell membranes, leading to a loss of their integrity.\textsuperscript{14,22−24} Moreover, several mechanisms have been proposed to explain the antimicrobial properties of composite films based on carbon nanotubes (CNT) including inhibition of electron transports, leakage and penetration of cell membrane...
Despite antimicrobial properties of MXenes have never been examined before, it is reasonable to assume that at least some of those mechanisms may work in MXenes, which were shown to destroy dye molecules in solution. Therefore, investigations of the mechanism of MXene’s interaction with bacterial cell membranes and its bactericidal activity are needed to determine the range of potential applications of these new materials.

Here, we present for the first time a report on the antibacterial behavior of Ti$_3$C$_2$T$_x$ MXene in the colloidal suspension. To better understand the health and environmental impacts of the new 2D carbides, the antibacterial activity of Ti$_3$C$_2$T$_x$ MXene toward two bacterial models, Escherichia coli (E. coli) and Bacillus subtilis (B. subtilis), was studied and compared with GO. The concentration dependent antibacterial activities were evaluated by cell viability assays together with scanning electron microscopy (SEM), transmission electron microscopy (TEM), and lactate dehydrogenase (LDH) release assay. On the basis of these results, we introduce MXenes as a new family of 2D antimicrobial nanomaterials. This will open a door for MXenes in the antibacterial applications and water purification industry.

**RESULTS AND DISCUSSION**

**Synthesis and Characterization of Ti$_3$C$_2$T$_x$.** Ti$_3$C$_2$T$_x$ suspension was prepared from multilayer (ML) Ti$_3$C$_2$T$_x$ “clay” by ultrasonication under a flow of argon (Ar) gas as described in the experimental section. Ti$_3$C$_2$T$_x$ synthesis was described in details elsewhere. GO was also synthesized by oxidizing natural graphite powders using H$_2$SO$_4$ and KMnO$_4$ according to the modified Hummers method and was used as a reference in this study. Figure 1 shows SEM images of Ti$_3$AlC$_2$ (Figure 1A), ML-Ti$_3$C$_2$T$_x$ (Figure 1B), and Ti$_3$C$_2$T$_x$ (Figure 1C) nanosheets dried on alumina wafer and photographs of their corresponding colloidal suspensions in water. The images clearly show the different appearance of the three materials after 10 min sonication. Both Ti$_3$AlC$_2$ and as-produced ML-Ti$_3$C$_2$T$_x$ show opaque gray color and their particles precipitated after 1 h and the SEM revealed well stacked nanosheets. On the other hand, delaminated Ti$_3$C$_2$T$_x$ formed dark green colloidal solution and the stacked layers were delaminated as observed from SEM (Figure 1C). The TEM micrograph in (Figure 1D) revealed thin, transparent flakes of delaminated Ti$_3$C$_2$T$_x$ nanosheets. Fluorine and oxygen were confirmed by energy-dispersive spectroscopy (EDS),
suggesting O- and F-containing surface terminations. Delaminated Ti$_3$C$_2$T$_x$ has highly exfoliated and smaller sheets, which are expected to provide a significantly higher surface area than Ti$_3$AlC$_2$ and ML-Ti$_3$C$_2$T$_x$, and an improved antimicrobial performance.$^{24}$ A typical XRD pattern of air-dried Ti$_3$C$_2$T$_x$ film is shown in Figure 1E. The presence of peaks corresponding to basal-plane reflections (00l) with c lattice parameter of 27–28 Å suggests the presence of water, and possibly Li ions, between the hydrophilic and negatively charged Ti$_3$C$_2$T$_x$ MXene nanosheets.$^{32}$ The sharp and intense peak (002) at 6.4° is at a much lower angle than typical of Ti$_3$C$_2$T$_x$ produced by etching in HF, having layers of water or solvated ions between MXene sheets. Peaks from 20 to 40° are still observed, which suggests a good periodicity between the stacked MXene layers.

**Antibacterial Activity.** In order to investigate the effect of delamination on the antibacterial efficiency of MXene, the inhibition effect of three materials (Ti$_3$AlC$_2$ (MAX), as-produced ML-MXene, and delaminated Ti$_3$C$_2$T$_x$ nanosheets) were examined against both E. coli and B. subtilis. The bacterial growth inhibition was determined by the colony counting method. Figure S1A (Supporting Information) shows the photographs of agar plates onto which control and bacterial cells were recultivated after treatment for 4 h with the same concentration of 100 μg/mL of nanomaterial. Figure S1B (Supporting Information) depicts the percentage growth inhibition of both bacterial strains exposed to the materials under study. MAX dispersion showed growth inhibition of only 14.39 ± 1.43% and 18.34 ± 1.59% for E. coli and B. subtilis, respectively. The ML- Ti$_3$C$_2$T$_x$ dispersion showed a little higher antibacterial activity compared with MAX with E. coli and B. subtilis growth inhibition of 30.55 ± 2.56% and 33.60 ± 2.89%, respectively. Whereas for the cells exposed to the colloidal solution of delaminated Ti$_3$C$_2$T$_x$ MXene, the loss of E. coli and B. subtilis cells viability increases to 97.70 ± 2.87% and 97.04 ± 2.91%, respectively, exhibiting much stronger inhibition. The three materials showed significant differences in their antibacterial activities against both bacterial strains. In particular, delaminated Ti$_3$C$_2$T$_x$ MXene has a much more pronounced antibacterial activity compared with those of MAX and ML-Ti$_3$C$_2$T$_x$ MXene and was used for further studies.

**Concentration Dependent Antibacterial Activity of Ti$_3$C$_2$T$_x$.** The antibacterial activity of Ti$_3$C$_2$T$_x$ against Gram (+) B. subtilis and Gram (−) E. coli was evaluated by measuring the growth curve and the cell viability after exposure of the bacteria to increasing concentrations of Ti$_3$C$_2$T$_x$ colloidal solutions. The optical density (OD) was monitored spectrophotometrically at 600 nm for pristine bacteria and bacteria treated with Ti$_3$C$_2$T$_x$ over different time intervals from lag phase (when individual bacteria are adjusting to the environment) to stationary phase (when their growth and death rates are equivalent). Bacteria (at 10$^7$ colony forming units (CFU)/mL) were treated with different concentrations of Ti$_3$C$_2$T$_x$ for 4 h, recultivated on agar plates, and evaluated by using the bacteria counting method. Figure 2 shows the typical photographs of E. coli or B. subtilis bacteria colonies after treatment with various concentrations of bacteria. As can be seen from both panels, the number of colonies significantly decreases with increasing concentration of Ti$_3$C$_2$T$_x$. The obtained results indicate the dose-dependent antimicrobial activity of Ti$_3$C$_2$T$_x$.

**Figure 2. Concentration dependent antibacterial activities of the Ti$_3$C$_2$T$_x$ in aqueous suspensions: Photographs of agar plates onto which E. coli (top panel) and B. subtilis (bottom panel) bacterial cells were recultivated after treatment for 4 h with 0 μg/mL (A), 10 μg/mL (B), 20 μg/mL (C), 50 μg/mL (D), 100 μg/mL (E), and 200 μg/mL (D) of Ti$_3$C$_2$T$_x$, respectively. Bacterial suspensions in deionized water without Ti$_3$C$_2$T$_x$ MXene material were used as control.**

both Gram (+) and Gram (−) bacteria. The bacterial cell loss gradually ascended with the increasing concentration of Ti$_3$C$_2$T$_x$. E. coli and B. subtilis showed 92.53% and 93.96% survival rate, respectively, at the lowest Ti$_3$C$_2$T$_x$ concentration of 2 μg/mL. By increasing the Ti$_3$C$_2$T$_x$ MXene concentration from 2 μg/mL to 20 μg/mL, the survival rate of E. coli and B. subtilis was decreased to 35.31% and 28.21%, respectively. More than 96% bacterial viability loss for both bacterial strains was observed at 100 μg/mL of Ti$_3$C$_2$T$_x$ and bacterial inhibition was increased to more than 99% at 200 μg/mL of Ti$_3$C$_2$T$_x$ (Figure 3). Additionally, the Ti$_3$C$_2$T$_x$ dispersions revealed a stronger influence on B. subtilis than E. coli at lower concentrations.

The obtained results are in agreement with previously reported data, where several nanomaterials showed a higher antibacterial activity against Gram (+) bacterial strains than Gram (−) bacteria and differences of the cell wall structure of two bacterial strains were reported as a possible reason for different sensitivities.$^{34}$ Gram (−) E. coli cells have negatively charged cellular membranes, as a function of the isoelectric point (pI) = 4−5. For the Gram (+) B. subtilis cells, the pI value of the membranes can reach 7, which produces a more negatively charged surface in culture medium.$^{35,36}$ Therefore, the higher negative charges of E. coli cells at pH 7 could explain their higher resistance against the direct exposure to Ti$_3$C$_2$T$_x$ substrate than B. subtilis cells in aqueous suspensions at pH 7. This could be attributed to the observed difference in the
antimicrobial activity against Gram (−) E. coli and Gram (+) B. subtilis. Recently, graphene-based materials were reported to show unique antibacterial properties and have become one of the most popular research subjects. Moreover, E. coli, as Gram (−) bacteria, are covered by a much thinner layer of peptidoglycan (thickness of 7−8 nm) but have an external protective lipid membrane, whereas Gram (+) B. subtilis lacks the external lipid membrane, but its thicker peptidoglycan cell walls are in the range of 20−80 nm. It was reported that the cell membrane of Gram (+) bacteria lacking the outer membrane were more easily damaged by direct contact with graphene nanowalls, as compared to the Gram (−) E. coli with the outer membrane. The hydrophilic Ti$_3$C$_2$Tx could effectively attach to bacteria, facilitating their inactivation by direct contact interaction.

In order to compare antibacterial activity of Ti$_3$C$_2$Tx with GO, both bacterial strains were treated with different concentrations of GO under the same experimental conditions. Figure 3 shows the viability of both E. coli and B. subtilis bacteria in control, which was taken as 100%, and exposed to 0−200 μg/mL of GO. For both bacterial strains, there were substantial differences in bacterial colonies on agar plates, indicating that the Ti$_3$C$_2$Tx MXene has a higher antibacterial activity as compared to GO in our experimental setup. Ti$_3$C$_2$Tx showed more than 98% cell inactivation to both bacterial strains at 200 μg/mL of Ti$_3$C$_2$Tx whereas GO induces about 90% inactivation at the same concentration (Figure 3).

To further evaluate the bactericidal properties of Ti$_3$C$_2$Tx MXene, the antibacterial activity is reported in terms of log reduction. The plate count experiments showed log 2.43 and log 2.21 reductions of viable E. coli and B. subtilis bacteria, respectively, as compared to the initial concentration of bacteria (10$^7$ CFU/mL) (Figure S2, Supporting Information). GO suspensions showed a log reduction of 1.02 and 0.97 for E. coli and B. subtilis, respectively, being less effective than Ti$_3$C$_2$Tx.

To evaluate the antibacterial activity of Ti$_3$C$_2$Tx in growth media, both bacterial strains were exposed to 200 μg/mL of Ti$_3$C$_2$Tx in LB media for 4 h. Figure S3 (Supporting Information) depicts the growth of bacterial cells in LB media in the presence of Ti$_3$C$_2$Tx. Figure S3A shows a significant decrease in log of bacterial growth when exposed to Ti$_3$C$_2$Tx. The viable cells count in growth media were 33.32% and 27.34% for E. coli and B. subtilis, respectively, in the presence of Ti$_3$C$_2$Tx as compared to that of control (see Figure S3B).

The effect of contact time on bactericidal activity of Ti$_3$C$_2$Tx (200 μg/mL) was further examined during the 4 h incubation period. Figure S4 (Supporting Information) shows the kinetics of antibacterial activity in terms of cell viability and log
reduction. The antibacterial activity increased with increasing contact time and cell viability decreased to 50% within 2 h of contact time and more than 98% cells viability loss was observed after 4 h. This relatively short contact time might also be advantageous for the application of Ti$_3$C$_2$T$_x$ as antibacterial agent.

The antimicrobial activity of Ti$_3$C$_2$T$_x$ nanosheets was further confirmed by bacterial regrowth curves using a second assay. Figure 4 shows the OD growth curves of *E. coli* and *B. subtilis* cells incubated with different concentrations of Ti$_3$C$_2$T$_x$. It was found that inhibition of both bacterial strains growth was dose dependent and the bactericidal activity increased with increasing Ti$_3$C$_2$T$_x$ concentration, which was in line with the number of colonies grown on the LB plates. Growth kinetics constants for both colonies were evaluated and are given in Table 1. It was found that the specific growth constant for *E. coli*, ($\mu_e$) decreased from 0.277 h$^{-1}$ to 0.068 h$^{-1}$ with increasing Ti$_3$C$_2$T$_x$ concentration from 0 to 200 $\mu$g/mL. For *B. subtilis*, a decrease in the growth rate constants ($\mu_b$) from 0.347 h$^{-1}$ to 0.134 h$^{-1}$ was observed with increasing Ti$_3$C$_2$T$_x$ concentration. With increasing Ti$_3$C$_2$T$_x$ concentration from 0 to 200 $\mu$g/mL, bacterial doubling time ($T_d$) was increased from 2.5 to 10.1 h and 2.0 to 5.16 h for *E. coli* and *B. subtilis*, respectively, showing a strong bactericidal effect.

**Bacterial Membrane Morphology Changes.** To understand the antibacterial effect of Ti$_3$C$_2$T$_x$ MXene, changes of morphology and membrane integrity of *E. coli* and *B. subtilis* cells due to the interaction with Ti$_3$C$_2$T$_x$ were further evaluated by SEM and TEM. As depicted by SEM images in Figure 5A, bacterial cells of both *E. coli* and *B. subtilis* cultured in the absence of Ti$_3$C$_2$T$_x$ were viable with no observed membrane damage or cell death. With increasing Ti$_3$C$_2$T$_x$ concentration from 0 to 200 $\mu$g/mL [control] (A), 50 $\mu$g/mL (B), and 100 $\mu$g/mL (C) of Ti$_3$C$_2$T$_x$, both bacteria suffered from prevalent cell lysis indicated by a severe membrane disruption and cytoplasm leakage (see the red circles at high magnification in panel C).

### Table 1. Specific Growth Constant and Doubling Time Obtained in the Batch Growth Tests for *E. coli* and *B. subtilis* Cells Treated with Different Ti$_3$C$_2$T$_x$ Concentrations

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<tr>
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<td>$\mu_b$ (h$^{-1}$)</td>
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Figure 5. SEM images of the *E. coli* (top panel) and *B. subtilis* (bottom panel) treated with 0 $\mu$g/mL [control] (A), 50 $\mu$g/mL (B), and 100 $\mu$g/mL (C) of Ti$_3$C$_2$T$_x$, at low and high magnification, respectively. Control bacterial cells were viable with no observed membrane damage or cell death and the higher magnification shows that the bacterium is protected by intact cytoplasmic membrane (panel A). At 50 and 100 $\mu$g/mL of Ti$_3$C$_2$T$_x$, both bacteria suffered from prevalent cell lysis indicated by a severe membrane disruption and cytoplasm leakage (see the red circles at high magnification in panel C).
from a prevalent membrane damage and cytoplasm leakage in the presence of 50 μg/mL of Ti$_3$C$_2$T$_x$, which is clearly observed at high magnifications (Figure 5B). Some bacterial cells still maintained the membrane integrity, but they were deformed. At 100 μg/mL of Ti$_3$C$_2$T$_x$, both bacteria suffered from prevalent cell lysis indicated by a severe membrane disruption and cytoplasm leakage (see the red circles at high magnification in Figure 5C).

Significant morphological changes in the cell structure could be attributed to detachment of the cytoplasmic membrane from the cell wall as confirmed by LDH release assay. The SEM observations were consistent with the bacteria colonies numbers in Figure 3. It is suggested that with increasing Ti$_3$C$_2$T$_x$ concentration both E. coli and B. subtilis were trapped or wrapped by the thin sheets of Ti$_3$C$_2$T$_x$ and subsequently formed agglomerates. This has been confirmed by spot EDS analysis on the surface of the bacterium (see Figure S5, Supporting Information). Similar observations were reported for graphene, GO, and CNTs, where the death of both the Gram (−) and the Gram (+) cells was ascribed to the disruption of their membranes and the leakage of their cytoplasm content after direct contact with graphene-based material. Liu et al. suggested that different aggregation/dispersion behavior of GO and rGO may have distinct effect in their antimicrobial activities [14].

Additionally, TEM images (Figure 6) were utilized to observe the cell wall and membrane damage, as well as the change of inner structure of the cells. TEM analysis of E. coli and B. subtilis before and after being exposed to 200 μg/mL Ti$_3$C$_2$T$_x$ showed a decrease in the number of bacterial cells in Ti$_3$C$_2$T$_x$ treated groups compared to the control. As Figure 6 shows, Ti$_3$C$_2$T$_x$ nanosheets were tightly adsorbed around the cells and even entered the cells (Figure 6, arrows a, c).

Meanwhile, the intracellular densities of both E. coli and B. subtilis decreased, revealing that they lost some intracellular substance. The attachment of Ti$_3$C$_2$T$_x$ MXene to the cellular membrane of both bacteria is clearly demonstrated by presence of the highly crystalline Ti$_3$C$_2$T$_x$ layers observed from the high resolution TEM (HRTEM) and the corresponding selected area electron diffraction (SAED) patterns, as well as the spot EDS analysis showing Ti signal on the surface of the treated bacteria (see Figures S6 and S7). In both E. coli and B. subtilis, the cell wall was stripped down after exposure to Ti$_3$C$_2$T$_x$ nanosheets (Figure 6, arrows b, d). Significant inner cell structure leakage was observed due to cell wall and membrane damage.

LDH release assay was used to quantitatively determine the extent of cell damage. Figure 7 shows the LDH activity in the supernatants after 4 h of incubation. Concentration dependent LDH release was observed as bacterial cells were exposed to Ti$_3$C$_2$T$_x$ nanosheets dispersions (Figure 7). The bacterial cells exposed to 2 and 10 μg/L of Ti$_3$C$_2$T$_x$ exhibited minimal LDH release for both E. coli and B. subtilis. However, LDH release increased significantly when bacterial cells were exposed to 200 μg/L solution of Ti$_3$C$_2$T$_x$, which showed cytotoxicity of 38.41% and 55.24% for E. coli and B. subtilis, respectively. This dose-dependent cytotoxicity shows that both the walls and the inner contents of the cell were damaged, suggesting that membrane disruption might be a major cell inhibitory mechanism.

Oxidative-Stress and Antimicrobial Activity of Ti$_3$C$_2$T$_x$ MXene. Some earlier studies have proposed oxidative stress as a common mechanism of antibacterial activity of several metal-, metal-oxide-, and carbon-based nanomaterials. Oxidative stress occurs when cells are exposed to elevated levels of ROS such as free radicals, O$_2^*$, •OH and H$_2$O$_2$. In particular, many previous studies have explored the generation of ROS on the surface of carbon, metal, and metal-oxide NPs like graphene, Ag, TiO$_2$, and ZnO. Although an agreement on the ROS production of different nanomaterials was difficult to reach among various studies, almost all engineered NPs including nonoxide nanomaterials appear to produce ROS under certain circumstances. For example, some studies detected ROS in TiO$_2$ nanoparticles suspensions under dark conditions, whereas other studies did not. A similar mechanism has been thought for antibacterial activity of iron nanoparticles.
oxide nanoparticles in which reduced iron species (Fe$^{3+}$/Fe$^{2+}$) reacted with oxygen to create ROS.

To identify if cellular oxidative stress may be induced by Ti$_3$C$_2$T$_x$, ROS dependent and independent oxidative stress was investigated in two separate abiotic assays. First, the production of superoxide anion (O$_2^{-}$) at different Ti$_3$C$_2$T$_x$ concentrations was monitored using XTT assay. As shown in the Figure S8 (Supporting Information), no noticeable absorption was detected at different Ti$_3$C$_2$T$_x$ concentrations revealing that MXene mediated no or negligible superoxide anion production and their role in Ti$_3$C$_2$T$_x$ antibacterial activity could be minimal. However, the production and impact of ROS other than superoxide anion needs to be discretely examined in future studies.

Second, oxidative stress mediated by Ti$_3$C$_2$T$_x$ was examined using glutathione oxidation assay. Glutathione is a tripeptide containing a thiol group, which serves as one of the major cellular antioxidants and is used as an indicator of the oxidative stress induced by different abiotic compounds. The oxidation of glutathione has been widely used as an indicator of the oxidative stress induced by different nanomaterials. Thiol groups (−SH) in glutathione can be oxidized to disulfide converting glutathione to glutathione disulfide. Moreover, direct contact of glutathione with nanoparticle surface also logically could lead to loss of glutathione by adsorption, or binding. In this study, glutathione was exposed to increasing concentrations of Ti$_3$C$_2$T$_x$ in a bicarbonate buffer and incubated for 4 h, after which the concentration of thiol groups was quantified by Ellman’s assay.

As shown in Figure 8, glutathione depletion was dependent on both Ti$_3$C$_2$T$_x$ concentration and incubation time. Although negligible glutathione loss was observed for the control samples in the absence of Ti$_3$C$_2$T$_x$, glutathione concentration was reduced from 97.5% to 61.7% when Ti$_3$C$_2$T$_x$ concentration was increased from 2 to 200 μg/mL, respectively (Figure 8B). It is unlikely that Ti$_3$C$_2$T$_x$ MXene itself can work as an oxidant for glutathione, but Ti$_3$C$_2$T$_x$ has reactive Ti−F groups on its surface, which are not stable at high pH, and also Ti$_3$C$_2$T$_x$ possesses a high negative surface charge, as shown by its −30 to −40 mV zeta-potential in aqueous solutions. Thus, both chemical reactions and physisorption are potentially possible. However, at the moment, there is no published data on interaction of Ti$_3$C$_2$T$_x$ with thiols.

It is important to note that MXenes also have good conductivity (>$2000$ S/cm measured on Ti$_3$C$_2$T$_x$ films$^{[38]}$), similar to or exceeding that of rGO. The mechanism in this case could be explained by formation of a conductive bridge over the insulating lipid bilayer, mediating electron transfer from bacterial intracellular components to the external environment and resulting in cell death.$^{[8,49]}

**Proposed Inhibition Mechanism of Ti$_3$C$_2$T$_x$ MXene.** Strong antibacterial property of Ti$_3$C$_2$T$_x$ may be partially attributed to the anionic nature of its surface. Ti$_3$C$_2$T$_x$ nanosheets have negatively charged surfaces. In addition, its high hydrophilicity may enhance bacterial contact to membrane surface resulting in inactivation of adhered microorganisms according to direct contact-killing mechanism. Moreover, hydrogen bonding between oxygenate groups of Ti$_3$C$_2$T$_x$ MXene and the lipopolysaccharide strings of the cell membrane could result in bacterial inhibition by preventing nutrient intake as recently proposed for GO nanosheets.$^{[10,50]}$ It is important to understand the interaction of MXene with cell membranes for the evaluation of MXene’s health and environmental impacts and to utilize it as biocide in disinfection industry. We have found the interesting antibacterial activity of Ti$_3$C$_2$T$_x$; however, still the interaction between MXene and bacterial cell membrane has to be investigated and fully understood. From the above LDH release assay, SEM, and TEM images, as well as glutathione oxidation assays, the antimicrobial mechanism of Ti$_3$C$_2$T$_x$ MXene nanosheets can be explained as follows: First of all, delaminated Ti$_3$C$_2$T$_x$ nanosheets with sharp edges have the capacity of adsorbing on the surface of microorganisms. It is also suggested that with increasing Ti$_3$C$_2$T$_x$ concentration, both E. coli and B. subtilis were trapped or wrapped by the nanometer-thin sheets of Ti$_3$C$_2$T$_x$ and subsequently formed agglomerates. Moreover, exposure of bacterial cells to sharp edges of Ti$_3$C$_2$T$_x$ as shown by the TEM image in Figure 1A, may induce membrane damage. The water contact angle on Ti$_3$C$_2$T$_x$ films was found to be 27° and its hydrophilicity may result in effective attachment of bacteria to Ti$_3$C$_2$T$_x$.$^{[51]}$ The antibacterial effects may also be attributed to strong reducing activity of MXene and its reactive surfaces.$^{[52]}$ The smallest Ti$_3$C$_2$T$_x$ nanosheets could permeate into the microorganism cell through direct physical penetration or via endocytosis. Finally, Ti$_3$C$_2$T$_x$ may also react with some molecules in the cell wall and cytoplasm of microorganism, disrupting the cell structure and leading to the death of the microorganism. Recently, several studies investigating the effects of carbon-based nanomaterials, such as graphene, GO, CNT, and fullerene, proposed a similar three-step antibacterial mechanism.
causing physicochemical damage to cell membranes depending upon the size of nanoparticles.\textsuperscript{10,12,14,23,41} 

Ti$_3$C$_2$T$_x$ has been used as a representative MXene in this study. However, there are now close to 20 members of MXene family that should be similarly screened for their potential use as antibacterial agents. Taking into account that other MXenes have different transition metals, such as Nb, Mo, V, and so forth,\textsuperscript{2,5,3–35} exposed on their surface, we can expect different chemical behavior as a function of the MXene composition and the surface termination (OH, O, F, etc.). Even Ti-based MXenes differ in chemical reactivity. For example, Ti$_3$C$_2$Tx oxidizes easier in the presence of oxygen and water than Ti$_3$C$_2$T$_x$\textsuperscript{32} Ti$_3$C$_2$T$_x$ produced by different methods has different functional groups on its surface, with more F on the surface of Ti$_3$C$_2$T$_x$ produced by etching in concentrated HF compared to that synthesized by extracting Al in diluted HF or LiF-HCl solution.\textsuperscript{6,37} Difference in surface chemistry may affect toxicity and antibacterial activity of MXenes. This study is the first step toward understanding interactions of MXenes with living matter and it is expected to open the door for extensive studies on other 2D carbides and nitrides of transition metals. Fine tuning of MXene surface functional groups, flake size, and conductivity, both in colloidal and membrane forms, may open a wide window for MXenes’ application in the antimicrobial coatings and water purification membranes.

CONCLUSIONS

Our studies demonstrate that Ti$_3$C$_2$T$_x$ in aqueous colloidal solution can stimulate antibacterial activity against Gram (−) E. coli and Gram (+) B. subtilis bacteria. Ti$_3$C$_2$T$_x$ antibacterial activity was dose dependent and exceeded that of GO. Direct contact with Ti$_3$C$_2$T$_x$ MXene can disrupt cellular membranes leading to cell damage and eventual death. We have focused this first study on the antibacterial properties of MXenes, but the cellular uptake and cytotoxicity of MXene should be studied to understand the health and environmental impact of MXenes. On the basis of these results, we introduce MXenes as a new family of 2D antimicrobial nanomaterials for their potential use in water treatment and biomedical applications.

MATERIALS AND METHODS

Synthesis, Delamination, and Dispersion of Ti$_3$C$_2$T$_x$. A colloidal solution of single- and few-layer Ti$_3$C$_2$T$_x$ particles was obtained by delaminating Ti$_3$C$_2$T$_x$ powders by ultrasonication, after etching Ti$_3$AlC$_2$ with LiF/HCl solution (Sigma-Aldrich) as described previously\textsuperscript{32} with minor modifications in the process. Briefly, the obtained ML-Ti$_3$C$_2$T$_x$ powder was dispersed in deaerated water with a weight ratio of ML-Ti$_3$C$_2$T$_x$:water of 1:250. The suspension was probe sonicated at 60% amplitude for 30 minutes (450 watts) while 3 sec pulse on and 1 sec off under flow of argon, and then centrifuged for 1 h at 3000 rpm to obtain the supernatant containing Ti$_3$C$_2$T$_x$ flakes. TEM, SEM, EDX, and XRD were used to study the structure, composition and morphology of the flakes.

Cell Preparation. The antibacterial properties of Ti$_3$C$_2$T$_x$ and GO colloids were evaluated using E. coli and B. subtilis as the model Gram (−) and Gram (+) bacteria, respectively. Glycerol stocks were used to inoculate defined overnight cultures in LB medium at 35 °C. Following that, 1 mL volumes of cell suspensions were subcultured and harvested at the exponential growth phase. Cultures were centrifuged at 5000 rpm for 5 min and pellets obtained were washed three times with phosphate buffered saline (PBS, Sigma-Aldrich) (pH 7.2) to remove residual macromolecules and other growth medium constituents. The cell pellets collected by centrifugation were resuspended in sterilized deionized water (DI) and diluted to approximate cell concentration of 10$^7$ CFU/mL. Gentamicin (50 μg/mL) was used as positive control. Water was used to replace PBS buffer for the antibacterial studies to prevent the aggregation of MXene in PBS during experiments. Figure S9, (Supporting Information) showed that cell viability of E. coli and B. subtilis was similar in DI and PBS during 4 h of incubation time.

Antibacterial Activity of Ti$_3$C$_2$T$_x$ (MXene) Nanosheets Dispersions. Antibacterial activity against each strain was determined by the colony count method and the measurement of OD. Batch assays were performed to compare the antibacterial activity of delaminated Ti$_3$C$_2$T$_x$ in colloidal solution with that of dispersions of ML-Ti$_3$C$_2$T$_x$ and Ti$_3$AlC$_2$. Delaminated Ti$_3$C$_2$T$_x$, ML-Ti$_3$C$_2$T$_x$, and Ti$_3$AlC$_2$ concentrations of 100 μg/mL were applied to both E. coli and B. subtilis and cell survival rate was counted by CFU/mL.

A second set of antibacterial activity tests was conducted by spread plate CFU counting. The bacteria (about 10$^7$ CFU/mL were incubated with different concentrations (2–200 μg/mL) of Ti$_3$C$_2$T$_x$ MXene for 4 h. Aliquots of the samples were withdrawn and CFU were counted by plating 40 μL of 10-fold serial dilutions onto LB agar plates. Colonies were counted after incubation at 35 °C and the cell survival rate was expressed as the percentage of the control and log reduction. The following equation was used to represent relative viability of cells:

$$ \text{relative cells availability} = \left( \frac{N_c}{N_m} \right) \times 100 $$

where $N_c$ is bacterial colonies of the control sample and $N_m$ are colonies for cells treated with Ti$_3$C$_2$T$_x$.

The log reduction was calculated using the following equation:

$$ \text{log reduction} = \log_{10} \left( \frac{N_c}{N_m} \right) $$

To examine the effect of MXene on bacterial regrowth, the batch assays were subjected to 2, 10, 20, 50, 100, and 200 μg/mL Ti$_3$C$_2$T$_x$. The batch assays were subjected to continuous shaking at 150 rpm and constant mesophilic temperature of 35 °C for 4 h. For controls, DI was added instead of Ti$_3$C$_2$T$_x$. The reaction mixture was then transferred to 15 mL tubes, each containing 10 mL of LB medium, and the tubes were inoculated on a shaking incubator at 150 rpm and 35 °C. Aliquots of the samples were withdrawn at specific time intervals and the value of OD at a wavelength of 600 nm was measured on a UV–vis spectrometer (Novaspec Plus). Bacterial regrowth curves were created by plotting OD values versus time and bacterial growth kinetics were studied. All experiments were performed as triplicates and average values were reported.

Antibacterial activity of Ti$_3$C$_2$T$_x$ in LB growth media was assessed by exposing the bacteria (about 10$^7$ CFU/mL) to 200 μg/mL of Ti$_3$C$_2$T$_x$ MXene for 4 h. Aliquots of the samples were withdrawn and CFU were counted as described earlier.

Lactase Dehydrogenase Release Assay. LDH release assay was used to determine the cell membrane activity of
MXene treated bacterial cells in colloidal solution using cytotoxicity detection kit (Roche Applied Science). The standard protocol assay was performed according to the manufacturer’s instructions. Briefly, E. coli and B. subtilis cells were treated with 2, 10, 20, 50, 100, and 200 μg/mL Ti₃C₂Tx in DI for 4 h. Following 4 h exposure to MXene, 50 μL of cell culture supernatant was transferred into sterile 1 mL centrifuge tubes. Then, 50 μL substrate mix was added and tubes were incubated at room temperature in the dark for 1 h. The reaction was stopped by the addition of 50 μL of stop solution. LDH release was quantified by measuring absorbance at 490 nm.

**Superoxide Radical (O₂•−) Assay.** The hypothetical possibility of superoxide radical anion (O₂•−) production was evaluated by monitoring the absorption of XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, Fluka). XTT can be reduced by superoxide radical anion (O₂•−) to form water-soluble XTT-formazan with the maximum absorption at 470 nm. XTT (0.4 mM) were dissolved in PBS solution at pH 7.0. Bacterial dispersions treated with Ti₃C₂Tx at different concentrations (1 μL) in DI were mixed with 1 μL of 0.4 mM XTT. The mixture was incubated in dark for 5 h; afterward, the mixture was filtered through a 0.45 μm polyethersulfone (PES) filter (Whatman) to remove Ti₃C₂Tx. The changes in absorbance at 470 nm were monitored with a UV–vis spectrophotometer.

**Abiotic Thiol Oxidation and Quantification.** The Ti₃C₂Tx (MXene)-mediated abiotic oxidation of glutathione was studied by quantifying thi oxidation following Ellman’s assay as described earlier. Briefly, 0.4 mM glutathione was prepared in a 50 mM bicarbonate buffer (pH 8.6) at a total volume of 250 μL. Following 4 h exposure to MXene, 50 μL of cell culture supernatant was transferred into sterile 1 mL centrifuge tubes. Then, 50 μL substrate mix was added and tubes were incubated at room temperature in the dark for 1 h. The reaction was stopped by the addition of 50 μL of stop solution. LDH release was quantified by measuring absorbance at 490 nm.

**Cell Morphology Observation with SEM and TEM.** SEM analysis was performed to observe the effect of Ti₃C₂Tx MXene on morphology and surface structure of the bacterial cells using FEI-Nova Nano SEM 650. SEM imaging of samples was accomplished using the following procedures: after the experiments, cells from the treated samples were fixed with 2.5% glutaraldehyde (Sigma-Aldrich) overnight at 4 °C, followed by washing with 0.1 M PBS (pH 7.4) and dehydration with a graded ethanol series (25, 50, 80, 100%). For SEM, samples were allowed to dry completely at room temperature and then coated with gold by sputtering (5 nm).

The ultrastructure of the bacteria was examined by TEM. The bacteria were pelleted and fixed overnight with a 4% formaldehyde–1% glutaraldehyde fixative. Following a wash with s-collidine buffer, the samples were postfixed with 1% osmium tetroxide for 1 h, dehydrated in graded concentrations of ethanol, and embedded in epoxy resin. The resin embedded tissue was polymerized at 60 °C overnight. Thick 1–2 μm and thin 90 nm sections were cut using a Leica EM UC6 ultramicrotome. Grids were stained with uranyl acetate and lead citrate stains. Ultrathin 90 nm sections were examined with a JEOL JEM-1230 transmission electron microscope operated at 80 kV. Digital images were acquired using an AMT digital camera system.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.6b00181.

Photographs of agar plates and results of inhibition studies, detailed cell viability and log reduction graphs, detailed TEM and EDS analysis. (PDF)

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**Notes**

The authors declare no competing financial interest.

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