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Biocatalytic membrane with acylase stabilized on intact carbon nanotubes for effective antifouling via quorum quenching

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ABSTRACT

This study developed biocatalytic membranes that can effectively control the surface biofouling based on the enzymatic quenching of bacterial quorum sensing. Acylase (AC) was immobilized and stabilized on intact carbon nanotubes (CNTs) via enzyme adsorption, precipitation, and crosslinking (EAPC) method, maintaining 66% of its initial enzyme activity for 200 days under rigorous shaking (200 rpm). This highly stable EAPC was anchored on the polyvinylidene fluoride (PVDF) microfilter using polydopamine coatings (EAPC membrane). The antifouling performance and mechanism of EAPC membrane was intensively evaluated under static incubation with a model bacterium of *Pseudomonas aeruginosa*. When compared with the PVDF membranes with and without free AC, the EAPC membrane enhanced the water permeability by 5-folds at an optimum loading of 0.40 g-CNTs/m^2 by effectively inhibiting the biofilm formation. The EAPC membrane with this optimal CNT loading did not increase the hydraulic resistance of membrane itself. In the lab-scale continuous filtrations, the EAPC membrane with its loading of 0.40 g-CNTs/m^2 demonstrated 1.6-fold delay of trans-membrane pressure increase compared to the PVDF membrane. It is anticipated that biocatalytic membrane based on quorum quenching nanobiocatalysis has a great potential in antifouling applications without changing the process configuration or additional treatments.

1. Introduction

Membrane filtration has become the main stream of advanced water treatment for various industry sectors [1]. Biofouling caused by the biofilm formation on the surface is the constant and expensive problem in the membrane technology [2]. So far, antifouling in the membrane process has been intensively studied via various methods such as physical removal [3,4], foulant-specific chemicals [5] and biocidal materials [6]. Biofilms are now considered as the natural way of microbial growth on the surface in aqueous environments [7,8], but the biofouling control still remains as a big challenge. Bacteria regulate various phenotypes such as biofilm formation, toxin production, exopolysaccharide production, and virulence factor in a cell-density dependent way, which are mediated by autoinducers (Quorum sensing, QS) [9–11]. Especially, *N*-acyl-homoserine lactone (AHL) type autoinducers generated by Gram-negative bacteria can be enzymatically degraded by acylase [12,13], lactonase [14] and oxidoreductase [15]. Recently, this enzymatic quorum quenching has been intensively studied to mitigate biofouling in the membrane filtration. In practice, carriers containing AHL degrading enzymes or whole cells were coupled with the membrane processes [10,16,17]. The biofilm formation is the surface phenomena with the sequence of cell attachment, microcolony formation, and maturation to three dimensional structures [18]. Therefore, the immobilization of quorum quenching enzymes on the membrane surface can be a direct and effective antifouling approach, which can be applied with no change of process configuration and additional treatments. Both activity and stability of quorum quenching enzymes would be main crucial factors to be considered for the successes of biocatalytic membranes. However, commercial membranes for the wastewater treatment such as polyvinylidene fluoride (PVDF), polysulfone (PS) and polyethersulfone (PES) are not suitable as a substrate for enzyme immobilization due to the lack of functional groups such as amine and carboxylate groups [10,19–23]. The surface treatments such as additional coating [24] and plasma treatment [25] have been adopted to introduce the functional groups for the enzyme attachments [26–28]. Build-up of additional coatings inevitably leads to the increase of intrinsic filtration resistance, and rigorous treatments can also damage the membrane performance. High surface porosity of membranes also restricts the surface area for the enzyme immobilization. Furthermore, harsh environment on the membrane surface such as solids accumulation and shear stress during system operation can easily cause the activity loss of immobilized enzymes.

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Nanobiocatalysis has evolved from simple combination of enzymes and nanomaterials for the enzyme immobilization to the effective development of synergetic interactions between enzyme immobilization methods with various nanomaterials. As a result, recent nanobiocatalytic approaches have demonstrated remarkable stabilization of enzyme activities compared to conventional enzyme immobilization approaches [29]. Several approaches of nanobiocatalysis have successfully demonstrated its potential in the long-term applications of enzyme systems with high enzyme loading and stability [30]. For example, acylase (AC), one of a quorum quenching enzymes, was successfully immobilized and stabilized on the magnetically-separable mesoporous silica [31] and carboxylated polyaniline nanofibers [32], both of which effectively mitigated the biofilm formation on the membrane surface. Recently, the simple dispersion of intact CNTs in an aqueous enzyme solution (enzyme adsorption), followed by enzyme precipitation and crosslinking, led to water-dispersible CNTs-based nanobiocatalyst with enhanced enzyme loading and stability [33].

In the present work, the quorum quenching AC was immobilized on intact CNTs by performing the protocol of enzyme adsorption, precipitation and crosslinking (EAPC). The activity and stability of EAPC-AC (called EAPC hereafter) were investigated. By having CNTs as the carriers of enzyme immobilization, EAPC would be easily filtered on the membrane surface of microfilters. In order to stably bind EAPC to commercial membrane materials, polydopamine was pre-coated on the membrane surface. The resulting EAPC membranes were characterized in terms of the inhibition of the biofilm formation and enhanced permeability considering the degree of filtration resistance due to surface EAPC loading.

2. Experimental section

2.1. Materials

AC (EC.3.5.1.14) from porcine kidney, *N*-acetyl-$_L$-methionine, sodium phosphate monobasic, sodium phosphate dibasic, Trizma hydrochloride (Tris-HCl), Trizma base, glutaraldehyde, ammonium sulfate, 2-mercaptoethanol, ethanol, *o*-phthaldialdehyde reagent solution, sodium tetraborate decahydrate, dopamine hydrochloride, sodium (meta) periodate ($NaIO_4$), *N*-hexanoyl-$_{DL}$-homoserine lactone (C6-HSL), 5(6)-Carboxyfluorescein diacetate *N*-succinimidyl ester (CFDA-SE) were purchased from Sigma Aldrich (St. Louis, MO, USA). CNTs (multi-walled, 30 ± 15 nm in outer diameter and 1–5 μm in length, purity > 95%) were purchased from Nanolab Inc. (Newton, MA, USA). Polyvinylidene fluoride (PVDF) disk-type membrane with a 0.45 μm pore size was purchased from Merck Millipore (Darmstadt, Germany).

2.2. AC immobilization on intact CNTs

AC was immobilized onto intact carbon nanotubes (CNTs) via the approach of enzyme adsorption, precipitation, and crosslinking (EAPC) [33]. CNTs solution (8 mg/mL) and AC solution (40 mg/mL) were prepared with phosphate buffer (PB, 100 mM, pH 7.0). Both solutions were mixed under shaking (200 rpm) for 1 h, and ammonium sulfate solution was added to induce enzyme precipitation (final concentration of 20% w/v). After incubation under shaking (200 rpm) for 30 min, glutaraldehyde was added to make a final concentration of 0.5% (w/v), and the mixture was incubated at 4 °C under shaking (50 rpm) for 12 h. All samples were treated with Tris-HCl buffer (100 mM, pH 7.0) under shaking (200 rpm) for 30 min to cap the unreacted aldehyde groups. In the case of enzyme adsorption (ADS) sample, the same volume of 100 mM PB was added instead of ammonium sulfate and glutaraldehyde. Finally, all samples were washed five times with 100 mM PB, and stored at 4 °C until use. For the comparison between ADS and EAPC, the amount of immobilized AC samples is represented by the weight of CNTs to be used for their immobilization.

2.3. Measurement of enzyme activity and stability

The AC activity was measured by the fluorescence-based assays based on the reaction of *o*-phthaldialdehyde with the amino group of $_L$-methionine, which is generated from the AC-catalyzed hydrolysis of *N*-acetyl-$_L$-methionine [31]. The AC-immobilized CNTs were mixed with *N*-acetyl-$_L$-methionine solution for the initiation of the enzyme reaction. During the enzyme reaction under shaking (200 rpm), 10 μL aliquot was taken from the reaction solution at appropriate time intervals and added to 140 μL of *o*-phthaldialdehyde solution in 50 mM sodium borate buffer (pH 9.5). Upon excitation at 340 nm, the emission intensity at 455 nm was measured using a microplate reader (Infinite M200 Pro, Tecan Trading AG., Männedorf, Switzerland.). The activity was calculated from the linear slope of the time-dependent increase in the emission intensity at 455 nm. One unit of AC activity is defined by the hydrolysis of 1.0 μmol of *N*-acetyl-$_L$-methionine per hour at pH 7.0 and room temperature. To evaluate the substrate diffusion in each immobilized enzyme system, the Michaelis constants (K_M) of free AC, ADS and EAPC were estimated by using Enzyme Kinetics Pro (ChemSW, Fairfield, CA, USA) that performs nonlinear regression based on the least-square method.

For stability measurements, the AC-immobilized CNTs were incubated in 100 mM PB or synthetic wastewater under shaking (200 rpm). Synthetic wastewater was prepared by following the protocol in previous studies [32,34]. The residual activity of each sample was measured using aliquots from the stock solution at different time periods of incubation. Relative activity was calculated by the ratio of residual activity at each time point to the initial activity.

2.4. Elemental analysis for estimation of enzyme loadings

The enzyme loadings of AC-immobilized CNTs were estimated by quantifying the nitrogen contents via elemental analysis. Tris-capping of un-reacted aldehyde groups was not performed to make enzyme the only source of nitrogen in the samples. AC-immobilized CNTs were excessively washed with pure Milli-Q water, and lyophilized using a freeze-dryer (FDU-2100 and DRC-1000, Eyela, Tokyo, Japan) for 24 h. The lyophilized samples were analyzed by elemental analyzer (Flash EA 1112, Thermo Fisher Scientific, Waltham, MA, USA).

2.5. Measurement of AHL autoinducer

The concentration of AHL autoinducers was measured by a colorimetric method, as described previously [35,36]. 2 mL of C6-HSL solution was supplemented with test samples including CNTs, ADS and EAPC, and then incubated under shaking (200 rpm) at room temperature for 2 h. After incubation, 40 μL of aliquot was taken and mixed with 50 μL of a hydroxyl amine dissolved in NaOH. After mixing same volume of ferric chloride in HCl and ethanol, 90 μL of mixture was added for color development. The absorbance was measured at 520 nm using a microplate reader for quantification of the AHL autoinducers.

2.6. Cell viability assay

Viability of bacterial cells was quantitatively evaluated using commercial Live/Dead staining kit composed of SYTO 9 and propidium iodide (LIVE/DEAD® BacLight™, Thermo Fisher Scientific, Waltham, MA, USA) [37,38]. Cell staining and measurement of fluorescence intensity was conducted according to the manufacturer's protocol provided. Green and red fluorescence intensities, which corresponds to viable and damaged cells, were recorded using a microplate reader (Infinite M200 Pro, Tecan Trading AG., Männedorf, Switzerland).

2.7. Fabrication of biocatalytic membrane

Dopamine hydrochloride solution (2 mg/mL) was prepared in