



# Membrane biofouling retardation by zwitterionic peptide and its impact on the bacterial adhesion

Si-Yu Wang<sup>1</sup> · Deng-Cheng Han<sup>1</sup> · Chao Song<sup>1</sup> · Meng-Na Li<sup>1</sup> · Muhammad Zaheer Afzal<sup>1</sup> · Shu-Guang Wang<sup>1</sup> · Xue-Fei Sun<sup>1</sup>

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## Abstract

Nanofiltration polyamide membranes naturally tend towards biofouling, due to their surface physicochemistries. Nisin, a type of short cationic amphiphilic peptide with antimicrobial properties, has been recognized as a safe antimicrobial for food biopreservation and biomedical applications. This study investigates the impact of nisin on the initial bacterial attachment to membranes, its anti-biofouling properties, and characterizes a non-monotonic correlation between nisin concentration and biofilm inhibition. Nisin was found to inhibit *B. subtilis* (G+) and *P. aeruginosa* (G-) attachment to both the nanofiltration membrane and the PES membrane. To determine the mechanism of action, we investigated the polysaccharides, protein, and eDNA as target components. We found that the quantities of polysaccharides and eDNA were significantly changed, resulting in bacterial death and anti-adhesion to membrane. However, there were no discernable impacts on protein. We postulated that nisin could prevent irreversible biofouling by decreasing adhesion, killing bacteria, and reducing biofilm formation. We examined membrane flux behavior through bench-scale cross-flow experiments at a set concentration of nisin ( $100 \mu\text{g mL}^{-1}$ ), with membrane behavior being confirmed using CLSM images. Results showed that nisin could enhance anti-biofouling properties through both anti-adhesive and anti-bacterial effects, and therefore could be a novel strategy against biofouling of membranes.

**Keywords** Biofouling · Anti-bacteria · Nisin · Polysaccharides · eDNA

## Introduction

Membrane biofouling has been considered as one of the most severe problems in nanofiltration, as it can seriously impair membrane high performance via reduction of water flux, resulting in high operation costs and energy requirements (Habimana et al. 2014; Wang et al. 2017). Biofilm formation, which starts with bacterial cells approaching and accumulating on the surface of the membrane, is a key factor in

biofouling. When the bacterial cells attach to the membrane surface, microcolonies are established and then develop into a continuous biofilm by embedding in a self-produced matrix of extracellular polymeric substances including polysaccharides, proteins, and eDNA (Ke et al. 2013). Once established, biofilms are very difficult to remove (Jiang et al. 2017; Yu et al. 2018). Traditional biofouling control in membrane-based systems is heavily reliant on the application of antibiotics and chemical disinfectants (Bereschenko et al. 2011; Shah and Mitch 2011). However, addition of chemical reagents to feed water and chemical cleaning of membranes are usually quite ineffective, and the biofilm layer is typically highly resistant to routine cleaning (Andersson and Hughes 2014; Michael et al. 2013).

Unlike the previously described chemical methods, biological methods aim to remove or to inactivate microorganisms by interfering with biofilm formation (Meng et al. 2017). Antibacterial peptides have unique component, which response in all organisms including animals, plants, bacteria, and even virus. Most of antibacterial peptides have amphipathic properties, as well as they are part of the oxygen-independent activity against bacteria (Bals and Wilson 2003,

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Si-Yu Wang and Deng-Cheng Han contributed equally to this work.

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✉ Xue-Fei Sun  
xuefeisun@sdu.edu.cn

<sup>1</sup> Shandong Key Laboratory of Water Pollution Control and Resource Reuse, School of Environmental Science and Engineering, Shandong University, Qingdao 266237, China

Kościuczuk et al. 2012). Nisin is an antibacterial peptide produced by *Lactococcus lactis* that exhibits a broad spectrum of inhibitory activity against bacteria (especially Gram-positive bacteria) (Li et al. 2018). Nisin exerts this bactericidal action by either partitioning into the bacterial membrane and then disrupting the physical integrity of the phospholipid bilayer and impacting barrier function, or translocating across the membrane and acting on internal targets (Hancock and Sahl 2006). Since its discovery, nisin has been deemed an alternative biopreservative, due to its low cellular toxicity at antimicrobial concentrations, which has made a remarkable impact on the food industry (Cotter et al. 2013; Hancock and Sahl 2006). Currently, studies are also focusing on the antimicrobial action of nisin and its potential biomedical applications (i.e., bacterial infections, cancer, oral diseases, and more), as an alternative to traditional antibiotics (Benmechemene et al. 2013; Shin et al. 2016). This is promising news in terms of biofilm formation and the medical industry, as bacteria can adhere to carriers that promote biofilm formation on medical devices and cause severe infection. In fact, the anti-adhesion effects of nisin have been pursued as a method for decreasing MRSA biofilm on medical devices and were shown to be effective (Okuda et al. 2013; Shin et al. 2016).

Although research groups have comprehensively studied the food biopreservation and biomedical applications of nisin, little is understood about the effects and mechanisms of nisin use to prevent membrane biofouling in water treatment. Thus, in this study, we investigated the potential of nisin as a biofouling control agent in water nanofiltration. We showed for the first time that low concentrations of nisin ( $100 \mu\text{g mL}^{-1}$ ) can decrease membrane biofouling by destroying both G+ and G- bacteria. We also demonstrated that nisin-treated membranes had reduced bacterial attachment and that nisin treatment induced extracellular polymeric substances (EPS) production changes in bacterial colonies, although the impacts widely varied depending on the type of bacteria and nisin concentration. The effectiveness of controlling membrane fouling with  $100 \mu\text{g mL}^{-1}$  nisin was further assessed in bench-scale cross-flow filtration units, which indicated that nisin exhibits great anti-biofouling performance via both anti-adhesion and anti-bacterial mechanisms of action. In addition, we found that bacterial attachment and EPS production responded to nisin concentration non-monotonically, indicating that multiple mechanisms are involved depending on nisin concentration.

## Materials and methods

### Chemicals

Nisin was purchased from Aladdin (Los Angeles, USA) ( $\geq 1000 \text{ IU/mg}$ ). Analytical grade inorganic salts including NaCl,  $\text{MgSO}_4$ ,  $\text{NaHCO}_3$ ,  $\text{CaCl}_2$ ,  $\text{NH}_4\text{Cl}$ ,  $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ ,  $\text{KH}_2\text{PO}_4$ , and

$\text{K}_2\text{HPO}_4$  were obtained from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Tryptone, yeast extract, and agar were obtained from Oxoid of Thermo Fisher Scientific (England, Basingstoke). Deionized water was used to prepare all treatment solutions. Nisin stock solution was prepared by dissolving 10 mg of nisin 100 ml of 0.01 M HCl, and was stored at 4 °C until experimental use.

### Preparation of bacteria

*Bacillus subtilis* (*B. subtilis*) ATCC 6051 (G+) and *Pseudomonas aeruginosa* (*P. aeruginosa*) PAO1 (G-) were used as model bacteria for the cell attachment assay, EPS quantification, and bench-scale cross-flow filtration. Prior to inoculation, the bacterial strains were transferred from stock cultures to LB media and aerobically incubated 30 °C (*B. subtilis*) and 37 °C (*P. aeruginosa*). All bacteria were subsequently transferred to fresh LB media (1 fresh single colony/bacterial type) and allowed to grow overnight with shaking at 30 °C, 200 rpm and 37 °C, and 200 rpm for *B. subtilis* and *P. aeruginosa*, respectively.

### Membrane preparation

The polyether sulfone nanofiltration membranes PES (Shanghai Speed Scientific Equipment Co., Ltd.) and tight nanofiltration membrane NF90 (Dow Filmtec, Sterlitech, S Kent, USA) were used. NF90 membrane is composed of a thin layer of fully aromatic polyamides with microporous polysulfone as supporting layer. The PES membrane was used in static attachment experiments and the NF90 membrane was used in bench-scale cross-flow filtration experiments. Both membranes were received as flat sheets and stored at 4 °C in deionized water prior to experimental use.

### Microtiter plate assay

Bacterial attachment potential to the PES membrane was conducted using a microtiter plate assay. Both short- (4 h) and long-term (24 h) experiments were conducted. PES membranes were divided into 15 mm × 10 mm pieces, sterilized by exposing to UV light for 30 min, and then placed into wells of 24-well polypropylene microtiter plates. Two hundred microliters of diluted bacteria suspension (inoculation level  $10^9 \text{ CFU mL}^{-1}$ ) was added to each well. The nisin stock solution ( $100 \mu\text{g mL}^{-1}$ ) was diluted with deionized water, and then was added to each well. The concentration of diluted solution is 0, 12.5, 25, 50,  $100 \mu\text{g mL}^{-1}$ . After 4 h and 24 h incubations at 30 °C and 37 °C, respectively, membranes were gently rinsed (2×) with an 0.85% NaCl solution to remove unattached bacteria. Attached cells on the membrane specimen were resuspended by sonicating each membrane specimen in 20 ml PBS for 20 min, followed by mixing for 15 s

using a vortex mixer. Then the plate count method was used to quantify attached bacteria and scanning electron microscopy (SEM) was also used to validate counts. Three replicates were included for each nisin concentration ( $n = 3$ ). The concentrations of each group are listed in Table S1.

### Extraction and quantification of polysaccharides, proteins, and eDNA

EPS was extracted from both *B. subtilis* and *P. aeruginosa*. Purification and quantification of EPS was carried out as described in the Dignac MF method (Wang et al. 2018). Bacteria at different concentrations of nisin were removed from LB media and resuspended in phosphate buffer solution (PBS). The bacterial suspension was heated at 70 °C for 10 min and then centrifuged at 12000×*g* for 15 min at 4 °C. Coomassie brilliant blue and sulfuric acid anthrone colorimetric assays were used to quantify protein and polysaccharide concentrations, respectively.

To quantitatively assess the impact of nisin on eDNA, bound EPS was extracted from *B. subtilis* and *P. aeruginosa* and then analyzed as follows: 3 M sodium acetate was added to the EPS extract, mixed with ethyl alcohol, and preserved overnight at 4 °C. After ultracentrifugation at 10,000×*g* for 15 min at 4 °C, the precipitate was preserved and evaporated with ethyl alcohol at room temperature. The precipitate was subsequently dissolved in water for further use. eDNA in the solution was measured at OD<sub>260</sub> and OD<sub>280</sub> for *B. subtilis* and *P. aeruginosa*, respectively.

### Total organic carbon TOC analyses

Total organic carbon (TOC) analyses were carried to determine the relationship between nisin concentration and bacteria growth. To determine TOC, *B. subtilis* and *P. aeruginosa* suspensions were diluted with select nisin concentrations showing in Table S1. TOC concentrations before and after nisin exposure were measured using Shimadzu TOC-V CPH Total Organic Carbon Analyzer.

### Evaluation of anti-adhesive and anti-biofouling properties

Anti-adhesive properties of nisin were evaluated using bench-scale cross-flow filtration. The NF90 membrane alone, without bacteria suspension exposure, demonstrated a constant water flux of 1 L min<sup>-1</sup>. During filtration, the water flux was measured every 10 min. After an initial water filtration period, the synthetic wastewater was changed, and the membranes were exposed to bacterial suspensions (previously exposed to nisin) at the same initial permeation flux for 240 min. Filtration was carried out at 25 °C. Effective membrane surface area was 43.24 cm<sup>2</sup>. Filtration units were disinfected

using ethanol prior to membrane insertion, and the membranes were sterilized by washing with deionized water, then exposing them to ultraviolet light for 30 min.

Membrane permeation fluxes were determined at room temperature. Water flux and permeate flux were both measured using bench-scale cross-flow units. The permeation flux ( $J$ ) was calculated using the following equation (Eq. 1):

$$J = \frac{V}{A \times \Delta t \times \Delta P} \quad (1)$$

where  $J$  (L m<sup>-2</sup> h<sup>-1</sup> bar<sup>-1</sup>) represents the permeation flux, and  $V$  (L),  $A$  (m<sup>2</sup>),  $\Delta t$  (h), and  $\Delta P$  (bar) represent the permeation volume, the effective membrane area, the permeation time, and the transmembrane pressure, respectively.

Bacterial adhesion was also evaluated. Bacterial adhesion rate was calculated using the following equation (Eq. 2):

$$R = \frac{(B-A) \times 100\%}{B} \quad (2)$$

where  $R$  represents the bacterial adhesion rate,  $A$  is the count of viable bacterial colonies after contact with the membrane sample, and  $B$  is the count of viable bacterial colonies without contacting the membrane.

### Staining and visualization

Confocal laser scanning microscopy (CLSM) is used to visualize anti-adhesion and anti-bacterial properties. The bacteria adhered to the NF90 membrane after cross-flow filtration were stained with LIVE/DEAD BacLight™ Bacterial Viability kits (Molecular Probes, Eugene, OR, USA), which consisted of two nucleic acid dyes that stain both live and dead cells: SYTO 9 and propidium iodide (PI). SYTO 9 stains both live and dead bacteria while PI only stains dead bacteria with damaged cell membranes. The excitation/emission maxima for dyes are 480/500 nm for SYTO 9 stain and 490/635 nm for PI. The viable damaged bacteria membrane fluoresced green, whereas dead cell stained red.

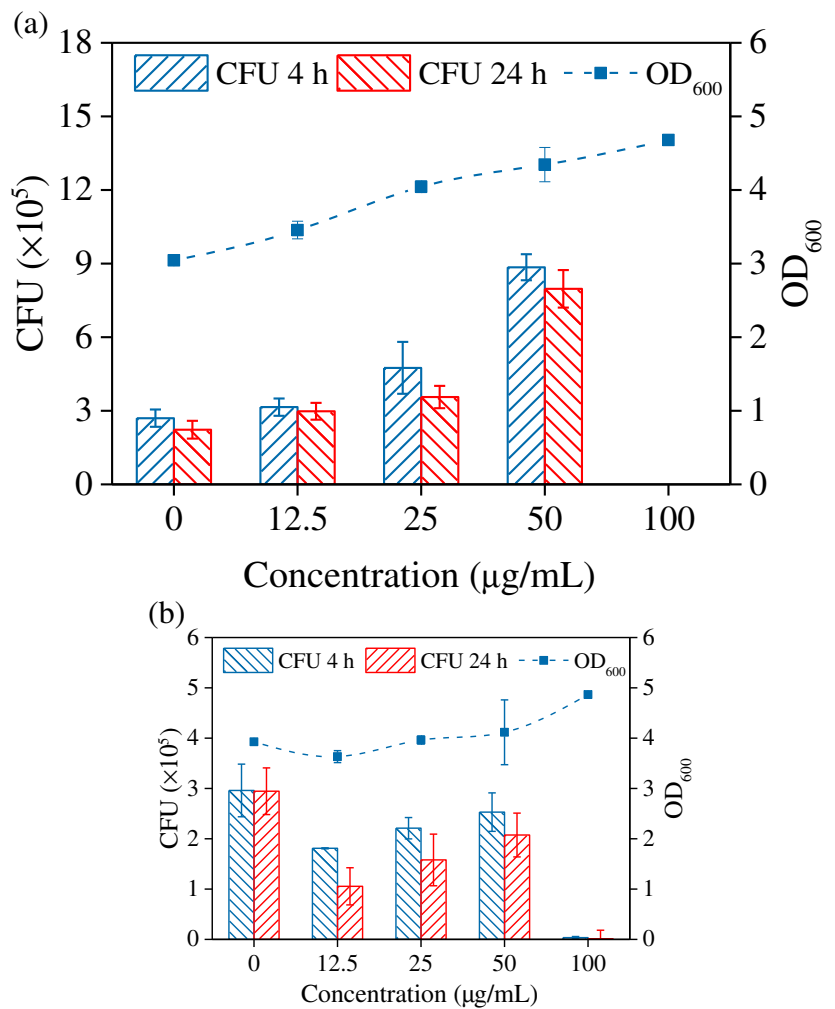
## Result and discussion

### Bacteria attachment (microtiter plate assay)

To investigate the characterize the effect of nisin on bacteria adhesion to PES membranes, exposed and unexposed *B. subtilis* and *P. aeruginosa* were incubated in a 24-well microtiter plate for short-term (4 h) and long-term (24 h) periods and attached bacteria were counted.

Figure 1 shows the amount of *B. subtilis* and *P. aeruginosa* bacteria attached to PES membranes with increasing concentrations of nisin, over short-term (4 h) and long-term (24 h)

**Fig. 1** Influence of nisin exposure on the attachment of **a** *B. subtilis* and **b** *P. aeruginosa* to PES membranes



exposure periods. Under long-term exposure settings, less bacterial attachment in the highest nisin concentration group compared to the control. Similar results were observed in short-term exposure settings. As nisin concentrations increased, a clear trend of decreased bacterial attachment was observed for *B. subtilis*. For *P. aeruginosa*, anti-adhesive effects seemed to be greater at lower concentrations of nisin (e.g., 12.5 µg mL<sup>-1</sup> and 25 µg mL<sup>-1</sup> showed 38.85% and 25.34% reductions in bacterial adhesion, respectively) than higher concentrations (e.g., at 50 µg mL<sup>-1</sup>, there was only a 14.53% reduction in bacterial adhesion). However, in *B. subtilis*, bacterial adhesion was significantly reduced with the increasing concentration (except in the highest concentration group (100 µg mL<sup>-1</sup>)). Therefore, low concentrations of nisin may partially enhance bacterial attachment. Differences in attachment between bacterial test organisms could be attributed to bacterial structure. Bag et al. found that nisin exhibited higher antibacterial efficacy against *B. cereus* than *S. typhimurium* (Bag and Chattopadhyay 2017). In this study, *P. aeruginosa* displayed a stronger attachment to nanofiltration membranes than *B. subtilis*. In fact, *B. subtilis*

has a more lipid-rich membrane wall and therefore is more likely to demonstrate higher sensitivity to nisin.

Compared to the control, the 100 µg mL<sup>-1</sup> nisin treatment notably decreased cell attachment to membrane. Indeed, for PAO1 and *B. subtilis* reductions were 98.82% and 100%, respectively. These results suggest that nisin can reduce bacteria attachment in short- and long-term exposures. It is worthy to note that when the exposure time was increased to 24 h, remarkably less bacterial cells attached to membranes in all nisin exposure groups. Thus, as exposure time is increased, the effectiveness of nisin also increased (up to 24-h exposure). Indeed, nisin has been shown to display direct antimicrobial activity under physiologically meaningful conditions. These results suggest *B. subtilis* and *P. aeruginosa* accumulation was decreased on PES membranes, and that decrease was mainly due to nisin’s bactericidal capacity. Nisin is type of cationic host-defense peptide. Therefore, positive charge may affect accumulation at negative microbial cell surfaces that contain lipopolysaccharides, and wall-associated teichoic acids in bacteria. Nisin can then contact the negative

surface of the membrane and insert into the membrane, initially straddling the hydrophilic head groups and the fatty acyl chains. Finally, nisin can disrupt the physical integrity of the bacterial membrane structure through break membrane function (Hancock and Sahl 2006). It shows that nisin can enhance the permeability of membrane because of negative inside. What is more, the rate of dissipation increases along magnitude of negative charge (Gao et al. 1991). This dissipation of bacteria membrane causes rapid efflux of amino acids and  $Rb^+$ , which results in membrane potential decrease. The transport of L-proline in membrane is blocked, and amino acids are released from bacterial cell. The cytoplasmic membrane is the primary target (Ruhr and Sahl 1985).

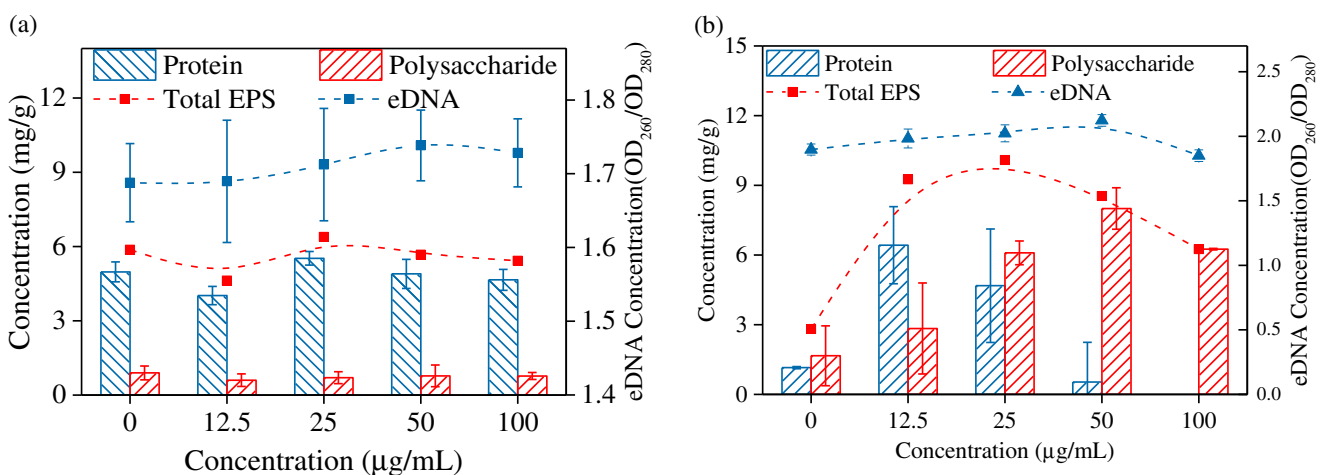
In this study, a large fraction of bacteria was killed in response to long-term attachment time, suggesting that the contact time of bacteria with nisin may be important.

To characterize the impacts of the nisin on bacterial attachment to PES membrane clearly, the SEM images was performed. The attachment of bacteria with and without nisin exposure was shown in the images. Indeed, compared with the control (Figs. S2a and S2e), the reduction of *B. subtilis* and *P. aeruginosa* in the nisin treated membranes was significant (Figs. S2c and S2g). As shown in Fig. S2, there were no apparent bacteria in the image boundary. Exposure to nisin was one of the obvious factors associated with decreased attachment (Figs. S2c and S2g). Furthermore, there were significant changes in all types of bacteria tested. Indeed, the bacillus-like bacteria in the control was distinctly different from the broken bacteria observed in the nisin-exposed culture. Under high magnification, the edge of bacteria was clearly visible in controls (Fig. S2d), but was obscure in the nisin-treated groups (Fig. S2h). Therefore, we hypothesized that nisin could both reduce the number of attached bacteria, and also kill bacteria directly.

## Impact of nisin on polysaccharides, proteins, and eDNA

Membrane biofouling is certainly related to EPS production. Polysaccharides, proteins, and extracellular DNA constitute EPS in bacterial biofilms. As shown in Fig. 2, polysaccharides and eDNA in both *B. subtilis* and *P. aeruginosa* responded to nisin exposure in a non-monotonic way. Indeed, nisin exposure changed the quantity of polysaccharides and eDNA differently in different concentration groups, which is consistent with the results from our attachment experiments. However, it is notable to point out that EPS proteins do not change in response to nisin exposure. These results suggest that nisin can change quantities of extracellular polysaccharides and eDNA, but do not have clear influences on protein. As previously mentioned, nisin can disrupt the physical integrity of phospholipids, which directly influences polysaccharides. These polysaccharide results are consistent with what was seen in the attachment experiments.

Extracellular DNA (eDNA) is another important component of biofilm. Previous studies have shown that bacteria can release eDNA via autolysis (Nagler et al. 2018). Furthermore, recent research has shown that eDNA and other kinds of extracellular polysaccharides are separated from each other in biofilms, with eDNA mostly being found in the Psl-free biofilm (Sugimoto et al. 2018). Therefore, eDNA may attribute to biofilm formation. Indeed, the coexistence of Psl and eDNA enhances bacterial attachment by forming a connected meshwork, increasing cell-to-cell interaction and biofilm compactness (Das et al. 2010; Ghafoor et al. 2011). Therefore, eDNA can create favorable conditions for cell adhesion to hydrophobic surfaces (Das et al. 2010). Accordingly, the presence of low concentrations of nisin can promote polysaccharides and produce eDNA, both of which have been proven to

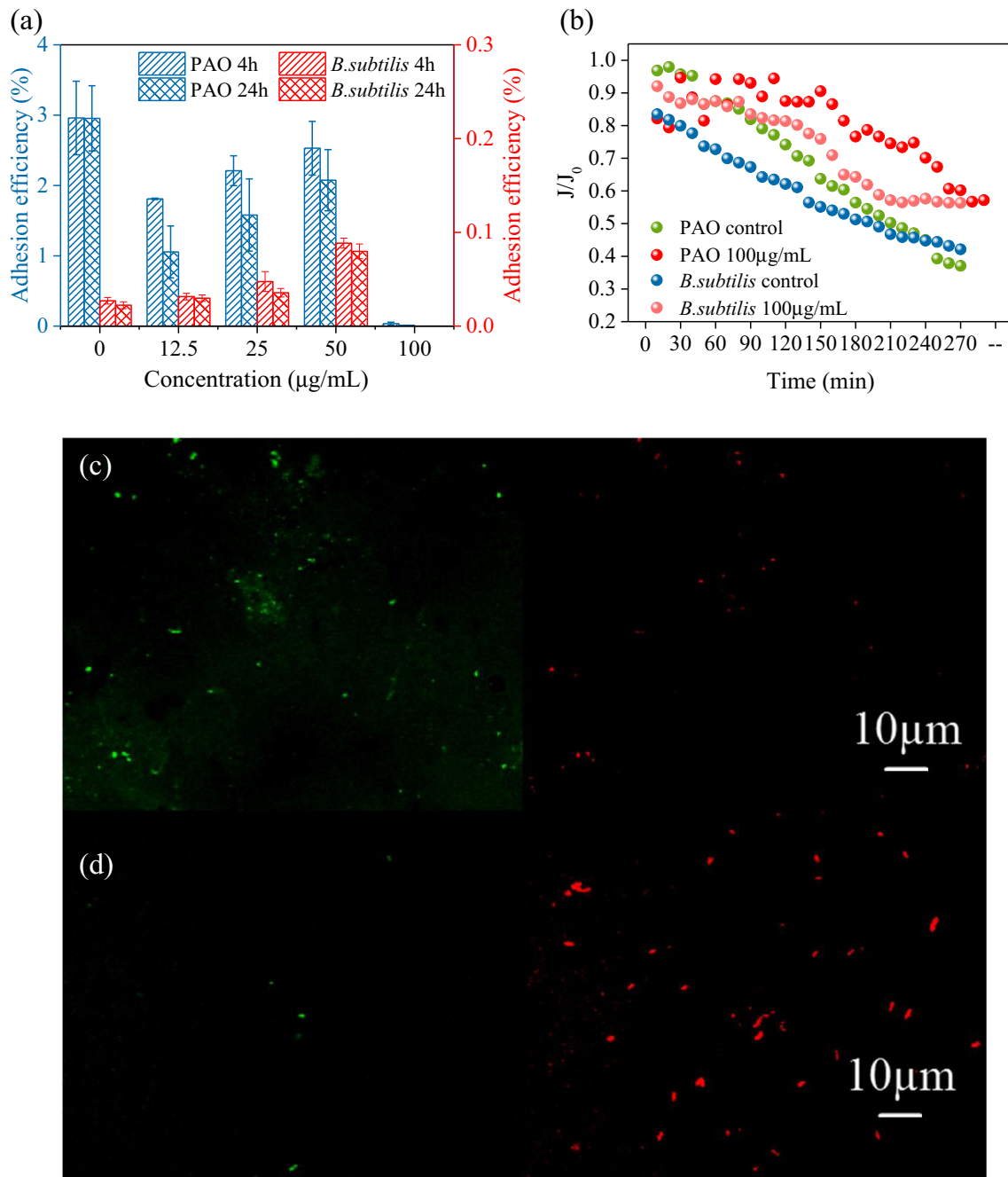


**Fig. 2** Effects of increasing concentrations of nisin on EPS production in **a** *P. aeruginosa* and **b** *B. subtilis*

enhance bacterial adhesion and surface aggregation due to the interactions of EPS products. This can also explain the results of the above attachment experiment. At high concentrations, nisin greatly reduced the quantity of polysaccharides and eDNA. However, it had no discernable impact on protein over the whole concentration range tested. These results suggest that decreased biofilm and bacteria attachment seen at high concentrations of nisin exposure is most likely due to the reduction of polysaccharides and eDNA.

### Nisin function in biofouling mitigation

The initial step of biofilm formation is bacteria adhesion. In this study, *B. subtilis* and *P. aeruginosa* were used as model bacteria to investigate effects of nisin exposure on bacterial adhesion to the membrane. Within 4 h, an obvious inhibition of initial bacteria attachment was observed in nisin-treated membranes. Furthermore, it is shown in Fig. 3a that the adhesion inhibition occurs in response to



**Fig. 3** Nisin function in biofouling mitigation. **a** Adhesion efficiency of the selected membranes. **b** Effects of nisin on NF90 membrane flux in *B. subtilis* and *P. aeruginosa*. CLSM images of permeated membranes of

unexposed *P. aeruginosa* (c) and nisin-exposed *P. aeruginosa* (d) (dead cells are shown in red, and live cells shown in green)

increasing nisin concentration in the 24-h attachment experiment. The anti-adhesion efficiency of untreated membrane was 2.96% for *B. subtilis* and 0.027% for *P. aeruginosa*. However, the membrane exposed to 100  $\mu\text{g mL}^{-1}$  nisin had very low bacterial adhesion efficiencies of 0.012% and 0% for *B. subtilis* and *P. aeruginosa*, respectively. In fact, there was statistically significant difference between the *B. subtilis* and *P. aeruginosa* in the presence and absence of nisin. As mentioned earlier, this is consistent with findings from previous research.

To evaluate anti-biofouling performance, we conducted a bench-scale cross-flow filtration experiment. During the cross-flow filtration, bacteria were adhered to the membrane surface via the permeate flow and held there by the drag force, which created a more favorable condition for biofouling. Flux during the fouling procedure was normalized to the water flux during the conditioning stage. Figure 3b shows the permeation flux of the membranes for both nisin-exposed and unexposed *B. subtilis* and *P. aeruginosa*. Significant flux decline was observed during the filtration of the synthetic wastewater without nisin for both *B. subtilis* and *P. aeruginosa*. Meanwhile, the permeate flux during the whole filtration of nutrient wastewater exhibited stable decreases when spiked with 100  $\mu\text{g mL}^{-1}$  of nisin. As the synthetic wastewater was filtered through the membrane, low nutrients and bacteria accumulated on the membrane surface, which may have supported biofilm formation, eventually causing a sharp decline in permeate flux.

Permeate flux decline lagged in both nisin-exposed bacteria groups, suggesting that bacterial accumulation on membrane was being limited. Clearly, nisin can limit bacterial adhesion on the membrane and kill bacteria, which presumably limited cell growth and repressed EPS production.

To confirm that nisin can have an anti-biofouling during water filtration, CLSM was conducted. Figure 3c, d (*B. subtilis* was shown in Figs. S4a and S4b) shows both live (green emission) and dead bacteria (red emission) after staining membranes with STYO9 and PI, respectively. The CLSM image showed that for all kinds of tested bacteria there were more live bacteria on control membrane than on the nisin-treated membrane. Indeed, compared to the control groups, the nisin-treated membrane had much more dead bacteria. Furthermore, the population of total bacteria on nisin-treated membrane was much less than control group in the visible horizon. The reduced bacterial attachment and increase of dead bacteria indicated less cell-cell and cell-membrane adhesion, which was responsible for permeate flux decline. These results suggest that the severe permeate flux decline observed without the presence of nisin could be attributed to the accumulation of bacteria and development of a biofilm, which was consistent with previous studies.

## Conclusion

In this study, the effects of nisin on bacterial attachment and on the biofouling of PES and NF90 nanofiltration membranes were investigated using *B. subtilis* and *P. aeruginosa* as model bacteria. Results indicated that the addition of nisin to the membrane feed-water is highly effective in decreasing membrane biofouling through reducing bacterial adhesion and killing bacteria. Due to differences in cell-wall structure, nisin had slightly different effects on G+ and G- bacteria. At a concentration of 100  $\mu\text{g mL}^{-1}$ , nisin can significantly reduce attachment of both *B. subtilis* and *P. aeruginosa* to membranes. In fact, low concentrations of nisin may partly enhance bacteria attachment. Furthermore, we observed concentration-dependent changes in the quantities of polysaccharides and eDNA, which is consistent with the results from attachment experiments. However, nisin exposure had no discernable impact on protein over the whole concentration range tested. Therefore, at high concentrations of nisin, the decreased biofilm and bacterial attachment observed is most likely due to the reduction of polysaccharides and eDNA. Finally, the bench-scale cross-flow experiment indicated that severe permeation flux decline occurred in the absence of nisin, which can be attributed to the accumulation of bacteria and the development of biofilm, which was consistent with previous studies.

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