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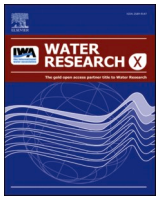

# Removal of urea in ultrapure water system by urease-coated reverse osmosis membrane

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ABSTRACT

Among the various substances found in the feed source for the production of ultrapure water (UPW), urea is challenging to remove because it is a small molecular weight molecule that is not easily oxidized and does not carry a charge under neutral pH conditions. Urease enzyme, found in various organisms such as plants and bacteria, catalyze the hydrolysis of urea into carbon dioxide and ammonia. In this study, urease was immobilized on the polyamide layer of a reverse osmosis (RO) membrane to remove urea in UPW systems. The removal efficiency of urea by urease-coated RO membrane showed up to 27.9 % higher urea removal efficiency compared to the pristine membrane. This increase in urea removal can be attributed to both physical and biological effects from the urease coating on the membrane. Firstly, urease on the membrane surface can act as an additional physical barrier for urea to pass through. Secondly, urea can be hydrolyzed by the enzyme when it passes through the urease-coated RO membrane. In a two-pass RO system typical for UPW production, the removal of urea by a urease-coated membrane would be enhanced by twofold. This overall method can significantly increase the removal efficiency of urea in UPW systems, especially when considering the compounded removal by the urease coating, rejection by RO, and additional reactions by other treatment processes. Moreover, urea in UPW systems can be removed without the installment of additional processes by simply coating urease on the existing RO membranes.

## 1. Introduction

With the increasing demand for semiconductors, key components of the fourth industrial revolution, the semiconductor industry has entered the starting point of the second super cycle (Voas et al., 2021). In the manufacture of semiconductors, ultrapure water (UPW) is used for the wet cleaning and wet etching steps, as impurities such as inorganic compounds, organic substances, pathogens, and suspended solids can significantly hamper the performance of transistors. UPW is produced through various processes combining adsorption, membrane filtration, ion exchange, and ultraviolet (UV) oxidation (Zhang et al., 2021).

Most organic compounds with relatively large molecular weights are removed during pre-treatment by reverse osmosis (RO) separation or UV oxidation (Jin et al., 2018). However, it is difficult to remove some dissolved organic compounds such as urea, trihalomethanes (THM), and isopropyl alcohol (IPA) to concentrations below 1 μg/L of total organic carbon (TOC), which is the standard from the Semiconductor Equipment and Materials Institute (SEMI) (Lee et al., 2016). Especially, urea has a small molecular weight, is resistance to oxidation, and does not carry a charge under neutral pH conditions (Choi and Chung, 2019). Therefore, the removal efficiency of urea in the existing RO process is less than 50 % due to its size and charge characteristics (Ray et al., 2020; Yoon and Lueptow, 2005). Moreover, the removal efficiency of urea through UV processes alone are known to be less than 10 % (Choi and Chung, 2019). In practice, there have been defective product incidents that were caused by the insufficient removal of urea in UPW systems (Rydzewski and Carr, 2003). Therefore, it is essential to develop a technology to effectively control urea in UPW.

Recently, research related to the use of urease, a urea decomposition enzyme that is an economical and environmentally friendly alternative to physicochemical urea removal processes, has been receiving attention. The urease enzyme, which is produced by various bacteria, plants, or fungi, is commonly found in the environment and in humans (Krajewska, 2009a). Urease is a metalloenzyme with two nickel ions per

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catalytic unit in the active site (Dixon et al., 1975; Todd and Hausinger, 1989). In the presence of the urease enzyme, urea rapidly hydrolyzes into $NH_3$ and $CO_2$ (Kistiakowsky and Rosenberg, 1952). Because of the aforementioned characteristics, urease was immobilized on polyethersulfone (PES) beads or graphene oxide (GO) sheets to control urea concentrations in applications such as blood purification for chronic kidney disease patients (Zhang et al., 2020, 2019). However, to apply these urease-decorated materials for urea removal in UPW systems, the installment of an additional process in the existing treatment train would be required, which is not only economically challenging but also difficult for operators to implement. Therefore, successful integration of urease enzymes to reduce urea contamination in UPW needs to be done without hampering the established treatment train in UPW systems.

In this study, urease was immobilized on the polyamide layer of a RO membrane to remove urea in the UPW system. Commercial RO polyamide membrane (BW30) was coated with the urease through the simple N-ethyl-N′-(3-(dimethylamino)propyl)carbodiimide/N-hydroxysuccinimide (EDC/NHS) chemistry. The amount of the urease immobilized was quantified by elution of the nickel present in the urease enzyme using nitric acid. The removal efficiency of urea by the urease-coated RO membrane was assessed using 10 ppm of urea in deionized water, mimicking UPW production system. The results of this study have direct potential applications for UPW systems, showing that urea removal can be improved without the installment of additional processes by simply coating urease on existing RO membranes.

## 2. Results and discussion

### 2.1. Membrane characterization

#### 2.1.1. Surface characteristics of the membranes

Among bi-nickel active sites of urease, one is responsible for binding and activating the urea, and the other is for the binding and activation of the water molecule (Amtul et al., 2002). Urea is selectively bonded with the bi-nickel active site of urease to be stabilized in a tetrahedral transition state in an orientation-specific mode (Benini et al., 1999). Therefore, three-dimensional placement of the urease, especially its bi-nickel active site, on the membrane, mediated through EDC/NHS chemistry, is important to preserve an effective enzymatic activity. Thus, the ratio between NHS:urease varied from 1:0.002, 1:0.02, 1:0.2, and 1:2 with a fixed concentration of urease of 10 g/L (the ratio between EDC:NHS was 2:5 at each condition). Afterwards, the pristine BW30 and urease-coated membranes were characterized through Fourier Transform infrared spectroscopy (FT-IR), water contact angle measurements, and scanning electron microscopy (SEM) imaging.

To understand the change in the surface functional groups after the immobilization of urease, FT-IR analysis was conducted. No clear difference in the peaks profile was observed between the pristine BW30 membrane and the urease-coated BW30 membranes compared to that of the powder form of urease (Fig. 1(a)). As the major peaks of the polyamide RO membrane are amide bonds, which are also found in urease, the peaks profile is not altered even after the immobilization of the urease. In addition, due to the penetration depth of FT-IR (up to 5 μm), most of the signal originates from the polysulfone support layer and thin surface coatings applied to the polyamide layer, such as the grafting of lysine or the deposition of polydopamine (Liu et al., 2019; Ye et al., 2015), are often not identifiable through FT-IR spectra. However, the effect of urease immobilization on the membrane surface can be noted by a change in the hydrophilicity of the membrane, which was analyzed by water contact angle measurements (Fig. 1(b)). Compared to the pristine membrane (61.5 ± 2.0), the contact angles of the urease-coated membranes decreased with the increasing NHS:urease ratio. Specifically, when the NHS:urease ratios were 1:0.2 and 1:2, water contact angles were 57.1 ± 1.5° and 53.5 ± 1.7°, respectively. The increase in hydrophilicity with increasing NHS:urease ratio can be attributed to the hydrophilic characteristics of the urease compared to the polyamide structure of the RO membrane (Krajewska, 2016).

To better understand the change in membrane surface chemistry resulting from the immobilization of urease on the surface, surface zeta potential of each membrane was also characterized (Fig. 1(c)). Surface charge of the urease-coated membranes was found to decrease to more negative values compared to the pristine membrane. In addition, the zeta potential of the surface also decreased with increasing solution pH. The pKa values of the ionizable groups of jack bean urease are known to be 5.3, 6.6 and 9.1, which are higher compared to the pKa of the pristine RO membrane (~3.6) (Krajewska and Ciurli, 2005). Therefore, from the change in surface zeta potential of urease-coated membrane, successful coating of urease was confirmed. This coating did not affect the surface morphology of the membranes, given the small size of the urease enzyme (~550 kDa, ~10 nm) (Cover et al., 2001), as noted by the SEM image analysis of the surface morphology (Fig. 1(d)).

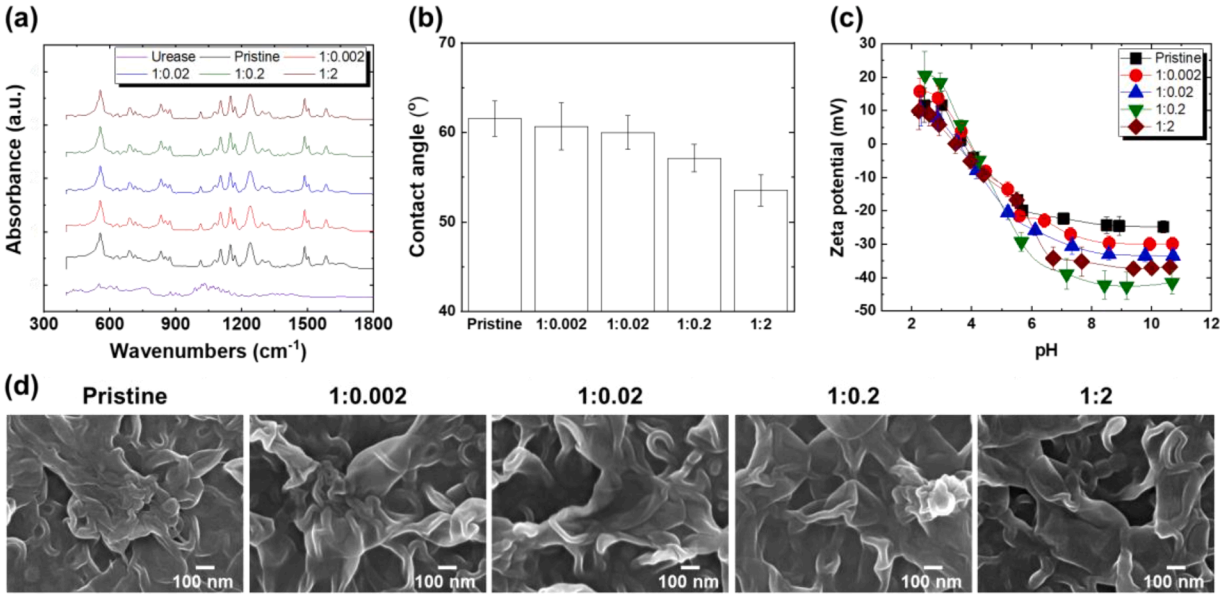


**Fig. 1.** Surface characteristics of the urease-coated membranes. (a) FT-IR spectra, (b) Water contact angle, (c) surface zeta potential, and (d) SEM imaging of the pristine and urease-coated membranes. The numbers indicate the EDC/NHS:urease ratios used for enzyme immobilization on the polyamide surface layer.

### 2.1.2. ICP-MS

To determine the optimum coating condition, nickel in the urease coating on the membrane surface was eluted using 2 % nitric acid and quantified through quadrupole ICP-MS analysis (Fig. 2). This result is interesting as the amount of urease coated on the membrane surface was dissimilar even though the concentration of urease for the immobilization was identical (10 mg/mL). Specifically, the amount of urease coated on the RO membrane surface was larger when the NHS:urease ratio was higher. These findings align with previous work by Zhang et al. on the immobilization of cellulase on anionic methacrylic acid:methyl methacrylate copolymer by EDC and NHS, who showed that the activity of cellulase was more efficient when the NHS to cellulase ratio was higher (Zhang et al., 2012). Similarly, the immobilization of urease on amino-functionalized beads or polysulfone membranes also increased when the ratio between glutaraldehyde-based activating solution to urease was increased (Alatawi et al., 2018; Poźniak et al., 1995). From the results of this and previous studies, a single urease molecule may occupy multiple active sites when the NHS:urease ratio is lower as urease is a macromolecule composed of 840 amino acids, which provide multiple sites per enzyme that can be cross-linked with the carboxylic group activated by the EDC/NHS chemistry, lowering the immobilization efficiency (Krajewska, 2009b). On the other hand, urease molecule develops relatively fewer conjugations when the NHS:urease ratio is higher and, therefore, more urease enzymes are able to be immobilized on the membrane surface.

## 2.2. Membrane performance

The effects of urease coating on the performance of the membrane were assessed using DI water and 2000 mg/L NaCl, respectively for pure water flux and salt rejection (Fig. 3).

The decrease in water flux and increase in salt rejection of the urease-immobilized membrane was thought to be contributed by the urease on the membrane surface acting as an additional layer that hinders the transport of water and solute. From a previous study, performance of the membrane was altered similarly when lysozyme was coated on the RO membrane surface (Tian et al., 2021). Moreover, in a case of the immobilization of 6-amino caproic acid (ACA) on the polyamide RO membrane, the flux declined while the salt rejection increased (Saeki et al., 2013).

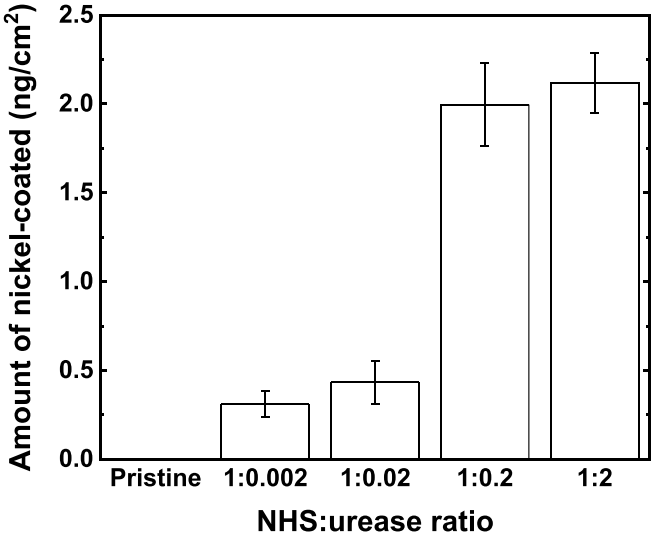


**Fig. 2.** ICP-MS analysis of the Ni contents eluted from the pristine and urease-coated membrane.

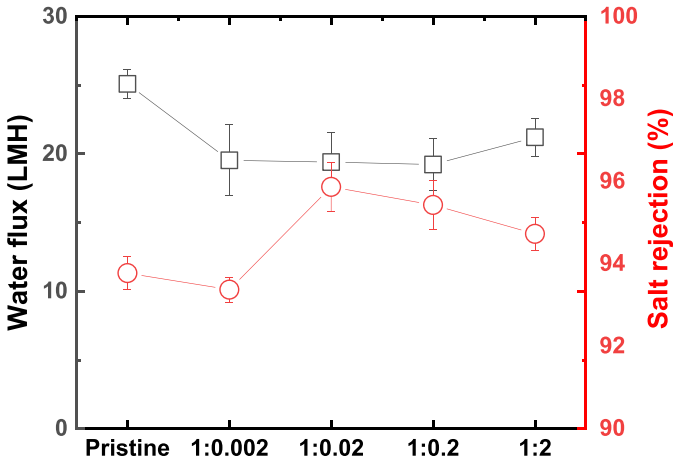


**Fig. 3.** Water flux and salt rejection properties of the pristine and urease-coated (at EDC/NHS:urease ratios of 0.002 to 2) membranes. Water flux was evaluated using DI water as the feed, while salt rejection was determined with a 2000 mg/L NaCl feed solution.

## 2.3. Urea removal efficiency

The rejection of urea by pristine and urease-coated RO membranes were assessed at different applied pressures as the hydrolysis of urea by the enzymatic reaction of urease would be dependent on the reaction time or velocity of the water going through the membrane layer.

The urease saturation curve, presenting the concentration of urea versus the initial reaction rate ($v_0$), is depicted in Fig. S1. The maximum rate ($V_{max}$) and the Michaelis constant ($K_m$) values of urease used in this study were 0.053 mmol urea/min and 37.607 mmol urea, respectively, from the Michaelis–Menten curve.

When the applied pressure was increased, the rejection of urea was also enhanced regardless of the pristine or urease-coated membrane. This phenomenon can be explained with the conventional solution-diffusion model explaining the transport of water and solute through the dense polymeric membrane (Wijmans and Baker, 1995). In the solution-diffusion model, water flux ($J_W$) follows the equation below:

$$J_w = \frac{D_w K_w^L C_{w0} v_w}{l \rho_w RT} (\Delta P - \Delta \pi) \quad (1)$$

where $D_w$ is the diffusion coefficient of water in membrane; $K_w^L$ is the sorption coefficient of water to the membrane surface; $C_{w0}$ and $v_w$ are the mass concentration of water in the feed solution and the molar volume of water, respectively; $l$ is the thickness of the membrane's active layer; $\rho_w$ is the density of feed water; $R$ is the ideal gas law constant, $T$ is the absolute temperature, $\Delta P$ is the transmembrane pressure, and the $\Delta\pi$ is the osmotic pressure difference between the feed and the permeate side of the membrane.

On the other hands, the solute flux across the membrane follows the below equation:

$$J_s = \frac{D_s K_s^L}{l} (C_{s0} - C_{sl}) \quad (2)$$

where, $D_s$ is the diffusion coefficient of solute in membrane; $K_s^L$ is the sorption coefficient of solute to the membrane surface; and $C_{s0}$ and $C_{sl}$ are the mass concentration of solute in the feed water and the permeate, respectively.

When comparing the water flux and solute flux, water flux is dependent on the applied pressure, while solute flux is affected by the concentration gradient of the solute. Therefore, the increase in the urea rejection with the higher applied pressure is due to the water flux increasing together with the applied pressure, while the solute transport is less dependent on the applied pressure, resulting in the increase in the

rejection rate of urea.

The removal of urea by the pristine membrane and urease-coated membranes at different applied pressure are depicted in Fig. 4. To better understand the underlying mechanism of urea removal by the urease-coated membrane, Table S1 shows the amount of total ammonia nitrogen (TAN) in the permeate of each condition at different applied pressures.

When comparing the removal of urea by pristine and urease-coated membranes, the removal of urea by urease-coated membranes was enhanced from 4.1 % to 27.9 % when compared to the pristine membrane. The highest urea removal was achieved at an applied pressure of 20 bar with a membrane coated at an EDC/NHS:urease ratio of 1:0.2 membrane, which resulted in a urea removal of 65.9 %.

This increase in urea removal efficiency can be a result of both physical and biological effects from the urease coating on the membrane surface. First, urease on the membrane surface can act as the additional physical barrier for urea to pass through and hinder passage of the urea, similar to how urea transport is inhibited by the fouling layer found on membrane surfaces (Courtney and Randall, 2022; Lee and Lueptow, 2001). Similarly, the grafting of m-phenylenediamine on the membrane surface using the same EDC-NHS chemistry, enhanced the rejection of urea from 16.8 % to 54.9 % by reducing the free hole volume in XLE RO membrane (Habib and Weinman, 2022). However, we speculate that the physical removal of urease-coated layer would be limited compared to that of the dense polyamide layer of RO membrane.

Secondly, urea can be hydrolyzed while it passes through the urease-coated RO membrane by the enzymatic activity of urease. As the urease used in this study has an enzyme activity of 1000 U/g, meaning 1 mmol of urea is degraded every minute by unit gram of urease, the amount of urease-immobilized on the membrane surface together with the contact time between urea and urease are the crucial factors affecting the hydrolysis efficiency of urea. When comparing the urea removal efficiency with the degree of urease immobilization, influenced by the NHS:urease ratio, removal of urea enhanced with increasing NHS:urease ratio due to the heightened urease enzyme immobilization on the RO membrane surface, as depicted in Fig. 2. This enhanced enzyme presence at higher NHS:urease ratio facilitated more efficient urea hydrolysis, contributing to the overall improvement in urea removal efficiency.

The performance of the urease-coated membrane should not be underestimated as the ppb-level of urea present in the UPW can hamper the quality of semiconductor, and therefore, it is necessary to develop multi-barrier process that can remove urea. When combined with the UV or vacuum-UV (VUV) treatment processes, urease-coated RO membrane has potential applicability to enhance the resilience of the UPW process against the contamination by urea. Moreover, UPW systems typically consists of a two-pass RO unit which would enhance the removal of urease-coated membrane by twofold. Considering the rejection of urea by the RO system would be between 20 %~50 %, and the removal of urea by UV (a typical part of the UPW unit) is around 10 %, the rejection together with removal of urea by urease-coated RO membrane significantly increase the removal efficiency of urea in UPW system (Choi and Chung, 2019). The stability of the urease-immobilized on the RO membrane surface was confirmed by quantifying the enzyme remained on the membrane surface after the operation. From the ICP-MS analysis, there was no statistically significant difference ($p$ value $> 0.05$) in nickel content eluted from urease-immobilized RO membrane before and after the operation as depicted in Fig. S2.

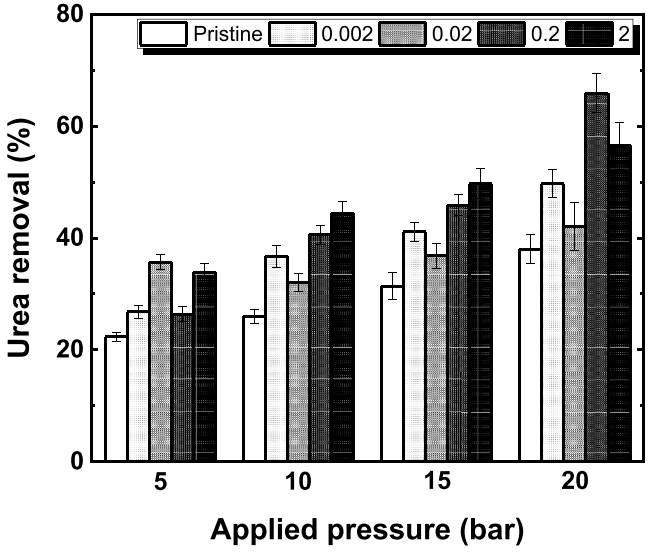


**Fig. 4.** Removal of urea by pristine and urease-coated membrane (NHS to urease ratio from 0.002 to 2) with varying applied pressure (5 bar to 20 bar).

## 3. Conclusion

In this study, urease was immobilized, through EDC/NHS chemistry, on the polyamide layer of a commercial RO membrane to remove urea in UPW systems. Successful immobilization of the urease was proven by quantifying the amount of nickel eluted from the membrane surface. When analyzing the water contact angle and zeta potential of the urease-coated membrane, it becomes more hydrophilic compared to the pristine membrane due to the charge characteristics of the urease immobilized on the membrane surface. The urease-coated RO membrane showed up to 27.9 % higher urea removal efficiency compared to the pristine membrane. This increase in the removal of urea was firstly contributed by the physical effect of urease coated on the membrane surface acting as an additional physical barrier for urea to pass through. However, urea was mainly hydrolyzed while passing through the urease-coated RO membrane by enzymatic hydrolysis considering relatively limited physical removal of urease-coated layer compared to the dense polyamide layer. Even though the removal of urea by urease-coated membrane was not complete, the results from this simple surface modification should not be underestimated since UPW systems consist of a two-pass RO system, which would double the additional removal provided by urease-coated membranes. Moreover, as the ppb-level of urea present in the UPW can hamper the quality of semiconductor, when urease-coated RO membrane is combined with other urea removal process, developed multi-barrier process would enhance the resilience of the UPW process against the urea contamination.

## 4. Materials and methods

### 4.1. Chemicals

Urea (ACS grade, > 99 %) was used as purchased from Fisher Scientific. 2-morpholin-4-ylethanesulfonic acid) (MES, low moisture content, >99 %), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, >98 %), N-Hydroxysuccinimide (NHS, 98 %), sodium phosphate dibasic heptahydrate (>99 %), and sodium phosphate monobasic monohydrate (ACS grade, > 98 %) were used as received from Sigma Aldrich. HEPES (99 % for biochemistry) was used as received from Thermo Fisher Scientific. Jack bean urease (CAS 9002-13-5, Sigma-Aldrich) was used. Sulfuric acid (95 %) was used as purchased from Samchun chemicals.

### 4.2. Coating urease on the membrane

A loose RO membrane, flat sheet BW30 (Filmtec) was used as model membrane. Before every experiment, the membrane was immersed in a 50 % isopropanol solution for at least 30 min. The membrane was then transferred to DI water for at least ten minutes. The rinse with DI water was repeated three times to completely remove the residual isopropanol solution. RO membrane was coated with the urease through the simple

N-ethyl-N′-(3-(dimethylamino)propyl)carbodiimide/N-hydroxysuccinimide (EDC/NHS) chemistry (Fig. 5). The membrane was placed on the glass to make active layer facing upwards, and customized silicon frame was put on the membrane to prevent leakage of the coating solution. The membrane was activated in MES buffer solution (0.1 mol/L, pH 5.0) containing 4 μM, 40 μM, 400 μM, and 4 mM EDC and 10 μM, 100 μM, 1 mM, and 10 mM NHS, respectively, at 37 °C for 1 h. Afterward, the membrane was washed with DI water to remove the residual chemicals. A 10 mM HEPES buffer solution (pH 7) with 10 g/L urease was then applied to react with the activated membrane surface under the same condition for 2 h. Finally, the urease-grafted RO membrane was rinsed with DI water to remove the excess enzyme remained on the membrane surface and stored in DI water.

### 4.3. Characterization of membrane surface

The presence of urease on the membrane was confirmed by Fourier-transform infrared spectroscopy (Perkin Elmer, USA) with a resolution of 1.0 $cm^{-1}$, in the range of 4000–400 $cm^{-1}$ by the absorbance mode. The hydrophilicity/hydrophobicity of the pristine and urease-coated membranes were also evaluated by the measurements of the static water contact angle with a SmartDrop (Femtobiomed, Korea). A minimum of twelve contact angle measurements on three different membrane coupons were acquired for each membrane. Changes in the membrane morpology after the urease coating were analyzed using SEM.

SurPASS Electrokinetic Analyzer with a flat surface cell (Anton Paar, USA) was utilized to analyze surface zeta potential through the measurements of streaming potential. Electrolyte solution with 1 mM KCl was used for streaming potential measurements. Zeta potential was measured with the pH range of 2–11 and the, pH of the solution was adjusted with HCl or NaOH after each measurement.

### 4.4. Quantification of Ni eluted from the membrane

Elution of the Ni in the urease coated on the membrane surface was followed by the methods described in a previous study (Chen et al., 2017). Membranes were immersed in 20 mL of 2 % nitric acid for 30 min. After physical removal of the nickel-free membranes, solutions were ready for ICP-MS analysis. Quadrupole ICP-MS (iCAP-Q, Thermo Scientific, Waltham, MA) was used to quantify the concentration of nickel. The system was calibrated using nitric acid solutions (2 %) with nickel concentrations ranging from 0 to 125 μg/L.

### 4.5. Kinetic characterization of urease

The kinetics of urease activity was measured in the 0.2 M phosphate buffer solution (pH = 7.0) containing 0.4 mg/mL of urease. To figure out the steady-state parameters of urease, the following urea concentrations were set: 0 mM, 20 mM, 40 mM, 100 mM, and 200 mM. After 30 min reaction time, to inhibit the activity of urease, 1 mL of the sample was taken and added to 1 mL of a 0.1 M sulfuric acid solution. For each urea concentration, the amount of urea in the solution was analyzed by LC-MS.

Through nonlinear least-square fitting of the measured initial reaction rates ($v_0$) values at various initial urea concentration to the Michaelis–Menten equation, steady-state parameters of urease, such as $K_m$ and $V_{max}$, were determined.

### 4.6. Membrane set up

A membrane system identical to the previous study was utilized to assess the efficacy of urease-coated RO membranes (Park et al., 2020). The stainless-steel membrane cell with the dimension of 7.7 cm×2.6 cm×0.3 cm ($l \times w \times h$) was connected to the membrane system. The retentate was recirculated back to the feed reservoir, and the permeate flux was measured continuously using a digital scale interfaced with a computer.

Urea rejection experiments were conducted to determine the effect of the urease coating with different EDC/NHS:urease ratios on the permeation of urea. 1 L of 10 ppm urea dissolved in the DI water was added to the 5 L feed tank. The feed velocity was 540 mL/min and the temperature of feed solution was maintained as 20 °C. An initial feed sample was taken right after the beginning of the experiments, and 10

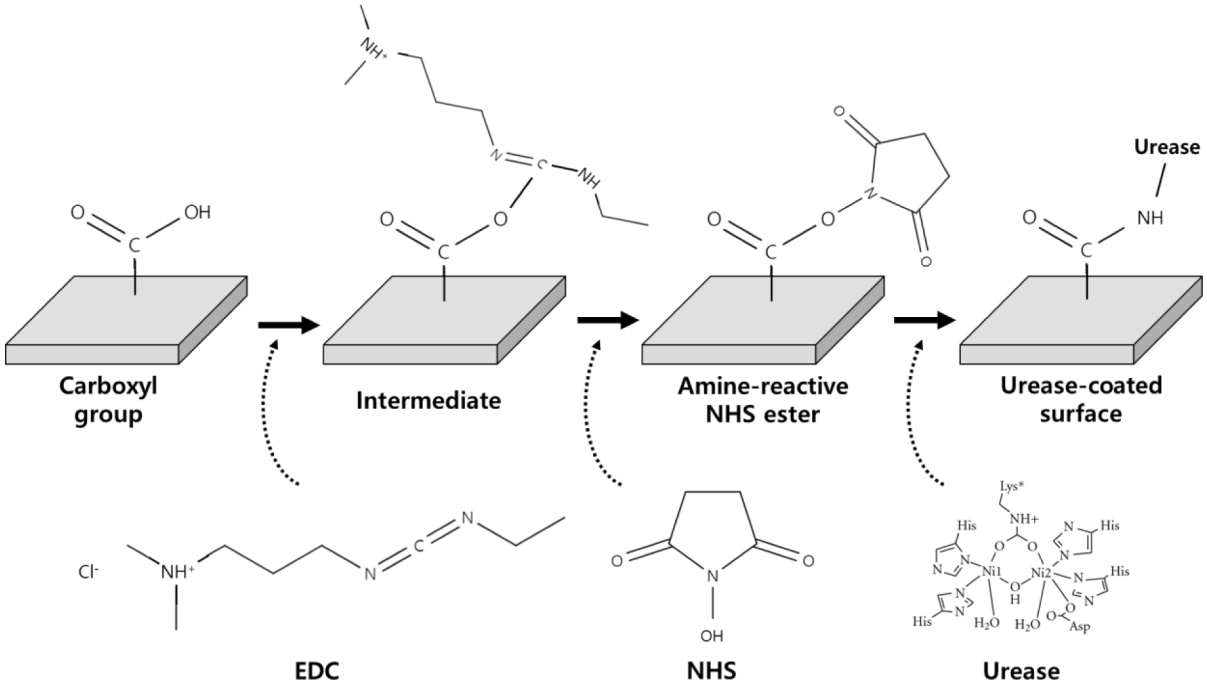


**Fig. 5.** Surface modification strategy to coat urease on the RO membranes through the EDC-NHS coupling reaction utilizes the native carboxylic groups of the polyamide layer for covalently binding of urease.

mL of the permeate samples were collected at varying operating pressure (5 bar, 10 bar, 15 bar, and 20 bar). The operating pressure was adjusted using a customized pressure regulator connected to nitrogen gas cylinder tank. In between the various operating pressure, the remaining volume of permeate in the tubing from the previous pressure was discarded to ensure that the permeate from the current operating pressure was collected.

### 4.7. Analytical method

The concentration of urea in each solution was analyzed by LC-MS2020 (Shimadzu, Japan) equipped with an electrospray ionization (ESI) interface. An HILIC (2.1 × 150 mm, 2.7 μm particle size, Agilent) column with acetonitrile and water as mobile phase was used. Specifically, mobile phase was the mixture of 0.1 % (v/v) formic acid in water and acetonitrile with volume ratio of solvents was 2:8. The system was calibrated using the DI water with urea concentration ranging from 100 to 1000 μg/L.

The concentration of total nitrogen was determined by Chromatropic acid methods (Humas 02022, Korea) and was measured by Hach spectrophotometers (DR 5000, USA). Potassium nitrate stock solution was used for the calibration standards for TN.

## CRediT authorship contribution statement

**Seung-Ju Choi:** Conceptualization, Investigation, Methodology, Formal analysis, Writing – original draft. **Lucas Crane:** Methodology, Formal analysis. **Seoktae Kang:** Conceptualization. **Treavor H. Boyer:** Resources, Supervision. **François Perreault:** Conceptualization, Supervision.

## 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.

## Data availability

Data will be made available on request.

## Acknowledgments

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.wroa.2024.100211.

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