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An enzymatic membrane reactor for oligodextran production: Effects of enzyme immobilization strategies on dextranase activity

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ABSTRACT

An enzymatic membrane reactor (EMR) with immobilized dextranase provides an excellent opportunity for tailoring the molecular weight (Mw) of oligodextran to significantly improve product quality. However, a highly efficient EMR for oligodextran production is still lacking and the effect of enzyme immobilization strategy on dextranase hydrolysis behavior has not been studied yet. In this work, a functional layer of polydopamine (PDA) or nanoparticles made of tannic acid (TA) and hydrolysable 3-amino-propyltriethoxysilane (APTES) was first coated on commercial membranes. Then cross-linked dextranase or non-cross-linked dextranase was loaded onto the modified membranes using incubation mode or fouling-induced mode. The fouling-induced mode was a promising enzyme immobilization strategy on the membrane surface due to its higher enzyme loading and activity. Moreover, unlike the non-cross-linked dextranase that exhibited a normal *endo*-hydrolysis pattern, we surprisingly found that the cross-linked dextranase loaded on the PDA modified surface exerted an *exo*-hydrolysis pattern, possibly due to mass transfer limitations. Such alteration of hydrolysis pattern has rarely been reported before. Based on the hydrolysis behavior of the immobilized dextranase in different EMRs, we propose potential applications for the oligodextran products. This study presents a unique perspective on the relation between the enzyme immobilization process and the immobilized enzyme hydrolysis behavior, and thus opens up a variety of possibilities for the design of a high-performance EMR.

1. Introduction

The enzymatic membrane reactor (EMR) is nowadays regarded as a green platform that enables the integration of bioconversion and membrane separation (Giorno et al., 2014; Giorno & Drioli, 2000). The EMR approach, in which the enzymes function as efficient biocatalysts in concert with a membrane separator for simultaneous product purification, has been increasingly reported for its various applications in both upstream and downstream processes (Jochems et al., 2011; Luo et al., 2020). One of the most significant applications of the EMR is the production of oligosaccharides – low molecular weight (Mw) carbohydrates with the number of sugar monomers intermediate of simple sugars and polysaccharides – which have high commercial value due to their specific chemical structures and unique physicochemical properties (Zhao et al., 2021). With increasing demand for oligosaccharides on the global

market, the production of oligosaccharides not only requires environmentally friendly processes but also a smart technology for precise control of product Mw during fabrication. The EMR is no doubt one of the ideal options for meeting both demands.

Traditional production of oligosaccharides introduces a considerable amount of hazardous chemicals, which potentially cause immune risks in practical usage of the products (Liu et al., 2019; Su et al., 2020). To address the undesired issues in production, our previous study used dextranase to convert polydextran to oligodextran while a membrane simultaneously functioned as a selective sieve to obtain the intermediate Mw oligodextran products (Su et al., 2018). The abovementioned work provided a strategy to tailor the Mw of oligedextran and thereby increase the product quality. Moreover, to obtain maximum amount of the target oligodextran products, the enzymatic hydrolysis should occur near the membrane surface for immediate removal of the target

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oligodextran from the reaction system and to avoid over-degradation. By this approach, products with narrow Mw distribution could be obtained. Enzyme immobilization on the membrane therefore offers a promising opportunity for better control of the overall process near the membrane surface.

Membrane modification is commonly carried out to make the membrane susceptible to enzyme immobilization (Oing et al., 2019). Polydopamine (PDA), a neurotransmitter that easily forms a thin coating layer by self-polymerization in alkaline aqueous solution, is reported to serve as a functional layer that enables the conjunction of enzymes and exposed catechol and quinone groups of the PDA layer (Alfieri et al., 2018). Based on the above theory, Zhang et al. established a versatile PDA coated membrane platform onto which dextranase was covalently attached (Zhang et al., 2018). Besides providing functional groups for the stable attachment of enzymes, the PDA coating improves the hydrophilicity of the membrane substrate, which contributes to increase the water permeability (Fan et al., 2017). In An alternative approach, Wang et al. (2018) developed a hierarchical coating layer on a membrane surface based on the secondary reaction between tannic acid (TA) and hydrolysable 3-amino-propyltriethoxysilane (APTES). The hierarchical TA/APTES nanosphere layer, which is rich in guinone groups, provides a hydrophilic, functional surface to which enzymes can readily attach (Wang et al., 2019). Zhou et al. (2020) further investigated the effect of the TA/APTES ratio on the enzyme loading efficiency and found that the enzyme loading could be greatly increased via TA/APTES surface modification, notably due to the occurrence of abundant quinone groups on the surface as well as the vast increase in surface area following the formation of the TA/APTES nanospheres.

Following membrane modification, glutaraldehyde (GA) is often introduced to form covalent bonds between the enzymes and the coating layer (Sigurdardóttir et al., 2018). The high activity between aldehyde groups on the coating layer and amine groups on the enzymes enables a high enzyme loading efficiency (Barbosa et al., 2014). Moreover, the GA molecules can easily react with the amino groups on different enzymes to form cross-linked enzymes aggregates (CLEAs). CLEAs are reported to maintain high enzyme stability and have therefore attracted considerable attention in commercial applications (Sheldon, 2007). Enzyme loading efficiency is also affected by the mode of immobilization. Incubating the modified membrane in enzyme solution is the most common immobilization strategy but in incubation mode, enzyme loading efficiency is often hampered by mass transfer limitations (Rana & Matsuura, 2010). Thus, the driving force of enzymes moving towards the modified membrane surface needs to be enhanced to improve the enzyme loading efficiency. A fouling-induced method, inspired by the mechanism of membrane fouling, has been proposed as a promising strategy to enhance enzyme concentration near the membrane surface (Luo et al., 2013; Morthensen et al., 2017).

The enzyme immobilization strategies described above provide various possibilities for the design of an EMR. In this study, we evaluated two membrane surface modification methods and two enzyme immobilization methods for the immobilization of dextranase on ultrafiltration (UF) membrane substrates. Thus, we coated the membrane substrates with either PDA or TA/APTES, followed by immobilization of dextranase via incubation or fouling-induced mode. Subsequently, we evaluated the respective strategies based on their performance in terms of production of oligodextran. Previous studies on dextranase immobilization have aimed at optimizing the hydrolysis rate of the enzymes (Bertrand et al., 2014; Shahid et al., 2019) but lack a discussion of tailoring the enzyme hydrolysis behavior to control the Mw of oligdextran. Therefore, besides focusing only on high enzyme loading and high enzyme activity retention upon immobilization, we also investigated the effects of the different immobilization strategies on the catalytic behavior of immobilized dextranase and compared the corresponding enzyme activity. Gel permeation chromatography (GPC) was used to analyze the components of the hydrolyzed oligodextran products in different EMRs, which illustrate the different hydrolysis

patterns of the immobilized dextranase. Based on the hydrolysis patterns of the immobilized dextranase, future applications of different enzyme immobilization strategies are proposed. Our work indicates multiple possibilities for the design of a high-performance EMR.

2. Materials and methods

2.1. Materials

Polyether sulfone (PES) membranes with molecular weight cut-off of 30 kDa were produced by EMD Millipore Corporation, USA. Dextran substrate (DXT70K) with Mw 70 kDa was provided by PharmaCosmos, Denmark. Tris (hydroxymethyl) aminomethane, dopamine hydrochloride, glutaraldehyde (GA, 25% v/v), tannic acid (TA), 3- aminopropyltriethoxysilane (APTES), dextranase (EC 3.2.1.11, dry powder from *Penicillium*. Sp.), Bradford reagent used for the protein assay and dextran benchmark with Mw 0.34, 5, 12, 25, 50 and 80 kDa were purchased from Sigma-Aldrich Co. Other chemicals were of analytic grade. Enzyme and substrate solutions were prepared in ultrapure water (generated from Millipore purification system).

Membrane modification with either dopamine or TA/APTES, enzyme immobilization and activity assay of immobilized enzymes were performed in a stirred cell (Amicon 8050, Millipore, USA) with an effective membrane surface area of 13.4 cm^2 .

2.2. Enzymatic membrane preparation by different immobilization strategies

2.2.1. Membrane modification

Dopamine or TA/APTES mixture was applied for surface modification of pristine commercial membranes. For dopamine modification, pristine membranes were incubated with 10 mL of 2 g/L or 4 g/L dopamine hydrochloride solution (pH 8.5, 10 mM Tris-HCl buffer) at 100 rpm and 25 $^{\circ}\mathrm{C}$ for different time-periods (1 h, 2 h or 4 h). Membrane modification by TA/APTES was carried out according to the work of Zhou et al. (2020): briefly, 2 g/L TA solution in Tris-HCl buffer (pH 8.5) was mixed with a 10 g/L APTES in EtOH solution at a volume ratio of TA/APTES = 8:1 to make 20 mL coating solution. Pristine membranes were then incubated in the TA/APTES coating solution at 100 rpm and room temperature (25 $^\circ\text{C})$ for 18 h. The TA/APTES modification introduced a layer of nanospheres on the membrane surface that is rich in quinone groups for enzyme immobilization by covalent bonding. After modification, the membranes were cleaned using running distilled water to remove the residual modifiers and then the modified membranes were installed into the Amicon cells for enzyme immobilization.

2.2.2. Enzyme immobilization

Enzyme immobilization on dopamine or TA/APTES modified membranes was carried out in incubation mode and fouling-induced mode. With dopamine modified membranes, 10 mL of 2 g/L dextranase solution (with 605–668 μ g soluble proteins) containing 1% (v/v) GA was placed in contact with the membrane surface in the Amicon cell. In the incubation mode, the enzyme solution was incubated with the membrane for 2.5 h at 100 rpm, after which the enzyme solution was recovered from the Amicon cell and stored for protein concentration measurements by Bradford assay. In the fouling-induced mode, the enzyme solution was filtered at 0.2 bar until all the solution was permeated from the cell. The permeate was collected for protein concentration measurements.

With the TA/APTES modified membranes, the enzyme immobilization occurred through covalent bonding between amino groups on the enzymes and the quinone groups on the coating layer, which formed via Michael addition and Schiff's base reaction. In the incubation mode, the enzyme solution (10 mL of 2 g/L dextranase) was added to the Amicon cell and the membrane was incubated for 2.5 h at 100 rpm. The enzyme



Fig. 1. (A) Dextranase distribution (in terms of protein amount) on membranes under different enzyme immobilization modes; (B) Schematic illustration of enzyme immobilization mechanism in the different modes; SEM images of (C) PDA modified PES 30 membrane; (D) PDA modified PES 30 membrane with GA-cross linked dextranase.

solution was recovered from the cell after the immobilization for protein concentration measurements. In the fouling-induced mode, 10 mL of 2 g/L dextranase solution was filtered at 4 bar and 500 rpm until all the solution was permeated from the cell. The permeate was collected for protein concentration measurements. After enzyme immobilization, each membrane was washed three times with 5 mL of pure water.

where c is the soluble protein concentration and V is the volume of the solution at the corresponding concentration. Subscripts *i*, *r*, *p* and *w* represent initial, recovered, permeate and washing solutions, respectively. The enzyme loading is defined as:

Enzyme loading(%) =
$$\frac{mass \ of \ immobilized \ dextranase}{mass \ of \ soluable \ dextranase} \times 100\%$$
Immobilizationefficiency(%) = $\frac{mass \ of \ immobilized \ dextranase}{mass \ of \ soluable \ dextranase} \times 100\%$

Enzyme immobilization experiments performed by the four independent methods were conducted in duplicates.

2.2.3. Enzyme loading determination

The protein concentration of the enzyme solutions was measured by the modified Bradford assay according to (Jankowska et al., 2021) 0–16 μ g/mL of bovine serum albumin (BSA) solutions were used for the calibration. Samples were diluted to be within the range of the protein calibration curve, as required. The enzyme solutions were mixed with Bradford reagent in a 1:1 volumetric ratio. After 5 min of incubation, absorbance was measured at 595 nm. Enzyme loading mass was calculated from the equation:

mass of immobilized dextranase =
$$c_i \times V_i - c_r \times V_r - c_p \times V_p - c_w \times V_w$$

2.3. Enzyme activity determination

2.3.1. Activity of immobilized and free enzymes

To measure the observed activity of the immobilized enzymes, 20 mL 4 g/L DXT70K solution was added to a 50 mL Amicon stirred cell (Amicon UFSC05001, Merck Millipore, USA) with the enzymatic membrane at room temperature and 100 rpm. Samples were collected at specified time intervals. To measure the activity of free enzymes, 1 mL of 2 g/L dextranase solution (or dextranase solution with 1% v/v GA) was introduced into 20 mL 4 g/L DXT70K solution for 90 min. Samples were collected every 5 min, then incubated in a boiling water bath to fully stop the reaction at specified time points. The reducing sugar content of



Fig. 2. (A) Enzyme activity; (B) dextran Mw variation and GPC chromatograms of dextran in a PDA modified EMR obtained under (C) incubation mode and (D) fouling-induced mode in an EMR designed using different immobilization modes.

all the collected samples was measured by using 3, 5-dinitrosalicylic (DNS) acid reagent, according to the method modified by Zhang et al. (2018). Specifically, 1 mL hydrolyzed samples were mixed with 1 mL DNS reagent and heated in a boiling water bath for 5 min. The samples were diluted 5 times by ultrapure water and measured at 540 nm. Immobilization yield, efficiency and activity recovery were calculated from the following equations (Sheldon & van Pelt, 2013):

$$Yield(\%) = \frac{immobilized \ activity}{starting \ activity} \times 100\%$$

Efficiency (%) = $\frac{observed \ activity}{immobilized \ activity} \times 100\%$

Activity recovery(%) =
$$\frac{observed \ activity}{starting \ activity} \times 100\%$$

The immobilized activity was determined by measuring the total residual enzyme activity after immobilization and by subtracting this activity from the total starting activity. The enzyme activity was defined as the amount of isomaltose (measured in μ mol maltose) generated after 1 min at 25 °C, using μ mol-isomaltose/min units. The enzyme activity tests of starting solution, residual solution and the immobilized dextranase were tested at 25 °C in duplicates.

The average Mw of the above samples was later tested in a Thermo Scientific - GPC system.

2.3.2. Enzyme kinetic parameter measurement

The Michaelis-Menten kinetic parameters K_m and V_{max} of enzymes

were determined by measuring the initial rates of the catalytic reactions using different substrates. 1.75 mg of dextranase dry powder (equivalent to around 32 μ g soluble protein) was mixed with 20 mL DXT70K substrate at various concentrations (namely 0.15625%, 0.3125%, 1.25%, 2.5%, 5%, 10%, 20%, 40%, w/v) for 3 min. To determine the kinetic parameters of GA-cross linked enzymes, 1% (v/v) of GA solution was introduced into the same reaction systems. Reducing sugars were then measured after the reaction to calculate the reaction rate. The experiments were conducted in triplicate. The values of the kinetic parameters were obtained by nonlinear curve fitting of the plot of reaction rate versus substrate concentration based on the Hanes–Woolf equation. The enzyme kinetic parameters were obtained from triplicate experiments.

2.4. Characterization of oligodextran products and membrane

2.4.1. Determination of oligodextran Mw

GPC was used to test the average Mw of oligodextran generated in the different reaction systems. 50 μL of each sample was eluted under 1 mL/ min in ultrapure water at 40 °C. A refractive index detector coupled with the G4000PWXL column from Shimadzu was used for testing the samples.

2.4.2. Membrane surface morphology

Scanning electron microscopy (SEM) was used to visualize the morphology of PDA modified PES membranes with immobilized enzymes. Here, samples with gold coating (Balzers PV205P, Switzerland) were investigated using an EVO40 microscope (Zeiss, Germany).

Table 1

Enzvi	ne immobilization effi	iciency, activit	v recoverv and	immobilization v	vield of the cross-linked	dextranase on the PDA	modified membrane.
2					J		

Immobilization mode	Parameter	Unit	Starting solution	Residual solution	On membrane
Incubation mode	Total enzyme activity ^a Observed enzyme activity Yield Efficiency Activity recovery	µmol-isomaltose/min µmol-isomaltose/min % %	11.56 ± 0.1 -	6.44 ± 0.0 -	$\begin{array}{c} 5.14 \pm 0.1 \\ 0.11 \pm 0.0 \\ 44.5 \pm 0.9 \\ 2.1 \pm 0.2 \\ 0.9 \pm 0.1 \end{array}$
Fouling-induced mode	Total enzyme activity ^a Observed enzyme activity Yield Efficiency Activity recovery	ymol-isomaltose/min µmol-isomaltose/min % % %	12.37 ± 0.0 -	3.07 ± 0.1 -	$\begin{array}{l} 0.9\pm0.1\\ 9.30\pm0.1\\ 0.62\pm0.2\\ 75.2\pm0.8\\ 6.7\pm2.1\\ 5.0\pm1.6\end{array}$

^a The total enzyme activity on membrane is calculated by total enzyme activity in starting solution subtract total enzyme activity in residual solution after immobilization; the observed enzyme activity on membrane was measured by terminology mentioned in Section 2.3.1.

3. Results and discussion

3.1. Enzyme immobilization on PDA modified membrane surface

3.1.1. Effect of enzyme immobilization mode on enzyme loading

Firstly, the effects of PDA coating parameters on enzyme loading were investigated (Table S1), and it was found that neither increased PDA concentration nor coating time significantly improved enzyme loading in incubation mode. A possible explanation is that the PDA layer might tend to form a brush-like surface that prevents the attachment of enzymes (Gao et al., 2011; Cai et al., 2012).

To improve enzyme loading efficiency on the membranes, we investigated methods to overcome the repulsion between the enzymes and the membrane coating layer. More enzyme-membrane contact could be achieved either by increasing the initial enzyme concentration or by applying pressure above the membrane. The latter strategy is known as fouling-induced enzyme immobilization. This method uses pressure to increase the enzyme concentration near the membrane surface (i.e. concentration polarization) (Luo et al., 2014). In the following study two different enzyme loading modes – incubation mode and fouling-induced mode – were compared.

The fouling-induced mode was applied to increase the enzyme loading efficiency on the PDA coated membrane surface. Fig. 1A illustrates the enzyme distribution on membranes prepared using two different immobilization modes. 49% (326.7 µg) of dextranase (in terms of protein mass) was found on the membrane surface when the foulinginduced immobilization mode was applied, whereas only 16% (107.8 µg) dextranase was loaded on the membrane surface in incubation mode. The proposed mechanisms are shown in Fig. 1B where GA forms covalent bonds between the enzymes and the PDA layer and simultaneously functions as an enzyme cross-linker to form CLEAs. In Fig. 1C and D. The CLEAs measured over 1000 nm in size, while the PDA particles (bright circles) had a diameter around 50 nm, which is similar to results reported by (Li et al., 2014). The coating layer weakened the total interaction (a sum of acid-base (AB), Lifshitz-van der Waals forces (LW) and electrostatic double layer interactions) between the enzyme aggregates and the modified membrane (Cai et al., 2017), which could result in most of the dextranase (81%) remaining in the solution after 2.5 h incubation. In the fouling-induced mode, however, the enzymes together with GA were filtrated towards the membrane surface by convective transport when the solvent passed through the membrane. From the perspective of adhesion energy, the strong driving force due to the filtration might overcome the static repulsion between the rough coating layer and the CLEAs. Under these circumstances the enzymes would not diffuse back to the bulk solution, but would instead contribute to an increase in local concentration at the membrane surface, with more efficient covalent bonding between enzyme and the membrane as the result. Consequently, a higher enzyme loading would be obtained on the membrane surface in fouling-induced mode than in incubation mode.

The result indicates that the applied pressure provides a driving force that overcomes the steric hindrance between enzyme clusters and the PDA coating, resulting in a higher enzyme loading efficiency.

The enzyme activity of the catalytic membranes was evaluated for 1260 min (21*h*) to observe the degradation efficiency of the immobilized dextranase (Fig. 2). With a higher enzyme loading, the enzymatic membrane in fouling-induced mode showed increasing activity within the first 120 min. Over the same reaction period (Fig. 2B), a rapid decline of dextran Mw was observed. By contrast, the enzyme activity in the incubation mode was low, and consequently, the accumulation of reducing sugars within the first 60 min was slow. Therefore, the observed peaks of isomaltose were not as obvious compared with the bulk dextran substrate (Fig. S1). In incubation mode, in accordance with the low activity, the decrease of dextran Mw was slow. Regarding the enzyme hydrolysis efficiency, the dextranase immobilized in fouling-induced mode outperformed those immobilized in incubation mode and led to a faster degradation of large dextran molecules.

Interestingly, when investigating the composition of the hydrolyzed oligodextran products in detail (Fig. 2C and D), the immobilized enzymes introduced by the different modes were found to have different hydrolyzing patterns. The dextranase (from Penicillium sp.) used in this study is reported to be an endo-glycosidic enzyme that randomly attacks the α -1,6 glycosidic bonds within large dextran molecules and releases shorter oligodextran until the hydrolyzed products become dimers. By contrast, exo-glycosidic enzymes degrade the dextran chains from the terminal side of the molecule to release end-products such as dimers or monomers (Khalikova et al., 2005). The GPC chromatograms in our study show that dextranase immobilized by incubation mode tended to produce end-products (single units of isomaltose) during the reaction and that the bulk of the large dextran molecules remained unattacked at the beginning. This finding indicates that the dextranase immobilized in incubation mode performed exo-hydrolysis so that products with a very broad Mw distribution were produced. By contrast, there was an overall Mw decline of the bulk dextran molecules on the membrane with fouling-induced enzymes while accumulation of end-products occurred during the hydrolysis. The results suggest that part of the foulinginduced dextranase on the membrane surface maintained the endohydrolysis pattern. Such a shift in hydrolysis performance of the immobilized dextranase has rarely been reported.

Immobilization efficiency, activity recovery, and immobilization yield are indicated in Table 1. The fouling-induced mode yielded a significantly higher immobilization yield (75.2%), efficiency (6.7%) and activity recovery (5.0%) compared to the corresponding values of the incubation mode (44.5%, 2.1% and 0.9%, respectively). Shahid et al. (2019) reported similar immobilization yield (34%–78%) of dextranase immobilized on an alginate matrix. The low activity recovery is due to a relatively large enzyme amount at the starting solution (605–668 μ g soluble proteins) and to the limited membrane surface that did not allow more enzymes to be immobilized. Secondly, the dextran macromolecules cannot easily penetrate the CLEAs, which leads to an activity



Fig. 3. (A)Enzyme activity and (B)dextran Mw variation in a TA/APTES modified EMR obtained under different immobilization modes; GPC chromatograms of dextran in TA/APTES modified EMR based on (C) incubation mode, (D) fouling-induced mode.

decline of the immobilized enzymes (Sheldon et al., 2021). Though only 5% of the initial enzyme activity was recovered in our work, the immobilized enzymes gradually catalyzed the dextran substrates into oligodextran products (Fig. 2B). The slower reaction enabled Mw tailoring during production, which offers a promising application for the EMR.

The fouling-induced mode exhibited higher enzyme immobilization efficiency and activity, which potentially could be applied at a larger scale to increase oligosaccharides productivity. However, during the 21 h enzyme activity test, around 10% of the immobilized enzymes in fouling-induced mode (30 μ g) leaked from the membrane surface, whereas no enzyme leakage was detected in the incubation mode. With incubation mode, in this regard, most enzymes were firmly immobilized via covalent bonding, which is beneficial for long-term usage due to reduced loss of enzyme to the surrounding environment.

3.2. Enzyme immobilization on TA/APTES modified membrane surfaces

Besides PDA, we applied TA/APTES surface modification for the immobilization of dextranase. Here, GA was not mixed with the dextranase because the reported results (in supplemental information Section B.2.4) suggested that non-cross-linked dextranase might perform better in hydrolysis. Applying similar procedures as described earlier, dextranase was immobilized using incubation and fouling-induced modes, respectively, on the TA/APTES coated membranes. The observed higher enzyme activity (Fig. 3) suggests a higher enzyme loading on the TA/APTES modified membrane surface in comparison to the PDA modified membrane surface. Moreover, according to a report

from Wang, Wang, et al. (2019), the TA/APTES nanoparticles have an average diameter of around 200 nm. Obviously, the spherical nanoparticles formed by TA/APTES are larger than the PDA particles (<50 nm) and form a larger asperity radius on the coating layer. The reported molecular size of dextranase from Penicillium sp. is around 67 kD (Larsson et al., 2003). Based on this molecular size, the diameter of a single and isolated enzyme molecule is estimated to be around 3.3 nm according to the protein size approximation from Erickson (2009) which assumes the enzyme has a spherical shape. Due to the small size of noncross-linked enzymes and relatively larger size of the TA/APTES particles, the TA/APTES coating layer is expected to provide a larger surface area on the membrane available to dextranase to anchor on. In the perspective of thermodynamics, the interaction energy between proteins and the membrane surface increases with a larger asperity radius of the membrane (Zhao et al., 2015). That is, enzyme adhesion on a larger particle (TA/APTES) surface should have a higher entropy (Li et al., 2019).

Regarding enzyme immobilization modes, as expected, the enzymes introduced by fouling-induced mode showed a relatively high enzyme activity (ca. 0.7 μ mol-isomaltose/min) particularly within the first 60 min of hydrolysis reaction. Correspondingly, the immobilized enzymes efficiently converted long chain dextran substrates into smaller units resulting in a rapid Mw decrease of dextran at the beginning of reaction (Fig. 3B). In the incubation mode, much lower enzyme activity was observed, leading to a slower dextran Mw decline. In a similar manner to the explanation discussed in Section 3.1, strong convection in the fouling-induced mode brought more enzymes towards the membrane surface, which enabled a higher enzyme immobilization efficiency and



Fig. 5. Specific activity of non-cross-linked and GA-cross-linked dextranase.

Fig. 4. Decreasing rate of dextran Mw in EMRs based on different immobilization strategies.

Table 2				
Kinetic parameters	in free	e and	cross-linked	dextranase.

Conditions	V _{max}	Km	
	(µmol-isomaltose/min/ mg)	(µM)	
Non-cross-linked dextranase (free dextranase)	$\textbf{292.1} \pm \textbf{13.9}$	$\begin{array}{c} \textbf{36.4} \pm \\ \textbf{1.9} \end{array}$	
GA Cross-linked dextranase (1% GA)	201.6 ± 16.3	35.6 ± 4.4	

higher activity.

Though the enzymes in the incubation mode showed a slower overall Mw decline, not much accumulated end-products (separated peak of isomaltose) were observed from the GPC chromatograms. The results indicate that the non-cross-linked enzymes attached to the TA/ATPES surface exerted the desired *endo*-hydrolysis when producing the oligo-dextran. When looking into the product composition generated by the fouling-induced enzymes, a sharp decline of the overall Mw of dextran substrates was observed at the beginning of the reaction (Fig. 3B), however without an immediate accumulation of end-products (i.e. isomaltose, Fig. 3D). The rate of end-product accumulation indicated that the appearance of end-products was due to an efficient degradation of dextran instead of due to *exo*-hydrolysis. With larger capacity of immobilized enzymes, the dextran substrate (70 kDa) was efficiently hydrolyzed into small units (around 8.9 kDa) even within the first 15 min reaction.

Additionally, enzyme activity loss was observed on the EMR after reusing the biocatalytic membranes in several reaction cycles (Fig. S3). Though covalent bonding is targeted, most of the enzymes loaded using fouling-induced mode are immobilized by adsorption due to the strong convection. The extent of leakage of immobilized enzymes from the support during the repeated cycles is similar to the one reported by da Silva et al. (2019).

3.3. Comparison of enzyme immobilization strategies

The previous sections indicate that EMRs based on different strategies exert different degradation behaviors (Fig. 4). Regardless of the modifiers, EMRs based on fouling-induced mode exhibited a significantly higher dextran Mw decreasing rate at the beginning of hydrolysis. On the other hand, TA/APTES coated membranes seemed to retain higher enzyme activity. Based on the analysis above, the TA/APTES layer provides a larger surface for enzymes to attach to and hence more dextranase is supposedly loaded onto its surface.

Another important factor affecting the activity of the immobilized enzyme is the CLEAs triggered by GA molecules (Migneault et al., 2004). The tight packing of the cross-linked dextranase might cause severe mass transfer issues when hydrolyzing the dextran substrates, and thus lower degradation rates (Verma et al., 2019). According to many studies of enzyme immobilization, enzyme activity decline due to aggregation is commonly observed (Nadar et al., 2016). Therefore, without GA molecules, the non-cross-linked dextranase on the TA/APTES should contribute to the high dextran Mw decrease rate. Enzyme kinetic studies may explain why the enzyme activity varies in different EMRs.

3.3.1. Enzyme kinetics

To better understand the hydrolysis efficiency of cross-linked and non-cross-linked dextranase, the effect of addition of GA cross-linkers on dextranase hydrolysis behavior was studied under room temperature (25 °C). In this experiment, the enzymes were not loaded onto a membrane but were directly mixed with the substrates. Kinetic parameters are given in Table 2. Higher V_{max} was observed in the reaction without GA cross-linked dextranase. The reason could be due to the location of the active center in the middle of the enzyme molecules (Zhang et al., 2018), which makes a part of the active sites inaccessible to the substrates upon cross-linking, thereby yielding a lower reaction velocity. Similar K_m values indicate similar affinity between substrates and the non-cross-linked dextranase and GA-cross-linked dextranase. Similar V_{max} and K_m change of the immobilized dextranase are found in research from El-Tanash et al. (2011). However, the Michaelis-Menten parameters only describe the reaction velocity of the enzyme at the beginning of the hydrolysis reaction (Ivanauskas et al., 2016; Johnson & Goody, 2011). The hydrolysis behavior over an extended period should also be investigated because, in real applications, the enzymes are generally expected to perform the hydrolysis during an extended run time.

Fig. 5 shows the specific enzyme activity of the GA-cross-linked and non-cross-linked dextranase during 90 min hydrolysis. The non-crosslinked dextranase gave very high specific enzyme activity in the beginning of the reaction, while the cross-linked dextranase showed lower and more steady specific enzyme activity. Thus the dextran substrates were almost fully degraded by the non-cross-linked dextranase within the first 5 min of reaction and the cross-linked dextranase resulted in a gradual Mw decline of the substrates (Fig. S6). When examined at the nanoscale, it has been reported that the structure of dextranase must change and form a tunnel-like space accommodating





Fig. 6. Illustration of different substrate mass transfer mechanisms on EMRs.

both water molecules and dextran substrate so that the necessary nucleophilic attack can occur to cleave the α-1,6 glycosidic bonds within the large dextran molecules. (Larsson et al., 2003). The aggregated dextranase might, however, be limited by steric hindrance so that it takes longer for this aggregated dextranase to change its structures for the degradation of dextran substrates. Hence, intermediate Mw oligodextran was observed during the reaction. The delayed hydrolysis behavior may actually offer a possibility for better tailoring the production of oligodextran because these intermediate sized products are desired for their particular bioactive functions (da Silva et al., 2019; Rastall, 2010). In an EMR that integrates both bioreaction and separation processes, slower and controllable degradation of substrate Mw that matches the removal rate of the target molecules would help in improving the quality of the products (Su et al., 2018). On the other hand, the non-aggregated dextranase exerts high activity, so they are usually used to tackle substrates at higher concentrations and scales (Su et al., 2020).

Regarding mass transfer, the scenarios of large dextran molecules accessing the enzyme active sites on the membrane surface could vary with different EMRs that lead to exo- or endo-hydrolysis (Fig. 6). Fixed on the membrane surface, the non-cross-linked dextranase should have more exposed active sites facing the bulk solution, whereas many active sites of the aggregated dextranase are expected to be shielded. The dextran substrate in this study has an average Mw of around 70 kDa, and a hydrodynamic radii of around 9 nm according to the Stokes-Einstein relationship (Ioan et al., 2001). With the fixed enzymes on the membrane surface, the hydrolysis is assumed to be dominated by the Brownian movement of dextran substrates (Blanco et al., 2017). On the TA/ APTES coated surface, there should be larger spaces between the noncross linked dextranase that allows random Brownian movement of the dextran molecules. More effective collisions between the substrates and exposed active sites would therefore have occurred during the hydrolysis, resulting in an efficient attachment of substrates and quick detachment of products. Due to less steric hindrance between the

enzymes and substrates, the immobilized dextranase could efficiently hydrolyze the dextran molecules (Frazier et al., 1997) exerting endohydrolysis that randomly attack the glycosidic bonds within the substrate molecules (Khalikova et al., 2005). However, the scenario around the CLEAs above the PDA coating layer might be different. The crosslinking led to high enzyme concentration in the CLEAs, and static compaction occurred among the enzymes, so there might be very limited space for whole dextran substrate to diffuse into the active sites for endohydrolysis (El-Tanash et al., 2011; Wang, Wang, et al., 2019). Instead, the limited space might only allow the terminal side of the large molecules to penetrate the CLEAs, leading to an exo-hydrolysis. Additionally, due to the larger size of CLEAs that have more binding sites for the substrates, once a large dextran molecule was attached on the CLEAs, it could hardly diffuse back to the bulk solution (Erhardt & Jördening, 2007), so the exposed active sites would continuously cleave the molecules until the smallest units, leading to the accumulation of endproducts during the intermediate process. Exo-hydrolysis thus has potential for the fabrication of low Mw oligosaccharides such as isomaltose (Zhou et al., 2019). The above analysis provides new insights into the working pattern of immobilized dextranase. With regard to specific products, the current study offers various selections of enzyme immobilization strategies.

3.3.2. Filtration performance of the EMR

We also evaluated the filtration performance of the membranes in terms of water permeability (Table S4). Both membrane modification and enzyme immobilization introduced extra filtration resistance to the membranes, which limits their separation performance in real processes. Therefore, the functional modification in combination with fouling-induced enzyme immobilization is proposed for application on a porous matrix, such as electrospun nanofibers (Jankowska, Zdarta, et al., 2021). The enzymatic matrix could then be coupled with a membrane for product separation. Due to the loose structure of the matrix, the enzymatic layer would not introduce much filtration

Table 3

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Summary	OT 6	enzyme	1mmobilization	strategies	- modifiers	enzyme	1mmobilization	mode	enzyme	aggregation
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Factors		Advantages	Disadvantages	Potential applications				
Modifier	PDA	Less severe membrane fouling	lower binding affinity to enzymes	Fabrication of EMR for simultaneous reaction and separation				
	TA/APTES	Higher binding affinity to enzymes	Severe membrane fouling	Fabrication of cascade EMR: TA/APTES coating on loose matrix materials for enzyme immobilization coupled with a membrane separator				
Enzyme immobilization	Incubation	Less enzyme leaking	Low enzyme loading efficiency	Long-term production				
mode	Fouling- induced	High enzyme loading efficiency	Severe membrane fouling Leakage of enzymes	Upscaling - handling substrates with higher concentration that increase total productivity				
Enzyme aggregation	Yes	Relatively constant reaction rate	Lower enzyme activity	Tailoring the Mw of oligosaccharides to improve product quality; production of low Mw saccharides (i.e. isomaltose)				
	No	High enzyme activity	Hard to control the reaction	Upscaling - handling substrates at a higher concentration that increase total productivity				

resistance above the membrane, and would therefore allow simultaneous enzyme reaction and products separation.

4. Conclusion

The current study evaluated different strategies of enzyme immobilization, each of which displayed different enzyme loading efficiency and activity. More interestingly, the type of catalytic activity of the immobilized enzymes was affected by the immobilization strategies.

The membranes coated by PDA or TA/APTES nanoparticles exhibited different surface morphologies and therefore different binding affinity to the enzymes. The 'fouling-induced' enzyme immobilization mode resulted in a higher enzyme activity, which therefore was adopted for a high-performance EMR. Furthermore, the enzyme kinetics of aggregated dextranase and non-aggregated dextranase was tested. Due to the GA cross-linker, the aggregated dextranase performed *exo*-hydrolysis on the membrane surface due to mass transfer limitations within the aggregated enzyme clusters. The filtration performance of the EMRs was compared to identify future applications of the EMRs. The above three factors – modifier, enzyme immobilization mode and enzyme aggregation - are summarized in Table 3.

This work focused on the effects of enzyme immobilization strategies on dextranase hydrolysis behavior, and presents an in-depth discussion on the corresponding mechanisms. The results suggest various possibilities for the design of a high-performance EMR for the production of oligosaccharides.

CRediT authorship contribution statement

Ziran Su: Conceptualization, Investigation, Methodology, Data curation, Writing – original draft, Writing – review & editing. Jianquan Luo: Conceptualization, Supervision, Writing – review & editing. Sigyn Björk Sigurdardóttir: Conceptualization, Methodology, Data curation, Writing – review & editing. Thomas Manferrari: Investigation, Methodology, Data curation. Katarzyna Jankowska: Investigation, Methodology, Data curation. Manuel Pinelo: Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carbpol.2021.118430.

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