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Preparation of affinity membranes using polymer phase separation and azido-containing surfactants

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Highlights

- We proposed a novel approach to prepare affinity membranes using click chemistry.
- Porous membranes were prepared using cellulose acetate by polymer phase separation.
- Membranes were functionalized with synthesized azido-containing surfactants.
- The azido groups on a membrane were conjugated with alkyne-containing ligands.
- The membranes showed affinity to a protein and an antibiotic of interest.

ABSTRACT

We propose a novel approach to prepare affinity membranes using azido-containing surfactants and click chemistry. Porous polymeric membranes were prepared using cellulose acetate via polymer phase separation in the presence of azido-containing surfactants. Thermally induced phase separation and nonsolvent-induced phase separation were used for membrane preparation. The azido groups displayed on the membrane surfaces were conjugated with nitrilotriacetic acid via Huisgen 1,3-dipolar cycloaddition to prepare membranes that displayed affinity toward a hexahistidine-tagged protein. Two types of phase separation successfully produced porous membranes with different microstructures but showed similar separation performances. In place of nitrilotriacetic acid, D-Ala-D-Ala was conjugated to the surface of the azido-functionalized membrane. The membranes functionalized with D-Ala-D-Ala showed high affinity toward vancomycin. The present approach leads to facile surface functionalization of polymeric materials and produces affinity membranes displaying various types of ligands on their surfaces.

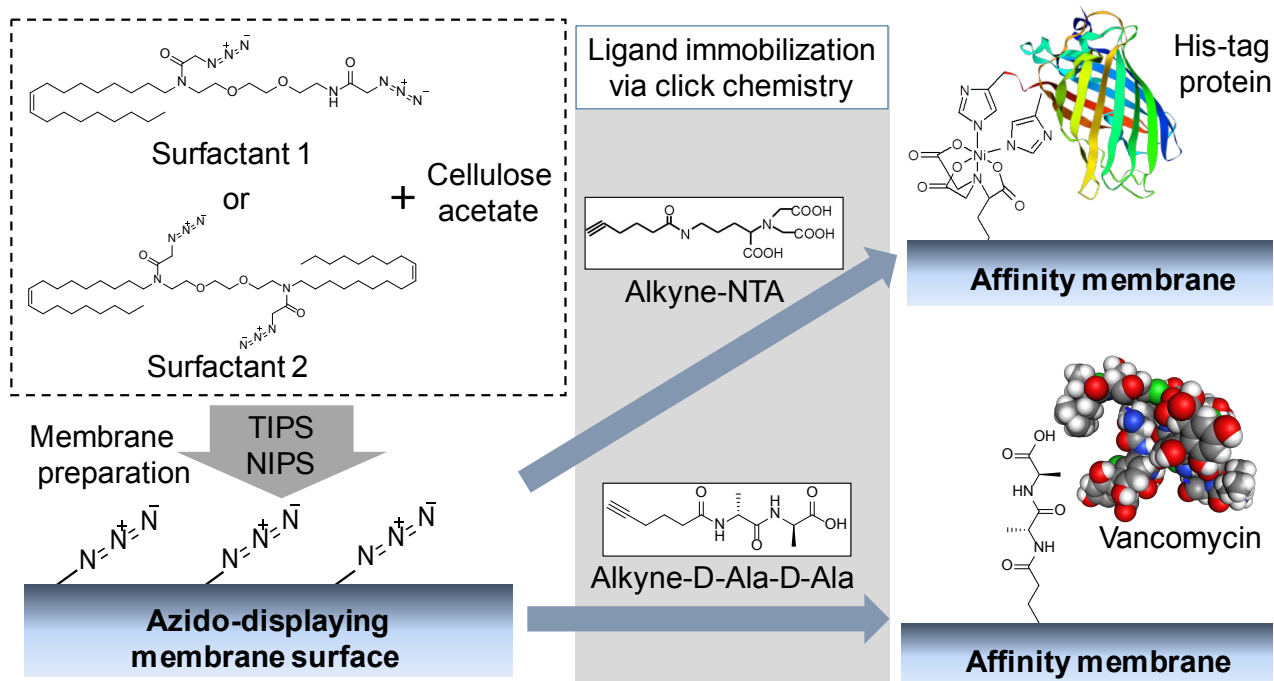
Keywords: affinity membrane, antibiotic, molecular recognition, protein, selective separation, surface immobilization

1. Introduction

The production of functional proteins plays an important role in the pharmaceutical, medical and food industries [1-3]. For example, the development of antibody drugs is a promising field in drug discovery, but a major drawback is the high production costs [4, 5]. Despite significant progress on protein engineering and the study of protein function, efforts that advance protein production and purification techniques at large scales are scarce [6-9]. Being scalable is one of important factors for protein production in industry. Membrane separation is scalable and already used in the food industry, e.g., production of drinking water and wastewater treatments. Membrane filtration and separation are also used in analysis and pretreatment for analyte preparation. Designing appropriate membrane processes for filtration aids high-throughput screening of functional substances [10], especially in drug discovery [11].

Because separation by a porous membrane is based on the sizes of substances, a rational strategy for selective separation of a target protein from a protein mixture is to integrate affinity on a membrane surface [10, 12-20]. In general, the surfaces of porous polymeric membranes are inert and stable immobilization of a ligand on an inert membrane surface is challenging. In our previous studies, we proposed the use of surfactant-like ligands in membrane preparation via polymer phase separation and succeeded in the preparation of affinity membranes, which displayed ligands on their surfaces [21, 22]. The prepared membranes showed remarkable affinity toward proteins of interest and successfully enabled selective purification of proteins from solutions containing a mixture of proteins.

These studies showed that polymer phase separation in the presence of surfactant-like ligands produced porous polymeric membranes where the surfaces were functionalized with ligands. However, selection of an organic solvent is an important factor for polymer phase separation and small amounts of additives (e.g., surfactant) to a polymer solution largely affect the behavior of the phase separation [23-28]. Moreover, dissolving surfactant-like ligands in a polymer solution can be challenging because some ligands are only water-soluble or an organic solvent impairs the functional properties of the ligands (e.g., protein-type ligand). In this study, to overcome these problems, we attempted a novel approach to prepare affinity membranes via phase separation using click chemistry. A porous polymeric membrane was prepared via phase separation in the presence of an azido-containing surfactant. Since the prepared membrane had azido groups on its surface, the azido groups were conjugated with alkyne-containing ligands in water via Huisgen 1,3-dipolar cycloaddition (one of the click reactions) [29-31], leading to ligand immobilization on the membrane surface [32]. Because ligand immobilization proceeded in water, a variety of water-soluble ligands are available for the preparation of affinity membranes. In this report, we successfully prepared azido-functionalized membranes that were transformed to membranes with affinity toward a hexahistidine-tagged (His-tag) protein [33] and vancomycin [34].



Scheme 1. Preparation of affinity membranes using azido-containing surfactants and Huisgen 1,3-dipolar cycloaddition. Azido-containing surfactants displayed azido groups on the surfaces of porous cellulose acetate membranes, which were available for ligand immobilization.

2. Experimental

2.1. Materials

N-(5-Amino-1-carboxypentyl)iminodiacetic acid (AB-NTA) and the HiLyte Fluor™ 555 Labeling Kit were purchased from Dojindo Laboratories (Kumamoto, Japan). 1-Bromohexadecane and oleoyl chloride were purchased from TCI (Tokyo, Japan). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (WSC) and acetonitrile were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Cellulose acetate and 2,2'-(ethylenedioxy)bis(ethylamine) were purchased from Merck (Darmstadt, Germany). 2-Azido acetic acid was purchased from Biosynth Carbosynth (Compton,

UK). Other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). D-Ala-D-Ala was purchased from Chem-Impex International Inc. (Wood Dale, IL, USA). Green fluorescent microspheres (100 nm in diameter) were purchased as a suspension from Micromod Partikeltechnologie GmbH (Rostock, Germany). The ultra-pure water used was produced by a Millipore water purification system (Elix UV 3 system, Millipore, Molsheim, France).

2.2. Synthesis of azido-containing surfactants (1 and 2) and alkyne-ligands

These are described in the Supplementary Material.

2.3. Preparation of recombinant proteins

His-tag GFP (hexahistidine-tagged enhanced green fluorescent protein) was prepared as reported previously [35]. Briefly, the gene fragment coding the N-terminus His-tagged GFP was cloned into the pBAD/thio-TOPO vector (Invitrogen, Waltham, MA, USA). Protein expression was induced in *Escherichia coli* strain Top10 (Invitrogen) by addition of 0.1% L-arabinose and subsequent incubation for 16 h at 27 °C. The His-tag GFP was purified using TALON metal affinity resin (Takara Bio Inc., Kusatsu, Japan).

2.4. Preparation of membranes via thermally induced phase separation (TIPS) [36]

Cellulose acetate (CA, 2.3 g) was mixed with triethyleneglycol (7.7 g) under a nitrogen

atmosphere at 55 °C for 2 h. A surfactant (115 mg) was added to the mixture and mixed at 160 °C. The CA solution was placed between two pieces of a glass plate (preheated at 160 °C) with 200 µm clearance, followed by quenching at room temperature (approx. 10 °C/min) to induce phase separation. After solidification of the polymer, the membrane was immersed in water to remove triethyleneglycol. The solution in the bath was replaced with fresh water once a day. The replacement was repeated for 3 days. The prepared membranes were kept in water until use.

Membranes were observed by a field-emission scanning electron microscope (FE-SEM, JSM-7500F, JEOL, Tokyo, Japan).

2.5. Preparation of membranes via nonsolvent-induced phase separation (NIPS) [37]

Cellulose acetate (CA, 10.6 g) was dissolved in a mixture of DMF and acetone (25 mL:25 mL). After stirring for 1 day, 0.3 mL ethanol solution containing an azido-containing surfactant (53 mg) was added to the CA solution. A nonwoven cloth (Precise™ SuperFlat AS 091, Asahi Kasei, Tokyo, Japan) was placed on a Teflon film. The CA solution was cast on the nonwoven cloth using an applicator (YBA-4 type, Yoshimitsu Seiki Co., Japan) with a gap of 200 µm. The cast CA solution was immersed in a coagulation bath of ethanol/water (1:1 in volume) at room temperature for 10 min. The solution in the bath was replaced with fresh water once a day. The replacement was repeated for 3 days. The prepared membranes were kept in water until use.

2.6. Surface functionalization of membranes via click reaction

An alkyne-ligand (0.42 mmol) was dissolved in 10 mL water containing 0.1 M sodium ascorbate and 4.4 mM CuSO₄. A pressure-driven syringe, which was equipped with a membrane filter holder (Swinnex[®], Millipore Corp., Bedford, MA, USA) and an azido-functionalized membrane (4.9 cm²) was filled with the solution. Five milliliters of the solution was filtered through an azido-functionalized membrane (4.9 cm²) and left at 25 °C overnight. The membrane was washed by filtrating 0.1 M HCl aqueous solution (5 mL × 3) and 1 mM NaOH aqueous solution (5 mL × 3).

2.7. Ni²⁺/NTA complex formation and filtration of the His-tag GFP solution

The NTA-functionalized membrane (4.9 cm² circle) was placed in a membrane filter holder. A 0.1 M NiSO₄ solution (5 mL) was filtered through the membrane using a syringe to load Ni²⁺ ions. Water (5 mL) was then filtered through the Ni²⁺-loaded membrane three times. A phosphate buffer (10 mL, 20 mM, pH 7.4) containing 0.5 M NaCl was filtered to equilibrate the membrane surface. The phosphate buffer solution (2 mL) containing the protein (e.g., 45 µg/mL His-tag GFP) was applied to the membrane using a syringe at a flow rate of approx. 1 drop/s. The passed fraction was collected (non-adsorption fraction). The phosphate buffer (10 mL, pH 7.4) was filtered through the protein-loaded membrane to remove unbound protein (passing fraction) and the bound protein was eluted by the phosphate buffer (5 mL, pH 7.4) containing 0.5 M imidazole and 0.5 M NaCl (elution fraction). The concentrations of GFP and Hilyte-labeled HRP were measured using a fluorescence spectrophotometer (FP-8200, Jasco, Tokyo, Japan).

2.8. Breakthrough curve

To study the GFP binding capacity of the membrane prepared with surfactant **2** via the TIPS method, a GFP solution (4 µg/mL) was applied to the membrane. Permeate fractions over specific volume intervals (1 mL) were collected and analyzed using a fluorescence spectrophotometer.

2.9. Filtration of the antibiotic solution

The phosphate buffer solution (2 mL) containing the antibiotics (45 µg/mL each) was applied to the D-Ala-D-Ala-functionalized membrane using a syringe at a flow rate of approx. 1 drop/s. The phosphate buffer (10 mL, pH 7.4) was filtered through the antibiotic-loaded membrane to remove unbound antibiotics (passing fraction) and the bound antibiotic was eluted by the phosphate buffer (5 mL, pH 7.4) containing 5 mM D-Ala-D-Ala (elution fraction). The concentration of the antibiotic was measured using a high-performance liquid chromatography (HPLC) system, LC-20AT (Shimadzu, Kyoto, Japan) equipped with a UV-vis detector SPD-20A (Shimadzu) and an Inertsil ODS-3 column (4 mm × 250 mm, GL Science, Tokyo).

3. Results and discussion

3.1. Preparation of azido-functionalized membranes and the observation of their surface

morphologies

We synthesized two different azido-containing surfactants (surfactants **1** and **2**, Scheme 1) to prepare azido-functionalized membranes. These surfactants consisted of two parts: a hydrophobic moiety (C18:1) for tethering to a polymeric membrane surface and azido groups for conjugating with alkyne groups of ligand molecules based on Huisgen 1,3-dipolar cycloaddition. Surfactant **1** had two azido groups and a single hydrophobic tail. Surfactant **2** had two azido groups and two hydrophobic tails (gemini-type surfactant). Both were designed to be tethered to a membrane surface.

Flat azido-functionalized membranes (approx. 200 μ m thick) were prepared using CA and the surfactants via polymer phase separation (TIPS and NIPS). CA was adopted as a membrane matrix because of its low nonspecific protein adsorption [38-42]. Our previous reports suggested that the surfactants are localized at an interface of polymer-rich/polymer-poor phases during phase separation because of their amphiphilic properties [21, 22]. The interface became the surface of a porous CA membrane, which played an important role in subsequent surface functionalization using ligand molecules.

SEM observations were performed to examine the morphology of the azido-functionalized membranes prepared (Fig. 1). All membranes had interconnected microporous structures, which were typical of membranes prepared via TIPS and NIPS. Cross-sectional images showed that the membranes prepared by TIPS had relatively uniform structures from the surfaces to the bottoms of the membranes without macrovoids. Those prepared by NIPS had heterogeneous structures composed

of dense polymer parts, macrovoids and thick fibers of supporting nonwoven clothes. It should be noted that there was no obvious difference in the membrane structure between surfactants **1** and **2**. A cellulose-based membrane swells in water to some extent and its surface morphology in water is different from that in a dry condition (SEM observation). The following experiments focused on the membranes “in aqueous solution conditions”.

The filtration of microspheres (100 nm in diameter) through the membranes provides approximate information on the effective pore sizes of the membranes. The membranes prepared with surfactants **1** and **2** via the TIPS method showed 70% and 71% rejection of the microspheres, respectively. The membranes prepared with surfactants **1** and **2** via the NIPS method showed 71% and 81% rejection, respectively. These results indicate that the effective pore sizes of the membranes were slightly smaller than 100 nm. The similar pore sizes of the membranes with surfactants **1** and **2** suggest that the structural difference between the surfactants did not affect the phase separation of cellulose acetate. These investigations revealed the successful preparation of flat membranes using CA and azido-containing surfactants via TIPS and NIPS.

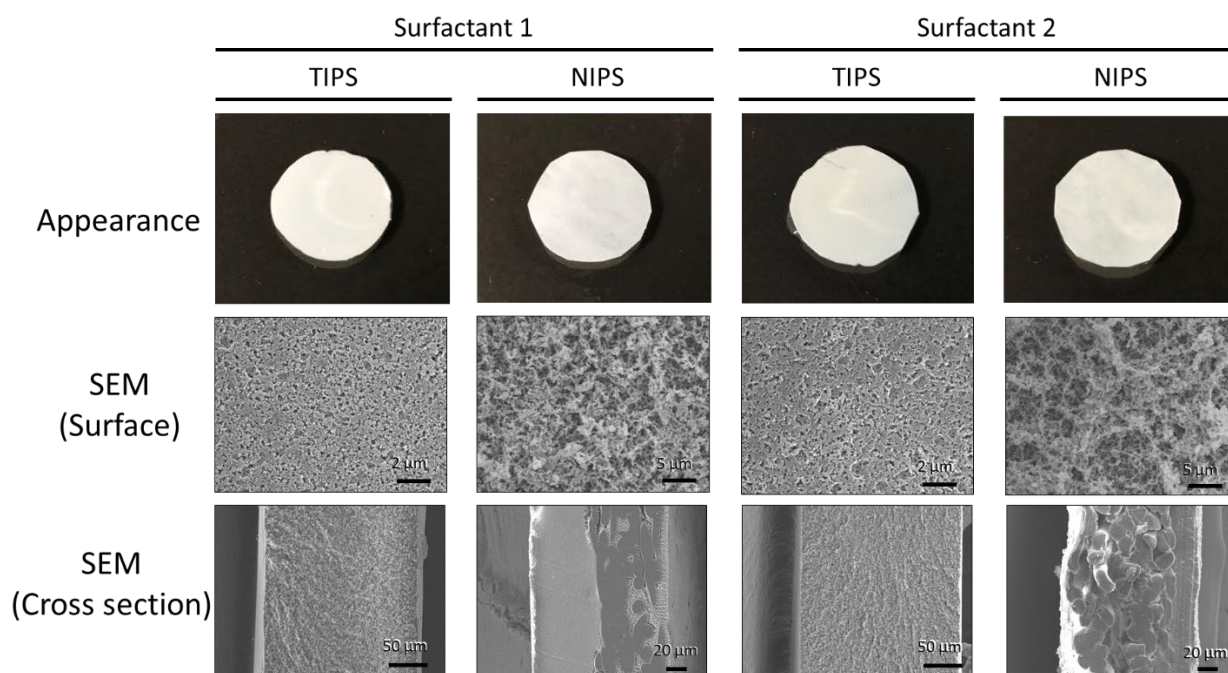


Fig. 1. Photographs and FE-SEM images of the surfaces and cross-sections of the azido-functionalized membranes.

3.2. Immobilization of alkyne-ligands via click chemistry

The azido groups on the membranes prepared were available for conjugation with alkyne groups via Huisgen 1,3-dipolar cycloaddition in water. We attempted the conjugation of nitrilotriacetic acid (NTA) to the azido groups to functionalize the membranes prepared from surfactant **1** or **2** via the TIPS and NIPS methods. Prior to conjugation, alkyne-containing NTA (alkyne-NTA, Fig. 1 and Scheme S5) was synthesized. Alkyne-NTA was conjugated with the azido groups displayed on the membrane surfaces in the presence of Cu^+ ions. Ni^{2+} ions were then added to the NTA-functionalized membranes to form the complex of Ni^{2+} /NTA on the membrane surfaces, which has high affinity towards proteins with a hexahistidine tag (His-tag) [33]. The conjugation and

complex formation procedures did not damage the membranes because these procedures were carried out in aqueous solutions under mild conditions.

An aqueous solution (2 mL) containing 90 μg His-tag GFP was loaded onto the Ni^{2+} /NTA-functionalized membranes. The membranes were washed and the protein eluted with a buffer solution containing 0.5 M imidazole. The contents of the His-tag GFP in fractions are summarized in Fig. 2. A protein separated by the membrane was supposed to maintain its conformation and property, because the His-tag GFP retained its fluorescence after the separation using the membrane. Despite the similarity of the molecular structures of surfactants **1** and **2**, the membranes prepared with the surfactants showed remarkably different His-tag GFP elution profiles. While a large part of the protein (more than 55 μg) passed through the membranes of surfactant **1** prepared via TIPS and NIPS, the membranes of surfactant **2** prepared via TIPS and NIPS effectively captured the His-tag GFP (more than 50 μg His-tag GFP) and released this protein in the elution fraction. The structural difference of the two surfactants was only the number of a hydrophobic carbon chain. The carbon chains of the surfactants were supposed to bind to a CA membrane via hydrophobic interaction. Thus, surfactant **2** bound more strongly on a CA membrane due to two hydrophobic carbon chains and displayed azido groups stably, which would result in the large capture amount of a target protein. Since surfactant **1** was less hydrophobic than surfactant **2**, surfactant **1** may detach from the surface of the membranes. This detachment may occur during membrane preparation, NTA-functionalization or filtration of a protein solution.

There was a small difference in the elution profile between the membranes prepared via TIPS and NIPS methods. The membrane prepared with surfactant **2** via TIPS showed a slightly higher capacity (57 μg) for His-tag GFP when compared with the membrane prepared via the NIPS method (52 μg). Indeed, a slightly lower content of His-tag GFP (16 μg) was observed in the passing fraction of the membrane prepared via the TIPS method when compared with that of NIPS (23 μg). Similar elution profiles were observed for membranes prepared with surfactant **1** via NIPS and TIPS methods. The difference in the protein-binding capacity is explained by the microporous structures of the membranes. The NIPS membrane had a thin fiber network on the surface and macrovoids in the cross-section, whereas the TIPS membrane had a relatively homogeneous dense structure on the surface and in the cross-section (Fig. 1). The high void ratio generally contributes to a high permeability of water and prevents membrane clogging. However, the macrovoids may decrease the effective surface area to capture a protein. These investigations showed that the membrane prepared with surfactant **2** via the TIPS method was appropriate for the affinity membrane. The following experiments were carried out using the membrane prepared with surfactant **2** via the TIPS method.

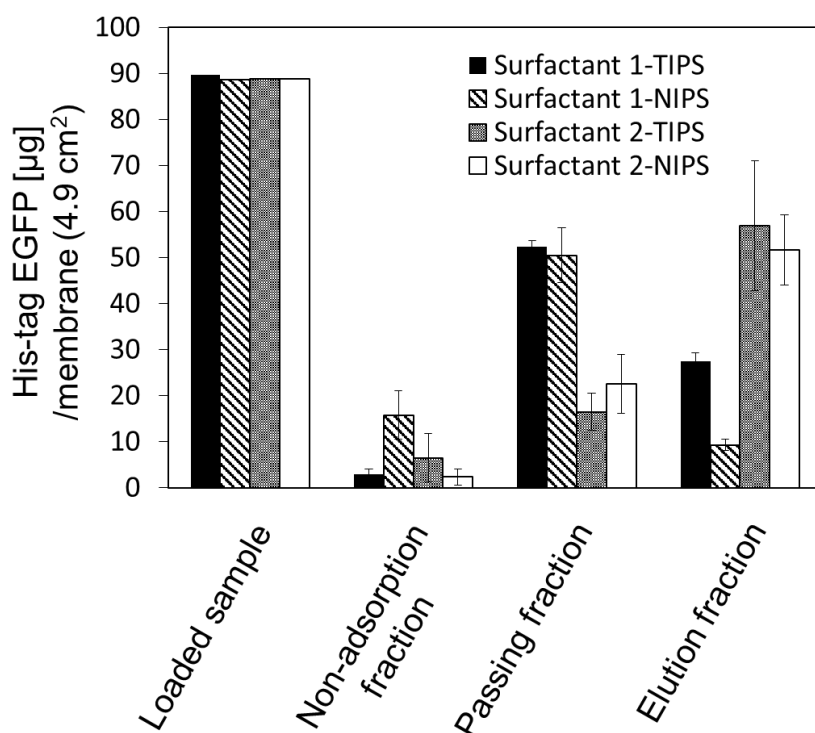


Fig. 2. Protein contents in fractions eluted from the membranes functionalized with alkyne-NTA for His-tag affinity separation. The definitions of non-adsorption fraction and passing fraction were described in section 2.7.

We then investigated the breakthrough of His-tag GFP through the NTA-functionalized membrane (Fig. 3). The maximal amount of adsorbed His-tag GFP was calculated to be $10.2 \mu\text{g}/\text{cm}^2$ ($50 \mu\text{g}/4.9 \text{ m}^2\text{-membrane}$), which concurs with the results presented in Fig. 2. In our previous study, a CA membrane prepared with a NTA-conjugated surfactant via the TIPS method showed a binding capacity of $17 \mu\text{g}/\text{cm}^2$ for His-tag GFP. The present study functionalized the surface after membrane preparation. Efficiencies of displaying azido groups on a membrane surface and reaction of the azido/alkyne groups may account for the decrease in binding capacity observed. This experiment also

ensured the durability of the membrane, because more than 20 times of the load and filtration of sample solution made no rupture of the membrane.

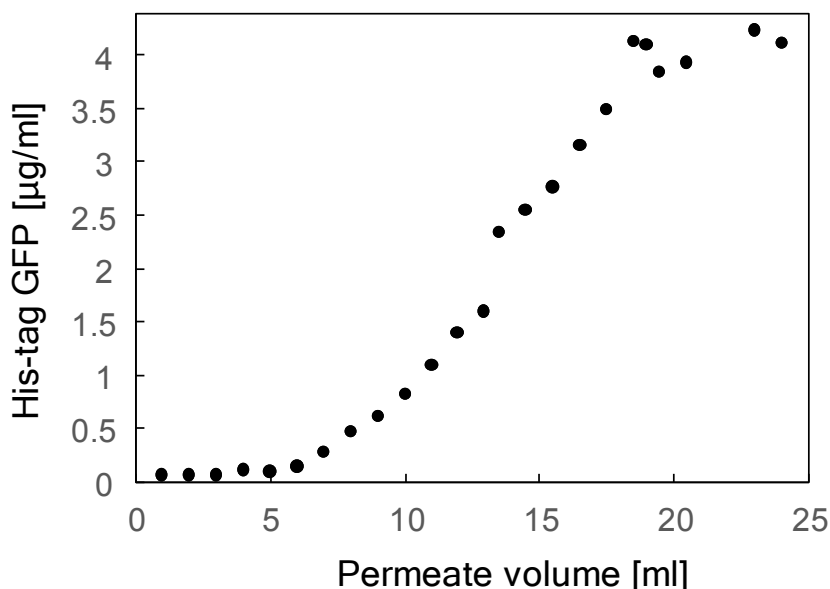


Fig. 3. Breakthrough curve for His-tag GFP through the NTA-functionalized membrane. The membrane was prepared with surfactant **2** via the TIPS method, followed by NTA-functionalization. One milliliter of the His-tag GFP solution ($4 \mu\text{g/mL}$) was repeatedly loaded onto the membrane (4.9 cm^2) at a steady flow rate [22].

3.3. Selective separation of the His-tag protein from a mixture solution

A NTA-functionalized membrane was prepared successfully from the azido-functionalized membrane using click chemistry. Here, we examined selective separation of a target protein from a protein mixture using the NTA-functionalized membrane. To prepare a protein without affinity to the membrane, horseradish peroxidase (HRP) was labeled with a red fluorophore, HiLyte Fluor™ 555.

HiLyte-labeled HRP and His-tag GFP were mixed in a buffer solution and loaded to the NTA-functionalized membrane. The amounts of proteins, which were eluted from the membrane, were quantified by measuring green and red fluorescence intensities. The results showed that the elution fraction contained only His-tag GFP and a negligible amount of HRP. The loaded HRP passed through the membrane and was found in the non-adsorbed and passing fractions (Fig. 4). These results demonstrated the selective separation of His-tagged proteins by the NTA-functionalized membrane.

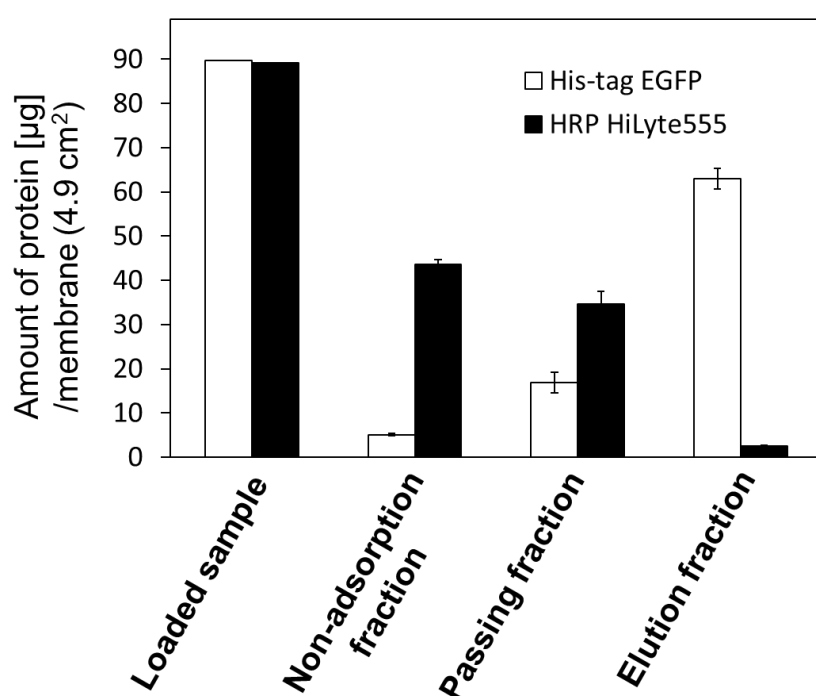


Fig. 4. Protein contents in fractions eluted from the NTA-functionalized membranes. The membrane was prepared with surfactant **2** via the TIPS method, followed by surface functionalization with NTA. The definitions of non-adsorption fraction and passing fraction were described in section 2.7.

3.4. Immobilization of a peptide via click chemistry

The above investigation proved that the azido-functionalized membrane can be easily transformed to an affinity membrane for His-tag protein separation. Finally, we employed a dipeptide (D-Ala-D-Ala) to functionalize the azido-functionalized membrane. Vancomycin, an antibiotic, recognizes and binds to D-Ala-D-Ala to prevent the synthesis of the bacterial cell wall. The removal of the antibiotic from pharmaceutical wastes and sewage offers health, economic and environmental benefits [43, 44].

We prepared alkyne-conjugated D-Ala-D-Ala (Scheme S6) and immobilized this dipeptide onto the membrane surface via Huisgen 1,3-dipolar cycloaddition in water. A mixture solution of vancomycin and ampicillin was loaded onto the membrane, followed by washing and elution with an appropriate buffer solution. A large part of vancomycin (37 μ g) was found in the elution fraction. In contrast, the majority of ampicillin (84 μ g) passed through the membrane and only a small amount of ampicillin was found in the elution fraction, suggesting negligible affinity of ampicillin toward the membrane. These observations indicated the successful separation of an antibiotic from a mixture.

Some peptides show specific affinity to a protein, an antibiotic and a physiologically active substance. Although these peptides are good candidates as ligands for these biologically active molecules, the solubility of peptides, which are soluble in water, is remarkably low in many organic solvents. Moreover, there is limited selection of a suitable organic solvent to dissolve a polymer for preparing a porous membrane, which can be available for phase separation. Thus, it is a rational

approach to immobilize a peptide ligand on the surface of a porous polymeric membrane in aqueous solution after the preparation of the membrane.

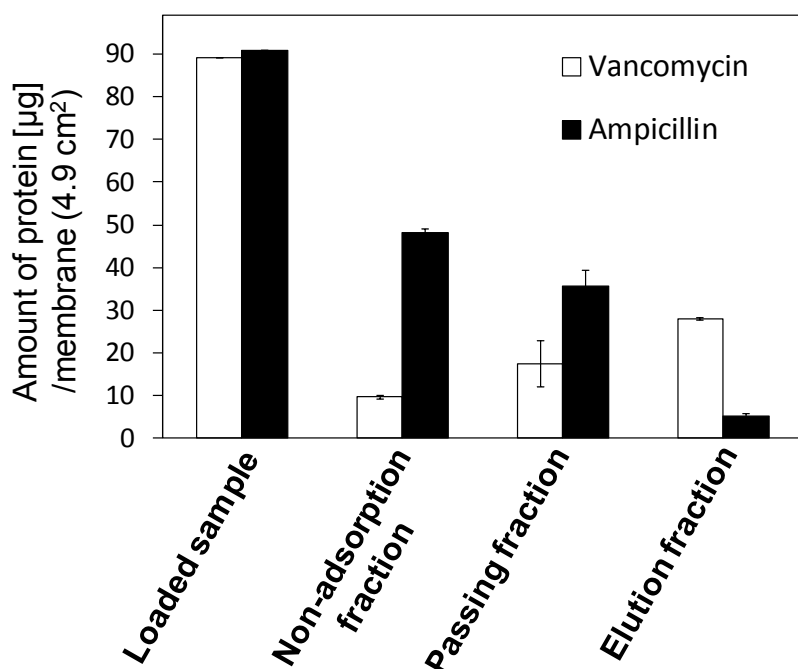


Fig. 5. Antibiotic contents in fractions eluted from the membranes functionalized with D-Ala-D-Ala.

The membrane was prepared with surfactant **2** via the TIPS method, followed by its surface-functionalization with D-Ala-D-Ala.

4. Conclusions

We synthesized azido-containing surfactants and prepared porous polymeric membranes via polymer phase separation, whose surfaces were functionalized with azido-containing surfactants. The azido groups on the membrane surfaces were successfully conjugated with NTA and D-Ala-D-Ala via Huisgen 1,3-dipolar cycloaddition to prepare membranes that had affinity toward a His-tag protein and vancomycin, respectively. Two different types of azido-containing surfactants synthesized and

two different types of phase separation were used for preparing affinity membranes. We found that the gemini-type azido-containing surfactant (surfactant **2**) and the TIPS method were appropriate for the high protein-binding capacity of the membrane. The present approach for the preparation of affinity membranes consists of two steps. The first step was the preparation of a porous polymeric membrane functionalized with an azido-containing surfactant using polymer phase separation. The second step involved immobilization of a water-soluble ligand to the membrane surface using click chemistry in an aqueous solution. Many ligands, which show selective binding properties to biomolecules and physiologically active substances, are soluble in water and are often unlikely to be soluble in organic solvents. Thus, it is rational to immobilize a wide variety of ligands on a membrane surface in water after membrane preparation, leading to a broad range of affinity membranes. The present approach should be useful for functionalizing the surface of porous membranes with natural and synthetic ligands.

CRedit authorship contribution statement

Kenta Morita: Experiments for membrane and synthesis, and writing the original draft. Shinano Takeda and Ayumi Yunoki: experiments for membrane and synthesis. Takane Tsuchii: filtration experiments of microspheres. Tstutomu Tanaka: preparation of His-tag GFP. Tatsuo Maruyama: supervision, conceptualization, writing and editing.

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Supplementary Material

Preparation of affinity membranes using polymer phase separation and azido-containing surfactants

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1. Experimental

1.1. Synthesis of surfactants **1** and **2**

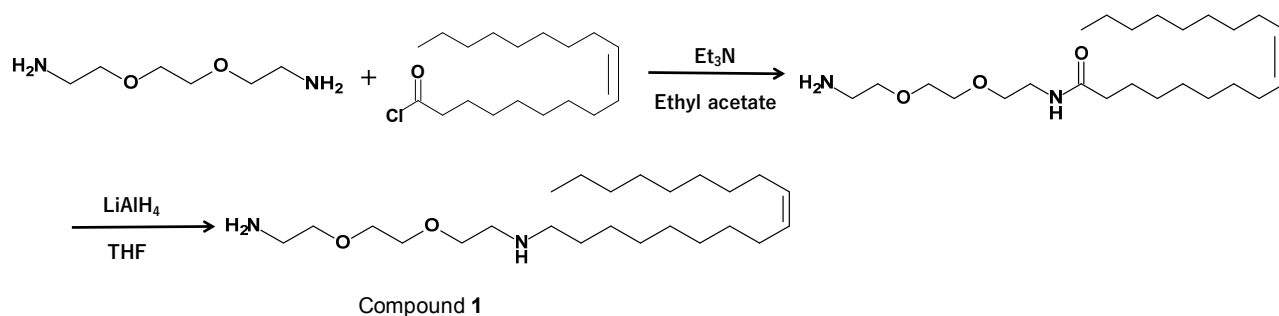
Surfactant **1**:

2,2'-(Ethylenedioxy)bis(ethylamine) (300 mmol, 44.5 g) was dissolved in ethyl acetate (30 mL) in a three-neck flask under N₂. After the mixture was heated to 60 °C, ethyl acetate (30 mL) containing oleoyl chloride (3 mmol, 903 mg) was added drop by drop to the mixture (Scheme S1). After refluxing for 20 h, the mixture was washed with an equivalent volume of water in a separatory funnel. The ethyl acetate phase was collected and evaporated, followed by vacuum drying for 24 h. The residue was dissolved in 30 mL dry THF at 60 °C under N₂ and a THF solution containing LiAlH₄ (19.5 mmol, 740 mg) was added. After stirring for 4 h at 60 °C, 10 mL ethanol was added to the mixture to terminate the reaction. After filtration using a filter paper, the solution mixture was evaporated (compound **1**). 2-Azido acetic acid (10 mmol, 1.01 g), *N*-hydroxysuccinimide (NHS, 10 mmol, 1.15 g) and *N,N'*-dicyclohexylcarbodiimide (DCC, 10 mmol, 2.06 g) were dissolved in 10 mL DMF and stirred for 20 h at 25 °C to prepare the 2-azido acetic acid NHS ester (Scheme S2). After filtration, a DMF solution containing the 2-azido acetic acid NHS ester was mixed with compound **1** and potassium carbonate (12 mmol, 1.66 g) for 20 h at 25 °C (Scheme S3). The solvent was evaporated. The dry residue was dissolved in chloroform (30 mL). The solution was washed with water (30 mL) twice. The chloroform phase was collected and evaporated overnight. The dry residue was dissolved in ethyl acetate (5 mL) and the solution was filtered, followed by vacuum drying

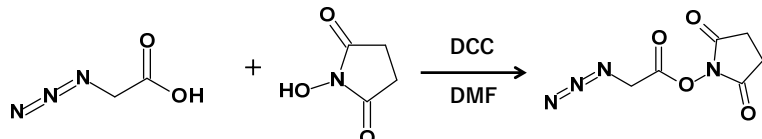
overnight. The product was identified by ^1H -NMR and DART-MS.

^1H NMR (500 MHz, CDCl_3): δ 0.88 (t, 3H, $J = 7.57$ Hz), 1.27 (m, 24H), 2.00 (m, 4H), 3.21–3.66 (m, 14H), 3.84–4.11 (m, 4H).

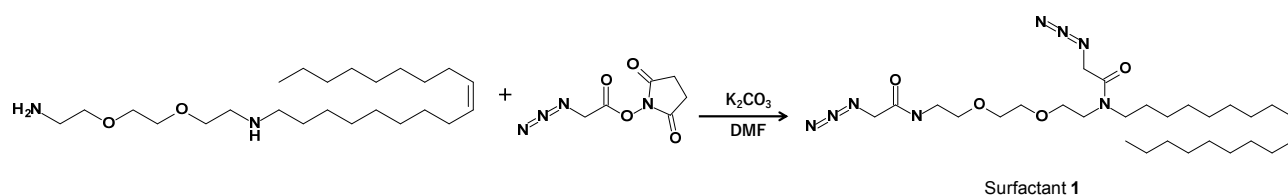
DART-MS, $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{28}\text{H}_{53}\text{N}_8\text{O}_4$, $m/z = 565.4$; found, 566.2.



Scheme S1. Synthesis of compound 1.



Scheme S2. Synthesis of the 2-azido acetic acid NHS ester.

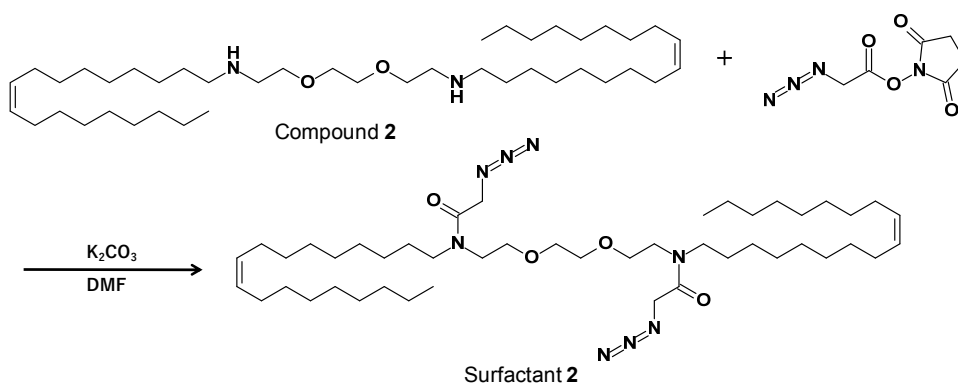


Scheme S3. Synthesis of surfactant 1.

Surfactant **2**:

Surfactant **2** was synthesized in a similar manner (Scheme S4). 2,2-(Ethylenedioxy)bis(ethylamine) (3 mmol, 445 mg) and potassium carbonate (6.6 mmol, 912 mg) were added in ethyl acetate (30 mL) in a three-neck flask under N₂. After the mixture was heated to 60 °C, ethyl acetate (30 mL) containing oleoyl chloride (6 mmol, 1.81 g) was added drop by drop to the mixture. The subsequent procedures were the same as those used to prepare surfactant **1**.

¹H NMR (500 MHz, CDCl₃): δ 0.88 (t, 6H, *J* = 6.70), 1.16–1.37 (m, 44H), 1.42–1.60 (m, 4H), 1.99–2.07 (m, 8H), 3.23 (t, 4H, *J* = 7.45 Hz), 3.37 (t, 4H, *J* = 5.75 Hz), 3.51–3.69 (m, 8H), 3.90–4.04 (m, 4H).



Scheme S4. Synthesis of surfactant **2**.

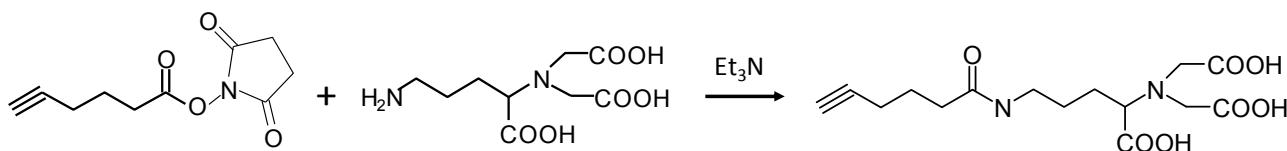
1.2. Synthesis of alkyne-ligands

Alkyne-NTA (Scheme S5):

NHS (17.2 mmol, 2.0 g) and WSC (17.2 mmol, 3.3 g) were added to dichloromethane (60 mL) containing 5-hexynoic acid (16.3 mmol, 1.83 g) and mixed at 25 °C for 15 h. The solution was

washed with 50 mL water three times and with 50 mL brine once. The dichloromethane phase was collected and dehydrated with MgSO_4 . The solvent was evaporated after filtration. The dry residue (138 mg), AB-NTA (0.55 mmol, 144 mg) and triethylamine (3 mmol) were dissolved in a mixture of DMF (6 mL) and water (2 mL). The solution was stirred under N_2 at 4 °C for 16 h. The solvent was evaporated after filtration. The dry residue was dissolved in 20 mL chloroform. The solution was washed with water (20 mL \times 2) and 0.1 M HCl aq. (20 mL \times 2). After dehydration using MgSO_4 overnight and filtration, the solvent was evaporated and the product (alkyne-NTA) was obtained.

^1H NMR (500 MHz, $\text{DMSO}-d_6$): δ 1.80 (q, 2H, $J = 7.69$ Hz), 2.28 (m, 2H), 2.76 (t, 2H, $J = 7.97$ Hz), 2.81 (t, 4H, $J = 8.24$ Hz), 2.85 (t, 1H, $J = 2.85$ Hz). MALDI-TOF-MS, $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{15}\text{H}_{22}\text{N}_2\text{O}_7$, $m/z = 358.3$; found, 358.9.



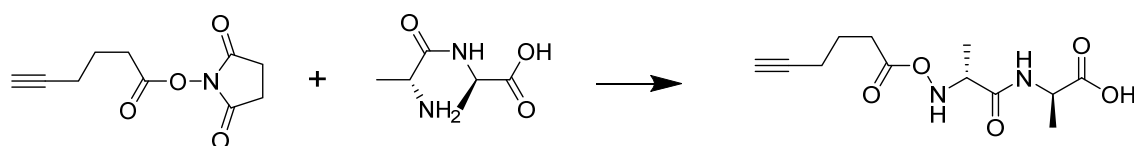
Scheme S5. Synthesis of alkyne-NTA.

Alkyne-D-Ala-D-Ala (Scheme S6):

NHS (1.3 mmol, 150 mg) and WSC (1.3 mmol, 249 mg) were added to 10 mL dichloromethane solution containing 5-hexynoic acid (1.3 mmol, 146 mg). The mixture was stirred at 25 °C overnight. The dichloromethane solution was washed with water (10 mL \times 3) and with brine (10 mL). The dichloromethane phase was collected and evaporated, followed by lyophilization

(5-hexynoic acid NHS ester). The residue obtained was added to a solution mixture (5 mL water and 15 mL acetonitrile) containing triethylamine (2 mmol, 202 mg) and D-Ala-D-Ala (1 mmol, 160 mg). The solution was stirred at 4 °C under N₂ overnight. The solution was evaporated and lyophilized (alkyne-D-Ala-D-Ala).

[M+H]⁺ calcd for C₁₂H₁₉N₂O₄, m/z=255.13; found, 255.04.



Scheme S6. Synthesis of alkyne- D-Ala-D-Ala.