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Deciphering the aggregation mechanism of bacteria (*Shewanella oneidensis* MR1) in the presence of Polyethyleneimine: Effects of the exopolymeric superstructure and polymer molecular weight

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ABSTRACT

Aggregation tests between bacteria and Polyethyleneimine (PEI) of low (600 g/mol) and high (750000 g/mol) molecular weight were performed in order to address the physico-chemical mechanisms underlying the interactions between cationic polymer and bacterial membranes. The selected strain, *Shewanella oneidensis* MR-1, produces a lipopolysaccharide (LPS) of various lengths depending on the growth conditions. Optical density, bioaggregate size, electrophoretic mobility measurements, TEM and AFM observations, and cell lysis tests (crystal violet release), were collected to describe the PEI-mediated aggregation of LPS-O-antigen-free and LPS-O-antigen-decorated bacteria. The results show that PEI of low molecular weight (600 g/mol) fails to aggregate bacteria, whereas PEIs of higher molecular weight (60000 and 750000 g/mol) lead to flocculation at low polymer concentrations. In addition, the LPS-O antigen bacterial superstructure is shown to act as a protective barrier, thus delaying the harmful effects of the cationic polymer. Despite this protection, the interaction of bacterial membranes with increasing concentrations of PEI leads to a series of deleterious processes including biosurface modification (peeling, membrane permeabilization and/or lysis), aggregation of bacterial cells, and complexation of PEI with both released biosurface fragments and cytoplasmic residues issued from lysis.

KEYWORDS

Aggregation - Flocculation - Polyethyleneimine - *Shewanella oneidensis* MR-1 - Lipopolysaccharide

I-INTRODUCTION

The aggregation of bacteria with polyelectrolytes is a crucial stage in many water treatment operations and biotechnological processes [1-4]. The intrinsic complexity of the microorganisms, their diversity, their intricate biointerphasial features (presence/absence and nature of their extracellular polymeric substances), make it difficult to assess the interaction mechanisms between the polyelectrolyte and the biocolloids. In a previous study, Krapf et al. [5] investigated the flocculation of *Shewanella oneidensis* MR-1 with polyethyleneimine of 60000 g/mol molecular weight (referred to as PEI60000 hereafter). *S. oneidensis* MR-1 has the particularity to produce varying lengths of lipopolysaccharides (LPS) according to the temperature of growth [6]. LPS is a major component of the outer membrane of Gram-negative bacteria and consists of a lipid A, included within the external leaflet of the outer cell membrane, a core oligosaccharide, and a polysaccharide referred to as O-antigen that protrudes towards the extracellular environment. In the case of *S. oneidensis* MR-1, the O-antigen part is absent at 30°C, but produced at 20°C with a length of about 30 to 50 nm. Therefore, controlling the temperature of growth allows for modulating the nature of the polymers protruding from the outer membrane. Those membrane-associated superstructures have been shown to represent a key-factor in the stability of bacterial aggregates [7, 8].

Among the various polyelectrolytes that are used in biotechnological processes, Polyethyleneimine (PEI) polymers are produced over a wide range of molecular weights corresponding to various applications. Low molecular weights, *i.e.* less than 2000 g/mol, are used to permeabilize bacterial membranes for gene transfection [9-11], for facilitating antibiotic penetration into the bacterial cell [12-14], and for bactericidal properties [15-19]. PEI of moderate to high molecular weights, *i.e.* ranging from 10000 to 750000 g/mol, are generally involved in the flocculation of bacterial sludges to improve the efficiency of dewatering in wastewater treatment processes [20, 21]. In that case, the optimal flocculation

concentration, addressed either by measurement of a filtration time [22] or that of an aggregate size [23], generally decreases with increasing polymer molecular weight in the 10000 to 100000g/mol range [2]. The aggregation of bacteria mediated by low PEI molecular weights (<2000 g/mol) has been shown to lead to longer filtration times, and the restabilization of bacterial suspensions at high polymer concentrations has not been observed [2, 22, 24]. It has also been reported that the binding of PEI with LPS leads to the release of associated material from the bacterial cell [25, 26], inducing bacterial membrane permeabilization [27]. The latter phenomenon is likely to influence the modalities of bacterial flocculation.

An improved understanding of the effects of PEI molecular weight, concentration and LPS on bacterial flocculation modes is critically needed to optimize the uses of PEI both in medical applications and wastewater sludge dewatering. In this work, we investigate the mechanisms underlying the flocculation of bacterial cells exhibiting or lacking the LPS O-Antigen superstructure with PEI of low (600 g/mol) and high (750000 g/mol) molecular weight, referred to as PEI600 and PEI750000, hereafter. Measurements of optical density, electrophoretic mobility and size of PEI-bacterial aggregates were carried out as a function of PEI concentration ranging from 0 to 100 mg/L. Transmission Electron Microscopy observations on ultrathin sections cut from resin embedded samples and Atomic Force Microscopy imaging of bacterial aggregates were used to observe the consequences of PEI/bacteria interactions. In addition, polymer deleterious effects were estimated *via* crystal violet releasing tests in the presence of PEI. The various stages of bacterial aggregation and flocculation are discussed here as a function of PEI concentration and molecular weight.

II-MATERIALS AND METHODS

II-1 Bacterial cultures

Shewanella oneidensis MR-1 bacteria were routinely cultured in Lysogenic-Broth (LB) (LB Broth Miller Difco TM), for 14 to 16 hours, at a defined temperature (either 20°C or 30°C). The cultures were grown in Erlenmeyer flasks (volume filling ratio of 1 to 5 in order to maintain identical oxygenation conditions), shaken at 160 rpm until stationary phase. After harvesting *S. oneidensis* MR-1 cultures bacterial cells were washed twice by alternating centrifugation (15 min, 5000 G), and cell pellets re-suspension in 1 mM KNO₃ solution. The final cell suspension density was then adjusted to an OD_{600nm} of 1 (*i.e.* 10⁹ cells/mL) with 10⁻³ M KNO₃ solution using a spectrophotometer (Safas Monaco, UV mC²). Throughout sample preparation, extreme care was taken to maintain the bacterial suspensions at their initial growth temperature.

II-2 Preparation of flocculated suspensions

The bacterial suspensions were divided into 100 mL aliquots and placed in polymethylmethacrylate (PMMA) reactors (5 cm in diameter) fitted with 4 PMMA baffles (0.5 x 6 cm). PEI (600 g/mol, Polysciences or 750000 g/mol, Sigma-Aldrich) was added under agitation to the bacterial suspensions using a micropipette (Eppendorf). After PEI addition, a two-step mixing procedure was adopted, *i.e.* a fast stirring stage (250 rpm for 3 minutes) followed by a slow stirring stage (60 rpm for 46 minutes). During the mixing procedure, both conductivity and pH were measured. At the end of mixing, the bacterial suspensions were placed in graduated Imhoff cones and residual turbidity (or optical density at 600 nm) was measured in the supernatant after a one-hour settling. Mixing and settling of the aggregated suspension were carried out at ambient temperature (~20 °C).

II-3 Physico-chemical characterization of bacterial aggregates

Electrophoretic mobility measurements were performed on the supernatant recovered after settling (diluted in 10^{-3} M KNO_3 solution when necessary) using a Zetaphoremeter IV (CAD Instrumentations, France). Details on the technique can be found in Krapf *et al.* [5]. The electrophoretic mobility measurements were performed in triplicate on at least 100 particles.

The size of bacterial aggregates was further determined before settling using a Sympatec Helos particle size analyser in the Fraunhofer approximation. To avoid multiple scattering in the measurement cell, the suspensions were first diluted in 10^{-3} M KNO_3 solution and homogenized by gentle hand-stirring to yield a 10% volume concentration. The results are volume-based and are represented as particle volume *versus* sphere diameter of equivalent volume.

Ultrathin sections of bacterial aggregates, cut from Epoxy resin embedded samples, were observed using a Philips CM20 transmission electron microscope operating at 80kV acceleration voltage. The embedding procedure is described in Lartiges *et al.* [28]. The damages of bacterial membranes caused by PEI were classified following the analysis of at least 100 bacteria. TEM micrographs of *Shewanella oneidensis* MR-1 bacteria (LPS O-antigen free and LPS O-antigen decorated) are presented in Figure S1 of the Supporting information section.

AFM images were recorded in air atmosphere using an MFP3D-BIO instrument operating in contact mode (Asylum Research Technology, Atomic Force F&E GmbH). The bacterial aggregates, taken at the end of both mixing periods, were first deposited onto glass-slide for 20 minutes, and then washed with deionized Milli-Q water to remove unsettled aggregates. The spring constant of silicon nitride cantilevers (MLCT-AUNM, Veeco Instruments SAS) was determined by thermal calibration ($\sim 10.4 \pm 1.7$ pN nm^{-1}). The geometry of the AFM tip was systematically controlled using a commercial grid for 3-D visualization (TGT1, NT-MTD

Compagny), the radius of curvature of the tip being in the 20-50 nm range. AFM images of *Shewanella oneidensis* MR-1 bacteria (LPS O-antigen free and LPS O-antigen decorated) are shown in Figure S1 of the Supporting information section.

II-4 Detection of membrane damages

The permeability of bacteria inner membrane was assayed by diffusion of crystal violet as follows. Aliquots of 1 mL bacterial suspension ($OD_{600} \sim 1$) were mixed with crystal violet (final concentration of 0.1 g/L) for 1 hour. The bacterial suspensions were then washed repeatedly in order to remove excess crystal violet (centrifugation at 13000 g for 3 minutes followed by redispersion of the cell pellets in fresh 10^{-3} M KNO_3 solution). The supernatant absorbance was measured after each centrifugation step by spectrophotometry (540 nm) until the amount of crystal violet released by the bacteria became negligible. PEI was then added to the bacterial suspension conditioned with crystal violet at a given concentration in the range of 0-100 mg/L. After an incubation of 50 minutes, bacterial cells were pelleted by centrifugation (13000 g for 3 minutes) and the violet crystal released from bacteria was measured by absorbance in the supernatant at 540 nm. Moreover, it was checked that a 100% release of crystal violet was obtained by lysing crystal violet-stained bacteria with glacial acetic acid at 0.1g/L. The relative amount of crystal violet released from PEI-treated bacteria was estimated from the percent ratio of absorbances measured in the supernatant of PEI-treated and acetic acid-treated bacterial suspensions.

III RESULTS

III-1 Aggregation of membrane-LPS O-antigen-free bacteria with PEI

Figure 1 compares the performance of PEI600 and PEI750000 for inducing the aggregation of LPS O-antigen free *S. oneidensis* MR-1, *i.e.* 30°C-grown bacteria with a reduced outer

membrane decoration. In the case of PEI600, the residual optical density increases regularly for PEI concentrations between 0 and 20 mg/L, and it gradually levels off at about 1.7 for polymer concentrations above 80 mg/L (Figure 1a). Concomitantly, the particle size slightly decreases from 1.2 μm to about 0.9 μm above 20 mg/L PEI concentration, thus revealing that the micron-size bacteria do not aggregate upon addition of PEI600. The electrophoretic mobility becomes slightly more negative with adding PEI600 up to a critical concentration of about 15 mg/L. With further increasing PEI concentration, a partial compensation of the negative charges carried by the cell membranes is observed without reaching, however, a complete neutralization at high polymer concentrations (Figure 1b). This observation may be explained as follows. The suspension pH increases from 6.5 to 9.8 during polymer addition (Supporting information, Figure S2). This implies a deprotonation of most PEI ionogenic groups at high pH [29] and thus the addition of weakly positive polymer at elevated concentrations, therefore rendering impossible the neutralization of all negative charges located at the biosurfaces. The minimum in electrophoretic mobility defines two concentration domains, denoted as C1 between 0 and 15 mg/L, and C2 above 15 mg/L.

The aggregation of LPS O-antigen-free *S. oneidensis* MR-1 with PEI750000 fits into a more conventional picture, defined by an initial steep decrease in residual optical density (OD) at low polymer concentration (after aggregate settling), and a nearly constant low OD value at higher PEI concentration (Figure 1c). In details, two PEI750000 concentration domains may be identified, *i.e.* from 0 to 5 mg/L and from 5 and 100 mg/L, which are hereafter referred to as D1 and D2, respectively. In the D1 domain, the aggregation of bacteria increases sharply with PEI750000 concentration where particle size reaches *ca.* 60 μm at the upper limit of D1 domain. Surprisingly, the electrophoretic mobility first sharply decreases towards more negative values with increasing PEI concentration from 0 to 5 mg/L (D1 domain). Then, it abruptly changes sign upon further increase in PEI concentration ($0.4 \cdot 10^{-8} \text{ m}^2 \text{ V}^{-1} \text{ S}^{-1}$ at about

20 mg/L), and it eventually decreases to zero value with further addition of PEI (Figure 1d). The electrokinetic behaviour observed in the D2 domain can be readily explained by a charge neutralization of bacterial surface following adsorption thereon of PEI chains of decreasing cationic charge as the solution pH rises from 6.8 to 9.5 at high polymer concentration (see the corresponding pH curve in Supporting information). In contrast, as the pH remains constant and equal to 6.8 in the D1 domain, the initial drop in electrophoretic mobility necessarily means that negatively charged sites at the bacteria surface have become electrokinetically-active following damages caused by PEI750000.

AFM micrographs (Figure 2) are consistent with the above aggregation features. Indeed, even though capillary forces may have induced the formation of a few loose aggregates during sample preparation and drying, LPS O-antigen-free *S. oneidensis* MR-1 treated with PEI600 appear generally dispersed. At a PEI600 concentration of 20 mg/L (figure 2a), spherical structures, about 180 nm in size, can be distinguished either fixed on the glass slide or associated with the cell surface that remains rather smooth otherwise. At higher PEI600 concentrations (*e.g.* 100 mg/L, Figure 2b), bacteria outer membranes become slightly granular.

In contrast, LPS O-antigen free *S. oneidensis* MR-1 treated with PEI750000 form compact aggregates (figure 2c). At low polymer concentration (D1 domain), the cell surface appears as mosaics of angular patches, approximately 80×200 nm in size. The width of those patches is about the order of magnitude of the diameter of a PEI branched structure for a molecular weight of 750000 g/mol, *i.e.* about 50 nm [30]. This suggests that AFM may allow direct visualization of either PEI750000 polymers on the surface of LPS O-antigen-free *S. oneidensis* MR-1 or the deformation of cell surface induced by the polymer. A few cell wall fragments deposited on the glass slide are also noteworthy (red squares in figure 2c). At

higher PEI750000 concentrations (D2 domain), bacteria merge into clusters with blister-like structures (Figure 2d).

TEM examination of ultrathin sections from resin-embedded samples completes the above AFM observations. As illustrated in Figure 3, bacterial cells treated with PEI600 essentially yield filamentous structures, whose aspects tend to indicate they are formed from membrane remnants, strewn with small 5 nm dark grains. This suggests that the bacterial membranes, weakened by their interaction with the cationic polymer, have not resisted against the resin embedding procedure. In contrast, bacterial cells appear better preserved upon interaction with PEI750000. In the D1 concentration domain, the damages caused by the addition of the high molecular weight polymer are reflected by the formation of membrane outgrowths and by the presence of small vesicles, about 20 nm in diameter, either retained or not on the bacteria surface (Figure 3c). Further increase in PEI750000 (D2 concentration domain) leads to severely damaged cells partially emptied from their cytoplasmic content, with large outgrowths remaining attached to the cell membrane (Figure 3d).

The deleterious effects of PEI on the permeability of bacterial cells were evaluated using crystal violet diffusion experiments (figure 4). For LPS O-antigen-free *S. oneidensis* MR-1 treated with PEI600, the release of crystal violet from bacterial cells strongly increases from 5% at low polymer concentration to reach a value of *ca.* 15% at 15 mg/L (C1 domain), slightly decreases until 50 mg/L, and then fluctuates significantly at higher PEI600 concentrations. The extent of damages seems slightly amplified when the bare bacteria are treated with PEI750000. Indeed, the diffusion of crystal violet reaches about 10% at a PEI concentration of 5 mg/L, and fluctuates between 12 to 20% in the D2 domain. The reduced release of crystal violet observed at low polymer concentrations suggests that some bacterial cytoplasmic membranes become altered from the very start of PEI addition. This can be the result of (i) an inhomogeneous distribution of polymer chains in the bacterial suspension, *i.e.*

some bacteria interact with many PEI polymers and others not, (ii) a strong enough interaction between a single PEI macromolecule and the bacterial membrane to alter the cytoplasmic membrane, (iii) an heterogeneity of cell membranes with some being more fragile.

III-2 Aggregation of membrane-LPS O-antigen decorated bacteria with PEI

In the presence of LPS O-antigen superstructure, the interaction behaviour of PEI polymers with *S. oneidensis* MR-1 becomes slightly more complex than that discussed in the preceding section for bare membranes (Figure 5). For PEI600, two aggregation regimes can be identified: for PEI concentrations lower than 20 mg/L (E1 domain), the residual optical density decreases to about 0.8 while particle size goes through a maximum at 1.8 μm . This marks a minor aggregation of bacteria accompanied by a linearly decrease in electrophoretic mobility towards more negative values (Figure 5b). In the E2 concentration domain, *i.e.* above a PEI600 concentration of 20 mg/L, the optical density continuously increases to reach 1.7 at high polymer concentration (Figure 5a), whereas the particle size remains constant at around 1 μm and a partial neutralization of bacterial charges can be further detected (Figure 5b).

On the other hand, for PEI750000, three concentration domains can be defined based on the evolution of the residual optical density (Figure 5c): the F1 domain where OD diminishes strongly for PEI concentration increasing from 0 to 5 mg/L, the F2 domain where OD drops even more abruptly for PEI concentration in the 5 to 15 mg/L range, and finally the F3 domain, where the optical density is low and roughly constant above a PEI concentration of 15 mg/L. The size of the aggregates basically increases with increasing polymer concentration, it peaks at 100 μm in the middle of the F2 domain, drops unexpectedly to 40 μm at the onset of F3, and then re-increases upon further polymer addition to reach about 150 μm at a PEI750000 concentration of 100 mg/L. In parallel, the electrophoretic mobility profile also shows the same three domains with two sharp extrema located at the limit

between F1 and F2 domains, and at the onset of F3 (15 mg/L), respectively, and eventually tends gradually to 0 at higher polymer concentrations, therefore indicating a neutralization of particle charges by PEI750000 (Figure 5d).

The AFM images of LPS O-antigen-decorated *S. oneidensis* treated with PEI of varying molecular weight appear rather different than those of bacteria devoid of O-antigen structure (Figure 6). On the whole, the images seem less resolved and even slightly blurred, which may result from a cell surface coating with the cationic polymers. At a PEI600 concentration of 20 mg/L, AFM imaging reveals bacteria with a relatively rough surface surrounded by a 50 to 60 nm halo. This layer becomes thicker at higher PEI concentration (100 mg/L), and the bacterial surface shows more pronounced patches of puffiness. TEM images shown in Figure 7 reveal wrinkled and deflated bacteria which suggests that, even though the PEI600 addition to the LPS O-antigen decorated *S. oneidensis* has obviously damaged the outer-membrane of bacterial cells, the presence of exopolymer coating may have protected the bacteria from the severe deteriorations previously evidenced for the bare bacteria.

In the case of high molecular weight polymer, PEI750000 concentrations pertaining to the F1 domain (*e.g.* 5 mg/L – Figure 6c) lead to the formation of ~250 nm outgrowths at the periphery of the bacterial cells. Clusters of bacteria with a slightly rough surface are observed in 20-30 mg/L range (Figure 6d), whereas the addition of higher amounts of PEI750000 to the suspension (Figure 6e - 90 mg/L - F3 domain) yields AFM images with barely recognizable cells that can be interpreted as severely damaged bacteria with possible extrusions of cytoplasmic material. The extent of membrane alterations is also observed to increase with PEI concentration (Figure 7), from almost intact bacteria in the F1 domain to obviously osmotic fragile cells with significant release of cytoplasmic content in the F3 domain.

As illustrated in Figure 8, the release of crystal violet from bacteria treated with PEI600 increases in the E1 domain to peak at 15 mg/L, and is roughly stable for higher polymer

additions. Such features are consistent with either an increased porosity of the bacterial cell membrane at low PEI600 concentration, or an increased number of bacteria exhibiting an altered membrane. A similar pattern can be inferred from the curve of crystal violet release in the case of PEI750000 (fig. 8b), with a significant increase of dye release between 0 and 25 mg/L where it reaches maximum at 20%. In both cases, the presence of LPS O-antigen superstructure appears to shift the release of crystal violet towards higher PEI concentrations.

IV DISCUSSION

IV-1 Flocculation mechanism of *Shewanella oneidensis* MR1 with PEI polymers

The aggregation of inorganic colloidal particles with polyelectrolytes of opposite charge is generally described as a two-stage mechanism [31]. Because of strong electrostatic interactions, the polyelectrolyte rapidly adsorbs onto the particle surface in a flat conformation [32], and the resulting charge neutralization causes the aggregation of otherwise repulsive particles. For highly charged polyelectrolytes and a particle size larger than that of the polymer molecule in solution, the surface-adsorbed polymers form charged patches amidst the regions of uncoated surface; the aggregation then proceeds from the attraction of oppositely charged domains residing on different particles [33]. In most studies dealing with inorganic particles, an optimum aggregation is obtained when the added polyelectrolyte just neutralizes the surface charge of particles, the optimum flocculation conditions being essentially independent of polymer molecular weight, and a particle charge reversal being observed in the presence of excess polyelectrolyte [33-35].

As a first approximation, bacteria -although most of them exhibit an heterogeneous permeable polymeric interphase [36, 37] - may be considered to behave similarly in the presence of cationic polyelectrolytes: the minimum amount of polymer required to initiate aggregation has been shown to be rather independent of molecular weight [2], and a

restabilization of bacteria suspensions is commonly observed at high polyelectrolyte concentration [38]. However, it has also been observed that PEIs of molecular weight below 800 g/mol do not generally induce bacteria aggregation [2], and that the optimum flocculant concentration does not systematically coincide with the isoelectric point of the bacteria, especially for rigid polyelectrolytes such as chitosan [1, 38].

Our findings are partly in line with literature results on the aggregation of bacteria with polyelectrolytes. The ability of low molecular weight PEI600 to aggregate both cell surface types of *S. oneidensis* MR-1 appears rather limited (Figures 1a and 5a). This implies the existence of a minimum chain length for bacteria aggregation to occur, a feature usually related to a physical bridging mechanism. If the optimum flocculant concentration is defined as the minimum polymer concentration required to achieve a low residual turbidity, the aggregation of LPS O-antigen-decorated *S. oneidensis* with PEI can be optimized around 15 mg/L for both PEI60000 and PEI750000 polymer systems (Figure 5c and see previous results obtained with PEI60000 in Krapf *et al.* [5]). In contrast, for bacteria with a "bare" outer membrane, the optimum flocculant concentration decreases with increasing PEI molecular weight from about 15 mg/L for PEI60000 [5] to 5 mg/L for PEI750000 (Figure 1c). Such dependence of optimal polymer concentration on molecular weight is consistent with a charge neutralization mechanism in the case of LPS O-antigen decorated bacteria, bridging effects in the case of LPS O-antigen free *Shewanella*. To make things even more puzzling, all flocculation optima basically coincide with sharp negative minima in electrophoretic mobility (Figures 1b-d and 5b-d). Moreover, for PEI750000, AFM imaging of aggregated LPS O-antigen-free *S. oneidensis* MR-1 suggests that a charge-patch neutralization mechanism might apply, the size of the approximately angular shapes possibly identifying with those of the polyelectrolyte patches on the bacterial surface.

Such questioning about the nature of flocculation mechanism cannot be simply resolved, but it reveals to the least the significant role of the bacterial interphase on aggregation. In all cases, the mild increase in electrophoretic mobility towards less negative or even positive values observed at high polymer concentration independently of molecular weight, evidences a charge neutralization of the bacterial surface cells. Such neutralization appears moderate, partly because of the concomitant increase in pH of the suspension and the associated deprotonation of PEI amine groups. At low PEI concentration, the initial drastic decrease of the electrophoretic mobility, that yields strong negative values close to the optimum dosage for flocculation, indicates a significant reorganization of the bacterial interphase upon interaction with the cationic polymer. Such rearrangement, which exposes additional electrokinetically active negative charges of the membrane to the outer solution, is consistent with deep structural damages detected by crystal violet release. Interestingly, assimilating *S. oneidensis* MR-1 to a cylinder 0.5 μm in diameter and 2 μm in length and PEI750000 to a disk of 50 nm in diameter [30], a simple calculation of surface coverage suggests that a 5 mg/L PEI addition enables twice the coating of the bacteria in suspension. The sharp electrophoretic minima could then well correspond to the peeling of the outer membrane in the case of LPS O-antigen-free *S. oneidensis* MR-1, and to the successive removal of superstructure and outer membrane for LPS O-antigen-decorated *S. oneidensis*. Similar calculations performed with PEI600 (about 2 nm in diameter) and PEI60000 (17 nm in diameter according to Park and Choi [30]), indicates that the amount of polyelectrolyte brought at the optimal flocculant concentration could coat more than ten times the bacteria surface.

In all cases, the aggregation of *S. oneidensis* seems to be mediated by the complexes formed between PEI and the bacterial polymers. This should generate a nonuniform charge

distribution at the surface of bacteria, obviously enhanced for higher molecular weight cationic polymer, which can then be invoked to explain the aggregation of the bacteria [39].

IV-2 Membrane alteration by PEI polymers

PEI polymers have been shown to strongly increase the membrane permeability of Gram-negative bacteria [12], cyanobacteria [4], and various eukaryotic cells [40]. Such permeabilization is identified by the release of cytoplasmic materials, the facilitated uptake of hydrophobic probes, or an increased cell susceptibility to antibiotics and detergents [12]. TEM examination of damaged membranes from resin-embedded bacteria revealed that the more permeable cells are generally characterized by slight undulations to severe alterations of the outer membrane [4, 12]. Molecular dynamic simulation [41] revealed that polyelectrolyte-mediated permeabilization of model experimental systems such as supported lipid bilayer [42, 43], multilamellar vesicles [44] or even living cells [45], proceeds with the formation of nanoholes. Interestingly, the interaction of lipid bilayers with cationic polyelectrolyte has also been found to provoke the peeling of vesicles [44] and authors therefore suggested that patches of the lipid bilayer might wrap around the cationic polyelectrolyte [43].

Overall, our results are consistent with these previous findings: the permeabilization of bacterial inner membrane is indicated by a release of crystal violet, and thin-section electron microscopy observations reveal not only strongly altered membranes but also the presence of membrane vesicles in the case of exposition to PEI750000, which could correspond to the cationic polyelectrolyte wrapped by the bacterial membrane (Figure S3 in Supporting Information). It should however be noted that some undulation, folding, and to some extent the formation of membrane vesicles, might be magnified by the embedding process.

IV CONCLUDING REMARKS

Unlike inorganic particles characterized by a neutralization of surface charges in the presence of polyelectrolyte, our results show that the interaction of a bacteria with PEI occurs on a three dimensional basis. The cationic polymer interacts and reorganizes the flexible exopolymeric superstructure and/or the bacterial membranes, thus leading to the exposure of supplementary negative charges to the solution. Similarly to a patch neutralization process, the overall charge compensation involves local excesses of positive charges brought by the polyelectrolyte chain and of negative charges originating from the bacterial interphase. Such heterogeneity in charge distribution is crucial for flocculation, which is supported by the observation that a too low PEI molecular weight does not trigger bacteria aggregation. Above the critical flocculant concentration, which corresponds to the overall neutralization of bacterial outer structures, the excess deterioration of the inner membrane in the presence of high PEI concentrations leads to a strong release of cytoplasmic material. The introduction of this additional material in the suspension implies a necessary weak restabilization of bacteria. On a practical basis, the latter phenomenon accounts for the severe decrease in filtration efficiency observed above optimal flocculant concentration [2, 23, 46]. From a more fundamental point of view, and in line with previous studies [47], our results make it clear that bacterial cells cannot be considered as hard and stable inert particles when interpreting flocculation phenomena.

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