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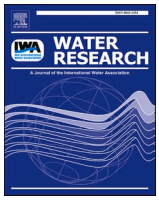

Understanding the ionic hydrogel-forming property of extracellular polymeric substances: Differences in lipopolysaccharides between flocculent and granular sludge

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ABSTRACT

An interesting and potential property of extracellular polymeric substances (EPS) is the hydrogel formation with calcium ions. Aiming at understanding the significant difference in the hydrogel formed between EPS from flocculent and granular sludge, a targeted investigation of the lipopolysaccharides (LPS), one of the important EPS components, was performed. LPS was isolated from the EPS of flocculent and granular sludge, and both the glycan and the lipid A parts of LPS were characterized and compared. The morphology of LPS-calcium (LPS-Ca) aggregates were visualized by the polymyxin B-based fluorescent probe. The LPS constituted about 25 % and 15 % of the EPS from flocculent and granular sludge, respectively. The flocculent sludge LPS showed a lower amount of glycans, shorter glycan chain length, lower molecular weight, and higher possibility of containing unsaturated lipids than the granular sludge EPS. The flocculent sludge LPS-Ca aggregates demonstrated invert structures with the water phase in between, contributing to the fluid-like property of the respect EPS-Ca. In contrast, with the remarkably different chemical structure, LPS-Ca aggregates from granular sludge displayed bilaminar multilayered morphology, contributing to the solid, self-standing hydrogel of EPS-Ca.

1. Introduction

Extracellular polymeric substances (EPS) have been considered essential for the biofilm structure. They are also the important matrix to form the three-dimensional microbial structure of flocs and granules. The role of EPS in different biofilms has currently attracted increasing attention. Great efforts have been made to distinguish the distinction between EPS from flocculent and granular sludge (Lv et al., 2014; McSwain et al., 2005; Zhu et al., 2015). The general approach is that, after EPS extraction from flocculent or granular sludge, the EPS composition and properties are analyzed, and differences as well as similarities are compared.

An interesting EPS property is the gel-forming property (Lin et al., 2013; Schambeck et al., 2020; Seviour et al., 2009). Seviour et al. (2009) studied the EPS sol-gel transition at different pH, observing that strong gel was formed by EPS from granules while not by any of the EPS samples from flocs. The ionic gel-forming property of EPS with $CaCl_2$ solution was studied by Lin et al. (2013) afterwards, it was reported that the mechanical property of EPS-Ca hydrogel from granular sludge was much stronger than that from flocculent sludge. The proposed explanations for gel formation were mainly based on the interactions between proteins and polysaccharides with calcium (Goh et al., 2014). It was believed that certain amino acid residues present in EPS could interact with calcium ions (Khalili et al., 2004; Leonard, 2013). On the other hand, Seviour et al. (2009) suggested that glycans were the dominant agents for forming a strong gel instead of proteins. Glycans contain functional groups such as hydroxyl and carboxylate groups, which can interact with calcium ions through ion-dipole or ion-ion interactions (Mierczyńska et al., 2015; Wang et al., 2018). It is noted that glycans include free polysaccharides and glycoconjugates such as glycoproteins and glycolipids. Different types of glycans might interact with calcium ions in a dissimilar way. Therefore, to understand the exact contribution of glycans to EPS hydrogel formation, it is necessary to investigate each type of glycans separately.

Very recently, it was reported that lipopolysaccharides (LPS, one type of glycolipids) were one of the important glycoconjugates in EPS extracted from flocculent sludge (Li et al., 2024). Previous studies indicated that LPS could be the key component for aggregate formation

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(Parikh and Chorover, 2007; Pazol et al., 2022; Rahnamoun et al., 2020). LPS is amphiphilic, with lipid A as the hydrophobic part and glycans as the hydrophilic part (including core oligosaccharides and O-antigen). Lipid A is relatively conservative. It interacts with calcium ions through the coordination of phosphate and/or carboxylate groups. In addition, the glycan part in LPS is extremely variable and could be crosslinked forming dimensional structures via calcium ions (Balantič et al., 2022; Leonard, 2013; Valentine et al., 2020). These findings indicate that there are active sites in LPS which can interact with calcium ions. What is the difference and similarity between the LPS from flocculent sludge and granular sludge and how does the LPS and calcium interaction influence the EPS and calcium hydrogel? These are important research questions to be answered for a better understanding of the gel-forming property of the EPS. To study this, LPS was isolated from the EPS of flocculent and granular sludge. Both the glycan part and the lipid A part were characterized and compared. The interaction of LPS with calcium ions was visualized by the specific staining. This study sheds light on the possible connections between LPS structures and composition with the EPS gel-forming property.

2. Material and methods

2.1. Extracellular polymeric substances (EPS) extraction from flocculent and granular sludge

The flocculent sludge was collected from two municipal wastewater treatment plants using the activated sludge process in Delft and Rotterdam, the Netherlands; and the granular sludge was collected from two municipal wastewater treatment plants using the aerobic granular sludge process (Nerada®) in Utrecht and Garmerwolde, the Netherlands. The collected raw sludge samples were first frozen and lyophilized afterwards for storage. The EPS was extracted from both the flocculent and granular sludge according to the previously reported extraction protocol (Li et al., 2021), i.e. 3.0 g of dried sludge was resuspended in 100 mL demi-water with adding 0.5 g of sodium carbonate. The mixtures were then heated at 80 °C for 30 min. After centrifuge, the supernatant was adjusted to pH 2.2 with 1 M HCl. The precipitates were collected, with a part of them being saved for the ionic hydrogel formation tests (the details refer to Section 2.3), and the rest being dissolved in 1 M NaOH till pH 8.5 and dialyzed in the dialysis bag with the molecular weight cut-off (MWCO) of 3.5 KDa before lyophilization.

These lyophilized EPS samples were dissolved in 0.1 M NaOH at a concentration of 1000 mg/L overnight before the determination of total carbohydrates content using the phenol-sulfuric acid method (Dubois et al., 1951) with glucose as the standard and the total proteins content using BCA assay with BSA as the standard (Smith et al., 1985).

2.2. Lipopolysaccharides (LPS) isolation from EPS

The LPS was extracted from those lyophilized EPS samples using the hot phenol-water method in accordance with the previous studies (Davis and Goldberg, 2012; DeLeo and Otto, 2008; Li et al., 2024). 10 mg of EPS samples was completely dissolved in 1 mL of a 1xPBS solution (pH=7.0). Then, 50 uL of proteinase K solution (10 mg/mL) was added and the mixture was heated at 65 °C for 3 h to cleave the peptides. An equal volume (1 mL) of water-saturated phenol solution (pre-heat at 68 °C) was added to the mixtures followed by vigorously shaking at 68 °C for 15 min. Suspensions were then cooled on ice with the addition of 1 mL of Milli-Q water before centrifugation at 12,000 rpm for 15 min. The tube was gently taken out from the centrifuge and the top aqueous layer was transferred into 10 mL of polypropylene tubes. The bottom layer of the phenol phase was re-extracted twice by an equal volume of Milli-Q water. All of the top layer solutions were mixed together and dialyzed against demi water in the dialysis tubing (MWCO 3.5 KDa) and lyophilized afterwards.

2.3. Interaction of both EPS and LPS samples with calcium ions

EPS-Ca hydrogel formation tests were conducted to assess the hydrogel formation capacity of EPS extracted from both flocculent and granular sludge (Felz et al., 2016). Part of the EPS acidic precipitates formed at pH 2.2 (the details refer to Section 2.1) were dissolved by adding 1.0 M NaOH till pH 8.5. The final concentration of the four EPS samples was at around 1.5 % (w/v). Afterward, those EPS solutions were transferred into plastic cylinders respectively. The size of the plastic cylinders was 23 × 15 mm (Diameter × Height), with the two ends sealed by the dialysis bag (MWCO 3.5 kDa). The plastic cylinders filled with EPS were put into a glass beaker containing 2.5 % calcium chloride solution (w/v) for 48 h with slow stirring. The calcium solution was changed every 12 h. After the gel formation tests finished, the EPS-Ca gel samples were collected for microscopic study.

Regarding the interaction of LPS with calcium ions, instead of dissolving the sample in 1.0 M NaOH, LPS was first dissolved in demi-water. The LPS solution was filled in the plastic cylinders with the size of Diameter × Height=10 × 10 mm, with the two ends sealed by the dialysis bag (MWCO 3.5 kDa). The plastic cylinders filled with LPS were put into a glass beaker containing 2.5 % calcium chloride solution(w/v) for 48 h with slow stirring. The LPS-Ca aggregates were collected for further microscopic study.

2.3. LPS characterization and visualization

2.3.1. LPS detection by SDS-page with PRO-Q emerald 300 staining

In this study, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to visualize the possible length of the glycan part in LPS according to Li et al. (2024), i.e., the lyophilized LPS samples were solubilized in miliQ water (1 mg/mL) before mixing with the loading buffer. 10 uL of LPS solutions and 8 uL of LPS standard (from *Escherichia coli* serotype 055:B5, 0.25 mg/mL) were loaded into the wells and separated on 12 % SDS gel with a 4 % stacking gel under reducing conditions at 200 V for 40 min. Afterwards, Pro-Q Emerald 300 staining of the gels was performed according to the protocol provided by Thermo-Fisher Scientific. The image of the gel was taken by a ChemiDoc MP imager (Bio-Rad, Hercules, CA).

2.3.2. LPS sugar monomers composition analysis by HPAEC-PAD

Sugar monomers in the four LPS samples were analyzed using High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) following the procedure described in (Felz et al., 2019). 1.0 mg of LPS samples were first hydrolyzed in 1.0 mL of 1 M HCl in a 105 °C oven for 8 h. After centrifugation, the supernatant was neutralized with 1 M NaOH and filtered through 0.22 μm PVDF filters. Filtrates were diluted with Milli-Q water in accordance with the concentration of the standard sugar monomers before being injected into the HPAEC-PAD. Seven neutral sugars (fucose, rhamnose, galactose, glucose, xylose, mannose, and ribose) and two amino sugars (galactosamine and glucosamine) were selected as standards in this study.

2.3.3. Functional groups identification by FT-IR

The functional groups of LPS were analyzed by Fourier Transform Infra-Red (FT-IR) spectroscopy over a wavenumber range from 4000 cm^{-1} to 600 cm^{-1}.

2.3.4. Morphology of LPS-Ca aggregates visualization using the polymyxin B-based fluorescent probe

Polymyxin B labelled with Rhodamine B was used to visualize the structures and morphology of LPS-Ca aggregates. The mechanism of the staining is that polymyxin B interacts with LPS molecules via their cationic l-α-γ-diaminobutyric acid (Dab) side chains and the hydrophobic residues (Manioglu et al., 2022). LPS-Ca aggregates which were obtained as described in Section 2.3 were washed with 1xPBS twice, followed by the incubation with polymyxin B labelled with Rhodamine

B solution (1.1 ug/mL) at room temperature for about 2 min. After centrifugation, the pellet was washed 4 times with 1xPBS to remove the residual probes. Finally, the samples were resuspended in 100 uL of 1xPBS for acquiring fluorescence imaging immediately. The images of polymyxin B with fluorescent probe Rhodamine B were captured with a CY3 filter. For each samples, various spots were chosen examined to observe the main features of these aggregations.

3. Results

3.1. The ionic hydrogel-forming property of EPS from flocculent sludge and granular sludge

EPS was extracted from two flocculent sludge samples and two aerobic granular sludge samples and their yields are listed in Table 1. The contents of total carbohydrates and proteins of each EPS are listed in the Supplementary Materials Table S1. It is noticed that the EPS yield from aerobic granular sludge is relatively higher than that from flocculent sludge. While looking at the hydrogel-forming property of EPS with calcium ions, the difference was apparent. Although both EPS from flocculent sludge and granular sludge exhibited the ability to form viscous hydrogels, the EPS-Ca hydrogel from flocculent sludge still looked like a fluid and was not able to self-stand at all (Fig. 1). In contrast, the EPS-Ca hydrogel from granular sludge became a solid piece reflecting the shape of the mould and able to self-stand completely.

3.2. LPS isolation and characterization

To understand the dissimilarity between the EPS-Ca hydrogels, a more targeted investigation by studying the specific EPS components was conducted. Lipopolysaccharides, which are an important type of glycolipids in the EPS, were selected for the investigation.

3.2.1. Yield of LPS isolation

The yield of LPS is listed in Table 1. It was interesting to see that the amount of LPS extracted from the EPS of granular sludge was much lower than that from flocculent sludge. Moreover, the total carbohydrates content of the extracted EPSs varied at 16 %−22 % (see Supporting Information Table S1), while the amount of LPS was already at 15 %−25 % of the extracted EPS. This implies that the amount of LPS might contribute significantly to the amount of total carbohydrates within the EPS. Additionally, their structure and property may influence the characteristics of EPS.

3.2.2. Glycan chain length distribution of LPS

LPS consists of lipid A and glycans (including core oligosaccharides and O-antigen, as shown in Supporting Information Figure S2). SDS-PAGE with Pro-Q Emerald staining was applied to investigate the chain length distribution of O-antigen which influences the molecular weight of LPS samples. O-antigen in LPS varies in their sugar monomer composition and the number of repeating units (Jann et al., 1975; Whitfield et al., 2020) . The more repeating units in the O-antigen, the longer the chain length is, and the higher molecular weight the LPS is. This can be displayed in the SDS-PAGE with the well-known “ladder--like” bands (Jann et al., 1975). Each band corresponds to the O-antigen with different repeating units. The fluorescence image is presented in Fig. 2. Clearly, the ladder-like pattern was displayed among each LPS sample, indicating that all of the LPS samples contained the O-antigen part. As the bands at the upper gel represent higher molecular weight O-antigen with longer sugar chains (Jann et al., 1975; Whitfield et al., 2020), it seems that the LPS from granular sludge should have relatively longer sugar chains.

Table 1
Yields of EPS and LPS from both flocculant and granular sludge.

Sludge type	Wastewater treatment plant	EPS yield (mg EPS/g VS sludge)	LPS yield (mg LPS/g VS EPS)
Flocculent sludge	Delft	164.3 ± 11.8	248.4 ± 4.4
	Rotterdam	299.8 ± 2.4	256.7 ± 7.3
Aerobic granular sludge	Utrecht	245.2 ± 11.9	146.4 ± 14.6
	Garmerwolde	223.7 ± 1.7	154.1 ± 13.7

3.2.3. Sugar monomer composition of glycans in LPS

To identify the monosaccharides in the LPS, quantitative analysis was conducted by HPAEC-PAD and the results were summarized in Table 2. In general, the total amount of sugar monomers in the LPS from granular sludge was higher than that from flocculent sludge, meaning that there is a higher amount of glycans in the LPS from granular sludge. This is in agreement with the findings from the SDS-PAGE analysis that the LPS from granular sludge has a longer sugar chain. Interestingly, for the LPS from flocculent sludge, the difference between the two samples is relatively big, i.e. the total amount of sugar monomers in the LPS from the sludge collected at the Rotterdam wastewater treatment plant is much higher and close to that of granular sludge. Whether it is due to the properties of the sludge in this specific wastewater treatment plant, or the change of the amount of sugar monomers between the LPS from flocculent sludge and granular sludge varies in a broad range, is an interesting topic to follow up.

To look at the detailed composition, the relative mole percentages of different sugar monomers were calculated and listed in Table 2. Firstly, there were a few sugar monomers that were more than 20 % (relative mole percentage) of the total amount of sugar monomers in the LPS. i.e. fucose, mannose and ribose in the LPS from flocculent sludge; fucose and mannose in the LPS from aerobic granular sludge. Judging from this bigger amount, probably, those sugars were part of the components in the O-antigen repeating units. Secondly, the significant difference between LPS from flocculent sludge and granular sludge was the amount of mannose and ribose: the LPS from granular sludge was rich in mannose while the LPS from flocculent sludge was rich in ribose. Mannose is C6 sugar while ribose is C5 sugar. Although mannose and ribose were found in both the core regions and the O-antigen of LPS (Samuel and Reeves, 2003), with more -OH groups and bigger molecule size, a higher mole percentage of mannose might increase the hydrophilic property and the glycan size, providing more opportunities for hydrogen bonding and intermolecular interactions. In fact, it was reported that the variations of sugar monomer composition in different polysaccharides affect the aggregate’s morphology (Charchoghlyan and Park, 2013; Miyoshi et al., 1998).

3.2.4. Functional groups related to the lipid A in LPS

The functional groups of LPS were investigated by FT-IR spectroscopy. The spectra are shown in Fig. 3, the peaks at the wavenumber of around 2920 and 2860 cm^{-1} were assigned to the vibration of the methylene ($-CH_2$), while peaks at around 2960 cm^{-1} and 1380 cm^{-1} were assigned to methyl ($-CH_3$) vibration. The hydrocarbon chains of fatty acids in lipid A are the main contributors to these peaks (Liu, 2021). The typical 1550 cm^{-1} peaks of the amide II groups (-NH) which were strong in EPS samples (EPS FT-IR spectra were shown in Supporting information Figure S4) disappeared in the LPS spectra, indicating that there was little protein present due to the pretreatment of protease before the LPS extraction. Thus, the vibrations at 1630 cm^{-1} were mainly from the amide I group (O = C—NH) of the N-acetylglucosamine parts in lipid A. The wavenumbers at around 1230 and 1217 cm^{-1} were from the phosphate groups (-P-O) (1230 cm^{-1} is metal ions bond phosphate and 1217 cm^{-1} is hydrogen bond phosphate), either attaching to N-acetylglucosamine in lipid A or Kdo (3-deoxy-d-manno-oct-2-ulopyranosonic acid) in the inner core.

Notably, there were some different peaks observed in the FT-IR spectra between the LPS from flocculent sludge and granular sludge: firstly, the relative peak intensity between the peak at 1040 cm^{-1} (the typical C—O-C bond of glycans) and the peak at 1630 cm^{-1} (amide I

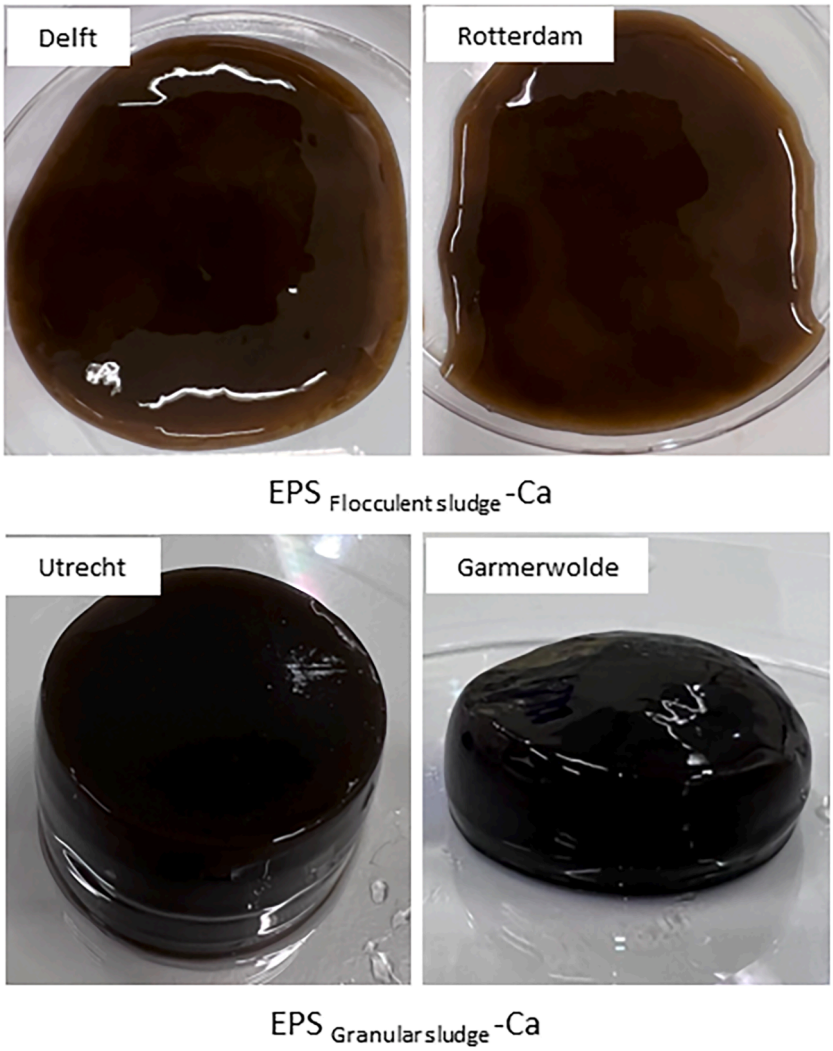

Fig. 1. EPS ionic hydrogel formation test by immerging EPS solution in calcium chloride solution ($CaCl_2$, 2.5 %, w/v). EPS concentrations were at around 1.5 %±0.3 % (w/v).

group of N-acetylglucosamine) is higher for the LPS from granular sludge than that of flocculent sludge (2 versus 1.6), suggesting that the LPS from granular sludge contain more glycans, which aligned with the aforementioned analysis.

Secondly, the wavenumber region of 1700–1750 cm^{-1} corresponds to the ester carbonyl (-C = O) absorption in lipid A (Valentine et al., 2020). Two peaks are presenting at 1735 cm^{-1} and 1718 cm^{-1} in the spectra of the LPS from granular sludge, with the absorbance intensity at 1735 cm^{-1} stronger than that at 1718 cm^{-1}. In comparison, these peaks are hardly seen in the spectra of the LPS from flocculent sludge. Given that the C = O groups can participate in hydrogen bonding interactions with water molecules or neighboring polar groups, (Brandenburg et al., 1997), the position and intensity of these carbonyl bands reflect the hydrogen bonding state of the ester carbonyl groups (Dreier et al., 2019), i.e. the peak at 1718 cm^{-1} attributed to hydrogen-bonded carbonyl groups, while the peak at 1735 cm^{-1} attributed to the non-hydrogen bonded (free) carbonyl groups (Urakawa et al., 2017). A stronger peak at 1735 cm^{-1} in granular sludge implies that the lipid exhibits higher density and/or the lipid layer is more densely packed, so that the carbonyl groups have little chance of being involved in the hydrogen bonding (Dicko et al., 1998).

Lastly, there are a few bands present in the spectrum of LPS from flocculent sludge below the wavenumber of 1000 cm^{-1} (bands with the peak at around 780, 865, 920, and 965 cm^{-1}), while this region in the spectrum of LPS from granular sludge is rather smooth. Interestingly, these bands correspond to the characteristic vibrations of trans-disubstituted alkenes (-CH_2) in unsaturated fatty acid chains. Compared to the saturated fatty acids, the unsaturated fatty acids with trans-disubstituted -CH_2 do not pack densely (Lewis and McElhaney, 2013). This could also explain why no strong peaks are appearing at 1735 cm^{-1} in the spectrum of the LPS from flocculent sludge.

In summary, the FTIR spectroscopy analysis demonstrates that in comparison to the LPS from granular sludge, the LPS from flocculent sludge contains fewer glycans and the possible presence of unsaturated fatty acids in lipid A which limits dense packing.

3.3. LPS-Ca aggregates visualization

To investigate the role of LPS in EPS-Ca hydrogel formation, the interaction between the LPS samples and calcium ions was investigated. The phase contrast microscopic images of LPS-Ca aggregates are shown in Fig. 4a. All of the LPS samples interact with calcium forming aggregates. However, the size of the aggregates is significantly different. The majority of the LPS-Ca aggregates from flocculent sludge is about 10–20 μm, distributing evenly in the aqueous solution. While the size of the LPS-Ca aggregates from granular sludge is much bigger, falls in the range of 100–400 μm.

To achieve targeted visualization of LPS, the polymyxin B based

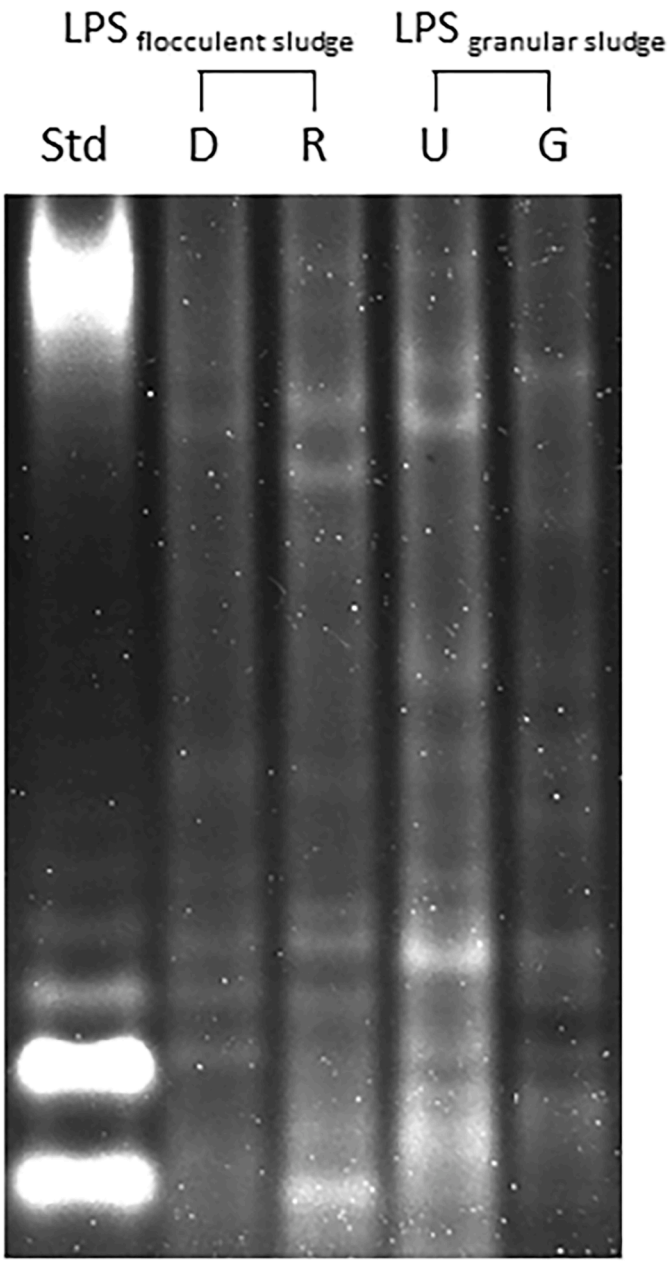

Fig. 2. SDS-PAGE with Pro-Q Emerald kit staining for lipopolysaccharides (LPS). Std, LPS standard from *Escherichia coli* serotype 055:B5. D, R, U and G: LPS samples were extracted from EPS in Delft, Rotterdam, Utrecht and Garmerwolde plants, respectively.

fluorescence probe was applied. Polymyxin B specifically binds to the lipid A in LPS. By labelling with rhodamine B as the fluorescence probe, the specific morphologies of LPS-Ca aggregates were clearly visualized with the fluorescence signal. There are some similar morphologies observed in all LPS-Ca samples (supplemental material Figure S4), however, significant differences were observed in these LPS-Ca aggregates as shown in Fig. 4b. LPS-Ca from flocculent sludge displayed the loosely packed inverted structure with water phase (black holes) in between the aggregated structure, with LPS-Ca $_{\text{Delft}}$ as inverted micelle and LPS-Ca $_{\text{Rotterdam}}$ as inverted rod, specifically. In comparison, LPS-Ca from granular sludge demonstrated the densely packed structure, with LPS-Ca $_{\text{Utrecht}}$ as bilaminar multilayered structure (onion-like) and LPS-Ca $_{\text{Garmowolde}}$ as spheres.

4. Discussion

4.1. Differences in LPS between flocculent and granular sludge influence the morphology of LPS-Ca aggregates

Lipopolysaccharides (LPS), one of the important EPS components, were specifically extracted from the EPS isolated from flocculent and granular sludge, characterized and compared. LPS consists of the glycan part and lipid A. Looking at the glycan part, the LPS from granular sludge contained more glycans, with longer glycan chain lengths and higher molecular weights. Regarding the lipid A part, it was more packed in the LPS from granular sludge. Moreover, there was almost no visible signal of unsaturated lipids observed in the FT-IR spectrum of the LPS from granular sludge. In comparison, there were clear signals of unsaturated lipids with trans configuration in the LPS from flocculent sludge. By applying the staining with polymyxin B-based fluorescent probe, bilaminar multilayered structures were observed in the LPS-Ca aggregates from granular sludge, while the inverted structures were observed in the LPS-Ca aggregates from flocculent sludge.

LPS are amphiphilic molecules, which can form a variety of aggregated structures in aqueous solutions. The type of those structures can be connected with the critical packing parameter (CPP) (Dopazo et al., 2021). This parameter is calculated as $CPP = \frac{V}{a_0 l_c}$, where V is the volume of the hydrophobic part of the molecule (in the current research it is the volume of the lipid A chains); a_0 is the area of the head group (here it is the optimal area of the glycan part); lc is the critical length of the hydrophobic tail (the critical length of the lipids).

According to the literature, the length of lipids in the LPS is relatively conservative (Wolny et al., 2011). Thus, lc can be assumed the same for LPS from both types of sludge. At this aspect, the CPP comparison can be simplified as $\frac{V_{lipidA}}{a_{glycans}}$, implying that the difference in the characterization of lipid A and glycans could influence the morphology of the LPS-Ca aggregates. In fact, there was a strong indication that the LPS from flocculent sludge has unsaturated fatty acid chains (with trans configuration). These chains introduce kinks in their structure due to double bonds, thus increasing the volume of the lipid A chains (Casares et al., 2019; Lewis and McElhaney, 2013). Furthermore, at the glycan part, the relatively shorter glycan chain length and lower molecular weight of the LPS from flocculent sludge might induce a lower optimal area a. Thus, with a higher volume (V) of lipid A and a lower optimal area (a) of glycans, $CPP_{\text{LPS from flocculent sludge}} > CPP_{\text{LPS from granular sludge}}$, favouring a less packed structure. Interestingly, according to Israelachvili (2011) and Parikh and Chorover (2007), if the aggregation structure is lamellar-like, $CPP \approx 1$ (e.g. Fig. 4b LPS-Ca Utrecht granules); if it is an invert structure, $CPP > 1$ (e.g. Fig. 4b LPS-Ca Delft and Rotterdam flocs); if it is sphere-like, $CPP < 1$ (e.g. Fig. 4b LPS-Ca Garmerwolde granules). When the different morphology of LPS-Ca in Fig. 2 is considered, the

Table 2
Relative mole percentages of sugar monomers and the total amount in weight of lipopolysaccharides.

Sludge type	Plant	Total (mg/g VS LPS)	Fucose (%)	Galactosamine (%)	Glucosamine (%)	Galactose (%)	Glucose (%)	Mannose (%)	Ribose (%)
Flocculent sludge	Delft	435.3	22.9	3.4	6.2	2.4	9.7	27.5	27.9
	Rotterdam	620.9	17.0	3.1	7.5	4.7	8.2	28.4	31.2
Aerobic granular sludge	Utrecht	693.6	24.0	4.2	7.7	4.6	10.2	40.4	9.0
	Garmerwolde	801.7	20.5	4.4	7.7	4.7	11.8	42.1	8.8

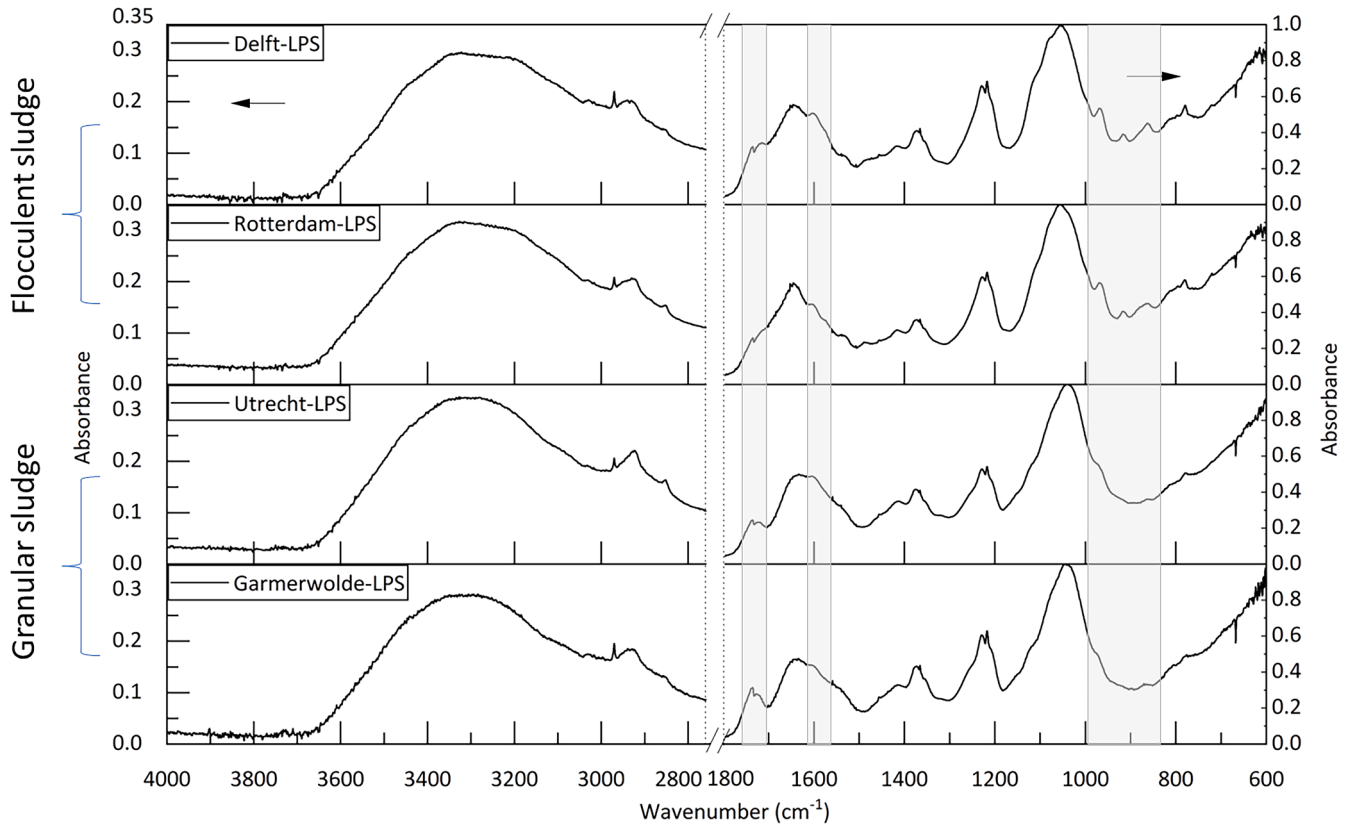

Fig. 3. FT-IR spectra of lipopolysaccharides (LPS) isolated from flocculent sludge (Delft and Rotterdam) and granular sludges (Utrecht and Garmerwolde) (wavenumber from 4000 to 2800 and 1800–600 cm^{-1}).

same conclusion that CPP $_{\text{LPS from flocculent sludge}}$ > CPP $_{\text{LPS from granular sludge}}$ can be drawn as well. Therefore, the difference in the morphology of LPS-Ca aggregates is indeed in agreement with the dissimilarity of the chemical structure and composition between the LPSs.

4.2. *LPS serving as an important component contributes to EPS properties*

LPS accounted for about 15 %−25 % of the total organic mass of the extracted EPS which is a significant fraction. LPS are amphiphilic molecules, forming a variety of aggregated structures in aqueous solutions. These aggregated structures could be stabilized by calcium ions through bridging adjacent phosphate groups in lipid A. The chemical structure and morphology of LPS-Ca might contribute to the property of EPS-Ca. Apparently, the compact bilaminar multilayered structure favors the formation of tightly packed structures that can self-stand, such as the EPS-Ca from granular sludge demonstrated. In contrast, the invert structure with the water phase in between the aggregation hinders the packing of the molecule, resulting in a fluid-like structure that is impossible to self-stand, such as the EPS-Ca from flocculent sludge displayed. Moreover, fatty acids are the components of lipids. Usually, unsaturated fatty acids have lower melting points and exist as a liquid at room temperature (Caliph et al., 2000; Jannin et al., 2008). Saturated fatty acids are comparatively more tightly packed together than unsaturated fatty acids. They have higher melting points and exist as a solid at room temperature (Siram et al., 2019). As another influencing factor, the high chance of unsaturated fatty acids in the LPS from flocculent sludge might increase the fluidity of EPS-Ca.

It is worth pointing out that, in the current discussion, assumptions and simplifications have been made to get a general overview of the connection between the characteristics of LPS and the structure of the EPS-Ca aggregates. To further understand the exact correlation, future research is needed to determine the chemical structure of lipids by GC–MS and the molecular formula of the glycan chains by NMR and MS. In addition, the conformation of amphiphilic molecules such as LPS are extremely sensitive to environmental factors such as the ionic strength, type of ions in the solution, the pH and temperature (Israelachvili, 2011), which are necessary conditions to be considered when studying the structure of EPS hydrogel.

5. Conclusion

Lipopolysaccharides (LPS), which is an important glycolipid component in the extracellular polymeric substances (EPS), constituted about 15 %−25 % of the EPS from flocculent and granular sludge. With a lower amount of glycans, shorter glycan chain length, lower molecular weight and high possibility of containing unsaturated lipids in the LPS from flocculent sludge, the LPS-Ca demonstrated an invert structure, contributing to the fluid-like property of the EPS-Ca. In contrast, with the remarkably different chemical structure, LPS-Ca from granular sludge displayed bilaminar multilayered morphology, contributing to the solid self-standing gel of EPS-Ca. The study of LPS sheds light on the properties of EPS and further developing EPS-based products.

CRediT authorship contribution statement

Ji Li: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation. **Xiaodi Hao:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Mark C.M. van Loosdrecht:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Yuemei Lin:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization.

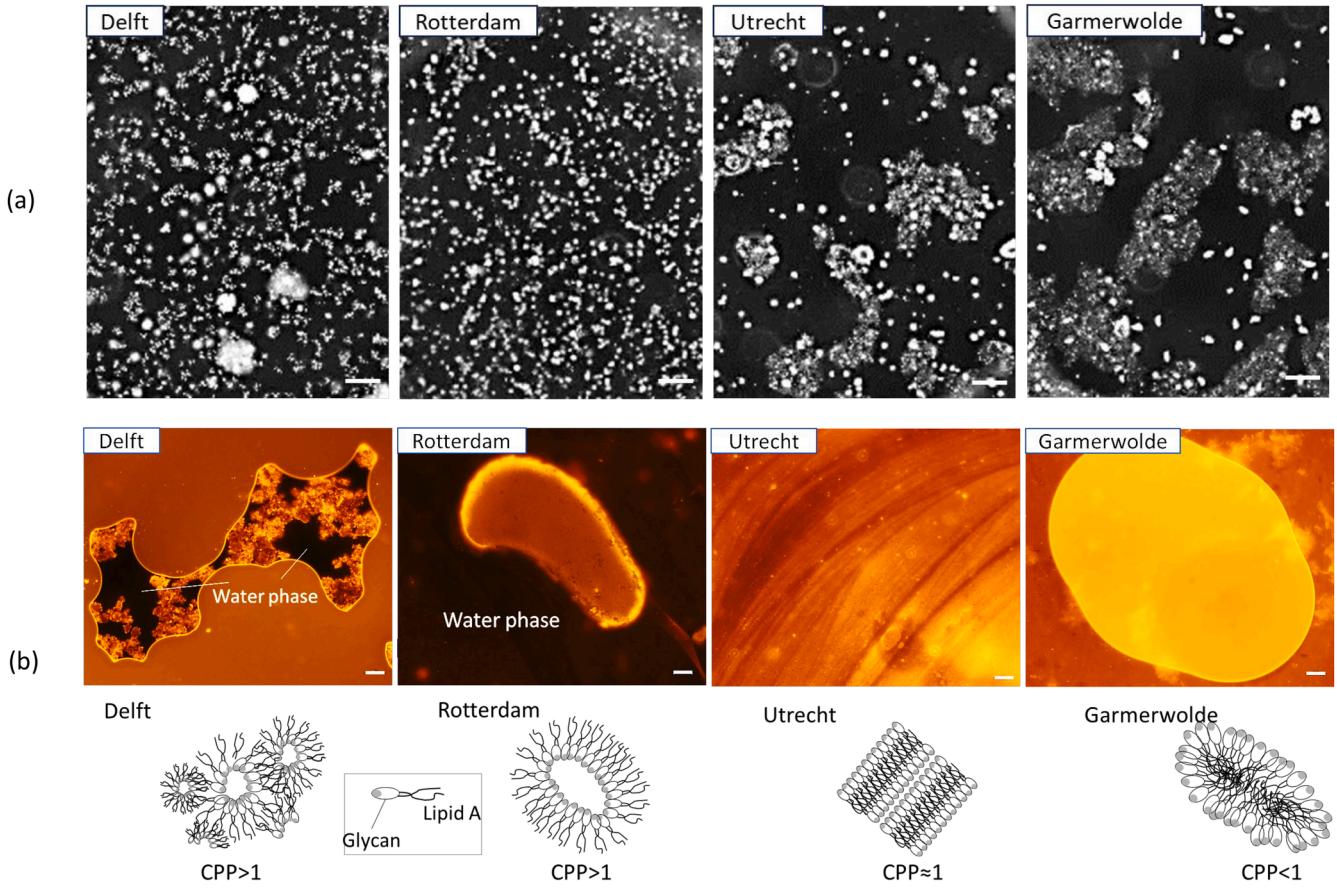

Fig. 4. The interaction between LPS and calcium ions (calcium chloride). **a).** phase contrast images showing the LPS-Ca aggregates. The size of LPS-Ca aggregates from flocs (Delft and Rotterdam) is much smaller than that from granules (Utrecht and Garmerwolde) (20–40 μm versus 200 μm). Scale bar: 100 μm. **b).** Visualization of LPS-Ca aggregates using polymyxin B labelled with Rhodamine B staining. The fluorescence images were captured with a CY3 filter. The fluorescent signal rich region indicate that binding of the lipid A in the LPS. Scale bar: 20 μm. The schematic diagram underneath the figures gives the possible formed structure and the concept of critical packing parameter (CPP) (more detailed information about CPP was discussed in Section 4.1).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2024.122707.

Data availability

Data will be made available on request.

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