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Graphic Abstract

[Diagram showing a bacterial ghost and 2D-FTIR-COS analysis results.]
Interaction between bacterial cell membranes and nano-TiO₂ revealed by two-dimensional FTIR correlation spectroscopy using bacterial ghost as a model cell envelope

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Abstract

The interaction between microorganisms and nanoparticles is a crucial step towards understanding the subsequent biological effect. In this study, the interaction between TiO$_2$ nanoparticles and bacterial cell membrane was investigated by Two-dimensional Correlation Fourier Transformation Infrared spectroscopy (2D-FTIR-COS) using bacterial ghosts (BGs), which are non-living bacterial cell envelopes devoid of cytoplasm. The synchronous map of 2D-FTIR-COS results indicated that the functionalities in proteins of BGs preferentially interacted with TiO$_2$ nanoparticles; whereas the interaction of TiO$_2$ nanoparticles with characteristic functionality in polysaccharides (C–OH) and phospholipids (P=O) were very weak or insensitive. This conclusion was further corroborated by settling of TiO$_2$ nanoparticles in the presence of pure protein, polysaccharide and phospholipid represented by bovine serum albumin (BSA), alginate and phosphatidylethanolamine (PE). Additionally, the asynchronous map of 2D-FTIR-COS indicated a sequential order of functionalities bonded to TiO$_2$ nanoparticles with the order of: COO$^-$ > aromatic C=C stretching > N-H, amide II > C=O, ketone. These findings contribute to deeper understanding of the interaction between TiO$_2$ nanoparticles and bacterial cell membrane in aquatic systems.

Keywords: Titanium dioxide nanoparticles; Bacterial ghosts; Two-dimensional FTIR correlation; Nanoparticle-cell membrane interaction
1. Introduction

Titanium dioxides (TiO$_2$) nanoparticles is one of the most widely used nanomaterials, with applications as cosmetics (Auffan et al., 2010), sunscreens (Nohynek et al., 2007), food additives (Weir et al., 2012) and photocatalysts (Hoffmann et al., 1995). The annual production of TiO$_2$ nanoparticles is rapidly increasing and estimated to reach 2.5 million metric tons by 2025 (Menard et al., 2011). Due to the increased production and application of synthetic TiO$_2$ nanoparticles, their release into the environment is inevitable. However, information regarding the TiO$_2$ nanoparticles toxicity, transport and fate in both natural and engineered systems is still scarce. Based on the existing studies, it is proposed that the interaction between nanoparticles and the membranes of microorganisms can be a critical initial process that precedes the toxicity pathways as well as influences the environmental fate of nanoparticles (Chen and Bothun, 2014).

To date, most related investigation on the interaction between cells and nanoparticles were mainly focused on how water chemistry, such as pH and ionic strength, affect the interaction between cells and nanoparticles (French et al., 2009; Ma et al., 2015). For example, solution pH determines the surface charges (i.e. zeta potentials) of both cells and nanoparticles, and thus influences the electrostatic interaction profile between the two objects. It was observed in many studies that low pH enhanced this interaction due to the nanoparticles being more positively charged while cells remaining negatively charged (Khan et al., 2011; Schwegmann et al., 2013). Salt ions can compress the electro-double layer of nanoparticles and cells, thereby reducing or eliminating the electro-double layer interaction. As a consequence, the commonly attractive van der Waals force becomes dominated and results in enhancement of cell surface and nanoparticle interaction under high ionic strength in water (Mukherjee and Weaver, 2010; Li et al., 2011; Shih et al., 2012). These
results could be well described by the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory due to
the fact that the sizes of both microorganism cells and nanoparticles aggregates are within the scale
of colloids. More recently, more comprehensive studies on the interaction between cell surface and
nanoparticle in the presence of natural organic matters (NOM) have been carried out to mimic
natural water environment (Lin et al., 2012). It was pointed out that the both bulk and
nanoparticle-bound NOM can inhibit the interaction between cells and nanoparticles due to the
delivery of negative charge to the surface of TiO₂ nanoparticles by NOM.

Approaches to probe the nanoparticle-membrane interaction are quite diverse, including by
atomic force microscopy (AFM) (Leroueil et al., 2007; Roiter et al., 2008), optical tweezers
(Ruscianno et al., 2009), and quartz crystal microbalance with dissipation monitoring (QCM-D)
(Keller and Kasemo, 1998; Zhang and Yang, 2011). QCM-D is the most extensively used tool for
nanoparticle-membrane interaction due to its ability to in situ detection of nanoparticles adsorption
on model cell membranes at a sensitivity level as low as tens of nanograms (Chen and Bothun,
2014). However, these techniques cannot distinguish which constituents or functionality of cell
membrane correspond to the interaction when a membrane with multiple constituents is applied.
Therefore, the binding affinities of cell surface constituents or functional groups to nanoparticles are
unexplored.

Fourier transform-infrared (FTIR) spectroscopy is a versatile technique that offers a
comprehensive insight into the molecular structure of principle constituents in bacterial cell
membranes, such as protein, polysaccharide and lipid (Mecozzi et al., 2009). Two-dimensional
correlation spectra (2D-COS), developed by Noda (1993), can be applied to resolve the overlapped
peaks by distributing the spectral intensity trends along a second dimension with the data set
collected as a function of a perturbation (e.g. time, temperature, concentration, etc.) (Noda, 1993; Dluhy et al., 2006). More importantly, it can also provide the information about the relative direction and sequential orders of structural variations in response to the perturbation. Thus, 2D-COS has been successfully applied to explore the interaction processes of NOM and TiO$_2$ nanoparticles (Chen et al., 2014). To the best of our knowledge, there is no literature reporting the application of 2D-FTIR-COS in the investigation on the interaction between nanoparticles and biological relevant components.

So far, the molecular mechanisms of the interaction between the NPs and bacterial cell membrane remain unclear, particularly, information on adsorption affinities of individual molecular constituents and functional groups is lacking. One of the major challenges is that cell membrane is dynamic and heterogeneous comprising multiple components such as phospholipid, protein, and polysaccharide (Chen and Bothun, 2014) that can lead to a more elaborate analysis of the mechanisms involved. Another challenge is that live cells undergoing metabolic process would secrete soluble microbial product (SMP) into the reaction solution and undoubtedly affect the interaction profile between cell surface and nanoparticles (Ni et al., 2011). A strategy to carry out nanoparticle-membrane interaction studies is to employ model membrane systems based on the phospholipid bilayer backbone of the cell membrane such as lipid vesicles (Hou et al., 2012; Lesniak et al., 2013; Chen and Bothun, 2014). Such systems can be further elaborated on by introducing other relevant components (i.e. protein and polysaccharides) that will make them more resemble the structure of cell membrane. Nevertheless, the synthesis of multi-components membrane architectures requires complex procedures and studies employing model membranes with embedded constituents such as protein and polysaccharide is still lacking to completely
explore the interaction mechanism.

Bacterial ghosts (BGs) have recently emerged as novel vaccine candidates owing to their properties of being non-living bacterial cell membrane structure (cell envelopes) devoid of cytoplasmic constituents, and maintaining the full cellular morphology and surface constituents of their living counterparts (Jalava et al., 2002; Kudela et al., 2010). Moreover, the BGs can be easily produced by genetic methods or chemical methods (Mayr et al., 2005; Amara et al., 2013b). Therefore, it will be advantageous to employ BGs to study the interaction mechanisms of nanoparticles and cell membrane.

The purpose of this study, therefore, is to investigate the interaction between TiO$_2$ nanoparticles and cell membrane, by 2D-FTIR-COS technique, with BG as a model system. The settling experiments of standard protein, polysaccharide and phospholipid with TiO$_2$ were carried out to further verify and support the results.

2. Material and methods

2.1 Cell cultures and TiO$_2$ nanoparticles.

*Escherichia coli* (*E. coli*) K-12 was used as model bacterium in this study. The bacterial cells were cultured in 50 mL Nutrient Broth ‘E’ (Lancashire, UK) with agitation at 200 rpm for 16 h. The cultures were then washed twice with sterile saline solution (0.9% NaCl) and resuspended in 50 mL sterilized saline solution with a cell density of ~2 × 10$^9$ colony forming unit per milliliter (cfu/mL). Degussa TiO$_2$ (P25, German) was used as a model TiO$_2$ nanoparticles in this study. The crystalline structures of the TiO$_2$ nanoparticles were identified through X-ray diffraction (XRD) analysis (Fig.
S1. Its crystal structure consists of 80% anatase and 20% rutile, with an average primary size of 20-30 nm as revealed by transmission electron microscopic (TEM) analysis (Fig. S2), which were consistent with the description of manufacturer and published literatures (Chowdhury et al., 2011; Tong et al., 2013a). A stock solution containing 10 g/L P25 solutions was used to prepare different concentrations of TiO$_2$ solutions.

2.2 BGs preparation and characterizations.

The BGs were prepared according to a chemical method named “sponge-like” protocol (Amara et al., 2013a; Amara et al., 2013b). This method based on using active chemical reagents in concentration between Minimum Inhibition Concentration (MIC) and Maximum Growth Concentration (MGC) for bacteria. The MIC and MGC of were determined according to a previous report (Andrews, 2001), and shown in Table S1. Four chemical reagents were used in this protocol and their applied concentrations are determined as 4 mg/mL for SDS, 0.02 M for NaOH, 1.05 µg/mL for CaCO$_3$ and 64 mM for H$_2$O$_2$. In brief, 50 mL of washed cells were incubated with SDS, CaCO$_3$ and NaOH for 1 h to produce micropores on the surface of bacteria cells. Then the mixtures were centrifuged at 4,000 rpm (Hermle Z323, Germany) for 10 min to evacuate the cytoplasmic constituent. The cell pellets were then washed with sterilized saline solution and resuspended in H$_2$O$_2$ solution for 30 min to guarantee the degradation of the residual DNA. Finally, the cells were collected by centrifugation at 4,000 rpm and resuspended in 60% ethanol to remove any soluble organic residual. Then BGs were harvested by centrifugation at 4,000 rpm and resuspended in 50 mL ultrapure water.

Light microscopy, scanning electronic microscope (SEM) and atomic force microscope (AFM) were used to observe the BGs as well as the normal bacterial cells. Detailed descriptions of these
works can be found in the supporting information. The DNA in the BGs and normal bacterial cells were, respectively, extracted using a Takara MiniBEST DNA Extraction Kit. DNA agarose gel electrophoresis (AGE) was also performed with 0.6% agarose gel at 80 V for 45 min in TAE buffer (40 mM Tris-acetate/1 mM EDTA, pH=8) to determine the existence of any residual DNA in the BGs. The concentrations of extracted DNA were determined via a Nanodrop spectrophotometry (Model 2000C, Thermo Scientific, Waltham, MA, USA).

2.3 Interaction of BGs with TiO$_2$ nanoparticles.

The BGs suspension was diluted to a concentration equivalent to $\sim 2 \times 10^8$ cfu/mL in all the experiments unless otherwise stated. A series of BGs suspensions containing different concentrations of TiO$_2$ nanoparticles ranging from 0 - 200 mg/L were prepared and the final solution pH was adjusted to circumneutral condition ($\sim 6.8$) using 0.01 M HCl and 0.01 M NaOH, which is close to the isoelectric point (IEP) of the TiO$_2$ nanoparticles (Huang et al., 2015). This pH condition can minimize the long-range electrostatic interaction (non-molecular interaction) and was environmental relevant (Parikh and Chorover, 2006). Then the suspensions were under vigorous agitation of 200 rpm for 8 h at 25 $^\circ$C under dark in an incubator. Finally, 20 mL of each suspensions was sampled and freeze-dried for the FTIR measurement.

2.4 Spectroscopic parameters.

An FTS-4000 Varian Excalibur series FT-IR spectrometer with attenuated total reflection (ATR) (Varian, Palo Alto, CA) was used to collect the infrared spectra. A mixture of the freeze-dried samples and 100 mg of potassium bromide (KBr, IR grade) were ground, homogenized and pressed.
The band from 4000 to 400 cm\(^{-1}\) were collected with an interval of 2 cm\(^{-1}\), and the ordinate was express as absorbance. Each spectrum was an average of 256 scans with automatic baseline correction. The obtained spectra were then smoothed using OMNIC 8.0 software for the subsequent analysis. The spectra of amide I region (1700-1600 cm\(^{-1}\)) were further analyzed to extract information regarding changes of the protein secondary structures by deconvolved spectra. A detailed description of the procedure can be found in the supporting information.

To assess the secretion of soluble microbial product (SMP) from normal cells of \textit{E. coli} K-12, which may influence the interaction profile between TiO\(_2\) nanoparticles and cell membranes, suspensions with 50 mL \(2 \times 10^8\) cfu/mL normal \textit{E. coli} K-12 cells were prepared under dark in the absence and presence of 100 mg/L TiO\(_2\) P25 and shaken at 25 °C. Three mL suspension was sampled and filtered through a 0.22 µm nylon membrane to remove cells or/and TiO\(_2\) nanoparticles at different time intervals. Then the filtrate was analyzed with a fluorescence spectrophotometer (F-7000, Hitachi, Japan) in excitation-emission-matrix (EEM) mode. Fluorescence EEM is a powerful tool to characterize SMP based on well-established principles (Hudson \textit{et al.}, 2007). For comparison, the fluorescence EEM of the bulk solution of BGs in the absence and presence of TiO\(_2\) nanoparticles were also monitored.

### 2.5 2D-FTIR-COS analysis.

In this study, the TiO\(_2\) nanoparticles concentration was applied as an external perturbation, and a set of concentration-dependent FT-IR spectra was obtained. Before conducting 2D-FTIR-COS, each FTIR spectrum was baseline-corrected and smoothed using Savitzky-Golay method (Wang \textit{et al.}, 2012; Chen \textit{et al.}, 2014). The practical computation of 2D-FTIR-COS was performed using
Matlab R2010a (Mathworks Inc., USA) (Noda, 1993; Chen et al., 2015). The synchronous correlation intensity can be constructed using the following equation:

\[ \phi(v_1, v_2) = \frac{1}{m - 1} \sum_{j=1}^{m} I_j(v_1)I_j(v_2) \]  \hspace{1cm} (1)

Asynchronous correlation can be calculated by:

\[ \phi(v_1, v_2) = \frac{1}{m - 1} \sum_{j=1}^{m} I_j(v_1) \sum_{k=1}^{m} N_{jk}I_j(v_2) \]  \hspace{1cm} (2)

Where \( m \) is the total number of the collected spectra, \( I_j(v) \) represents the intensity of the \( j \)th spectrum collected at a specific band or wavenumber of \( v \) (denoting with a subscript of “1” or “2”). The term \( N_{jk} \) corresponds to the \( j \)th column and the \( k \)th row element of the discrete Hilbert-Noda transform matrix, which is defined as:

\[ N_{jk} = \begin{cases} 0 & \text{if } j = k \\ \frac{1}{\pi(k-j)} & \text{otherwise} \end{cases} \]  \hspace{1cm} (3)

The sign of the synchronous peaks \( \phi(v_1, v_2) \) reflects simultaneous changes in intensities measured at \( v_1 \) and \( v_2 \) in response to perturbation (Dluhy et al., 2006). A positive sign, \( \phi(v_1, v_2) > 0 \), indicates the intensities change in the same direction (either increase or decrease simultaneously), while the trend is reversed for \( \phi(v_1, v_2) < 0 \). The sign of the asynchronous peak
\( \phi(v_1, v_2) \) reflects the sequential order of the intensity change measured at \( v_1 \) and \( v_2 \) in response to perturbation. If \( \phi(v_1, v_2) \) and \( \phi(v_1, v_2) \) have the same sign, the intensity change at \( v_1 \) occurs predominantly before \( v_2 \) while the rule is reversed if \( \phi(v_1, v_2) \) and \( \phi(v_1, v_2) \) have the opposite sign.

2.6 TiO\textsubscript{2} precipitation with standard protein, polysaccharide and phospholipid.

To further verify the role of the key biomolecular constituents in cell membrane in the interaction with TiO\textsubscript{2} nanoparticles, a series of settling experiments were performed according to previous studies (Lin et al., 2012; Ma et al., 2015). Bovine serum albumin (BSA), sodium alginate and phosphatidylethanolamine (PE) were used as standard biomolecular constituents to represent protein, polysaccharide and phospholipid, respectively. The mixed suspensions of 100 mg/L TiO\textsubscript{2} nanoparticles and biomolecular constituents with concentrations ranging from 0 to 200 mg/L were prepared (pH=6.8) and subjected to settling experiments for 11 h, and their individual suspensions were also conducted as control. At different settling time, 1 mL aliquot of the mixed and individual suspensions were taken from the top of the suspension and immediately transferred into cuvettes to measure absorbance at 660 nm (A\textsubscript{660}). The settling curves were plotted using the ratio of absorbance at a given time point (A\textsubscript{t}) to the initial absorbance (A\textsubscript{0}) as coordinate and time as abscissa.

3. Results

3.1 BGs as model cell envelope.

To examine whether normal cells will secrete any SMP into the bulk solution, fluorescence EEM was applied to show the spectra of SMP secreted by normal cells of \textit{E. coli} K-12 cells in the
absence and presence of TiO$_2$ nanoparticles (Fig. 1). The peaks at Ex/Em of 230/340 nm (peak T1) and 280/340 nm (peak T2) are reported to be associated with the tryptophan-like protein, (Hudson et al., 2007) which is a common fluorescent SMP secreted by bacterial cells. For E. coli cells under dark (Fig. 1a), the peak intensities increased along with time (Fig. S3a), suggesting that SMP was secreted into the bulk solution. With the addition of TiO$_2$ nanoparticles, the peak intensities still exhibited an increasing trend (Fig. 1b) along with time, while lower than those of sole E. coli cells at each time point (Fig. S3a). This difference was due to the released SMP adsorbing on the TiO$_2$ and thus led to a decrease of SMP concentration in the bulk solution. Therefore, the investigation of interaction between TiO$_2$ nanoparticles and cell membrane will certainly be affected by the presence of these release metabolic products. For example, it has been well-recognized that protein absorbed on nanoparticles surface to form nanoparticle-protein ‘corona’ (Mahon et al., 2012; Lesniak et al., 2013; Saptarshi et al., 2013), which would ultimately determine the interaction profile of nanoparticles with the biological membrane systems, rather than the pristine surface of the unmodified nanoparticles. For example, a previous study found that protein adsorption onto nanoparticles reduced their ability to adhere to cell surface (Lesniak et al., 2013). Therefore, employing model cell envelopes such as BGs, which lack metabolic activities, are of important merit to reveal an unbiased nanoparticle-membrane interaction mechanism.

To avoid the interference of SMP, BGs as model cell envelope was applied as model cell membrane in this study. Fig. 2 compares the SEM and AFM images of normal bacterial cell and BGs. The SEM and AFM images proved that the BGs were in good conditions and still maintained the 3D structure with micropores on the surface which were consistent with previous studies on the morphology of BGs (Amara et al., 2013b). Besides, the crystal violet stained BGs could be
observed by light microscopy (Fig. S4), which was indicative of stable cell envelopes structure. Furthermore, the agarose gel electrophoresis results shown that no observable band on the lane of the BGs compared with the normal bacterial cells of *E. coli* K-12 (Fig. S5). The DNA concentrations for the BGs and normal bacterial cells were 1.7 ± 1.5 and 78.5 ± 1.3 ng/µL, respectively (Table S2). These results indicated the genomic DNA in the as-prepared BGs had been substantially degraded.

Additionally, the fluorescence EEM analysis of the bulk solution of BGs in the absence and presence of TiO$_2$ nanoparticles shown that fluorescence intensity at 230/340 and 280/340 nm were insignificant and remained constant along with time (Fig. S3b). This implied that the as-prepared BGs had lost metabolic activity and no SMP was produced due to the evacuation of the cytoplasmic constituents, which would allow us to avoid the interferences induced by the presence of SMP in the nanoparticles-membrane interaction studies.

### 3.2 2D-FTIR-COS analysis on the interaction between TiO$_2$ nanoparticles and BGs.

The FTIR spectra of the BGs as a function of TiO$_2$ nanoparticle concentrations are shown in Fig. S6. The spectral variations mainly occurred in the 1700 – 1000 cm$^{-1}$ region, where the absorption changed significantly. An increase in TiO$_2$ nanoparticles concentrations caused the characteristic bands changing to various degrees, indicating changes of vibrational structures in BGs by interacting with the TiO$_2$ nanoparticles. However, some of the bands strongly overlapped, and enhancement of the spectral resolution is needed to understand how individual IR band is subjected to the perturbation.

2D-FTIR-COS analysis can allow enhancing the spectral resolution by spreading overlap
peaks in a second dimension, and as a result simplifying the interpretation of one dimension spectrum. Fig. 3 illustrates the synchronous and asynchronous FTIR maps of BGs with TiO₂ nanoparticles as the perturbation. The FTIR regions of bacteria, corresponding to the wavelength ranges of 1000-1200, 1200-1400, and 1500-1700 cm⁻¹, could be roughly assigned to polysaccharide, phospholipid, and protein, respectively (Schmitt and Flemming, 1998). Detailed spectral assignments are presented in Table 1. In synchronous maps (Fig. 3b) most autopeaks, which locate on the diagonal, appear in the protein region, suggesting that protein mainly responds to concentration perturbation. A prominent peak at 1000 cm⁻¹ was due to the increased TiO₂ concentration (Fig. S7) (Kiwi and Nadtochenko, 2005). Therefore, the cross peaks located at 1000 cm⁻¹ in the synchronous and asynchronous map will not be taken into consideration. Closer observation of the protein region (Fig. 3a) shows that all the cross peaks, which locate off the diagonal, exhibit positive signs, indicating that their intensity change in the same direction.

The asynchronous map can provide information on the sequential order of specific structural response to perturbation based on the signs of the cross peaks. In this study, red color indicates a positive sign, while blue color indicates a negative sign in the asynchronous map (Figs. 3c and 3d). Likewise, most of the cross peaks of the asynchronous map located in protein region (Fig. 3d). Specifically, four characteristic cross peaks were observed at the bands of 1612-1674, 1565-1612, 1550-1565 and 1400-1500 cm⁻¹ (Fig. 3c), and the four bands were assigned to C=O stretching, COO⁻ symmetric stretching, N-H (amide II) and aromatic C=C stretching, respectively. (Kiwi and Nadtochenko, 2005) The signs of the cross peaks (Table 1) indicate that sequential order of the bonding affinities of these bands with TiO₂ nanoparticles follow the order: COO⁻ → aromatic C=C stretching → N-H, amide II → C=O, ketone. It must be noted that the phospholipids and
polysaccharides might also contain functional groups such as COO\(^{-}\), C=O and aromatic C=C. However, in terms of their abundance in bacteria, the protein are rich in these functionalities, while the characteristic functional moieties for polysaccharide and phospholipid are C-O and P=O, which did not response to the perturbation. Results herein could roughly imply that the protein play a major role in the binding process of cell membrane to the TiO\(_2\) nanoparticles.

### 3.3 Settling experiments.

To further confirm the interpretation of the 2D-FTIR-COS results, a series of settling experiments were conducted using the selected protein, polysaccharide and lipid with TiO\(_2\) nanoparticles. The settling curves of the test substances at concentrations ranging from 0 to 200 mg/L with TiO\(_2\) were shown in Figs. 4a, b and c. Significant biomolecule-type-dependent settlings were observed. Only protein mixed with TiO\(_2\) shows precipitation behavior at concentrations higher than 10 mg/L, suggesting that protein interact with TiO\(_2\) much stronger than polysaccharide and phospholipid. The photos of settling experiments of the three test substances at concentration of 100 mg/L are provided in Fig. 5. Additionally, comparison of the A\(_{660}\) (absorbance at 660 nm) value of the test substances before and after mixed with TiO\(_2\) were also calculated by the following equation:

\[
\Delta A = A_{\text{mixture}} - (A_{\text{test substance}} + A_{\text{TiO}_2})
\]  

(4)

where \(A_{\text{mixture}}\) is the A\(_{660}\) of the mixture, and \(A_{\text{test substance}}, A_{\text{TiO}_2}\) represent the individual A\(_{660}\) of the test substances and TiO\(_2\). In principle, a positive \(\Delta A\) indicates interaction between TiO\(_2\) nanoparticles and test substances as a result of forming test substances-TiO\(_2\) hetero-agglomeration.
(Rieger et al., 2004; Lin et al., 2012). Conversely, zero or negative $\Delta A$ indicates no or very weak interaction between TiO$_2$ and the test substances. As shown in Fig. 4d, the $\Delta A$ had positive value at concentrations higher than 5 mg/L and exhibited an increasing trend with increasing concentrations, whereas no significant variations were observed with increasing polysaccharide and phospholipid concentrations mixed with TiO$_2$ nanoparticles. This suggests that TiO$_2$ nanoparticles preferentially interacted with protein and consequently formed larger hetero-agglomeration; while the interaction between TiO$_2$ nanoparticles and polysaccharide and phospholipid were weak or negligible.

4. Discussion

4.1 Increasing understanding of nanoparticle-cell membrane interaction.

Understanding the interaction between nanoparticle and cell membrane is a crucial step toward predicting subsequent biological effects (Hou et al., 2012). As aforementioned, this interaction has been explored using various techniques and biological systems (e.g. cells and lipid bilayer) (Chen and Bothun, 2014). However, it is less clear what cell surface molecules are involved in the interaction (Chen and Bothun, 2014; Ma et al., 2015). This work for the first time specifically investigated the nanoparticle-cell membrane interaction using BGs as a model cell membranes at molecular level with 2D-FTIR-COS technique. The results of this study, revealed by the 2D-FTIR-COS, demonstrated that cell membrane functionalities of protein preferentially interacted with TiO$_2$ nanoparticle; whereas the interaction of TiO$_2$ nanoparticle with C–OH (polysaccharide) and P=O (phospholipid) were very weak or insensitive to IR. Although this adds to the limited literature regarding the roles of bacterial cell envelope biomolecules in the nanoparticle-membrane
interaction, this finding is contradictory to previous reports. Jiang et al. (2010) previously reported that, in addition to protein, the lipopolysaccharide (LPS) also shown adhesive ability to metal oxides nanoparticles via hydrogen bonding with the O-antigen part (polysaccharide) using pure LPS extracted from *E. coli*. This discrepancy may have three possible explanations. First, the FTIR technique is insensitive to detect hydrogen bonding (Parikh and Chorover, 2006). Second, the level for LPS in *E. coli* K-12 bacterial cell envelope is much less than those for the phospholipid and protein, which are 4.73 and 4.83 times, respectively, higher than LPS level in terms molar ratio (Gmeiner and Schlecht, 1980). Third, the BGs maintain the phospholipid backbone structure with embedded protein, which may possibly compete with the LSP for absorption sites on the nanoparticles surfaces and thus leading to insignificant changes in the 2D-COS-FTIR response in the polysaccharide region. Whereas the study using extracted pure LPS only qualitatively represent the tendency of LPS to interact with nanoparticles regardless the three-dimensional structure of cell envelope. The current study cannot exclude the possibility of the interaction between LPS (polysaccharide) and TiO$_2$ nanoparticles when nanoparticles approach the bacterial surface. Nevertheless, our major findings herein indicate that protein plays dominant role in the interaction between TiO$_2$ nanoparticles and bacterial cell membrane. This was corroborated with the results from the settling experiments using standard protein, polysaccharide and phospholipid, which indicated the protein shown remarkable ability to form hetero-agglomeration with nanoparticles. Additionally, the asynchronous map of 2D-FTIR-COS indicates the propensities of functionalities bonded to TiO$_2$ nanoparticle followed as: COO$^-$ > aromatic C=C stretching > N-H, amide II > C=O, ketone.

Knowledge on the interaction capacity and sequences of different biomolecules and functional
groups of cell membrane to nanoparticles is supposed to bring new insight into the
nanoparticle-membrane interactions and help to explain the toxicity of nanoparticles. For example,
the nano-toxicity may possibly depend on the adhesion of nanoparticles on the cell membrane
protein. Indeed, the cell membrane protein are suggested to be protected by the cell surface
polysaccharide polymers and thus unlikely to interact with large particles. The interaction between
the polysaccharide and large particle surface is unlikely to induce toxicity because this is similar to
the manner in which bacteria adhere to large surfaces through the surface polymers in natural
environment (Neu and Marshall, 1990). In contrast to their bulk counterparts, nanoparticles have
extremely small sizes and therefore very likely to be able to travel across the gap between the
surface polysaccharide polymers and reach the cell membrane surfaces. However, there exists
consensus that nanoparticles with high surface energy typically tend to aggregate to form
micro-scale agglomerates due to the unspecific interaction, thereby lowering their surface energy.
Thus the interaction behavior between nanoparticles and cell membrane has been frequently
elucidated as nanoparticles agglomerates with the cell membrane under static water condition and
underlying mechanism could be interpreted by the DLVO theory which considers the sum of
electrostatic and van der Waals interaction. This study conduct experiment under circumneutral
condition which is close to the IEP of TiO$_2$ nanoparticles and thus tend to aggregate according to
DLVO theory. In this case, there arises a concern that the results obtained from the micro-scale TiO$_2$
agglomerates did not realize the understanding in nano-scale. Nevertheless, it is important to note
that the DLVO theory were based on the assumption of steady-state behavior of agglomerates and
under static water conditions. We must recognize, however, that possible disruptions of aggregates
due to the force induced by water flows (i.e. friction and lubrication or shear force) should be
considered (Min et al., 2008; Nel et al., 2009). More importantly, natural and engineered aquatic systems typically under flowing condition. Therefore, the influence was environmental relevant and expected because of relative high agitation speed (200 rpm) were applied in this study. As a result, there should be likelihood of single nanoparticle or rafts from multiple particles directly bonding to the cell membrane surface protein due to their high propensity to interact with protein in the TiO$_2$-water-BGs system. This may lead to conformational changes of the protein and could be a possible reason for nanoparticle cytotoxicity. In this study, the changes in secondary structures of the protein in the BGs were characterized by the infrared self-deconvolution with second derivative resolution enhancement and with curve-fitting (Fig. S8). Results showed a decrease in $\alpha$-helix contents and increase in the unordered and aggregate strands contents after exposed to nanoparticles (Table 2), indicating that the protein secondary structures were significantly changed and partial protein unfolding occurred after interacting with TiO$_2$ nanoparticles (Wu and Narsimhan, 2008). This is in accordance with the results of a previous study (Jiang et al., 2010), which also suggested that the protein damaged when exposed to TiO$_2$ nanoparticles and as a consequence leading to loss physiological activities.

4.2 Significance for understanding the transport and fate of TiO$_2$ nanoparticles in natural system.

In fact, apart from NOM and bacteria, protein are ubiquitously present in aquatic environments, particularly in wastewater-impacted water, as a result from microbial metabolism or anthropogenic input (Hudson et al., 2007; Meng et al., 2013). TiO$_2$ nanoparticles is increasingly being used in commercial products and it will be inevitably released into aquatic environments. Therefore, TiO$_2$ will finally meet NOM, bacteria and protein, which are likely to influence their transport. A
A previous study observed significant change in the aggregation state and deposition of TiO$_2$ nanoparticles in the presence of bacterial cells or/and NOM due to the changes of surface properties (Chowdhury et al., 2012). Nevertheless, how the protein affect the transport and fate of TiO$_2$ nanoparticles has seldom been considered. Our results herein suggested that the protein of the cell envelope or in the bulk solution play a critical role in the interaction with TiO$_2$ nanoparticles. Thus, the actual transport and fate of TiO$_2$ nanoparticles could be altered because the strong binding role of protein, considering the ubiquitousness of soluble protein in aquatic systems, especially in the anthropogenic-impact urban river with high SMP input from the waste water treatment plants (WWTPs) effluent.

### 4.3 Implications for the transport and fate of TiO$_2$ nanoparticles in engineered system.

As the products of human activity, commercial TiO$_2$ nanoparticles have a high likelihood of entering municipal sewage that flows to centralized WWTPs, in which biological treatments were typically applied. It is very plausible that the majority of TiO$_2$ nanoparticles will be attached to the cell surface proteins of the activated sludge (mainly microorganisms) therein and their fate will be accompanied with the activated sludge. Given that the activated sludge could end up being as agricultural land amendments (fertilizers), placed in landfills, incinerated, or dumped into oceans (Kiser et al., 2009), the subsequent ecological impact and relative risk assessment remain unexplored and should be taken into consideration and examined in the future. In addition, the high propensity of proteins or COO$^-$$^-$-rich substances to interact with TiO$_2$ nanoparticles provides a plausible clue for their removal in industrial wastewater where tremendous amount of TiO$_2$ nanoparticles waste are produced. Microorganisms/protein or COO$^-$$^-$-rich substances could act as coagulant to remove TiO$_2$ nanoparticles wastes by sequential treatments of coagulation, flocculation
and sedimentation (Serrao Sousa et al., 2017).

4.4 Technological aspects

The elucidation of nanoparticle-membrane interaction is beneficial for the design of novel nanoparticles which can work effectively in the presence of bacteria in water and wastewater treatment. Furthermore, the nanoparticle properties (i.e. sizes and shapes) will also influence the nanoparticle-cell membrane interaction (Tong et al., 2013b; Lin et al., 2014); thus more types of TiO$_2$ nanoparticles with different sizes and morphologies (i.e. nanotubes, nanorods and nanosheet, etc.) should be examined in the future. On the other hand, variation in bacterial cell envelope structure profiles (i.e. lipids with different tail length or degree of saturation, the levels of outer membrane protein, and lipopolysaccharide, etc.) could be manipulated via genetic approaches using relative mutants (Gao et al., 2012; Huang et al., 2015). BGs derived from the cell envelope-related mutants will enable one to determine the role of the interested gene products in the interaction between cell membrane and nanoparticles. Additionally, as membrane construction of Gram-positive and the Gram-negative bacteria are different, further studies using the BGs derived from Gram-positive bacteria (Abrams and McNamara, 1962) are therefore warranted. In general, the approach using BGs as model cell membrane combined with the 2D-FTIR-COS technique would provide an ideal platform to reveal the bionano surface interaction mechanism at molecular level.

5. Conclusions

The interaction between TiO$_2$ nanoparticles and bacterial cell membrane was investigated at molecular level using 2D-FTIR-COS analysis and BGs as model cell envelope. The main
conclusions are:

- The synchronous map of 2D-FTIR-COS results shown that the functionalities in proteins of BGs have high propensity to interacted with TiO₂ nanoparticles, whereas the interaction of TiO₂ nanoparticles with polysaccharides (C–OH) and phospholipids (P=O) were not detected under the test condition.

- The asynchronous map of 2D-FTIR-COS suggested a sequential order of functionalities bonded to TiO₂ nanoparticles with the order from high to low: COO⁻ > aromatic C=C stretching > N-H, amide II > C=O, ketone. These findings highlighted the role of protein in the interaction mechanisms between nanoparticles and bacterial cell membrane.

- Co-settling of TiO₂ nanoparticles with pure biomolecules (i.e., protein, polysaccharide and phospholipid) also highlighted the high propensity of protein molecules to interact with TiO₂ nanoparticles.

- 2D-FTIR-COS analysis using BGs as model cell membrane were shown to be a promising approach to investigating the molecular mechanisms by which nanoparticles interacting with bacterial cell membrane.

- This study could enhance our current knowledge on interaction mechanism of TiO₂ nanoparticles with bacterial cell membrane in water and has important implication for the nanotoxicity as well as the transport and fate of TiO₂ nanoparticles in the natural and engineered systems.

**Associated content**

**Supporting Information**
Additional detail information including protocols for light microscopy observation, scanning electronic microscopy (SEM), transmission electron microscopy (TEM), atomic force microscopy (AFM), X-ray diffraction patterns (XRD) and curve-fitted FTIR spectra; and Tables showing the minimum inhibition concentration and maximum growth concentration for *E. coli* K-12 (Table S1), and DNA contents of BGs and normal bacterial cells (Table S2); and Figures showing the XRD pattern (Fig. S1) and TEM image (Fig. S2) of Degussa P25TiO₂ nanoparticles, fluorescence intensity evolution (Fig. S3), light microscopy images (Fig. S4), Agarose gel electrophoresis results (Fig. S5), FTIR spectra of BGs with TiO₂ nanoparticles (Fig. S6) and TiO₂ nanoparticles alone (Fig. S7), second derivative resolution enhanced and curve-fitted amide I region for protein secondary structure of BGs (Fig. S8).

### Acknowledgments

The project was supported by a research grant (GRF14100115) of the Research Grant Council, Hong Kong SAR Government. The project was also support by a Direct Grant from the Research Committee and a Technology and Business Development Fund (TBF15SCI008) from the Office of Research and Knowledge Transfer Services of The Chinese University of Hong Kong, and the research grant (41573086 and 41425015) of National Science Foundation of China to G.Y. Li and T.C. An. P.K. Wong was also supported by CAS/SAFEA International Partnership Program for Creative Research Teams.
Figure Captions

**Fig. 1** Fluorescence contour plots of the SMP secreted by (a) *E. coli* cells under dark; (b) *E. coli* K-12 under dark with the presence of TiO$_2$ nanoparticles (100 mg/L).

**Fig. 2** SEM and AFM images of the normal *E. coli* K-12 cell (a, c) and bacterial ghost (b, d), respectively.

**Fig. 3** Synchronous (a, b) and asynchronous (c, d) 2D-FTIR-COS maps generated from the 1700-1300 cm$^{-1}$ region (a, c) and 1700-1000 cm$^{-1}$ region (b, d) of the FTIR spectra of BGs with the increasing TiO$_2$ nanoparticles concentrations.

**Fig. 4** (a) Settling curves of standard protein, (b) polysaccharide, (c) phospholipid with 100 mg/TiO$_2$ nanoparticles; and (d) the difference of absorbance at 660 nm of the test substances before and after mixed with TiO$_2$ nanoparticles.

**Fig. 5** Photos of the settling experiments of test substances with TiO2 nanoparticle.
References


functionalized gold nanoparticles between water and lipid bilayers as model cell membranes.

*Environmental Science & Technology* 46(3), 1869-1876.


Kiwi, J. and Nadtochenko, V., 2005. Evidence for the mechanism of photocatalytic degradation of...
the bacterial wall membrane at the TiO$_2$ interface by ATR-FTIR and laser kinetic spectroscopy.

*Langmuir* 21(10), 4631-4641.


Table 1. 2D-FTIR-COS result on the assignment and sign of each cross-peak in synchronous and asynchronous (in the brackets) map of BGs with increasing TiO$_2$ nanoparticle concentrations (Signs were obtained in the upper-left corner of the maps).

<table>
<thead>
<tr>
<th>Region</th>
<th>Position (cm$^{-1}$)</th>
<th>Possible assignment</th>
<th>Protein</th>
<th>Sign</th>
<th>Phosphate</th>
<th>Polysaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>1612 - 1674</td>
<td>amide I, C=O stretching</td>
<td>+</td>
<td>(+)</td>
<td>+ (+)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1565 - 1612</td>
<td>aspartate or glutamate COO$^-$ symmetric stretching</td>
<td>+</td>
<td>(+)</td>
<td>+ (+)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1550 - 1565</td>
<td>amide II, N-H, C-N of protein</td>
<td>+</td>
<td>(+)</td>
<td>+ (+)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1400 - 1500</td>
<td>aromatic C=C stretching, C-H bend from CH$_2$</td>
<td>+</td>
<td>(+)</td>
<td>+ (+)</td>
<td></td>
</tr>
<tr>
<td>Phosphate</td>
<td>1200 - 1250</td>
<td>P=O from phosphate</td>
<td>0</td>
<td>+ (0)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Polysaccharide</td>
<td>1100 - 1170</td>
<td>C-O stretching of polysaccharide</td>
<td>0</td>
<td></td>
<td>0</td>
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</table>
Table 2. Band assignments for protein secondary structures of BGs and changes of the protein secondary structures exposed to increasing TiO$_2$ nanoparticles concentrations estimated by the curve fitting of the amide I region (1600 -1700 cm$^{-1}$) from the FT-IR spectra.

<table>
<thead>
<tr>
<th>Secondary structures</th>
<th>Wavenumber (cm$^{-1}$)</th>
<th>TiO$_2$ concentrations (mg/L)</th>
</tr>
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<tbody>
<tr>
<td>Aggregated strands</td>
<td>1625-1610</td>
<td>0 %</td>
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<tr>
<td>β-Sheet</td>
<td>1640-1630</td>
<td>25</td>
</tr>
<tr>
<td>Unordered</td>
<td>1645-1640</td>
<td>50</td>
</tr>
<tr>
<td>α-Helix</td>
<td>1657-1648</td>
<td>100</td>
</tr>
<tr>
<td>3-Turn helix</td>
<td>1666-1659</td>
<td>150</td>
</tr>
<tr>
<td>Antiparallel β-sheet/aggregated strands</td>
<td>1695-1680</td>
<td>200</td>
</tr>
</tbody>
</table>
Fig. 1

(a) *E. coli* K-12 under dark

(b) *E. coli* K-12 under dark with TiO$_2$ nanoparticles
Fig. 2
Fig. 3
Fig. 4

Protein

(a) $A/A_0$

Polysaccharide

(b) $A/A_0$

Phospholipid

(c) $A/A_0$

(d) $\Delta A$ (cm$^{-1}$)

Protein Polysaccharide Phospholipid
Fig. 5

<table>
<thead>
<tr>
<th>Time</th>
<th>TiO₂⁺</th>
<th>Protein</th>
<th>TiO₂⁺</th>
<th>Polysaccharide</th>
<th>TiO₂⁺</th>
<th>Phospholipid</th>
</tr>
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<tbody>
<tr>
<td>0 h</td>
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<td>1 h</td>
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<td>3 h</td>
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<td>11 h</td>
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</table>
Highlights

- Interaction between bacterial cell membranes and nano-TiO$_2$ were studied at molecular level.
- Bacterial ghosts devoid of cytoplasm were used as model cell membrane.
- 2D-COS results suggested a sequential order of functionalities bonded to nano-TiO$_2$.
- Protein played the most important role in the interaction mechanism.