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Polyethyleneimine-Mediated Flocculation of *Shewanella oneidensis* MR-1: Impacts of Cell Surface Appendage and Polymer Concentration

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ABSTRACT

In wastewater treatment plants, optimizing bacterial flocculation and bacterial sludge dewatering requires a detailed understanding of the concomitant biological and physico-chemical processes governing the action of flocculating agent on living cells. Here we investigate the interactions between polyethyleneimine (PEI, 60000 g/mol) and *Shewanella oneidensis* MR-1 lacking or not the lipopolysaccharide (LPS) O-antigen surface structure. Flocculation tests were performed on bacteria with/without LPS O-antigen after being exposed to 0-100 mg/L PEI concentrations. Measurements of electrophoretic mobility and bacterial aggregates size were complemented by transmission electron micrographs and atomic force microscopy images. While low PEI concentrations (< 20 mg/L) lead to flocculation of both bare and LPS O-antigen-decorated bacterial strains, the lysis of bacterial membranes occurred at larger polymer concentrations for the latter, which highlights the protective role of LPS O-antigen against harmful PEI-mediated membrane alterations. Depending on polymer concentration, two types of bacterial aggregates are identified: one that solely integrates bacterial cells, and another that includes both cells and cell residues resulting from lysis (membrane and/or LPS fragments, and inner cell content materials). The latter is expected to significantly contribute to water entrapping in sludge and thus lower dewatering process efficiency.

Keywords: Flocculation - Polyethyleneimine - Bacteria - *Shewanella oneidensis* MR-1 - Lysis - Permeabilization

1. Introduction

The polyethyleneimine (PEI) macromolecule is used in numerous applications, ranging from coagulation/flocculation of bacterial wastewater sludge (Legrand et al., 1998) to permeabilization of gram-negative bacteria membranes (Alakomi et al., 2006) leading to bacterial lysis (Beyth et al., 2008). In municipal wastewater treatment plants, the activated sludge process consists in the use of bacterial flocs for digesting the wastewater organic matter. After the settling of those aggregates to yield the

clarified water, most of the bacterial sludge is recycled to biological treatment processes, while the remaining sludge is conditioned with the addition of high molecular weight polymers such as PEI, and/or ferric chloride and lime (Deneux-Mustin et al., 2001). The procedure, that is followed by a mechanical dewatering step, results in the final collection of a bacterial cake containing at best about 70% water. Such a high water content leads to severe difficulties in terms of storage, and sludge biological post-treatment (incineration). In order to optimize the efficiency of dewatering procedure and reduce water content in bacterial sludge, detailed knowledge on the necessarily coupled biological and physico-chemical action modes of PEI on living bacterial cells is critically needed.

Bacterial communities in sludge wastewater are highly diversified and structurally organized in aggregates (Bourrain et al., 1999; Martins et al., 2004; Chaignon et al., 2002). Several studies pointed out that extracellular polymeric substances (EPS) produced by bacteria act as ligands between cells, which maintains a mechanical cohesion of the bacterial consortium (Jorand et al., 1995; Keiding and Nielsen, 1996). The presence of negative charges carried by the EPS (*i.e.* extra-cellular polysaccharides including lipopolysaccharide (LPS)) favors the binding of multivalent counter-ions that, in turn, bridge EPS chains belonging to adjacent cells, thus ensuring a given stability and strength of the formed flocs. These ions may further lead to an increase of the overall osmotic pressure of the system and thus to the trapping of interstitial water, which lowers dewatering treatment efficiency (Mikkelsen and Keiding, 1998; Curvers et al., 2009). In addition, the presence of extracellular polymeric substances seems to significantly impact sludge dewatering because EPS play a key role in controlling the erosion of bacterial flocs under shear flow conditions (Mikkelsen and Keiding, 1998). The amount of added flocculent is another important factor which impacts wastewater sludge dewatering. With increasing flocculent concentration, dewatering process is first improved before reaching a maximal efficiency (Marinetti et al., 2010). Waite suggested that increasing the concentration of high molecular weight cationic polymer in bacterial suspensions, results in an increase of floc size and an enhancement of dewatering efficiency (Waite, 2006). He further underlined that for some critical state of floc compactness, water cannot flow through pores of flocs anymore, but instead has to bypass the floc

structure. Depending on the size of the bacterial aggregates, this can significantly lower the efficiency of dewatering process.

Although the types of associations between the flocculent and the sludge remain unclear, the aforementioned study suggests that there is some optimal floc size, reached for an optimal dose of polymeric flocculating agent, that leads to the best dewatering yield. Such dose, corresponding to an 'ideal coagulation state' in terms of dewatering, is not well-defined. The major reason for this is the critical lack of knowledge on the nature of / coupling between the bio-physico-chemical processes that determine the destabilization mechanisms of bacterial suspensions and the aggregates state after exposure to cationic polymers such as PEI. Many studies attempted to characterize flocs with the help of structure modelling and concept of fractal dimension (Martin et al., 2004; Chaignon et al., 2002; Guan et al., 1998) but few focus on the very action of polymeric flocculating agent on bacterial sludge. In addition, the alterations of bacterial membranes after contact with PEI and the corresponding impact on flocculation processes are hardly considered (Vaara, 1992; Nian et al., 2008). Nikaido (1989) showed that the LPS structure at the periphery of bacterial membranes exhibits a low permeability, thus acting as a protective barrier against potentially harmful (macro)molecules. Any disturbance in LPS organization was further shown to affect the overall bacterial permeability (Nikaido, 2005). The binding of PEI to LPS leads to a release of stabilizing counter-ions (Vaara, 1992; Nian et al., 2008), which, in turn, results in a disorganization and possible removal of LPS fragments. This is accompanied by a reordering of phospholipids that come in replacement of removed LPS (Mikkelsen and Keiding, 2002), which explains how PEI causes membrane permeabilization, and possible cellular material release (Arrington et al., 2008). All these elements unambiguously question the validity of studies where bacteria are viewed as hard (impermeable), stable inert particles when in contact to PEI. In that respect, interpretations of electrokinetic data on bacterial cells according to hard sphere models are necessarily approximate (Duval and Gaboriaud, 2010) and so are interpretations of PEI-cell electrostatic interactions according to standard DLVO theory (Duval et al., 2011). After PEI conditioning, the bacterial membranes may not remain intact, some inner bacterial materials may be released to the outer solution,

thus resulting in major deviations from oversimplified theoretical DLVO predictions (Mikkelsen and Keiding, 2002). Finally, the effects of EPS on the flocculation of LPS-coated gram negative bacteria remain poorly known.

In this study, we investigate the mechanisms governing the destabilization of bacterial suspensions after addition of PEI (molecular weight 60000 g/mol). The analysis is carried out for *Shewanella oneidensis* MR-1 (Myers and Nealson, 1988), that have the particularity to produce various lengths of LPS O-antigen depending on its temperature of growth (Korenevski et al., 2002). We have chosen two growth temperatures, resulting in the maximal (20°C) and minimal (30°C) length of produced LPS. This strategy allows us to work with cells of identical genotype but different phenotypes in terms of surface structure.. The work aims at elucidating the effects of PEI on bacterial membrane, subsequent bacterial flocculation, and structure of formed flocs for strains exhibiting or lacking the LPS O-antigen surface structure. The analysis is based on systematic measurements of electrophoretic mobility and size of PEI-bacterial aggregates over a large range of PEI concentrations, and it is complemented by TEM and AFM images of flocs.

2. Materials and methods

2.1 Bacterial culture and cell conditioning

Shewanella oneidensis MR-1 (Myers and Nealson, 1988) was cultured at 20°C or 30°C for 14 to 16 hours in Lysogenic-Broth (LB Broth, Miller, Difco™), under agitation (160 rpm), in Erlenmeyer flasks with a volume filling ratio of 1 to 5 so as to maintain identical oxygenation conditions. Bacterial cells were harvested in stationary phase and washed twice in 1 mM KNO₃ solution (prepared in deionised water, Millipore-MilliQ, 18.5 MΩ/cm) by centrifugation (15 min, 5000 G). At each stage of the preparation, the bacteria suspensions were kept at their corresponding growth temperature (i.e. 20°C and 30°C respectively). Finally, samples were resuspended in 1 mM KNO₃ solution, and the optical density at 600 nm was adjusted to 1 (ca. 10⁹ cells/mL).

Shewanella oneidensis MR-1 was selected because it produces varying length of LPS O-antigen depending on growth temperature (Korenevski et al., 2002). At 30°C, the produced LPS chains do not include the O-antigen part whereas at 20°C, the length of O-antigen is maximal (between 30 nm and 50 nm) (Supplementary data, Figures A-B). It is recalled that LPS consists of 3 distinct parts: lipide A, core and O-antigen that decorates the membrane.

2.2 Preparation of flocculated suspensions

Flocculation tests were carried out at room temperature, in 100 mL polymethylmethacrylate (PMMA) reactors (5 cm diameter) fitted with 4 PMMA baffles (0.5 x 6 cm). Polyethyleneimine (PEI) (60000 g/mol, purchased from Polyscience Europe GmbH) was used as a flocculating agent. Various PEI concentrations (0-100 mg/L) were tested (batch experiments). The suspensions were then mixed using a rectangular blade located at one-third of the reactor height from the bottom. The mixing procedure consisted of two steps: a fast stirring stage, 250 rpm for 3 minutes, followed by a slow stirring stage, 60 rpm for 46 minutes. Conductivity and pH measurements were monitored during mixing and AFM observations were made straightaway after this mixing step. Finally, suspensions were settled in graduated Imhoff cones for 1 hour. The residual turbidity (or optical density) of the supernatant was measured spectrometrically at 600 nm on tenfold diluted samples in 1 mM KNO₃. Sedimented materials were collected after the settling of the suspensions and were then analysed by TEM. The integrity of cell membranes after exposure to PEI was evaluated from crystal violet adsorption/release experiments, as detailed in Supplementary data.

2.3 Electrophoretic mobility

Electrophoretic mobility measurements were performed for the supernatants containing unsettled bacterial flocs using a Zetaphoremeter IV (CAD Instrumentations, France). The cells in the supernatant were first diluted in 1 mol/L KNO₃ solution in order to carry out electrokinetic measurements at fixed ionic strength over the whole range of PEI concentrations. Electrophoretic mobilities of bacterial

aggregates were determined from the reflection of a laser beam and particle trajectories tracked with a charge-coupled device camera. Recorded images were then processed (Zetaphorometer 4.30, CAD Instrumentation) to calculate mobility from the displacement of particles subjected to a constant direct-current electric field (800 V/m). Three cycles were recorded to carry out at least 100 measurements of aggregate mobility for each PEI concentration investigated. Three independent assays were performed according to the aforementioned procedure.

2.4 Size measurements

The fact that size of bacterial aggregates was measured before the settling avoided artifacts due to possible change in aggregate structure. The measurements were performed with a Sympatec Helos particle size analyser using the Fraunhofer approximation. Suspensions were diluted in 1 mol/L KNO₃ solution and gentle stirring allowed an homogenisation of suspensions before they were brought to the analysis cell through a peristaltic pump. For each experiment, duplicate measurements were performed. The reported sizes refer to the diameter of hard spheres equivalent to those of the targeted aggregates in terms of diffracted light intensity (Chaigon et al., 2002).

2.5 Transmission electron microscopy

The settled aggregates were embedded in an epoxy resin for observation using a Philips CM20 transmission electron microscope operating at 80kV acceleration voltage. To that end, a classical inclusion procedure was adopted (Lartiges et al., 2001). The cohesion of bacterial aggregates was ensured by the addition of 2% glutaraldehyde for 4 hours. Aggregates were then contrasted in 2% osmium tetroxyde solution for 1 hour. Between each step of preparation, the excess of reactive was removed by washing. Then, samples were dehydrated upon successive additions of acetone at final concentrations of 10, 20, 40and 60% (v/v), with 5 min delay between each step, and then at concentrations 80% (15 min), 90% (2x20 min) and finally 100% (v/v) (3x20 min). Subsequently, the acetone was gradually exchanged with epoxy resin monomers (Kit Embed 812, Euromedex). Finally, the

samples were placed in molds, and resin monomers were polymerized at 60°C for 12 hours. The resin-embedded samples were cut in ultrathin sections (1 μm) using an ultramicrotome (ultra-cut E, Reichert-Jung). These sections were then stained with uranyl acetate and lead citrate to enhance resolution of organic matter (Reynolds, 1963) before positioning on copper grids. To properly address the damages of bacterial membranes caused by PEI, analysis was performed on at least 100 bacteria.

2.6 Atomic force microscopy

Before the sedimentation step, the bacterial aggregates were deposited onto glass slides. After 20 minutes, the surface coated with aggregates was washed with Milli-Q water. The samples were then immediately transferred into the AFM cell and AFM images were recorded in air atmosphere using an MFP3D-BIO instrument operating in contact mode (Asylum Research Technology, Atomic Force F&E GmbH, Mannheim, Germany). Silicon nitride cantilevers of conical shape (MLCT-AUNM, Veeco Instruments SAS, Dourdan, France) were used, and their spring constants, denoted as k , were determined following thermal calibration method (Vadillo-Rodrigues et al., 2004), providing k values of $\sim 10.4 \pm 1.7$ pN nm⁻¹. Prior to any experiment, the geometry of the AFM tip was systematically controlled using a commercial grid for 3-D visualization (TGT1, NT-MTD Compagny, Moscow, Russia) and curvature of the tip in its extremity was found to lie in the range ~ 20 to 50 nm.

3. Results

3.1 PEI-mediated flocculation of bacteria devoid of LPS O-antigen surface structure

For PEI concentration ranging from 0 to 17.5 mg/L (regime A1, Figure 1A), optical density of the supernatant decreases from 1.2 to 0.2, and particle size increases up to ~ 25 -30 μm . These trends typically reflect a PEI-mediated aggregation of the bacteria considered in this section. When increasing PEI concentration from 17.5 mg/L to 40 mg/L (regime A2, Figure 1A), optical density rises up to 1.1, a value comparable to that measured before addition of PEI, and aggregates size clearly decreases down to 15 μm . Finally (regime A3, Figure 1A), for PEI concentrations above 40 mg/L, optical density drops to

~0.2 whereas the size of aggregates increases again to *circa* 30 μm , which highlights a second aggregation stage.

The electrokinetic measurements performed on bacteria devoid of LPS O-antigen are reported in Figure 1B as a function of PEI concentration. Similarly to Figure 1A, the three regimes A1-A2-A3 can be clearly distinguished. Indeed, electrophoretic mobility remains roughly constant in regime A1 ($\sim -0.6 \cdot 10^{-8} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}$) and then decreases (in magnitude) to ~ 0 at the onset of regime A2. This is explained by a gradual neutralization of negative charges carried by bacterial particles (isolated cells and/or aggregates) following adsorption of PEI (cationic polymer). For further increase of PEI concentration (regime A2 to regime A3), the mobility increases in magnitude to finally reach a constant value of $\sim -0.4 \cdot 10^{-8} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}$ in regime A3. This suggests the presence of negatively charged bacteria and/or bacterial aggregates in the medium at this high PEI concentration. Measurements of the suspension pH and conductivity (Figure 1C) as a function of PEI concentration provide qualitative information on the permeabilization state and integrity of bacterial membranes. In details, pH continuously increases for polymer concentration corresponding to regime A1 and A2. Comparison with pH data obtained in the absence of bacteria (Supplementary data, Figure C) demonstrates that pH is solely determined by PEI when added in concentrations larger than 40 mg/L. For lower polymer content in solution, PEI is adsorbed onto the bacterial membranes and bacterial residues, thereby leading to a lower pH as compared to that for situation where bacteria are absent. Quantitative inspection of conductivity data measured as a function of PEI concentration in the presence and absence of bacteria (Figure 1C and Supplementary data, Figure D, respectively) further suggests that interactions between PEI and bacteria/bacterial aggregates result in the release of ions and/or charge carriers (*e.g.* cell residues) possibly originating from deterioration of cell membranes. This result, that is supported by AFM and TEM images discussed below, is confirmed by crystal violet absorbance/release tests. A three-fold increase of crystal violet amount in the supernatant within the regime A2 of polymer concentration (Supplementary data, Figure E), pictures a significant permeabilization of bacterial membranes.

Figure 2 shows typical images obtained by TEM (panels A1.a, A2.a, A3a) and AFM (panels A1.b, A2.b, A3.b) for bacterial aggregates formed after exposure of bacteria to PEI in regimes A1, A2 and A3. For PEI concentrations corresponding to regime A1 (≤ 17.5 mg/L), both AFM and TEM (panels A1.a,b) suggest that bacterial cells are organized in aggregates where the membrane structure of constituting bacteria do not appear significantly damaged. Nevertheless, protruding spherical vesicles of about 200 nm in diameter may be clearly identified on the outer bacterial membranes (see red squares indicated in Figures 2A1.a and 2A1.b). The fact that those vesicles are visible in both AFM and TEM images attest that they are not the result of resin-embedding artifacts. Detailed observations reveal that $\sim 30\%$ of the bacteria within the aggregates exhibit such vesicles at their outer periphery. Upon a further increase in PEI concentration (regime A2, Figures 2A2.a and 2A2.b), bacteria remain organized in aggregates form, the aspect of which considerably differs from that in regime A1: aggregates are more compact and the identification of individual bacteria within a given aggregate becomes difficult. Severe damages of the bacterial membranes now confer the aggregates a puffy appearance. TEM micrographs point out the presence of spherically wrapped cell envelope residues (50 to 100 nm in diameter) located at the outermost regime of the bacterial aggregates (see red squares in Figure 2A2.a). 75% of the aggregated cells show this type of membrane damage. Finally, for sufficiently high PEI concentrations (> 40 mg/L, regime A3), it becomes impossible to distinguish individual cells within a given aggregate (Figure 2A3.b). Aggregates now appear much more inflated than in regime A2 and numerous cell material residues may be observed (Figure 2A3.b). In addition, long filamentous structures, possibly associated with fragments of released cytoplasm (see Figure 2A3.a), are identified and suggest that the bacterial lysis is now complete.

3.2 PEI-mediated flocculation of bacteria with LPS O-antigen decorated membrane

The dependence of optical density, size, electrophoretic mobility, pH and conductivity on PEI concentration are depicted in Figure 3 for suspensions of bacteria that exhibit LPS O-antigen surface appendage. This figure constitutes the pendant of Figure 1 discussed in the previous section for bacteria

devoid of LPS-O antigen surface structure. Three regimes of PEI concentration that correspond to peculiar evolutions (discussed below) of the aforementioned parameters with increasing PEI concentration can be distinguished B1 (≤ 20 mg/L), B2 (20 mg/L $<$ PEI concentration ≤ 60 mg/L) and B3 (PEI concentration ≥ 60 mg/L), respectively.

B1 represents the regime where bacterial aggregation takes place, as judged from the decrease of the optical density from 1.1 to 0.2 and the increase of aggregate size up to ~ 80 μm for PEI concentration ~ 20 mg/L (Figure 3A). Upon further increase in PEI concentration (regime B2), the optical density remains approximately constant (around 0.2) before slightly increasing to 0.4 at 60 mg/L PEI concentration. Aggregate size (Figure 3A) abruptly drops to ~ 40 μm for a ~ 20 mg/L PEI concentration and does not significantly vary with further increase in PEI content within regime B2. Finally, for PEI concentrations higher than 60 mg/L (regime B3), the optical density is constant and sharply drops to a 0.2 value at the highest PEI concentration investigated. Moreover, the size of aggregates decreases to about 25 μm . Regarding electrokinetics (Figure 3B), the electrophoretic mobility dramatically increases (in magnitude) from $\sim -0.5 \cdot 10^{-8} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}$ to $-2 \cdot 10^{-8} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}$ for an increase in PEI concentration from 0 to 20 mg/L (regime B1). This indicates that the bacterial aggregates carry an apparent volume charge density that becomes increasingly negative upon the addition of cationic PEI up to a concentration of 20 mg/L. Above a PEI concentration of 20 mg/L, the aggregate mobility dramatically decreases in magnitude (regime B2) and even changes sign at 60 mg/L before stabilizing around zero for PEI concentration in the 60-80 mg/L range. This suggests neutralization of negatively charged groups located within/at the surface of bacterial aggregates following PEI adsorption.

Membrane permeability experiments (Supplementary data, Figure E) measured by the release of intracellular crystal violet in the supernatant of bacterial suspensions slightly increases from 5 to 10 % within the regime B1 of PEI concentration, and further increases up to $\sim 16\%$ for PEI content ~ 30 mg/L. The absorbance then levels off or even slightly decreases with further increase of PEI concentration. The release of crystal violet for PEI concentration in the range 0-30 mg/L, reflects a significant

permeabilization of the cell membranes. This permeabilisation goes in pair with the release of ions and/or charged cell residues that contribute to an increase in conductivity (Figure 3C), which is further supported by conductivity measurements carried out in the absence of bacteria (Supplementary data, Figure D). Finally, similarly to the situation for O-antigen-free bacteria, the adsorption of PEI onto outer bacterial membrane and/or LPS-O antigen appendage results to a lower pH (Supplementary data, Figure C) as compared to that of solutions where bacteria are absent. For excess of PEI, suspensions with or without bacteria have similar pH values.

Figure 4 presents typical TEM and AFM observations of PEI-mediated aggregates of bacteria exhibiting (before introduction of PEI) LPS-O antigen surface structure in regimes B1 (panels B1a,b), B2 (panels B3a,b) and B3 (panels B3a,b). In agreement with the results discussed above (Figure 3A), Figures 4B1.a and 4B1.b underline the presence of bacterial aggregates for PEI concentrations lower than 20 mg/L (regime B1). Closer examination of TEM micrographs reveals that about 75% of bacteria exhibit superficial damages that take the form of protruding membrane residues (red squares in Figure 4B1.a). One may further note the presence of spherical outgrowths (~200 nm in diameter, Figure 4B1.b) at the surface of bacterial aggregates. Some of these residues may be clearly identified at the glass surface that supports the aggregates. With increasing PEI concentration to 60 mg/L (regime B2), TEM and AFM observations (Figures 4B2.a and 4B2.b, respectively) still display bacterial aggregates but the damages of cell membranes are now amplified (see red squares in panels 4B2.a,b), both in number and in severity. About 50 to 70% of the bacterial cells now present at their outer periphery a dramatic loss of membrane integrity visible as spherical nodules leading to membrane breaking. For PEI concentrations above 60 mg/L (regime B3), the aspect of bacterial aggregates is significantly different as compared to that at lower PEI concentrations. Indeed, the previously mentioned spherical outgrowths at the cell membrane have evolved to large asperities (see red square in Figure 4B3.a), and for some of the bacteria, an entire loss of the cytoplasmic content is recognizable as a rough thin film on the AFM glass slide (see red square in panel 4B3.b), that likely consists of released cell materials. The analysis of TEM

micrographs indicates that more than 60% of the bacteria exhibit membrane breakup/perforation events and release of their inner cytoplasmic content.

4. Discussion

4.1 PEI-mediated flocculation of bacteria devoid of LPS O-antigen surface structure

The dependence of optical density, aggregate size, electrophoretic mobility, pH and conductivity on PEI concentration (Figure 1) clearly demonstrates the succession of three distinct regimes for the formation of bacterial aggregates upon gradual addition of PEI. For PEI concentrations lower than 17.5 mg/L, bacterial cells are present in the form of aggregates and cell membranes do not display severe damages. The occurrence of bacterial aggregation at such PEI concentrations is fully consistent with the corresponding dramatic decrease (in magnitude) of the electrophoretic mobility measured for the aggregates. Indeed, this decrease indicates that the negative bacterial charges are gradually neutralized by adsorbed (and positively charged) PEI chains at the bacterial surfaces, thus leading to a decrease of the interparticular repulsive interactions that favor aggregation *via e.g.* the formation of PEI bridges. For larger PEI concentrations (17.5-40 mg/L), the electrophoretic mobility remains, at first, close to a zero value and then increases in magnitude for PEI concentration in the 30-40 mg/L range (regime A2, Figure 1B). A zero electrophoretic mobility indicates that the neutralization of bacterial charges is complete. The important coverage of bacterial surfaces by PEI chains likely leads to (i) increased repulsive interactions between bacterial aggregates, and (ii) a decrease in probability for a free PEI chain to bind PEI-uncoated bacterial membranes. In turn, these processes are sufficient to prevent further growth and sedimentation of aggregates, in line with data of Figures 1A and 1B. The increase in aggregate mobility (in magnitude) for PEI concentrations around 30-40 mg/L necessarily implies the beginning of a bacterial lysis *via* the deterioration of the membranes after interaction with PEI. In turn, the overall electroneutrality of bacterial flocs surface is no longer achieved and the mobility of these flocs and that of released cell residues become negative. In line with this erosion, the liberation of inner cell residues concurs with the observed increase in conductivity and solution pH. In the third and last regime of PEI

concentrations (>40 mg/L), a second aggregation step occurs, as indicated by a decrease in optical density and increase in aggregate size (Figure 1C). For such PEI concentrations, bacterial lysis is significant, leading to a drastic disruption of bacterial membrane, thus inducing the release of a substantial amount of inner cell material, as supported by TEM and AFM imaging (Figures 2A3.a,b). A increase in pH (in the absence of PEI) would not lead to cell lysis or permeation but, instead, would induce a change in the state of the polymer fringe (Gaboriaud et al., 2008). Indeed, due to a modification of the charge carried by the peripheral structure of the cell, polymer swelling would take place. As shown by Dague et al. (2006) for bacteria of similar type than the ones used in our study, an increase in pH leads to an increase of the magnitude of cell electrophoretic mobility. For basic pH (values between 8 and 11), electrophoretic mobility reaches approximately $- 2.8 \cdot 10^{-8}$ and $- 3.8 \cdot 10^{-8} \text{ m}^2 \text{ V}^{-1} \text{ s}^{-1}$, respectively for bacteria covered by polymeric shell and devoid of polymer surface appendage. Comparatively, at similar pH values, we obtained electrophoretic mobility values close to zero, meaning that the very effect of PEI on bacterial membrane is observed. In agreement with previous literature (Salt et al., 1995; Cordes et al., 1990) that evidences the possible aggregation of RNA, DNA or organits *via* binding with PEI, the second aggregation of interest here is probably that of released bacterial cell materials and wrapped membranes (clearly observable in Figures 2A3.a,b). Those newly formed aggregates probably adsorb onto surfaces of already present bacterial aggregates, *via* PEI-mediated bridges, leading to an increase of size, to a decrease in optical density or, equivalently, to sedimentation.

4.2 PEI-mediated flocculation of bacteria with LPS O-antigen decorated membrane

Similarly to the case of bacteria devoid of LPS O-Antigen, the aggregation of cells with LPS O-antigen surface appendage takes place at low PEI concentrations (regime B1, 0-20 mg/L). However, the aggregate size of about 80 μm at 20 mg/L PEI concentration is about three times larger than that of aggregates consisting of bare bacteria at similar PEI concentration. The propensity of bacteria with LPS-O-antigen outer membrane to form larger aggregates might be explained by the associated steric hindrance of PEI chains to adsorb onto LPS decorated-membranes and by their ensuing larger

availability for binding neighboring particles. The biocolloidal residues observed on AFM and TEM images (spherical outgrowths, Figure 4B1.a,b) likely originate from the complexation of LPS O-antigen appendages with PEI and the subsequent release of those structures in the solution and on the surface of the AFM glass slide. Because LPS appendages contribute to the screening of negative charges carried by bacterial outer membranes (see details in Gaboriaud et al. (2008) and Clements et al. (2008)), the elimination, even partial, of LPS O-antigen from the bacterial surface by the PEI at sufficiently high concentration (Figure 4B1.a,b), leads to an enhanced exposition of negative membrane surface charges to the outer solution, which is consistent with the observed increase (in magnitude) of aggregate electrophoretic mobility (Figure 3B). With increasing PEI concentrations up to 60 mg/L, the aggregation of bacteria with LPS O-antigen decorated-membranes occurs according to a mechanism similar to that invoked for bacteria without LPS O-antigen. Namely, PEI adsorbs onto the bare bacterial membranes (from which LPS O-antigen has been removed), which leads to a neutralization of negative bacterial surface charges, to a lowering of interparticular electrostatic energy barrier and to aggregation. This process results in 40 μm aggregate size which is comparable to that of aggregates consisting of bacteria free of LPS-O antigen within regime A2 of polymer concentration (Figure 1A). The decrease in aggregate size from $\sim 80 \mu\text{m}$ down to $\sim 40 \mu\text{m}$ when increasing PEI concentration from ~ 20 to 60 mg/L (Figure 3A) can be directly related to the evolved nature of the bacterial surface within this range of polymer concentration, passing from fully to partially LPS-covered membranes (with the limit of membranes free of LPS). In addition, the release of LPS O-antigen within regimes B1 and B2 induces superficial damages that weaken and permeabilize free LPS O-antigen membranes, thus contributing to a conductivity increase and a significant release of crystal violet (Supplementary information, Figure E). The main difference between cell lysis and permeation is related to membrane integrity. Permeation renders possible exchanges (for example, ions or nutrients) between cell cytoplasm and external environment, through the cell membrane. This explains why crystal violet release is more significant than for the case of bare bacteria. Observed damages are shaped as spherical vesicles which are reminiscent of those resulting of a stress applied on the LPS membrane, as described by Schooling and

Beveridge (2006). Finally, for PEI concentrations larger than 60 mg/L, the bacteria suffer from severe damages that ultimately lead to entire cell lysis. The corresponding increase in optical density is due to the corresponding release in the medium of cell residues and of their PEI-mediated aggregated forms. These biocolloidal residues bound to most of the macromolecules, thus avoiding large bacterial aggregates to form. This explains in fine why there is only a slight increase in optical density at large PEI concentrations as compared to that observed for the nude bacteria situation where all introduced PEI macromolecules are available for cell binding and thus forming large aggregates. As a matter of fact, the PEI chains bound to cell residues become less available for contributing to bacterial cells aggregation.

5. Conclusions

As a conclusion, Figure 5 provides a schematic illustration of the various processes discussed above that govern the PEI-mediated aggregation of LPS O-antigen free bacteria and that of bacteria exhibiting such surface appendage. The results evidence a major impact of bacterial surface surstructure on aggregation. In particular, lower amounts of PEI are needed to flocculate bacterial cells decorated by LPS O-antigen as compared to the situation observed for bare bacteria. The steric hindrance encountered by PEI chains in the vicinity of LPS decorated-bacteria prevents the cationic polymer from significantly adsorbing onto the cell surfaces and instead promotes the interactions between PEI-free bacterial outer membrane *via* the formation of interbacterial polymer bridges. In contrast, PEI readily adsorbs onto individual bacterial membranes devoid of LPS O-antigen barrier, thereby lowering their probability to bind *via* the polymer bridges. Another aspect is the difference between the PEI amounts required for lysis of bacteria with and without LPS O-antigen. Lysis of the latter is complete for all bacteria observed with a 40 mg/L PEI concentration whereas only 60% of LPS O-antigen decorated cells are lysed after exposure to 60 mg/L PEI. This reflects the protective role played by LPS O-antigen against harmful action of PEI. Indeed, PEI reacts first with a significant amount of LPS O-Antigen surface structures to permeabilize and fragilize the cell membranes and then contribute to an efficient lysis of the so-become bare cell membranes. Evidence of such role played by LPS is the observation of similar

aggregation processes for bacteria that are devoid of LPS O-antigen and those that lack this appendage after preliminary action of PEI.

The current study highlights the complexity of the mixed physico-chemical and biological processes that must be considered for appropriately understanding the aggregation processes as they occur *e.g.* in wastewater sludge treatment. In that respect, special attention is here devoted to identifying the toxic impact of flocculating polymer agent on bacterial surface structures that exhibit or not LPS O-antigen appendage, and the resulting consequences for cell aggregation. In wastewater sludge treatment, the flocculating agent is usually added in excess as compared to the content of bacteria. Our study demonstrates that such procedure likely leads to full cell lysis, which, in turn, increases the dispersion of biocolloidal residues throughout the medium and potentially diminishes dewatering efficiency. Possible alternatives for avoiding such failure in wastewater sludge treatment would be a careful control and optimization of flocculating agent concentration in order to reach conditions of bacterial flocculation without massive and undesirable cell lysis. An abacus could be constructed in order to correlate sludge organic content with optimal flocculent dose. Organic content could then be measured via the use of spectrometry techniques, and flocculent concentration subsequently adjusted. Further investigations are surely needed, in particular viscosity measurement and mechanical characterisation of bacterial/PEI aggregates would inform on the relationship between aggregate structures and easiness in dewatering processes. Moreover, an adjustment of PEI concentration to lowest possible values leading to cell lysis would make the water treatment plant avoid the use of PEI excess, which is environmentally unfriendly and expensive. Because bacterial sludge composition is highly variable from one treatment plant to another, it is necessary that previously mentioned abacus are constructed for each plant. In a forthcoming publication, the here-reported analysis will be complemented by the investigation of impacts of PEI molecular weight on bacterial cell aggregation processes. In addition, rheological and nanomechanical analyses (Polyakov et al., 2011) carried out on our model bacteria with and without LPS O-antigen will be given in order to achieve a local, quantitative understanding of the impacts of PEI and cell surface appendage on the water retention properties of bacterial sludge.

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Supplementary data

Details on (i) the surface structure of free and decorated LPS O-antigen *Shewanella Oneidensis* MR-1 bacteria (Figures A, B), (ii) crystal violet adsorption/release experiments, (iii) the effect of PEI on pH and conductivity of KNO₃ electrolyte solution in the absence of bacteria (Figure C and D), (iv) the extent of crystal violet release with varying PEI concentration for both bacteria analysed in this study (Figure E). This material is available free of charge via the Internet at <http://sciencedirect.com>.

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Figure captions

Figure 1. Dependence of **A)** optical density and aggregates size, **B)** optical density and electrophoretic mobility (μ); **C)** pH and conductivity, on concentration of PEI added to the solution initially containing isolated free LPS O-antigen bacteria.

Figure 2. Typical TEM and AFM observations (three panels at the top and bottom, respectively) of free LPS O-antigen bacteria flocculated with **A1.a)** 17.5 mg/L, **A2.a)** 37.5 mg/L and **A3.a)** 90 mg/L PEI, and with **A1.b)** 2.5 mg/L, **A2.b)** 35 mg/L and **A3.b)** 100 mg/L PEI. TEM and AFM observations were performed on at least 100 cells and 10-20 cells, respectively.

Figure 3. Dependence of **A)** optical density and aggregates size, **B)** optical density and electrophoretic mobility (μ); **C)** pH and conductivity, on concentration of PEI added to solution initially containing LPS O-antigen decorated bacteria.

Figure 4. Typical TEM and AFM observations (three panels at the top and bottom, respectively) of LPS O-antigen decorated-bacteria flocculated with **B1.a)** 5 mg/L, **B2.a)** 20 mg/L and **B3.a)** 90 mg/L PEI, and with **B1.b)** 5 mg/L, **B2.b)** 35 mg/L and **B3.b)** 90 mg/L PEI. TEM and AFM observations were performed on at least 100 cells and 10-20 cells, respectively.

Figure 5. Schematic illustrations of the 3 aggregation regimes for free LPS O-antigen bacteria upon increase of PEI concentration: **A1)** aggregation, **A2)** lysis and flocs erosion and **A3)** aggregation of cell residues. Schematic illustrations of aggregation regimes for LPS O-antigen decorated bacteria : **B1)** aggregation, release of LPS O-antigen surface appendage, **B2)** PEI-mediated binding of neighboring LPS O-antigen free bacterial membranes and **B3)** lysis.