

Bacteriophage-based strategies for biofouling control in ultrafiltration: *in situ* biofouling mitigation, biocidal additives and biofilm cleanser

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Abstract

The ability of bacteriophages to infect and destroy specific bacteria makes them promising antimicrobial agents in industrial processes. In this study, potential strategies of bacteriophage-facilitated biofouling control during membrane ultrafiltration (UF) were investigated through use of the model T4 bacteriophage and the model host bacterium, *Escherichia coli*. In the dead-end filtration mode, phages were immobilized on the membrane surface to serve as biocidal agents and inhibit the propagation of bacteria *in situ*. After 6 h of filtration, a 36% flux reduction was observed for the T4-functionalized membrane in comparison to 71% for the non-functionalized membrane. Surface modification of the membrane using O₂ plasma treatment resulted in increased numbers of bound phage and enhanced biofouling resistance of the membrane. Introducing the phage into the feed of a cross-flow filtration system effectively mitigated the water flux reduction of the membrane caused by bacterial growth. By modifying the concentration of phage additives, the growth of bacteria was delayed, inhibited, or eliminated. Phage treatment changed the structure of biofilms on the membrane surface and facilitated *in situ* biofilm cleaning. A 20% greater recovery in water flux was observed for the biofilm-contaminated membrane following phage-assisted cleaning when compared to the membrane cleaned via a physical washing process. Taken together, these results show that bacteriophage assisted anti-biofouling strategies have the potential to mitigate biofouling in membrane processes in an environmentally friendly manner.

Keywords: ultrafiltration membrane; biofouling control; bacteriophage; biofilm; membrane cleaning

1 Introduction

Severe shortages of fresh water will lead to a growing demand for effective water reuse and desalination strategies. As a family of advanced water purification techniques, membrane processes have increasingly been employed over recent years. Ultrafiltration (UF) is a membrane technique driven by low pressure, that generally removes colloids, microorganisms and organic compounds from water. Compared with its counterparts (coagulation, advanced oxidation, disinfection, etc.), there are no extra chemicals required during UF. Furthermore, UF units are available in various sizes, which allows a tighter control of energy consumption based on the specific application [1]. Currently, UF is considered as an indispensable pre-treatment for reverse osmosis (RO), the core technique for industrial process water production (such as in food manufacturing [2], mining [3] and landscape water recycling [4]) and the ideal standalone setup for household/outdoor water purification. Nonetheless, as with other membrane techniques [5, 6], UF suffers from irreversible fouling caused by microorganism propagation. Chemical cleaning (e.g., chlorine) is commonly applied in commercial membrane processes to mitigate biofouling; however, polymeric membrane materials are vulnerable to chlorine-based oxidative damage. Even worse, it has been reported that chemically-washed membranes can be more prone to bacterial attachment [7].

Incorporating functional materials into the membrane matrix to reduce sorption of organic compounds and bacterial growth is a promising method for biofouling mitigation. Polymers and nanoparticles (NPs) possessing anti-fouling properties, such as hydrophilic compounds (polysulfobetaine [8, 9], polyethylene glycol [10], silica NPs [11], etc.), low surface energy

polymers (such as polydimethylsiloxane [12] and perfluorooctanoate [13]) and biocidal agents (e.g., quaternary ammonium [14], metal NPs, graphene oxide [15, 16]), have all been applied in the design of anti-biofouling membranes. Although appreciable improvements have been reported in laboratory research, few of them have been applied in a large-scale setup due to the high cost of materials, sophisticated fabrication procedures, potential emergence of antibiotic-resistant bacteria and general health concerns.

Biological anti-biofouling materials, such as enzymes and peptides [17, 18], are of growing interest in anti-biofouling membrane fabrication, because of their effective anti-microbial properties, low cost and low toxicity to humans. Bacteriophages (phages) are another appealing option. Bacteriophages exist naturally in soil and marine environments; they are one of the most common organisms in the biosphere. Bacteriophages inactivate host bacteria by binding to the host surface, injecting their genetic material into the bacterial cells and then multiplying inside the cell until the cell membrane breaks. It has been reported that one lysed bacterial cell is able to release 300 new bacteriophages for further infection [19].

The isolation and large-scale propagation of bacteriophages stimulated the application of phage in diverse anti-biofouling fields [20, 21]. A bacteriophage suspension can effectively disperse and eradicate biofilm formed on a solid surface [22, 23]. Bacteriophage coated metal probes are able to detect the appearance of pathogens on medical equipment [24] and in food [25]. Phage coated polymeric materials can be used as wound dressings to prevent bacterial infection [26, 27] and phage functionalized platforms can be used in the design of biosensors [28]. In other

applications, phage-coated paper [29] and cellulose membranes [30] have been used as protective packages to prevent contamination of foods by bacterial pathogens. In water treatment processes, phages have been employed as indicators of disease-causing bacteria [31], been added to anaerobic/aerobic processes to inhibit the growth of undesirable bacteria [32] and been blended with sludge to improve its dewaterability [33].

The effective anti-microbial properties of bacteriophage may be exploited to mitigate biofouling in membrane processes, with the aim of increasing membrane lifetime and reducing operating costs. Goldman et al. [34] were the first to report the use of bacteriophages to alleviate biofouling of membranes used in water filtration. The water permeation flux of an UF membrane was improved by 40-60% by addition of bacteriophages to the feed water (*P. aeruginosa*, *A. johnsonii* and *B. subtilis* and their lytic phages, $N_{bacteria}:N_{phage} = 1:50\sim70$). However, no specific investigation on the optimization of phage dosage was reported. Phage treatment was recently used to achieve considerable water flux recovery (78%) of hollow fiber membranes in a membrane bioreactor (MBR) [35]. To date, these are the only two studies that examined the impact of phage on the performance of the membrane filtration process; hence, our understanding of phage activity in these systems is very limited in comparison to that of other biocidal agents. To broaden the application of bacteriophage-based biofouling control in membrane processes, more potential strategies and operational protocols need to be explored.

While phage has been shown to be effective as biocidal additives during membrane filtration and as biofilm cleansers for membrane washing, the potential implementation of phage for *in situ*

membrane biofouling control has not been previously explored. Phage-based *in situ* biofouling control requires anchoring of phages on various surfaces via physical adsorption [36, 37] or covalent binding [26, 38] methods. Immobilizing phages onto membranes via such strategies is expected to mitigate the dilution effect during the filtration process and ensure that most of the phages effectively contact bacteria that approach the membrane surface.

In this study, T4 phage and its host bacterium, *E. coli*, were selected as model biocidal agent and model bacterium, respectively, to investigate bacteriophage-assisted biofouling control in the UF membrane filtration process. The *in situ* anti-biofouling potential of bacteriophages was investigated by anchoring T4 phage on the membrane surface to mitigate the water flux reduction of a dead-end UF process. In a cross-flow filtration system, the T4 phage was introduced as a biocidal agent in the feed. The performance of T4 in preventing membrane flux decline and the effectiveness of phage treatment for membrane biofilm removal (i.e., membrane cleaning) were investigated. This study demonstrates that phage functionalized anti-biofouling membranes and phage-based additives/cleaners could be economical and environmentally friendly approaches to relieve biofouling problems in membrane filtration processes.

2 Materials and Methods

2.1 Chemicals and media solutions

N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) (crystalline), N-Hydroxysuccinimide (NHS) (98%), ethylenediamine (ED) ($\geq 99.5\%$, GC), 2-(N-morpholino) ethanesulfonic acid (MES) buffer ($\geq 99\%$) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic

acid (HEPES) (BioPerformance Certified, $\geq 99.5\%$) buffer were purchased from Sigma-Aldrich (Oakville, ON, Canada).

All solutions were prepared in deionized (DI) water produced by a Milli-Q ultrapure water purification system (Millipore, Billerica, MA, USA) and were sterilized before application.

SM buffer, containing 5.8 g/L sodium chloride (NaCl) (BioXtra, $\geq 99.5\%$), 0.12 mg/L magnesium sulfate (MgSO_4) (anhydrous, ReagentPlus[®], $\geq 99.5\%$) and 5% (v/v) 1-M Tris-base, with the final pH adjusted to 7.5 using 1M hydrochloric acid (HCl) (ACS reagent, 37%), was used as the phage propagation medium. Luria Bertani (LB) (Microbiologically tested) medium prepared at a concentration of 20 g/L was used for bacterial culture. Synthetic wastewaters were prepared by adding a carbon source to DI water. For the dead-end filtration process, 5 g/L glucose ($\geq 99.5\%$, GC) was used as the C source, whereas for the cross-flow filtration process, 5 g/L LB was used. The following salts were added to each medium to prepare the synthetic wastewaters: 0.6 g/L disodium phosphate (Na_2HPO_4) (NIST SRM 2186II), 0.3 g/L potassium dihydrogen phosphate (KH_2PO_4) (anhydrous, HPLC LiChropur[®]), 0.1 g/L ammonium chloride (NH_4Cl) (BioUltra, $\geq 99.5\%$), 0.5 g/L sodium chloride (NaCl) (BioXtra, $\geq 99.5\%$), 0.12 g/L magnesium sulfate (MgSO_4) (anhydrous, ReagentPlus[®], $\geq 99.5\%$) and 0.01 g/L calcium chloride (CaCl_2) (anhydrous, $\geq 96.0\%$). The final solution had a pH of 6.5 ± 0.2 and calculated total ionic strength of 27.5 mM. The synthetic wastewater had conductivities of $1660 \pm 20 \mu\text{S cm}^{-1}$ (OAKTON conductivity meter, CON 11 series, Vernon Hills, IL USA) for the glucose-containing solution and $1740 \pm 20 \mu\text{S cm}^{-1}$ for the LB-containing solution. An autoclave and membrane filtration

(0.22 μm) were used to sterilize the LB-containing and glucose-containing synthetic wastewaters, respectively.

2.2 Membrane modification

Three types of membranes were used in a preliminary infectivity test: (i) pristine poly(ether sulfones) membrane (PES), (ii) PES membrane treated with oxygen plasma (PES-O₂) and (iii) O₂-PES membrane treated sequentially with EDC/NHS crosslinker and ED (PES-O₂-ED). The PES membrane (Sepro PES 30) (MWCO: \sim 20 kDa) was purchased from SEPRO Separation Solutions (Carlsbad, CA, USA). Membrane samples were stored in DI water at 4 °C and dried in a desiccator overnight before use. To prepare a PES-O₂ membrane, the dry UF membrane sample was placed on a quartz disk which was then placed directly under a live electrode in a vacuum chamber with the chamber walls acting as the grounding electrode. The chamber was then evacuated using a two-stage rotary vane pump (Alcatel Adixen Pascal Series 2005SD, Ideal Vacuum Products LLC, NM, USA) until a pressure approximately 10 mtorr (\sim 1 Pa) was reached, after which the chamber was filled with a mixture of compressed oxygen (99.993%, 5 sccm) and argon gas (99.999%, 250 sccm) to a pressure of 1.2 - 1.3 torr (160 -173 Pa). Then, 20 W of continuous radio frequency power was applied to each sample for 60 s of plasma treatment.

An amine-carrying chemical, ED, was used to prepare an -NH₂ functionalized membrane (PES-O₂-ED) by modifying the PES-O₂ membrane surface. The PES-O₂ membrane was fixed to the bottom of a Falcon™ polystyrene 12-well microwell plate (Thermo Fisher Scientific, Waltham, MA, USA) with the active layer immersed in 2 mL EDC-NHS solution (consisting of 4 mM

EDC, 10 mM NHS, and 0.5 M NaCl in 10 mM MES buffer) for 1 hour to convert the carboxyl groups on the PES-O₂ membrane into intermediate amine-reactive esters. Then, the EDC/NHS modified membrane was rinsed with DI water and contacted with 2 mL of 10 mM ED solution (dissolved in DI water with 10 mM HEPES buffer and 0.15 M NaCl) for 30 min. Finally, the membrane was rinsed with sterilized DI water (2 mL) 3 times to remove the unreacted ED.

2.3 Propagation of bacteria and T4 phage

The K12 strain of *Escherichia coli* (*E. coli*) and the T4 bacteriophage (ATCC[®] 11303-B4[™]) were used in this study. Culturing of *E. coli* was performed as described in our previous work [39, 40].

A phage stock of 10¹² PFU/mL (PFU=plaque forming units) was prepared and adjusted to 10¹⁰ PFU/mL for all experiments using dead-end filtration. The concentration of phage suspension used in the cross-flow test varied from 10⁸ to 10¹⁰ PFU/mL. The propagation of T4 was based on our earlier work [38, 41]. In brief, 100 µL of T4 phage stock was added to 5 mL of the overnight-cultured *E. coli* suspension and incubated at 37 °C on a platform shaker (120 rpm) for 20 min. Then, the mixed suspension was added to 200 mL of *E. coli* (mid-exponential growth phase) and the entire suspension was incubated overnight. This suspension was then centrifuged at 5,000g for 10 min to remove agar and bacterial debris, followed by a purification of supernatant using the PEG8000/NaCl aqueous two-phase method [42]. The obtained phage suspension was further centrifuged at 16,000g for 1 h. Then, the supernatant was discarded and the precipitated phage was resuspended in SM buffer. To determine phage titer, ten-fold serial dilutions were prepared and plated with the Double-Layer Agar (DLA) method by mixing 100 µL of a diluted phage

suspension together with 100 μL of *E. coli* (mid-exponential growth phase, $\text{OD}_{600} = 0.3 \pm 0.1$) in 3 mL of molten soft agar, which was then spread over LB plates. The LB agar plates were incubated overnight at 37 °C and the plaques were counted the following day.

2.4 Phage immobilization on the membrane surface

Before the phage immobilization, PES, PES-O₂ and PES-O₂-ED membranes were exposed to UV in a fume hood for 10 min sterilization. A membrane coupon with a surface area of 3.8 cm² was cut and placed at the bottom of a Falcon™ polystyrene 12-well multiwell plate (Thermo Fisher Scientific, Waltham, MA, USA) with the active side in contact with 1 mL of 10¹⁰ PFU/mL T4 phage suspension for 24 h at 4 °C. The phage suspension was discarded and replaced with 1 mL of SM buffer. Finally, the membrane samples were lifted vertically with a tweezer and rinsed three times with 1 mL of SM buffer ejected from a pipette to remove any unattached or loosely-attached phage.

2.5 Characterization of membranes

X-ray photoelectron spectroscopy (XPS) was used to investigate the changes in elemental composition and chemical bonding on the membrane surface before and after plasma treatment as well as phage coating. The analysis was carried out using a monochromatized Al-K α X-ray source $\nu = 1350$ eV with a spot size of 400 μm (ThermoFisher Scientific K-Alpha, Waltham, MA, USA). A minimum of three positions were selected on each membrane surface to obtain the average atomic percentage of elements on the membrane surfaces. The surface morphology of the membranes was observed by FEI Quanta 450 Environmental Scanning Electron Microscope (FE-

ESEM) (FEI Company, USA). All membrane samples were coated with platinum (~4 nm) before observation. The pore size distribution on the membrane surface was analyzed using the ImageJ 1.50i software.

For the observation of bacteria on the membrane surface, the sample was contacted with 2.5% glutaraldehyde (in 0.1 mM sodium cacodylate buffer) for 15 min at room temperature to fix the bacterial cells [43]. Then, the sample was rinsed twice with a 0.1 mM sodium cacodylate buffer solution and dehydrated sequentially with ethanol/DI water solutions (30%, 50%, 70% and 90%) for 10 min each. The samples were dried for at least 3 h before applying a platinum (4 nm) coating.

2.6 Infectivity test (lysis circle) of UF membranes

100 μ L of mid-exponential phase *E. coli* K12 bacteria (10^7 CFU/mL, $OD_{600} = 0.30 \pm 0.01$) was mixed with soft molten agar (immersed in 54 °C water bath after autoclaving), poured onto an LB agar plate and left for 10 min to solidify. The phage-functionalized membrane coupons were placed on the bacteria-LB agar plates with the active side facing down. The entire plate was incubated at 37 °C for 18 h to observe the formation of lysis rings around the membrane coupons. The average lysis diameter for five replicate experiment is reported.

2.7 Anti-biofouling efficiency of phage-modified surface in dead-end and cross-flow filtration modes

The *in situ* biofouling control potential of the T4-functionalized membrane was observed via a dead-end filtration system. In an Amicon Dead-end Filtration Cell, 20 mL of *E. coli* K12 (10^5

CFU/mL) suspension was filtered using pristine and phage-functionalized membranes to allow the deposition of bacterial cells. Then, the feed was changed to synthetic wastewater to promote the replication of bacteria that attached to the membrane surface in the first stage. The filtration process was continued for at least 6 h under 30 psi (2.07 bar) pressure and the water flux of the membrane was recorded. The membrane sample (effective surface area: 3.1 cm²) was compacted for 30 min to achieve steady permeation flux. Therefore, time 0 in Fig. 4 was 30 min after the beginning of synthetic wastewater filtration. The average results of triplicate experiments are reported.

A standard bench-scale UF cross-flow filtration system (Fig. S1) was used to investigate the anti-biofouling performance of the T4-functionalized membrane. First, the membrane sample (effective surface area: 33 cm²) was compacted with 5 L of sterilized DI water for 8 h to achieve steady permeation flux under a pressure of 30 psi (2.07 bar), a flow rate of 0.5 LPM and at a controlled temperature of 25 °C. The feed solution was then switched (without flow interruption) to synthetic wastewater inoculated with *E. coli* K12 (final concentration in the feed tank: 10⁵ CFU/mL). The filtration process was continued for 24 h at 25 °C. The average results of triplicate experiments are reported.

The predation relationship between the selected model phage and bacterium was observed using a batch test. 100 µL of prepared *E. coli* and 100 µL T4 phage stock were inoculated into 2 mL of synthetic wastewater (with LB as nutrient source) to achieve the desired concentration ratios ($N_{E.coli} : N_{T4\ phage} = 10^6\ \text{CFU/mL} : 10^8\ \text{PFU/mL}$, $10^6\ \text{CFU/mL} : 10^9\ \text{PFU/mL}$ and $10^6\ \text{CFU/mL} : 10^{10}$

PFU/mL). The same mixed suspension was prepared in all 12 wells of a Falcon™ polystyrene 12-well multiwell plate (Thermo Fisher Scientific, Waltham, MA, USA) and the plate was incubated at 25 °C at 100 rpm on a platform shaker (New Brunswick Scientific Co., Inc. NJ, USA) and every hour, the optical density (OD₆₀₀) of one well was measured by UV/VIS spectrophotometry (UV/VIS Agilent Cary 8454 spectrophotometer, Santa Clara, CA, USA) to obtain a bacterial growth curve over 12 h. The experiment was conducted in triplicate. A sterile solution of 0.9% NaCl (100 µL) mixed with 100 µL T4 phage stock served as the negative control.

2.8 T4 phage treatment for a biofouled membrane

The effect of phage treatment on the removal of biofilm from a membrane surface was investigated by growing biofilm on a PES membrane, then washing the biofouled membrane with DI water or a phage suspension while observing changes in water flux. To conduct the experiment, 20 mL of an *E. coli* suspension (10⁶ CFU/mL) was filtered through a pristine PES membrane to deposit bacteria on the membrane surface. Then, without removing the biofouled membrane coupon from the Amicon Dead-end Filtration Cell, 5 mL synthetic wastewater (LB as carbon source) was added to the cell which was placed for 24 h at 37°C on a platform shaker at 100 rpm. Next, the synthetic wastewater was discarded, and the cell was gently washed with DI water three times to remove loosely bound bacteria. To wash the biofilm, 5 mL of DI water (control) or 10¹⁰ PFU/mL of T4 suspension was added into the cell containing the membrane sample. This single dose of phage treatment at a concentration of 10¹⁰ PFU/mL was shown to be effective at removing biofilm in an earlier study [44]. Studies examining the use of bacteriophage at different doses to control or remove biofilms are summarized in Table S1. The entire cell with membrane and DI

water (or phage suspension) was placed on a platform shaker at 100 rpm for 2 h at room temperature. The whole filtration cell with membrane was once again rinsed three times with DI water. Finally, the water permeation flux of the membrane was examined with both DI water and synthetic wastewater under the same conditions as described in Section 2.7. The biofilm morphology on the membrane surface was observed by SEM after removing the membrane from the cell. The average of duplicate experiment is reported.

2.9 Statistical Analysis

The data was analyzed via the Student's t-test (Excel) assuming a two-tailed and homoscedastic/heteroscedastic distribution (determined by the F-test, $p < 0.05$) to determine any statistically significant differences. P values of < 0.05 were considered significant.

3 Results and Discussion

3.1 Immobilization of T4 phage on the different membranes

Due to their hydrophobic nature, the sulfone and ether composed backbones of pristine PES membranes are favorable to the binding of protein moieties mainly via hydrophobic interactions. Oxygen plasma treatment has been applied to produce negatively charged functional groups (carbonyl ($-C=O$), hydroxyl ($-OH$) and carboxylic acid ($-COOH$)) on different substrates [45]. The PES- O_2 membrane was expected to provide more stable anchoring of phages via hydrogen bonding and acid-amine covalent bonding which leads to amide linkages [36]. Although there is no precise model regarding the charge of bacteriophages, it has been hypothesized that most of

the tailed phages present a net negative charge with negatively charged heads and positively charged tails [46]. According to this hypothesis, a positively charged surface may lead to a “head-down, tail-up” orientation of T4 phages and perform more effectively in the inactivation of bacteria (since tailed phages use tails to capture the host bacteria). Therefore, in addition to the pristine PES and PES-O₂ membranes, EDC/NHS-ED treated PES-O₂ membranes were also prepared to develop a positively charged surface (with -NH₂ functionalities) to facilitate immobilization and effective orientation of phage on the membrane. Since it was reported that overnight contact offered complete and sufficient immobilization of phages on various substrates via both physical adsorption and covalent binding [36], the phage suspension was placed in contact with membrane samples for 24 h to achieve complete immobilization.

XPS analysis of the membrane surfaces (PES, PES-O₂ and PES-O₂-ED) was performed to confirm whether the -COOH and -NH₂ functionalized surfaces were successfully further functionalized by O₂ plasma and ED treatment. The O element content on the PES-O₂ surface increased by approximately 3.9% in comparison to that on the PES surface ($p < 0.05$) (Table 1). The O1s spectra were further analyzed to observe changes in functional groups on the membranes. The pristine PES membrane contained mainly O=S=O (532.3 eV) and C-O-C (533.8 eV) functional groups [47] from the polyethersulfone backbone (Fig. 1A). The O₂ plasma treatment led to the appearance of new oxygen-containing functional groups, HO-C=O (533.1 eV) and C-OH (534.3 eV), which accounted for 21.6% of the total O spectra (Table S2). The increase of oxygen-containing functional groups was also confirmed by analysis of chemical bond distribution based on the C1s spectra across the different membranes (Fig. S2, Table S3). The weak N1s peaks on

the pristine PES and PES-O₂ membranes were unexpected and their appearance might be ascribed to impurities introduced during fabrication of the commercial membrane (Fig. 1B). After ED coating, the peak intensity of N1s strongly increased and the N content on the PES-O₂ membrane increased from 1.2 % to 3.8 % (p<0.05) (Fig. 1B, Table S4). The existence of N-C=O (400.2 eV), -NH₂ (399.5 eV) and -NH₃⁺ (402.5 eV) functional groups [48, 49] on the PES-O₂-ED membrane confirmed the covalent binding between -NH₂ groups of ED and -COOH groups on the membrane. The predominant functional groups on PES-O₂ (-COOH) and PES-O₂-ED (-NH₂) membranes confirmed successful membrane surface modification and these three types of membranes were expected to demonstrate different extents of phage binding.

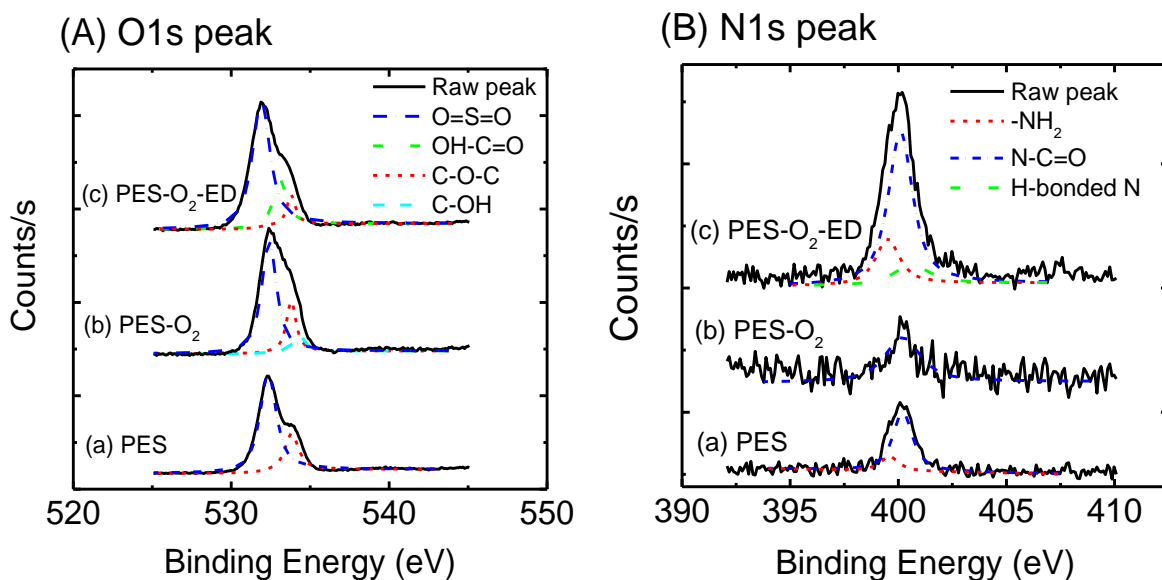


Figure 1. X-ray photoelectron spectroscopy (XPS) (A) O1s spectra and (B) N1s spectra for the pristine membrane (PES), oxygen plasma treated membrane (PES-O₂) and PES-O₂ after ED coating (PES-O₂-ED).

Table 1. Elemental content of the different membranes before and after T4 immobilization

Membrane	C (%)	O (%)	S (%)	N (%)	P (%)
PES	71.6 ± 3.8	21.1 ± 2.8	6.1 ± 0.3	1.4 ± 0.6	-
PES-O ₂	67.9 ± 0.5	25.2 ± 0.6	5.5 ± 0.1	1.2 ± 0.2	-
PES-O ₂ -ED	69.6 ± 0.3	20.5 ± 0.4	5.2 ± 0.2	3.8 ± 0.1	-
PES+T4	63.6 ± 3.6	27.7 ± 3.6	1.4 ± 0.1	5.8 ± 0.5	1.5 ± 0.1
PES-O ₂ +T4	64.0 ± 0.2	25.7 ± 0.3	2.4 ± 0.1	5.9 ± 0.6	1.9 ± 0.2
PES-O ₂ -ED+T4	62.7 ± 0.1	20.2 ± 0.8	4.4 ± 0.5	4.8 ± 1.1	1.3 ± 0.4

The changes in elemental composition of the membrane surface after T4 immobilization (PES+T4, PES-O₂+T4 and PES-O₂-ED+T4) were evaluated (Table 1). Phages are rich in nitrogen and phosphorus found in the capsid proteins; hence, N and P peaks were considered as markers for the presence of phage on the membrane surfaces. We observed a significant increase in the atomic percentage of N on PES and PES-O₂ membranes (p<0.05) after T4 immobilization, along with a similar decrease of S on both membranes. This result may be linked to the phage coverage that reduced the exposure of C-S/S=O=S bonds of polyethersulfone on the membrane. Because of the amine content in the ED monomer and -SO₃ groups in the EDC/NHS crosslinker, the N and S content are not good indicators of T4 immobilization on the PES-O₂-ED membrane.

The characteristic P2s peak appeared with an atomic P content of 1.3% on the PES-O₂-ED membrane, confirming the attachment of phage. The P peak was also observed on the T4 immobilized PES and PES-O₂ membranes, which confirmed the successful immobilization of T4 across different membrane surfaces. There was no significant difference in the T4 quantity on the three types of membranes based on the atomic P content. However, since the P content was considerably lower than that of other detected elements on all membranes, it may not be an accurate measure to quantify the amount of attached T4 phage. The quantity of attached T4 phage on the different membranes was further verified via measurement of a lysis zone and SEM observation.

The binding of the phages to the membrane surfaces was further verified by the bacterial lysis experiment. Membranes (3.8 cm² coupons) functionalized with T4 phages were placed on top of a lawn of *E. coli* on agar plates (with the phage functionalized surface facing down) and incubated overnight. Immobilized phages were expected to recognize and infect their host bacteria, thereby producing bacteria-free zones. There was no obvious lysis circle observed on the control membranes (Fig. 2A-C). In contrast, 4.5 ± 2.0 mm, 7.0 ± 3.5 mm, and 2.8 ± 1.5 mm lysis rings presented around T4 functionalized PES, PES-O₂ and PES-O₂-ED (Fig. 2D-F) membranes, respectively. These results indicate that the immobilized phages on all three types of membranes maintained the capability to bind and inactivate microorganisms on the membrane surface. The larger lysis circles around selected membranes may indicate a larger quantity of effectively oriented phages (“head-down, tail-up”) on the surface that could yield faster phage propagation and

diffusion. Therefore, it was concluded that PES and PES-O₂ surfaces are more favorable to the effective binding of T4 phages.

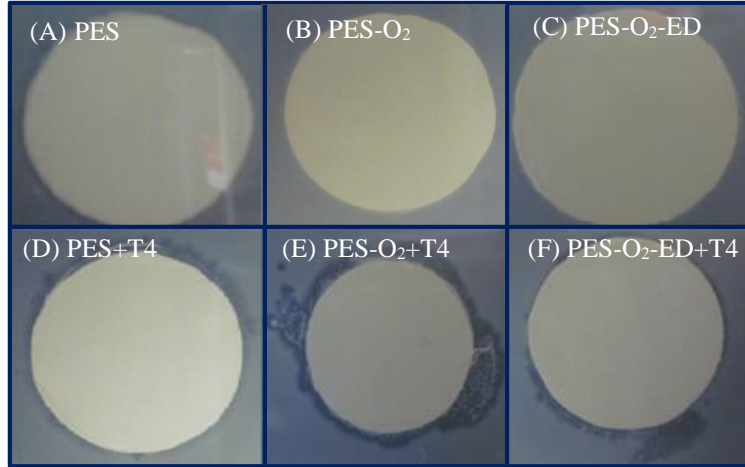


Figure 2. Plaque assays of phages immobilized on (A) pristine PES, (B) PES-O₂, and (C) PES-O₂-ED membranes. The lysis rings were measured after the membrane coupons (3.8 cm²) were placed in contact with *E. coli* lawns for 18 h at 37 °C.

The membrane surface morphology and the immobilized phage density were further observed by SEM. The plasma treatment increased the average pore size of the PES membrane (from 16.5 ± 2.7 nm to 22.5 ± 1.4 nm) (Fig. 3A-B). Most of the bacteriophages had an ellipsoid shape (Fig. 3D-F). The size of phages ranged from 45-189 nm. Theoretically, the PES and O₂-PES membranes should retain all phages on their surface in the filtration process; however, a small quantity of phages was observed on the permeate side, likely forced through by the high pressure used in the UF process and some unavoidable defects (collapsed pores, folds, uneven chemical materials, etc.) found in pristine membranes (tested phage rejection: 99.93% for PES-O₂ and

99.99% for PES membranes at 30 psi in the dead-end filtration module with a phage suspension of 10^8 PFU/mL). The phage densities, calculated from SEM images, on PES, PES-O₂ and PES-O₂-ED membranes, were 1.5 ± 0.3 particles/ μm^2 , 2.0 ± 0.3 particles/ μm^2 and 1.3 ± 0.2 particles/ μm^2 , respectively. The difference between PES and PES-O₂-ED membrane was not significant; however, the PES-O₂ surface showed the highest degree of phage anchoring among these membranes. Although the quantity of phage attached on the membrane surfaces was lower in comparison to that reported in the literature (4.5 ± 0.7 particles/ μm^2 , immobilized on a glass slide) [38], the observation of bacterial lysis showed that there was a sufficient quantity of phages to inhibit bacterial reproduction and prevent biofouling *in situ*.

The high antimicrobial activity of a membrane can be attributed to the combined effect of phage quantity and favorable phage orientation. Due to its small size, it was difficult to distinguish the orientation of T4 phage via SEM, therefore, fluorescence microscopy was used to confirm the favorable orientation of T4 phages based on the bacterial capture efficiency (Fig. S3). In comparison to control membranes, phage-functionalized membranes showed a significant increase in bacterial attachment, which can be attributed to the bacterial recognition and capturing ability of the phage. Both the bacterial lysis experiment and fluorescence microscopy observation inferred that there were sufficient quantities of phage on the membrane surface to effectively capture the bacteria and inhibit their growth.

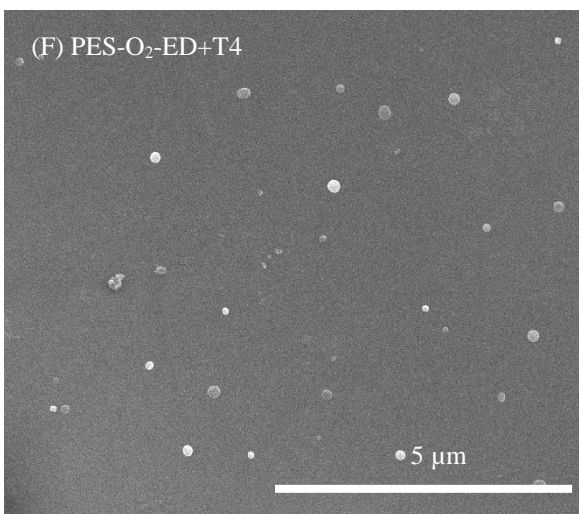
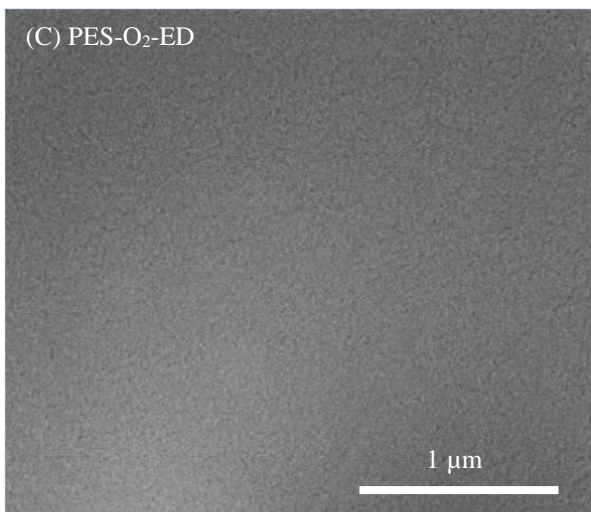
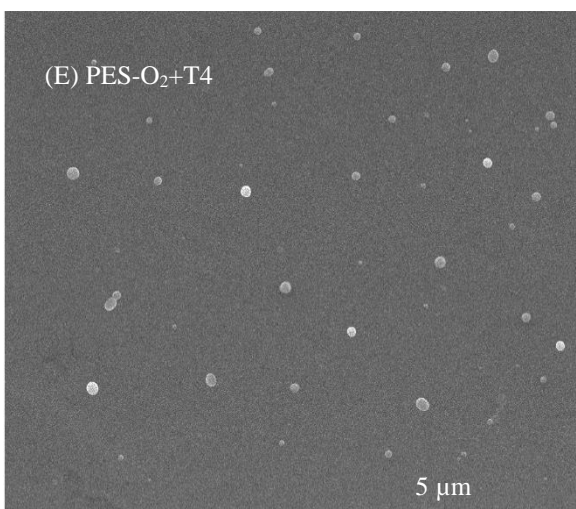
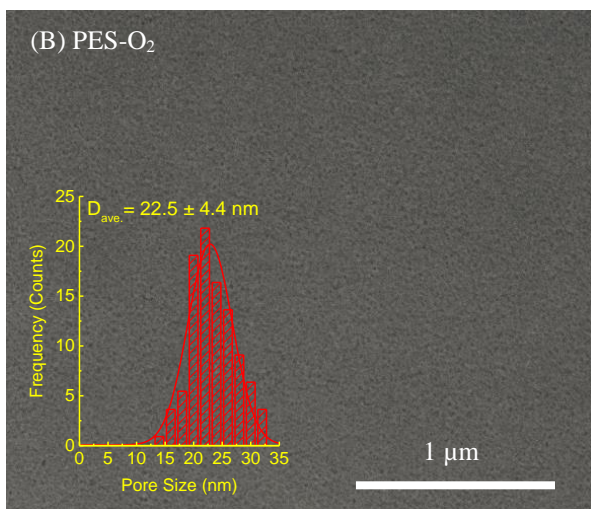
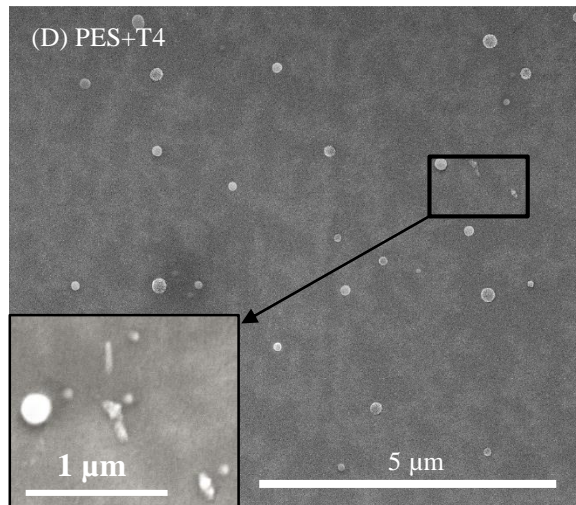
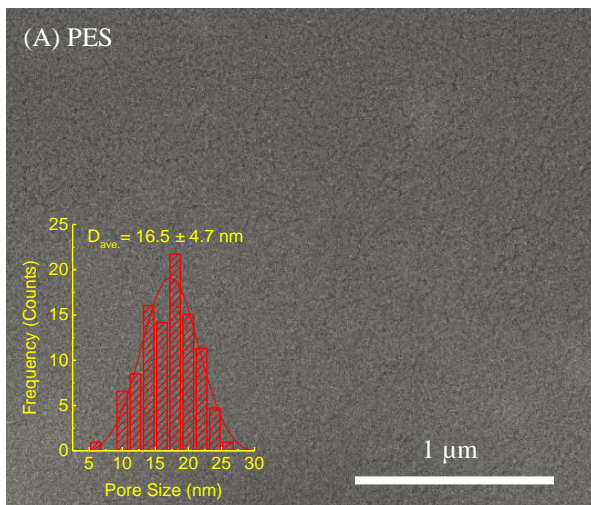


Figure 3. SEM micrographs of the different membranes before (Fig. 3A-C) and after (Fig. 3D-F) T4 immobilization.

Application of plasma treatment to produce more oxygen-rich functional groups, thereby facilitating phage anchoring, was also investigated with different polymeric surfaces (polyhydroxyalkanoate [36], cellulose [37], polyethylene and polytetrafluoroethylene [26]). The O₂ plasma treated surface exhibited superior phage binding in comparison to other treatment methods, such as polyacrylic acid grafting and EDC/NHS cross-linking; however, few explanations were given. The surface free energy and surface charge of the different membranes were measured to better understand the interaction between phages and the surfaces (Fig. S4A). A higher surface free energy usually indicates a stronger molecular attractive force of the membrane surface [50]. The O₂-plasma treated membrane exhibited the highest surface energy among the three types of surfaces, which may be one reason for the observed higher attachment of phages to the membrane surface. The surface energies of the PES and PES-O₂-ED membranes were not significantly different. The T4 phage used in this study had an overall negative charge ($\zeta = -25.9 \pm 0.2$ mV, in SM buffer, pH~7.5) [38]. The zeta potentials of the PES, PES-O₂, and PES-O₂-ED membranes under the same pH conditions were -22.5 ± 0.3 mV, -41.3 ± 4.8 mV and -13.03 ± 3.1 mV (Fig. S4A), respectively. Since the quantity of phage attached to the membrane surface is inversely proportional to that of the membrane zeta potential, this indicates that electrostatic interaction is not the predominant mechanism controlling phage attachment to the membrane surface. Rather, the high surface energy, the covalent bonding between carboxyl groups of the PES-O₂ membrane and the amine groups in the protein structure of the phage [36],

and the hydrophobic interaction between the PES membrane and phage may be strong enough to immobilize the phage. Since there was no obvious advantage to using the PES-O₂-ED membrane with respect to phage binding and antimicrobial activity, only the PES and PES-O₂ membranes were selected for further study.

3.2 Bacteriophage-assisted *in situ* biofouling control in a dead-end filtration process

In situ biofouling control via phage functionalized membranes was evaluated using a static biofouling assay with a lab scale dead-end UF setup. During this process, the same quantity of bacteria was deposited on different membrane surfaces and nutrient solution continuously flowed through to assist biofilm development. The water flux of the biofouled PES membrane sharply decreased in the presence of nutrient broth and only 27.8% of the initial flux remained after 6 h of filtration (Fig. 4A, Fig. S6A). By contrast, flux reduction of the PES+T4 membrane was much slower and a 35.2% reduction of initial water flux was observed under the same experimental conditions. As glucose and salt ions are unable to be rejected in the UF process, reduction of membrane flux should be mainly caused by bacterial growth, as well as the crystallization of salt and accumulation of nutrients (glucose) on the membrane. The T4 phage immobilized on the membrane surface served as *in situ* biocide to lyse attached bacteria and then continuously release new phage to prevent the growth of bacterial colonies on the membrane. The same inhibitory effect of the T4 phage on biofouling was observed with the PES-O₂ membrane (Fig. 4B, Fig. S6B). After 7 h of filtration, only 28% flux reduction was observed on T4 immobilized PES-O₂ membranes while this value was 62.2% in the absence of T4. It should be noted that phage

binding had an impact on the water flux of the membrane (the initial water flux of the membrane decreased $13.4 \pm 4.2\%$ and $9.5 \pm 2.7\%$ on PES and PES-O₂ membranes after T4 functionalization, respectively) (Fig. S5). Therefore, the benefits of phage immobilization on preventing the flux reduction caused by biofouling was observed after 0.5 h and 5 h of filtration on the PES (Fig. S6A) and PES-O₂ membranes (Fig. S6B), respectively. The mitigating effect of the T4 phage on membrane flux reduction indicates the strong potential of applying T4 phage to control biofouling *in situ* on the UF membrane in a dead-end filtration process.

The larger quantity of phages led to a lower extent of flux reduction ($((Flux_{at\ time\ 0} - Flux_{at\ time\ 6\ h}) / Flux_{at\ time\ 0} * 100\%)$ for the PES-O₂+T4 membrane in comparison to that of the PES+T4 surface. Nonetheless, since the T4 phage immobilization on pristine PES surface already shows appreciable *in situ* biofouling control, it was selected for further investigation of biofouling mitigation in a cross-flow filtration mode.

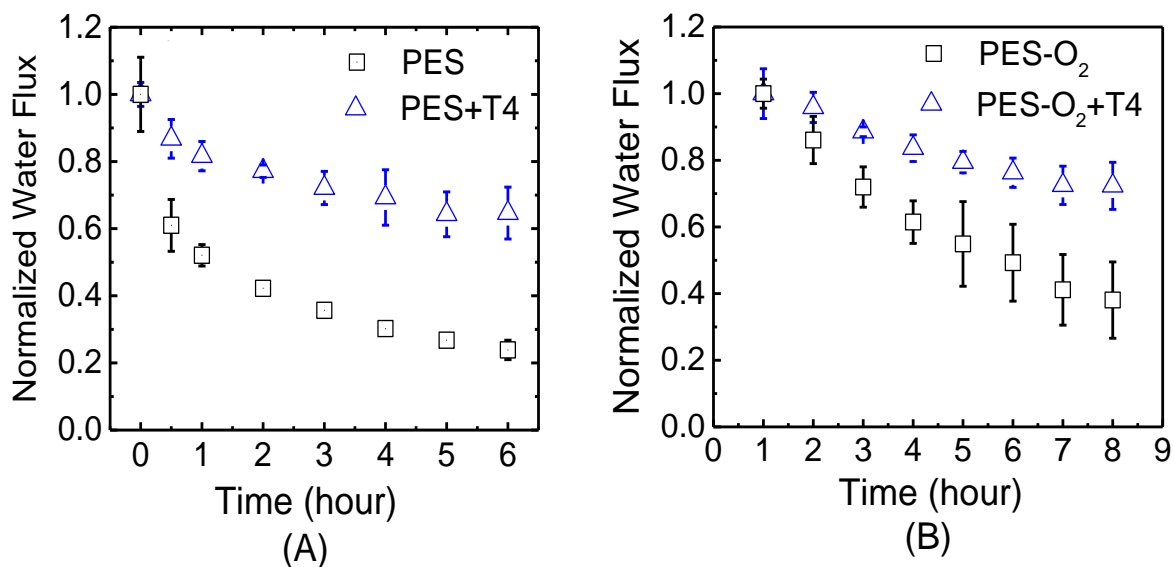


Figure 4. Water permeation flux of the (A) PES and T4-immobilized PES membranes, (B) PES-O₂ and T4-functionalized PES-O₂ membranes in a dead-end filtration system at 30 psi. The values are normalized to the water flux of the membrane at time 0 h ($Flux_{\text{of time } t} / Flux_{t=0}$).

3.3 Bacteriophage associated biofouling control in a cross-flow filtration process

The biofouling resistance of the phage-functionalized membrane was further investigated under dynamic cross-flow filtration using synthetic wastewater (containing *E. coli*) as a feed solution. Although a similar initial water flux was observed on the PES+T4 membrane in comparison to that of a PES membrane (Fig. 5A), the phage-functionalized membrane exhibited a lower flux reduction caused by the growth of bacteria in the feed solution. However, after 7 h, the flux of the PES+T4 membrane (blue curve) began to decrease more rapidly and the difference in flux between the two membranes became insignificant after 20 h of filtration (data not shown). The

significant flux reduction indicated that the growth rate of bacteria in the nutrient solution was higher than the bacterial-lysis speed of the T4, thus, the T4 load was not sufficient to control the growth of the *E. coli*.

In contrast to the dead-end filtration model in which newly released T4 phage were all maintained on the membrane surface, the T4 phage in the cross-flow filtration system could detach from the membrane surface and release into the retentate solution due to the strong shear force. To address this issue, the anti-biofouling potential of the T4 phage was further evaluated by injecting T4 phage directly into the feed together with bacteria and nutrients (Fig. 5A, PES with T4 in the feed, red curve). When T4 phage was spiked in the feed, a delayed rapid-flux-reduction was noted (after 9 h) in comparison to that observed for the case where phage was immobilized on the membrane surface (after 7 h). In the crossflow filtration mode, directly spiking phage as a biocidal additive in the feed tank was shown to be a more effective anti-biofouling strategy than immobilizing the phage on the membrane.

Although T4 immobilized on the PES membrane or suspended in the feed solution mitigated the flux reduction of the membrane caused by biofouling, this effect was only maintained for a short time. It was hypothesized that the predation relationship between bacteriophage and bacteria is a key factor that controls the flux reduction caused by biofouling. Therefore, the anti-microbial activity of T4 phage at different multiplicities of infection (MOI) was investigated (Fig. 5B). In a phage free suspension, the exponential growth of *E. coli* started at approximately 4 h. When T4 phage was added and the ratio reached $N_{E.coli}:N_{T4\ phage} = 1:10^2$ (10^6 CFU/mL: 10^8 PFU/mL) (the

same ratio as that injected in the cross-flow filtration process), the growth rate of *E. coli* was inhibited and exponential growth was delayed by ~ 3 h. When the concentration of phage further increased to $N_{E.coli}:N_{T4\ phage} = 1:10^3$ (10^6 CFU/mL: 10^9 PFU/mL), no significant growth of bacteria was observed after 12 h of culturing (according to OD₆₀₀). When $N_{E.coli}:N_{T4\ phage} = 1:10^4$ (10^6 CFU/mL: 10^{10} PFU/mL), the OD₆₀₀ value nearly reached 0 (decreased from 0.033 to 0.005) at 12 h and no regrowth of bacteria was observed after one week. These observations confirmed that the concentration of T4 phage was a key factor in controlling the growth of the host bacterium *E. coli*.

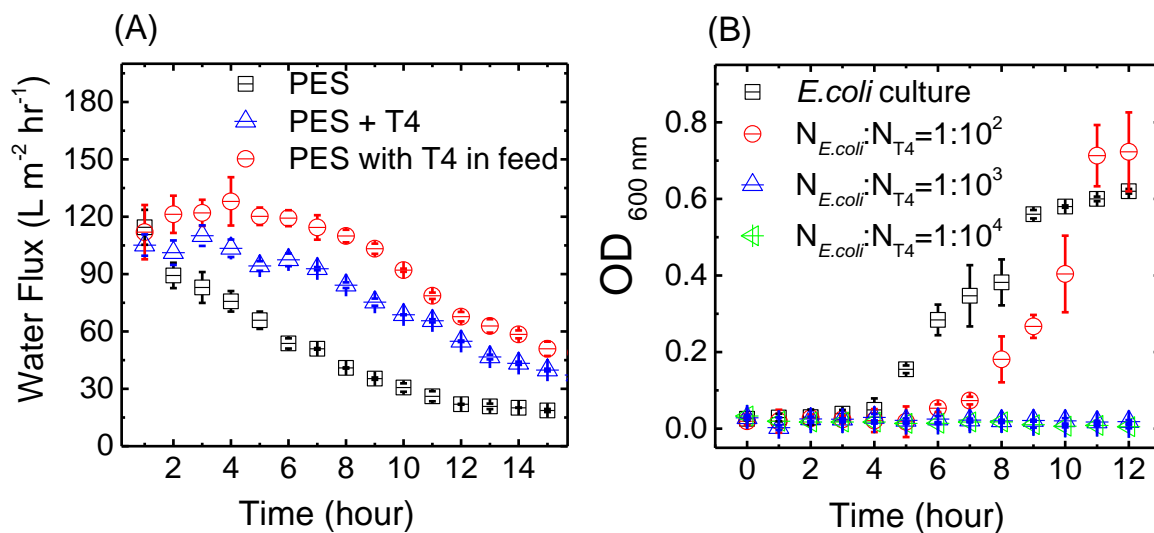


Figure 5. (A) The permeation flux of the PES (black) and T4 immobilized PES membranes (blue) in a cross-flow filtration system. The red curve shows the water permeation flux of PES with T4 inoculated in the synthetic wastewater ($N_{E.coli}:N_{T4\ phage} = 10^5$ CFU/mL: 10^7 PFU/mL). (B) Growth

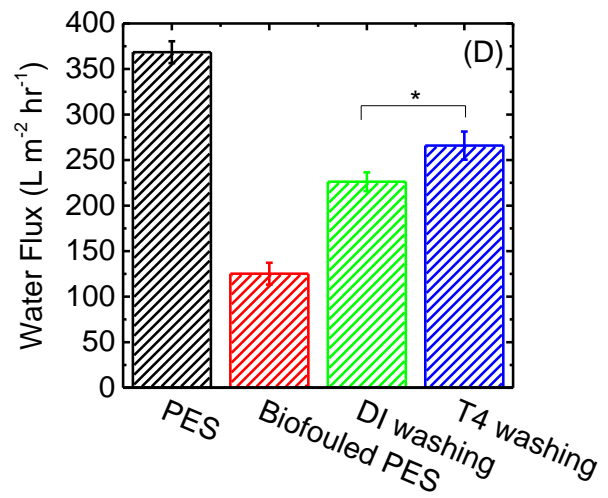
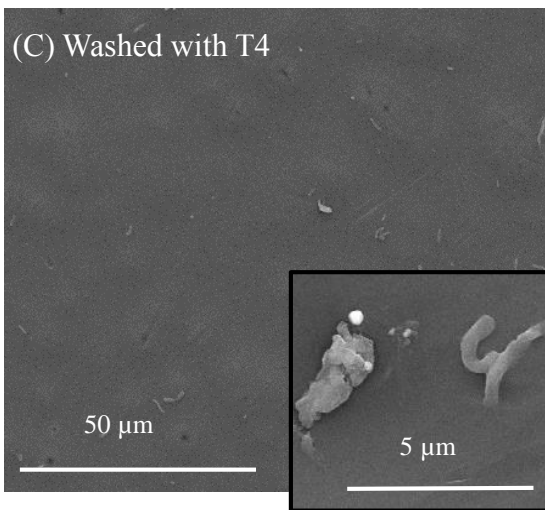
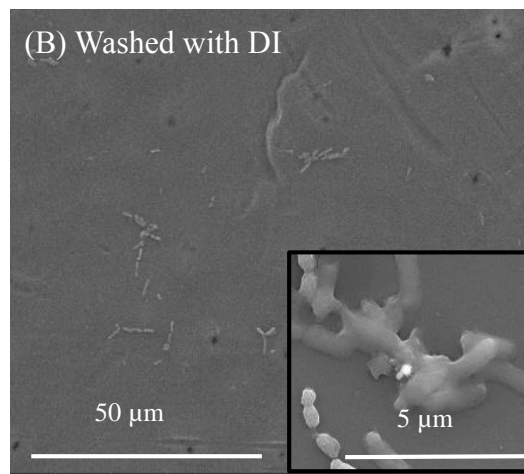
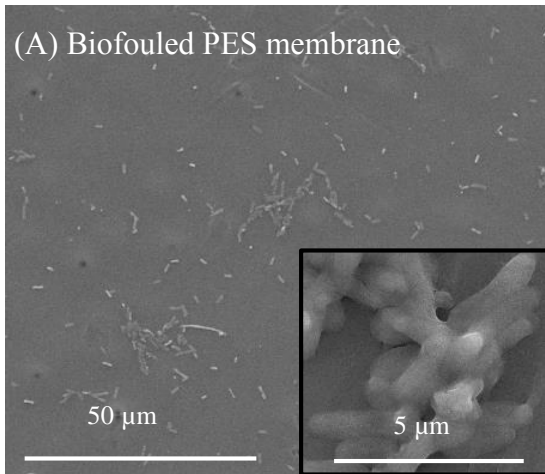
curve of *E. coli* (CFU/mL) at different phage concentrations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4 Bacteriophage assisted cleaning of a biofouled membrane

Phage-assisted cleaning is another promising method to control biofouling in a membrane process. The Delftia lytic phage (DTP1) (10^{12} PFU/mL) was reported to be capable of disturbing the extracellular polymeric substance (EPS) matrix formed on hollow fiber membrane by a drug-resistant bacterial strain [35]. Although 78% water flux recovery was observed after phage therapy, no control experiment utilizing DI water washing/backwashing was reported in this study, without which, we cannot determine the exact contribution of the phage treatment. Herein, the potential of phages to destroy biofilms was studied with a dead-end filtration model (as the dead-end system accumulates bacteria and forms biofilm faster and it better mimics the membrane washing in practical application in comparison to a cross-flow system).

After 24 h of incubation (experimental protocol described in section 2.7), considerable *E. coli* colonies were formed on the PES membrane surface (Fig. 6A). The biofouled PES membrane only maintained 34% of the original water permeation flux in comparison to that of the pristine membrane (Fig. 6D). When DI water was applied to clean the biofouled membrane (Fig. 6B), the quantity of bacterial cells on the membrane surface was significantly reduced which could be attributed to the strong shear force; however, some EPS encased bacteria were still present. 58% of the water permeation flux (from 125 to 226 LMH) was recovered after the DI washing (Fig.

6D). When the T4 phage suspension was applied as cleaning agent, the number of bacterial cells showed a similar reduction to that of the DI water washed membrane (Fig. 6C). Approximately 75% of the water permeation flux of the membrane was recovered after phage spiking (Fig. 6D). After DI water and T4 suspension washing, membrane samples were continuously challenged with bacteria-containing synthetic wastewater to observe changes in flux (Fig. 6E). The T4 phage washed membrane experienced a smaller flux reduction rate and nearly 30% higher flux was observed with the T4 washed membrane (125 LMH) in comparison to that of the DI washed membrane (90 LMH), after 4 h of filtration. This result infers that some phage remained on the membrane surface after phage spiking and demonstrates that phage could serve as an effective biocide to mitigate the growth of bacteria in membrane filtration processes.



- Membrane washed with DI water
- Membrane washed with T4 suspension

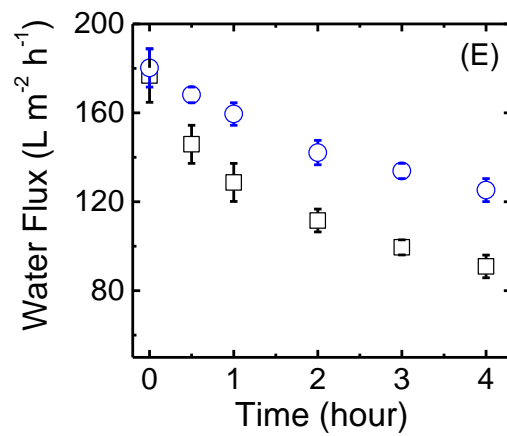


Figure 6. SEM images of (A) the *E. coli*-contaminated membranes, the *E.coli*-contaminated membrane after (B) DI water washing and (C) T4 suspension washing. The water permeation flux of different membranes evaluated in a dead-end filtration system with (D) DI water and (E) synthetic wastewater as feed, respectively.

The investigation of dead-end and cross-flow filtration along with phage treatment exhibited potential in application of bacteriophage for the control of membrane biofouling via diverse strategies: (i) inhibiting the growth of bacteria when used *in situ* on the membrane surface, (ii) preventing bacterial propagation in the feed solution and (iii) degrading biofilm formed on the membrane surface in a manner analogous to a cleanser. A high concentration of bacterial suspension (10^6 ~ 10^8 CFU/mL) was applied in this study to simulate the long-term filtration experiments. Due to the low-cost and easy accessibility of phages, the stability and efficacy of these phage-associated anti-fouling strategies can be maintained conveniently by regeneration of phage-coating and re-spiking phage suspension into the feed tank. However, as with other phage-based anti-biofouling coatings, the long-term filtration experiments with real water/wastewater effluents would be necessary to evaluate the performance of the phage-based strategies for their real-life applications in membrane biofouling control.

Although the application of bacteriophage-assisted biofouling control in practical membrane processes faces challenges, especially regarding its high specificity of bacteria recognition, its instability of immobilization on surfaces, and certain sacrifices in terms of water flux, more solutions continue to be proposed and investigated. The use of a “phage cocktail” has been proposed as an effective strategy to mitigate biofouling caused by multiple types of bacteria on food packaging surfaces [37] and in wastewater filtration processes [34]. To improve the immobilization of phages, a lipid nanovesicle capsule [51] was prepared to encase bacteriophages and maintain their activity under complex immobilization conditions. This approach could improve phage activity following incorporation into the membrane polymeric structure. In comparison to other antimicrobial materials, such as engineered polymers [52], carbon- [53, 54] and metal-based NPs [55, 56], phage-based biocides may have a lower bacteria-inactivation efficacy. However, they still hold incomparable advantages such as being an easily obtained resource (isolated from specific wastewater sample), having a low inherent toxicity, being self-replicating and self-limiting (the growth of phage rely on the host bacteria), and possessing the ability to co-evolve with phage-resistant bacterial mutants [57, 58]. Phage-associated biofouling control strategies and protocols applied for membrane processes still merit further exploration.

4 Conclusion

Developing a natural material- (peptide, enzyme, bacteriophage, etc.) based anti-biofouling membrane for water filtration has gained growing interest. Here, three types of phage-based biofouling control methods were proposed as potential strategies to reduce UF membrane

biofouling in different filtration modes; namely, (i) phage functionalization of the membrane surface for *in situ* biofouling mitigation, (ii) phage spiking of the feed-solution as a biocidal additive during filtration, and (iii) phage spiking post-filtration for biofilm removal.

In the dead-end filtration process, the phage-immobilized PES membrane mitigated up to 50% flux reduction caused by the growth of bacteria on the membrane surface. The *in situ* biofouling control property of the PES membrane was enhanced by increasing the quantity of immobilized phages via oxygen plasma treatment on the membrane. During the cross-flow filtration process, bacteriophages served as biocidal additives. Direct spiking of phage at a concentration of $N_{E.coli}:N_{T4\ phage} = 1:10^2$ (10^6 CFU/mL: 10^8 PFU/mL) effectively inhibited the rapid growth of bacteria cells in the feed and delayed flux reduction of the PES membrane up to 9 hours. The bacterial growth rate was controlled by varying the multiplicity of infection. Moreover, phage assisted biofilm cleaning disturbed the biofilm matrix and enhanced water flux recovery rate by 20% as compared to the physical washing process.

With growing concerns regarding the spread of antibiotic-resistant microorganisms, alternative biofouling controls will play a non-negligible role in membrane processes. The three approaches proposed in this study contribute to the investigation of novel bacteriophage-associated anti-biofouling membranes and the development of environmentally friendly membrane cleaning/maintenance protocols, which can further broaden the contribution of membrane processes in wastewater reclamation and increase the future fresh water supply.

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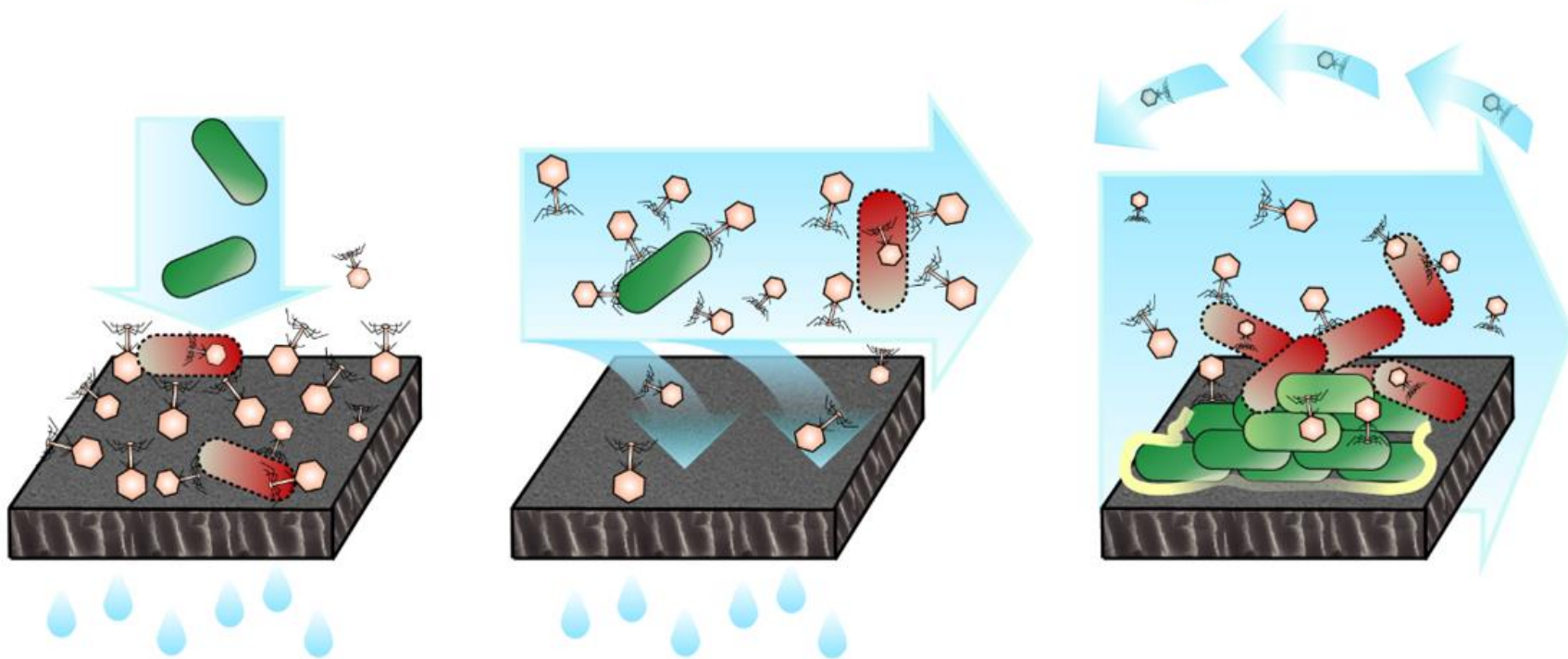
Coulombe of the Plasma Processing Laboratory, McGill University, for the plasma treatment.

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(A) *In situ* biofouling mitigation

(B)

(C) Biofilm

Scheme: The bacteriophage-assisted anti-biofouling strategies used in UF membrane process.

Supplementary Data

Bacteriophage-based strategies for biofouling control in ultrafiltration: *in situ* biofouling mitigation, biocidal additives and biofilm cleanser

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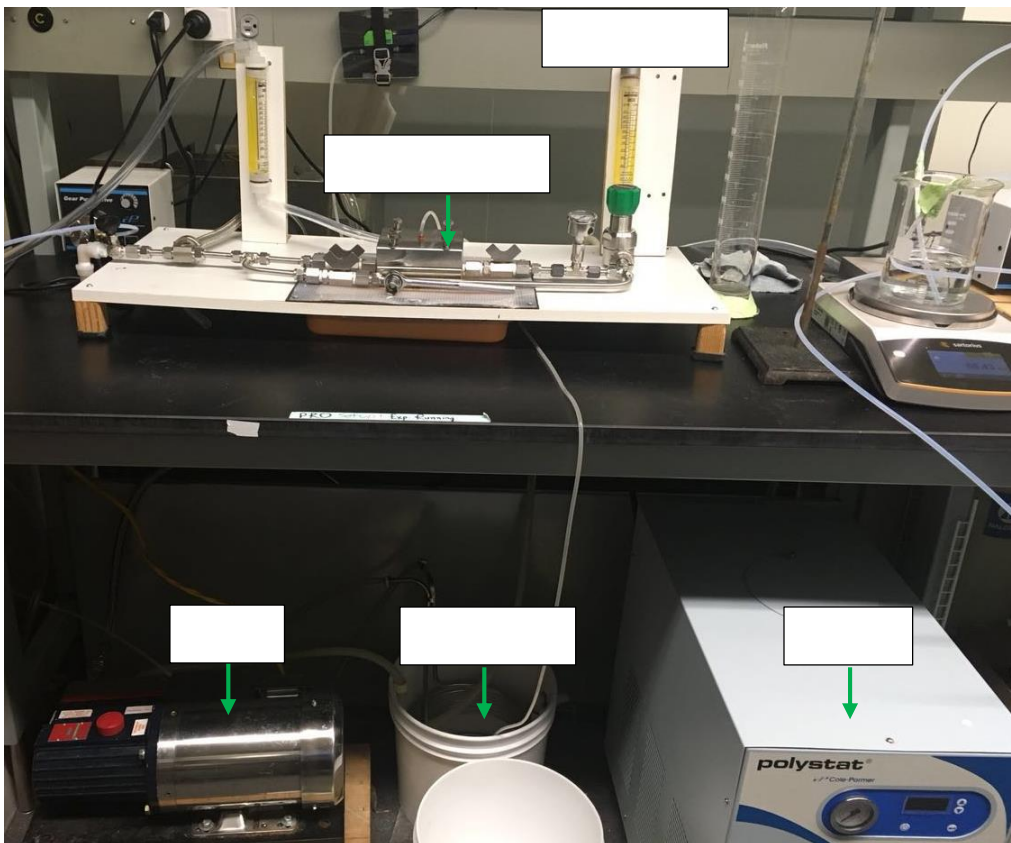


Figure S1. Image of the laboratory-scale UF cross flow filtration setup.

Table S1. Studies examining the use of bacteriophage at different doses to control or remove biofilms

Concentration	Phage and biofilm	Result	Ref.
10 ² -10 ⁷ PFU/mL	Combining T4 phage and cefotaxime together to eradicate <i>E.coli</i> biofilm formed on the microtiter plates of the Calgary biofilm Device/MEBEC Assay.	Increasing phage concentration from 10 ⁴ to 10 ⁷ PFU/mL, the minimum biofilm eradication concentration of cefotaxime decreased from 128 to 32 µg/L.	(Ryan et al. 2012)
10 ⁸ PFU/mL	SAP-26 phage was applied together with rifampicin (0.6 mg/L) to treat 24 hr old <i>S. aureus</i> biofilms formed on the polystyrene microtiter plates.	The addition of SAP-26 reduced biofilm cells by ~ 1 log.	(Rahman et al. 2011)
10 ⁸ PFU/mL	Phage P100 was applied to control <i>L. monocytogenes</i> biofilms on stainless steel surfaces.	The disaggregation of biofilms was observed; while, viable cells were still present.	(Montañez-Izquierdo et al. 2012)
10 ⁹ PFU/mL	Phage P100 was applied to control <i>L. monocytogenes</i> biofilms on stainless steel surfaces.	P100 reduced biofilms formed by <i>L. monocytogenes</i> strains by 3.5–5.4 log/cm ² .	(Soni and Nannapaneni 2010)
10 ¹⁰ PFU/mL	<i>P. aeruginosa</i> phage M4 was applied to reduce biofilm on neutral hydrogel coated <i>Lubri-sil</i> all-silicone 16 French Foley catheters.	Phage treatment showed effective reduction of biofilm. The regrowth of biofilms occurred over time but could be prevented via a retreatment with a phage cocktail.	(Fu et al. 2010)

10 ¹⁰ PFU/mL	T4 bacteriophage was applied to reduce the 24 hr old <i>E.coli</i> biofilm on PES membrane surface.	Phage assisted biofilm cleaning enhanced water flux recovery rate by 20% as compared to the physical washing process.	This study
10 ¹² PFU/mL	The Delftia lytic phage (DTP1) (10 ¹² PFU/mL) was applied to disturb biofilm formed on hollow-fiber membrane filters.	A 78% water flux recovery was observed after phage therapy. No control experiment utilizing DI water washing/backwashing was reported in this study to understand the exact contribution of phage spiking.	(Bhattacharjee et al. 2015)

As summarised from the literatures (Table S1), a phage suspension with a concentration of 10⁴~10⁸ PFU/mL exhibits a certain efficacy in biofilm cleaning; reducing biofilm bacteria and disaggregating the biofilm structure. However, their antibiofouling functions are limited and extra treatment or combination of bacteriophages with other biocidal agents are required to achieve a “fully effective” biofilm removal. Increasing the concentration of phages to 10⁹~10¹⁰ PFU/mL showed effective biofilm cleaning efficacy; while, there is still a possibility of bacterial regrowth. With further increase of phage concentration, the regrowth potential of bacteria was reduced. Using a multi-phage “phage cocktail” has been suggested (Fu et al. 2010) as a potential means to prevent the regrowth of bacteria. The possible regrowth of bacteria at the phage concentration of 10¹² PFU/mL has not been investigated in the literature.

Table S2. Area ratio for suggested attribution of XPS O 1s spectra of different membranes

Membrane	O=S=O (532.3 eV)	HO-C=O (533.1 eV)	C-O-C (533.8 eV)	C-OH (534.3 eV)
PES	76.9%	-	23.1%	-
PES-O ₂	60.7%	15.8%	17.7%	5.8%
PES-O ₂ -ED	72.9%	18.9%	8.2%	-

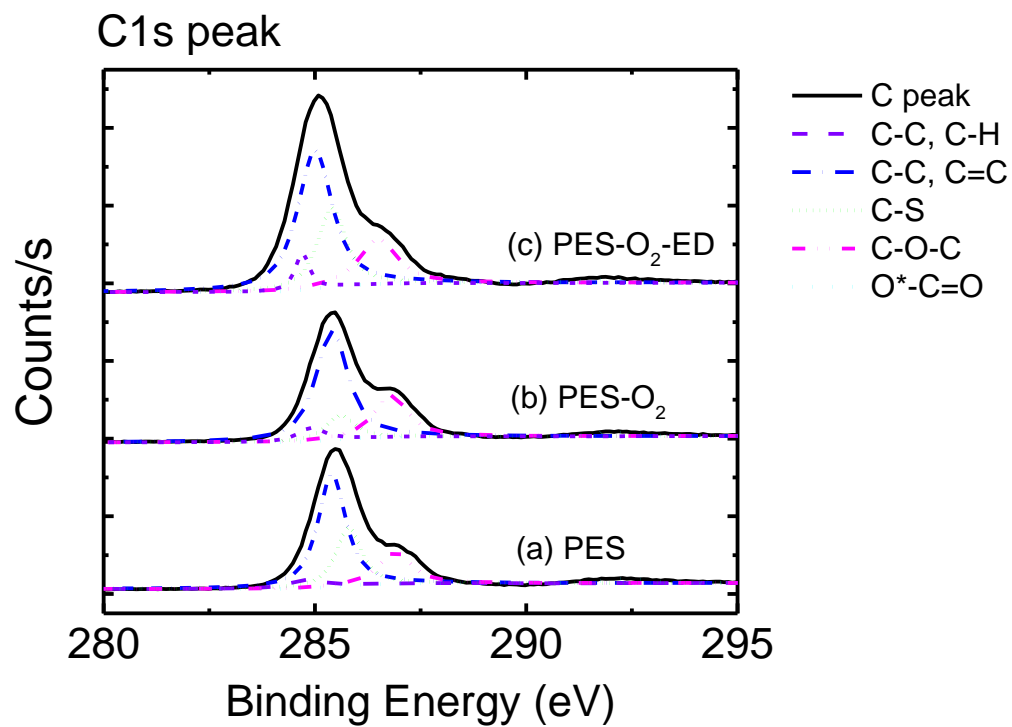


Figure S2: XPS C1s spectra of the pristine membrane (PES), oxygen plasma treated membrane (PES-O₂) and PES-O₂ after ED coating (PES-O₂-ED).

Table S3. Area ratio for suggested attribution of XPS C 1s spectra of different membranes

Membrane	C-C, C-H	C-C, C=C	C-S-C	C-O-C (286.8 eV)
	(284.8 eV)	(285.4 eV)	(285.8 eV)	O*-C=O (288.5 eV)
PES	4.0%	51.1%	25.3%	19.6%
PES-O ₂	6.1%	57.0%	11.3%	25.6%
PES-O ₂ -ED	5.5%	50.7%	23.5%	20.3%

Table S4. Area ratio for suggested attribution of XPS N 1s spectra of different membranes

Membrane	-NH ₂	N-C=O	H-bonded N
	(399.5 eV)	(400.2 eV)	(402.5 eV)
PES	26.9%	73.1%	-
PES-O ₂	-	100%	-
PES-O ₂ -ED	9.4%	88.4%	2.1%

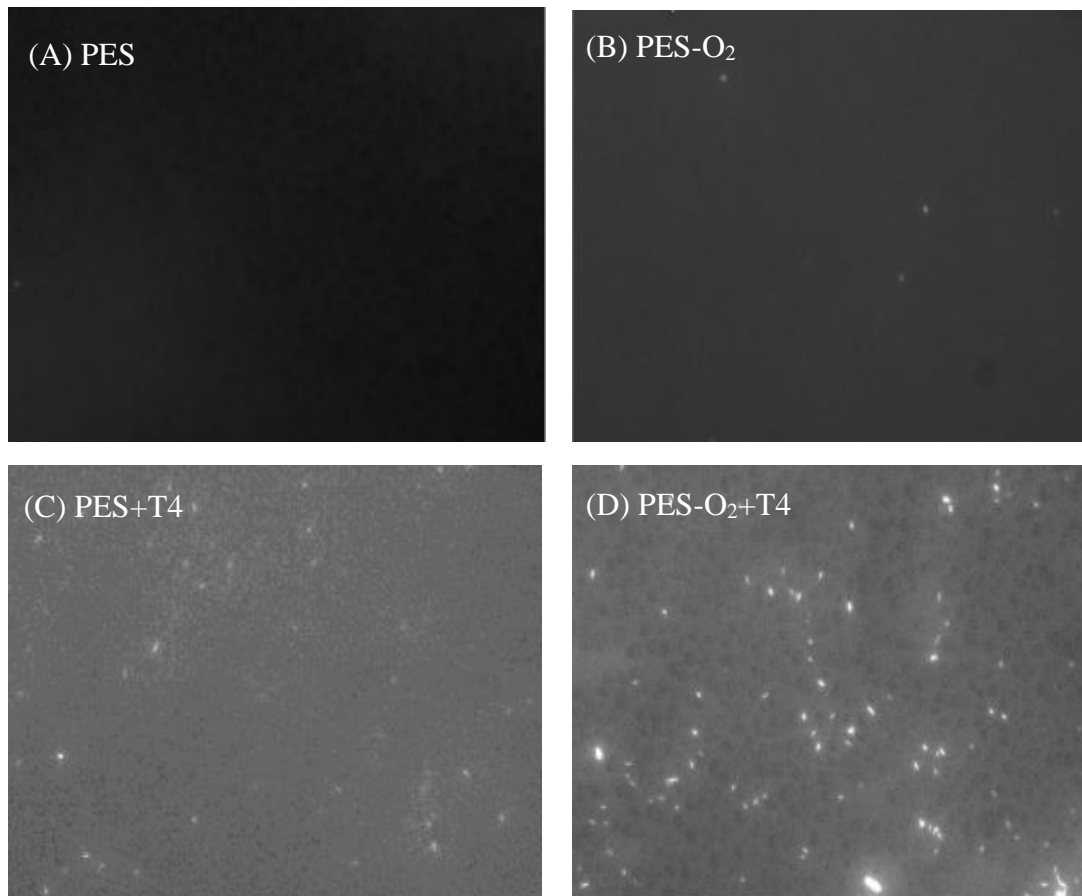


Figure S3. Bacteria capture efficiency of phage, as observed by representative fluorescence microscopy images of *E. coli* attached to (A) PES, (B) PES-O₂, (C) PES+T4 and (D) PES-O₂+T4.

Fluorescence microscopy (Olympus IX71, Tokyo, Japan) equipped with an Evolution VF cooled monochrome CCD camera (1392x1040 resolution with 4 x 4 binning) was used to visualize the density of immobilized phage on the membrane surface. In this experiment, T4 immobilized membrane with a surface area of 3.8 cm² was contacted with 1 mL of 10⁷ CFU/mL of *E. coli* K12 GFP (marked with green fluorescent protein) for 10 min and washed subsequently with PBS buffer. During rinsing, membranes were kept in the vertical position to ensure that all loose bacteria were washed away from the surface. The samples were then dried in air to completely remove the remaining drops of buffer. Then, samples were placed between a glass slide and cover slip with a drop of glycerin and observed under a microscope with a 40× magnification objective. At least 30 locations were randomly imaged from each sample. The number of captured bacteria per image was analyzed using the software, ImageJ 1.50i.

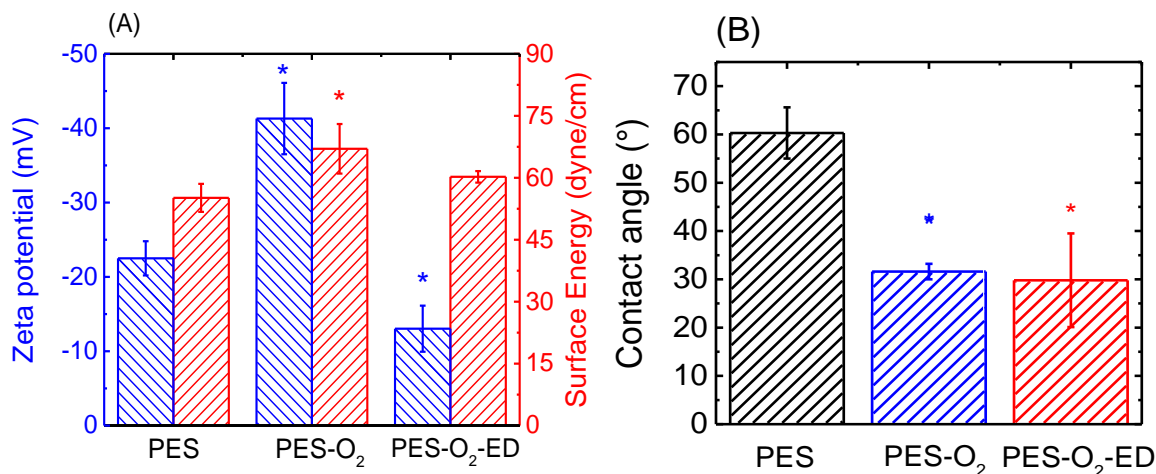


Figure S4. (A) The surface zeta potential and the surface energy and (B) the water contact angle of the different membranes. Asterisks (*) indicate a statistically significant difference between the PES and PES-O₂ or PES-O₂-ED membranes ($p < 0.05$).

The electrokinetic analyzer (EKA) (Anton Paar, Graz, Austria) was applied to measure the surface charge of each membrane at pH 7.3 (the same pH used for phage immobilization) with 1 mM KCl (pH was adjusted using 1 M NaOH and 1 M HCl) as the feed solution. The average results of triplicate experiments are reported. The surface free energy of each membrane was evaluated by measuring the contact angle of the membrane surface with three different liquids: DI water, diiodomethane, and glycerol. The contact angle was measured by using the equipment (VCA, AST Products, Inc., MA, USA) via the sessile method. The surface free energy value was then calculated with the system's SE-2500 software via the Geometric theory model. At least 5 locations were randomly selected and measured from each sample.

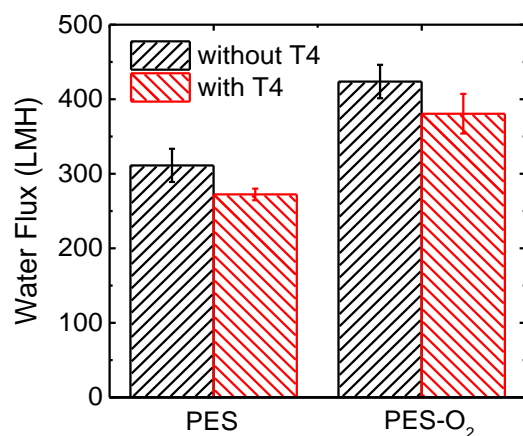


Figure S5: The water permeation flux of PES and PES-O₂ membranes before and after T4 phage immobilization (tested with DI water in a dead-end filtration cell under 2.07 bar pressure). The experiment was conducted in triplicate.

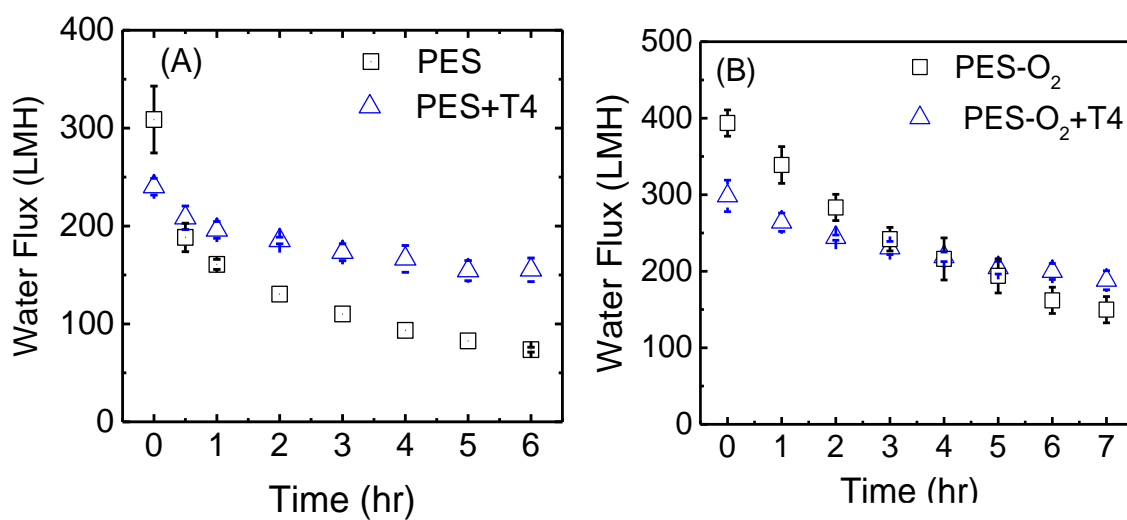


Figure S6: The water permeation flux of PES and PES-O₂ membranes before and after T4 phage immobilization (tested with the synthetic wastewater in a dead-end filtration system under 2.07 bar pressure).

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