**Fluorescence-assisted real-time study of magnetically immobilized enzyme stability in a
crossflow membrane bioreactor**

A. Y. Gebreyohannes^d, T. Geens^a, A. Kubarev^a, M. Roeffaers^a, W. Naessens^c, T. Swusten^b, T.

Verbiest^b, I. Nopens^c, Suzana P. Nunes^d, I. F.J. Vankelecom^a

^a Membrane Technology Group, Division cMACS, Faculty of Bioscience Engineering, KU Leuven, Celestijnenlaan 200F, PO Box 2454, 3001 Leuven, Belgium

^bMolecular Imaging and Photonics, Department of chemistry, KU Leuven, Celestijnenlaan 200D, box 2425, 3001 Leuven, Belgium

^cBIOMATH Department of Data analysis and mathematical modelling, Ghent University, Coupure Links 653, 9000 Gent, Belgium

^dKing Abdullah University of Science and Technology (KAUST), Biological and Environmental Science and Engineering Division (BESE), Advanced Membranes and Porous Materials Center (AMPM), 23955-6900 Thuwal, Saudi Arabia

(*Corresponding author) E-mail: ivo.vankelecom@kuleuven.be
Tel: +32-16-321594

Abstract

The detailed structures and distribution of enzymatically active magnetic-responsive dynamic layers (Enz^{SP}) were investigated for the first time in a crossflow superparamagnetic biocatalytic membrane reactor (XF-BMR^{SP}). The trade-off between a higher mass transfer rate, lower fouling tendency, and preventing washout of the dynamic layer, highly depends on the balance of the various forces that act on the Enz^{SP}. The real-time visual inspection of the system was realized through the design and fabrication of an adapted crossflow system, guided by computational fluid dynamics (CFD) simulations. Time-resolved images of the dynamic layer under a broad range of operational conditions was obtained using fluorescence microscopy.

The deposition, dispersion and stability of the dynamic layer was mainly governed by the external magnetic force. The shear force did not cause significant particle washout when a buffer solution was recirculated without permeation even under a turbulent flow regime (6.4 cm/s $\sim$ Re =5200). The removal of the external magnetic force after initial magnetic immobilization of the Enz^{SP}, or the substitution of a smooth flow velocity by the propagation of an impulse flow, significantly affected the stability of the dynamic layer.

Although membrane fouling occurs at the membrane-solution interface, where a laminar flow regime prevails, most membrane fouling models are based on turbulent flow. Therefore, the detailed, time-resolved images obtained here can provide solid foundation for the development of theoretical models that can describe the membrane fouling under the more representative laminar flow regime.

List of abbreviation

APTMS	3-Aminopropyltrimethoxysilane
BCA	Bicinchoninic Acid
BMR ^{SP}	Superparamagnetic biocatalytic membrane reactor
CFD	Computational fluid dynamics
CMC	Carboxymethyl cellulose
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
EMR	Enzyme membrane reactor
Enz ^{SP}	Enzyme functionalized superparamagnetic nanoparticles
Fluo ^{SP}	Fluorescence marked superparamagnetic nanoparticles
GA	Glutaraldehyde
M ^{SP}	Stimuli-responsive “smart” polymeric membrane
NP ^{SP}	Superparamagnetic nanoparticles
Pe	Peclet number
PEG	Polyethylene glycol
PVDF	Polyvinylidene fluoride
PZC	Point of zero charge
TMP	Transmembrane pressure
XF-BMR ^{SP}	Crossflow superparamagnetic biocatalytic membrane reactor

1. Introduction

A transition in demographic, food, energy and environmental sectors evokes the need for sustainable development in order to limit global warming and preserve our way of life. To limit the use of polluting fossil fuel resources, energy conversion from biomass is a topic gaining more importance on global scale [1]. Bioethanol has proven to be effective as a fuel or as an additive in gasoline [2]. Lignocellulosic biomass, which accounts for up to 50% of the biomass dry weight, is a very attractive source of bioethanol, since it does not compete with food and feed production in land and water use.

Biomass is saccharified from cellulose to glucose through hydrothermal, chemical, physico-chemical and biological techniques. Most of these methods are not yet implemented on large scale [1]. The improvement of chemical and biocatalytic approaches is vital for the evolution from petrochemical-based to bio-based chemicals [3]. Enzymatic/biocatalytic saccharification is more beneficial since it has low operational cost, mild operation conditions (pH 4.8, 45-50°C) with limited undesired product formation. However, challenges like switching from batch to continuous processes, and handling real feed stocks have to be taken into consideration [2].

The main problems in the application of enzymatic batch processes are (i) the inhibition of the reaction by the products themselves and (ii) the downstream separation [4]. Therefore, interest arises in high substrate loading feed-batch and continuous process operations in combination with immediate removal of the hydrolysis products [5]. This can be achieved by enzymatic membrane reactors (EMR) [6-8]. Lignocellulose valorization using EMR comes with simplicity,

energy savings, reasonable solid loading rate and modularity [9]. However, the free enzyme has to be kept inside the EMR and must be retained by the membrane, while the products permeate through. This limits the possibility to use a high-flux membrane process, such as microfiltration. As an alternative, the enzyme can be covalently immobilized on the membrane surface [10, 11]. However, cellulose fouling is a serious drawback, requiring frequent membrane cleaning [12] and many enzyme immobilization strategies do not allow membrane cleaning without affecting the enzyme. Recently, an innovative reversible immobilization strategy, which can allow the removal of the enzyme to clean the membrane, was developed. This strategy enhanced the lifetime of both membrane and enzyme and lowered the operating costs [9, 13, 14].

This novel concept is based on the reversible magneto-responsive immobilization of enzymes through superparamagnetic nanoparticles (NP^{SP}) to form enzyme-immobilized NP^{SP} , referred as Enz^{SP} . The Enz^{SP} s are subsequently immobilized on the surface of a magnetizable membrane using an external magnetic force. The concept is also referred to as a superparamagnetic biocatalytic membrane reactor (BMR^{SP}). However, such a system was until now only investigated in a dead-end set-up.

Compared to dead-end systems, crossflow systems have lower fouling propensity, which makes them industrially more relevant. A high crossflow velocity results in higher tangential shear stresses over the membrane surface, thereby making foulant deposition less detrimental with less occurrence of irreversible fouling. On the other hand, high crossflow rates impart high pumping costs [15].

The main intention of adding a dynamic layer of Enz^{SP} is also to limit the extent of membrane fouling. Hence, the BMR^{SP} concept in a crossflow set-up can be useful, since it would allow to operate a system at relatively low crossflow velocity. It is thus highly imperative to investigate to what extent the combination of these fouling mitigating methods is attractive. In addition, for BMR^{SP} , the residence time of the substrate is an important factor that determines the effectiveness of the reactor productivity. Higher crossflow rates will result in lower contact times between the enzyme and the substrate, although the mass transfer is enhanced. A reasonably low crossflow rate is therefore preferential to reach high degrees of conversion. Yet, a crossflow BMR^{SP} system creates the risk of Enz^{SP} wash-out. The trade-off between a higher mass transfer rate, lower fouling tendency and preventing Enz^{SP} wash-out, highly depends on the balance of the various forces that act on the Enz^{SP} . This problem is an important research gap between BMR^{SP} in dead-end and in crossflow configuration. Indeed, it is challenging to compose a force balance on a superparamagnetic nanoparticle layer. Full coverage of the membrane by Enz^{SP} is assumed to be made by packing multi-layers of Enz^{SP} . Therefore, the force balance in a crossflow BMR^{SP} should be described on top of the superparamagnetic layer.

The aim of this paper is to take the first steps towards the investigation of a BMR^{SP} in crossflow operation. Since the crossflow velocity might result in lifting and wash-out of the Enz^{SP} , it is imperative to keep a good balance between the various forces and the magnetic force, which is expected to be the main force that keeps the Enz^{SP} immobilized on the membrane. Therefore, this work involves the design of a crossflow module based on different design criteria, and operation under a broad spectrum of operational conditions. The real time dynamics of the Enz^{SP} layer was *in-situ* observed using fluorescence microscopy. The data from the *in-situ* microscopic

study was used to determine a qualitative force balance on the Enz^{SP}, which will eventually be used to design a first crossflow (XF)-BMR^{SP}.

2. Materials and Methods

2.1 Materials

Dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) were acquired from Arcos. Poly(vinylidene fluoride) (PVDF, 534 kDa). Carboxymethyl cellulose (CMC), glucose, cellobiose, cellotriose, glutaraldehyde (GA; 25 % v/v%) and sodium tetraborate were obtained from Sigma-Aldrich (Belgium); methoxy(polyethyleneoxy)-propyltrimethoxysilane (PEG Silane), (3-aminopropyl) trimethoxysilane (APTMS) from ABCR chemicals. Alexa FluorTM 488 and bicinchoninic acid (BCA) were from ThermoFisher Scientific. The enzyme cellulase (NS WG009), was kindly provided by Novozymes (Table 1).

Table 1: Properties of the cellulase.

Primary Activities	Endoglucanase, 1,4 Beta-glucosidase, Xylanase
Side Activities	Exoglucanase, Xylosidase
pH Range (Optimal)	4.5-6
Temperature range (Optimal)	35-60°C
Primary Mode of Action	Hydrolyzes 1,4 Beta-D-glucosidic linkages in cellulose

2.2 Superparamagnetic Nanoparticle Preparation

The superparamagnetic nanoparticles (NP^{SP}) are used both as a filler inside a mixed-matrix membrane to form the superparamagnetic polymeric membrane (M^{SP}) and as a carrier for the enzyme to form the superparamagnetic enzyme (Enz^{SP}) [9].

The NP^{SP} , which were used as a nanofiller in the membrane to give the membrane superparamagnetic properties, were coated with PEG. To immobilize enzymes on the NP^{SP} surface, reactive amine groups were introduced through silylation of the ferric oxide particles with APTMS. Detailed description of the particle preparation is given elsewhere [2, 13].

In order to provide a suitable microenvironment, both the model enzyme used here (cellulase) and the NP^{SP} were dissolved/dispersed in a 50 mM sodium acetate buffer, pH 4.5. To prepare 20 g/L NP^{SP} stock solution, a given mass of NP^{SP} was dispersed in the sodium acetate buffer and sonicated till a uniform dispersion was formed. Thanks to the APTMS coating, the NP^{SP} exhibited an excellent colloidal stability when dispersed in the sodium acetate buffer at a relatively high particle concentration (20 g/L). 120 μL of the dispersion was added to an empty vial. The buffer was removed with an external magnet. GA was used as a crosslinker between the primary amine on the NP^{SP} and a primary amine enzyme in a later step [9]. 1 mL of GA at 5, 10, 20 and 25 v/v% was added to the vial and mixed using a vortex. The closed vials were put on a custom-made mechanical rotator in which the mixture reacted for a minimum of 2 h. The GA activated NP^{SP} were washed 5 times with water to remove unreacted GA.

Subsequently, 2.4 mg of GA activated NP^{SP} were mixed with 2 mL of enzyme in a vial. The mixture was reacted overnight on a mechanical rotator at room temperature. After the incubation, the

Enz^{SP} was washed five times with the sodium acetate buffer to remove unreacted cellulase. The particles suspended in the same buffer were stored at 4°C until further use.

The cellulase loading on the NP^{SP} is determined by the bicinchoninic acid (BCA) analysis using a Pierce© BCA protein assay kit [2].

2.3 Fluorescence Labeling of NP^{SP}

Alexa FluorTM 488 is an amine reactive dye that will react with the amine group of NP^{SP} or Enz^{SP} to form a fluoresce-labelled NP^{SP}, named here as Fluo^{NP}. In order to fluorescence label the APTMS coated NP^{SP}, the standard protocol given by Thermofisher Scientific for aminated oligonucleotide fluorescent-marking was adapted. The reaction mixture contains 250 µg of the Alexa FluorTM dye, 14 µL DMSO, 75 µL sodium tetraborate buffer pH 8 and 160 µg of aminated NP^{SP} in an Eppendorf tube. The incubation was performed at room temperature in a centrifuge at a speed of 1000 rpm for 6 h. Since the particles tend to settle during the stirring, the settled particles were manually dispersed every 30 min during the first 3 h. The Fluo^{NP} were stored at -18°C and protected from light.

2.4 Membrane and Module Preparation

2.4.1. Mixed Matrix Membrane Preparation

The superparamagnetic polymeric membrane (M^{SP}) was prepared by phase inversion [16] as detailed in [9] using 12 wt% PVDF dissolved in DMF containing 0.33 wt% PEG coated NP^{SP}.

2.4.2. Membrane filtration set-up

The module was designed and custom-made based on computational fluid dynamic (CFD) model-based simulations. During this optimization, three criteria were pursued: (i) a Reynolds number well below the laminar limit, (ii) the absence of dead zones and (iii) of recirculation streams. Simulations were performed using the simpleFOAM and pimpleFOAM solvers of OpenFOAM (v2.2.2, OpenFOAM foundation). The objective of this crossflow membrane module is the *in-situ* observation of NP^{SP}/Enz^{SP} using a fluorescence microscope (Olympus BX 51), while performing filtrations. This objective results in many different design criteria:

i. Transparency: a transparent module makes possible for the light to reach, illuminate or excite the sample. Thus, polymethylmethacrylate (PMMA) was the material chosen.

ii. Size of the module: high-magnification, high-resolution can be frequently better reached at shorter distance. Therefore, in order to study the surface of the membranes with the best possible magnification and resolution, the PMMA layer and the height of the flow cell have to be as thin as possible so that the distance between the objective and the sample is the shortest possible. In addition, the module has to be small enough to fit under the microscope together with the permanent magnet situated underneath the membrane module. It also needs to have a small membrane surface area in order to limit the size and amount of the M^{SP} and NP^{SP} respectively. Design criteria i and ii will result in a compact crossflow membrane module with low flow cell volume.

iii. Laminar regime: a laminar regime is less studied in literature, since it is less effective to diminish fouling. Since the concept of BMR^{SP} is a fouling mitigation strategy by itself, employing high crossflow rates that could give rise to a turbulent flow regime is not necessary. Moreover,

filtration cells with laminar flow regime are generally not available in literature, while membrane fouling especially at the membrane-solution interface is often accompanied by a laminar flow regime. Therefore, the Computational Fluid Dynamics (CFD) simulation was tuned to design a filtration cell that can be operated under laminar flow regime.

iv. The implementation of filtration: The driving force for the filtration is a transmembrane pressure (TMP). The module is designed to withstand a pressure of 4 bar. This sets a limit to the minimal thickness of the PMMA layers, which is also leak-free. Criteria i and iv result in a trade-off between magnification and mechanical strength.

v. Use of metal-free materials i.e. safe to work in the presence of 2.35 T magnetic field strength.

Using all these criteria, the magneto-responsive crossflow membrane module was designed using the computer program Inkscape. The module is made of three different PMMA layers: the bottom part (a) which contains the permeate chamber (8 mm), the middle (e) with a 2 mm thin PMMA plate and the 8 mm-thick top layer (f) to provide mechanical strength to the overall structure (Figure 1). All these parts and the permeate chamber in the bottom layer were cut/engraved using a laser cutter (Trotec, speedy 100 R, from Fablab-KU Leuven). The inlet and outlet points were located in the middle layer while the permeate outlet was located in the bottom layer.

Owing to the thin middle PMMA layer, the membrane surface was visible under fluorescence microscopy. In between the top of the O-ring (b) and the middle layer (e), a 1 mm rubber layer (d) was used to prevent leakage under the applied TMP. The flow cell has 1.33 cm³ volume and 13.3 cm² active membrane surface area.

Figure 1. Inkscape design of the PMMA layers and the fabricated transparent crossflow module: a: permeate chamber; b: o-ring; c: membrane; d: 1mm rubber layer; e: 2 mm PMMA layer (feed chamber); f: top PMMA layer (microscope window); g and h: feed inlet outlet; i: permeate outlet.

2.5. Filtration experiments

2.5.1. *In-situ* visualization in crossflow BMR^{SP}

The NP^{SP}s were injected as homogeneously as possible in an open crossflow module. The module was closed by adding the middle and top PMMA layer and then mounted on top of a permanent Nd magnet of 2.35 T (length 7 cm; width; 4 cm). Due to the magnetic field gradient, the NP^{SP}/Enz^{SP}s were attracted towards the surface of the membrane and form a dynamic layer of particles on the top of the M^{SP}. The slide holder of the fluorescence microscope (Olympus BX 51) was removed to fit the crossflow module together with the magnet in between the stage and the objective (Nikon Plan 10/0.30) (Figure 2). An objective with a magnification of 10X was found to be the only objective compatible within the size of the XF-BMR^{SP} setup. The light source (X-cite Series 120

PC) goes through specific optic filters to excite the sample. A Canon camera (Canon EOS 5D mark II) was used to record the images from the biocatalytic bed. On top of the camera, a lens (LMscope, APS-C standard; magnification 2.2X) was mounted inside the microscope. The pictures made, covered a membrane surface of 9.8 mm by 6.4 mm and were later on adjusted for brightness and contrast using FIJI tool [17].

Figure 2. Image and schematic representation of the crossflow BMR^{SP} under the fluorescence microscope.

2.5.2. Background

To implement Enz^{SP} in a crossflow system, the particles have to form a static layer on the top of the membrane. First, the Enz^{SP} is dispersed in the bulk medium above the membrane. Subsequently, due to the presence of an external magnetic field gradient, the Enz^{SP} was attracted towards the surface of the membrane to form a biocatalytic coating layer on the membrane.

Since the stability of this dynamic layer is highly imperative, a force balance gives clear insights into which parameters play the most important roles. Generally, the various force fields can be divided into two main categories: i) forces as a result of the particle-particle interactions and ii) external forces on an individual particle e.g., magnetic and drag force.

The *in-situ* observation was used to qualitatively determine the force balance under various operating conditions i.e. varying feed pH (4.5-7.5), nanoparticle concentration, crossflow rate ((1-100 mL/min), magnetic field strength and feed foulant concentration. To circulate the feed in the module, an HPLC pump (Azura P 4.1S, Knauer) that can provide up to 10 mL/min constant flow or a peristaltic pump that can provide upto 100 ml/min were used. During filtration, the retentate was re-circulated back to the feed tank, while permeate was collected in a separate tank. The filtration of model foulant (CMC) or sodium acetate buffer was done under constant TMP supplied by a pressure controller (GI, A5 T) located along the retentate line (Figure 2). Continuous monitoring of the TMP was executed by a manometer (Eriks, Econ, max 6 bar) while the mass of permeate over time was measured by a balance (Mettler Toledo classic plus) connected to a computer to automate the measurement. In order to keep the biocatalytic bed on the surface of the membrane, a permanent magnet was put underneath the filtration cell. A permanent magnet was also put on the sidewall of the feed tank in order to evaluate possible particle washout that would eventually be re-circulated back to the feed tank.

Prior to each experiment, the membrane was stabilized by filtering demineralized until a steady-state pure water permeability was achieved. To evaluate the stability of the Enz^{SP}, pictures at three main positions on the surface of the membrane, i.e. inlet, center and outlet of the crossflow system along the x-axis were taken over a minimum time of 30 min.

After optimization of the *in-situ* observation conditions, the *in-situ* foulant degradation and subsequent cellulose valorization of the XF-BMR^{SP} was evaluated. In order to reach the optimal working temperature of the cellulase, the feed tank was immersed in a thermo-regulated water bath at 50°C. Then, 14.4 mg of Enz^{SP} was magnetically dispersed over the surface of the membrane resulting in a 1.1 mg/cm² surface coverage. The module is further filled with the feed solution of 0.5 wt% CMC in a sodium acetate buffer (pH 4.5). In order to evaluate the performance difference, a parallel neutral system that contained neutral NP^{SP} was used.

2.6. Hydrolysis product quantification

The amount of product formed in the continuous biocatalysis, i.e. permeate samples collected over time from the crossflow BMR^{SP}, was quantified by high-pressure liquid chromatography (HPLC). Before injection, the samples were filtered through syringe filter (25 mm, 0.2 µL Polyethersulfon VWR international) on a 5 mL syringe (B Braun). A 5 µL sample was injected on a MetaCarb 67H column (length 300 mm; diameter 6.5 mm; temperature 35°C) inside an Agilent 1200 series HPLC equipped with a refractive index detector. The eluent was a 0.0275 v/v% dilution of 96% H₂SO₄ with a velocity of 0.5 mL/min. Given the retention time and measured peak area of glucose, cellobiose and cellotriose, the product concentrations are calculated from a known response factor.

3. Results and discussion

3.1. Qualitative Force Balance

In a XF-BMR^{SP}, it is crucial to find the force balance, which can ensure the stability of the Enz^{SP} magnetically immobilized on the M^{SP}. The effect of the various factors on the force balance was then qualitatively evaluated in order to find feasible conditions for a

sustainable operation of the XF-BMR^{SP}.

3.1.1. Influence of crossflow velocity

The crossflow velocity is one of the most important factors that determines the stability of the biocatalytic bed. A broad range of crossflow velocities (v^{xf}), based on a prior calculation of Reynolds numbers were examined. These crossflow velocities are the mean v^{xf} , under the assumption that the longitudinal velocities in the module are homogeneous over the cross-section in the middle of the flow cell.

A mixture of 2.6 μg Fluo^{NP} and 14.4 mg Enz^{SP} was dispersed on 13.3 cm² M^{SP} and was immobilized on the surface of the membrane by a 0.1 T magnetic field. The lowest v^{xf} examined was 0.06 cm/s while the highest was 6.4 cm/s without permeation, while circulating sodium acetate buffer at pH 4.5 (Figure 5 and Figure S2). The absence of movement of the Fluo^{NP} dots over time (Figure 5 a-c) until a v^{xf} of 0.6 cm/s indicated that Enz^{SP} stayed magnetically immobilized on the M^{SP}. As of a v^{xf} of 0.9 cm/s, some of the Fluo^{NP} dots slowly moved over time. This can be observed in Figure 5 d-i and in Figure S2 for v^{xf} of 1.2-3.1 cm/s. This movement could mainly be due to the transition from a laminar to a turbulent flow regime. The CFD simulation predicted that the transition from laminar to turbulent regime would start from a v^{xf} of 1.4 cm/s, while the experimental observation was at 0.9 cm/s. Compared to initial time pictures, new fluorescent dots appeared or the colour intensities of the existing ones changed after 15 and 40 min of feed recirculation. The newly appearing dots could be due to the auto-fluorescence nature of the M^{SP} (see section 3 of supporting information), which became visible due to movement of the

biocatalytic bed. However, compared to the applied v^{xf} (0.9 cm/s), the distance over which the Fluo^{NP} moved, i.e. $\sim 0.017 \mu\text{m/s}$, was negligible.

At a v^{xf} of 6.4 cm/s, although the microscopically observed region (centre of the filtration cell) remained intact, it was macroscopically visible that a membrane without Enz^{SP} coverage appeared at the outlet of the cell. At the highest v^{xf} i.e. 6.4 cm/s, the biocatalytic layer seemed to crack and open. The green line observed in Figure 5 h and i is the M^{SP} in between the two slices of Enz^{SP} layer. Nevertheless, although application of such a very high v^{xf} generally is not desired, the effect of the magnetic field (0.1 T) prevailed over the v^{xf} . To conclude, a v^{xf} of 0.6 cm/s is shown to keep the Enz^{SP} in place. This value could slightly increase when a permeation instead of simple recirculation is employed, which will add up on the magnetic field force,.

Figure 5. Effect of crossflow velocity over time on the biocatalytic Enz^{SP} layer.

A mixture of 2.6 μg Fluo^{NP} and 14.4 mg Enz^{SP} was dispersed on 13.3 cm² M^{SP} and fixed by a magnetic field (0.1 T at the surface of the membrane). A sodium acetate buffer of pH 4.5 was the feed in the absence of TMP. Observations were made through the green FITC filter i.e. emission at 535 nm/40 and excitation 480nm/30.

3.1.2 Location inside the flow cell

When varying the v^{xf} , it was clear that some regions of the biocatalytic layer were more susceptible to the v^{xf} . In order to further elucidate this phenomenon, the biocatalytic bed was evaluated at three different positions at a v^{xf} of 0.6 cm/s. The observed positions were: A inlet zone, B middle and C as outlet zone (see inset of Figure 6). All three locations are aligned on

the inlet-outlet axis. According to the CFD simulations, this zone is a relatively uniform and laminar region at v^{xf} higher than 0.9 cm/s. Since the amount of NP^{SP} was only 2.4 mg, full coverage of the surface of the membrane was not achieved. As a result, visualization of the auto-fluorescent membrane was sufficient to evaluate any change in the biocatalytic bed. Figure 6 shows that no visible change in the biocatalytic bed could be observed at positions A and B, when a sodium acetate buffer at pH 4.5 was recirculated along the surface of the membrane, at a v^{xf} of 0.6 cm/s without permeation, while a slight fade in the fluorescence was detected at position C. In good agreement with the CFD simulation, there was no significant drift of the dynamic layer of NP^{SP} along the inlet-outlet axis.

Figure 6. Observing the stability of the biocatalytic bed at different positions of the XF-BMR^{SP} containing 2.4 mg NP^{SP} dispersed on 13.3 cm² of membrane along the x-axis, while recirculating sodium acetate buffer of pH 4.5 at 0.6 cm/s without permeation. Observations were made through the green FITC filter i.e. emission at 535/40 and excitation 480/30.

3.1.3 Influence of time

Although neither NP^{SP} nor Enz^{SP} were significantly relocated by the v^{xf} , the observation time of 40 min (Figure 5 and 6) may not be sufficiently long to draw substantiated conclusions. Hence, the long term stability of the biocatalytic bed was evaluated at two different v^{xf} .

Figure 7 shows a series of pictures taken over 3 h when a sodium acetate buffer was recirculated at v^{xf} 0.6 cm/s without permeation. Except for a slight variation, all pictures appeared to be similar. Hence, the NP^{SP} layer was stable over a reasonable period. The slight difference in between the various pictures could mainly be due to a change in the axial drift of the microscopy setup, which shifts the image from the focus of the camera. It is highly imperative to mention that, although the mass of NP^{SP} in Figure 6 and 7 were the same, the NP^{SP} layer was better dispersed over the surface of the membrane in Figure 7 due to sonication of the NP^{SP} suspension prior to magnetically dispersing them over the surface of the membrane.

In another experiment, a sodium acetate buffer was continuously recirculated over a layer containing 14.4 mg of Enz^{SP} mixed with 2.6 μg Fluo^{NP} at 0.9 cm/s v^{xf} for about 2 h. In this case, the movement of the Fluo^{NP} can be indeed detected. However, again due to the stable immobilization by the magnetic field, the movement of the biocatalytic bed was not as fast as the applied v^{xf} (Figure 8).

Figure 7. Long-term stability of the biocatalytic bed while recirculating sodium acetate buffer pH 4.5 at 0.6 cm/s over 2.4 mg NP^{SP} magnetically dispersed on 13.3 cm² membrane surface without permeation. Observed through the green FITC filter i.e. emission at 535/40 and excitation 480/30.

Figure 8. Long-term stability of the biocatalytic bed while recirculating sodium acetate buffer pH 4.5 at 0.9 cm/s over 14.4 mg Enz^{SP} mixed with 2.6 µg Fluo^{NP}, magnetically dispersed on 13.3 cm² membrane surface without permeation. Observed through the green FITC filter i.e. emission at 535/40 and excitation 480/30.

3.1.4. Influence of pH

Considering the various forces to which the biocatalytic bed is subjected, those resulting from particle-particle interactions are as important as the external forces, i.e. magnetic and drag forces, discussed so far. They are both van der Waals and electrostatic forces, and are highly sensitive to the microenvironment surrounding the particle. In particular, the electrical interactions are very susceptible to changes in pH and ionic strength of the medium. pH influences the surface potential of the particles, as indicated by the point of zero charge measurement earlier, which may play a major role in the state of particle aggregation and their response to the external magnetic field.

The effect of the pH was evaluated at three different pH values (4.5, 5.75 and 7.5) at v^{xf} of 0.6 cm/s over 2.4 mg of NP^{SP} without permeation.

Since the point of zero charge value of the NP^{SP} was 7.4, the particles were expected to form bigger aggregates at pH 7.5 relative to the other pH values. Subsequently, the susceptibility of bigger aggregates to the applied magnetic field will be relatively small. As a result, instability of the biocatalytic bed, was expected at the v^{xf} , for which the biocatalytic bed was stable over long period of time. Unlike what was expected, there was no significant change observed in the biocatalytic bed at the three pH values after 40 min of recirculating the respective buffers (Figure 9).

According to Tsouris and Scott [18], analysis of the various forces on paramagnetic particles indicated that the magnetic force is less dependent on the inter-particle distance and outweighs the effect of the van der Waals and electrostatic interactions. The magnetic susceptibility of superparamagnetic materials is several orders of magnitudes higher than that of paramagnetic particles (10^5 compared to 10^{-3}) [19]. The NP^{SP} will thus be more responsive to

the external magnetic force than the particle-particle interaction. This could best explain the negligible influence of pH on the stability of the nanolayer.

Figure 9. Influence of pH when 2.4 mg of NP^{SP} was magnetically dispersed on 13.3 cm², while recirculating sodium acetate buffers at v^f of 0.6 cm/s without permeation. pH 4.5 and 5.75 were observed through green FITC filter i.e. emission at 535/40 and excitation 480/30 and at pH 7.5 observation was made through red CAL i.e. emission at 630/60 and excitation 580/20.

3.1.5 Influence of magnetic field

The magnetic force is crucial to withstand v^f in order to maintain an intact biocatalytic layer. In order to confirm whether keeping the magnetic field after the initial dispersion was essential, the magnet was removed after magnetically dispersing 2.4 mg NP^{SP}.

Even though a conservative v^f of 0.3 cm/s was applied, Figure 10 clearly shows that the NP^{SP} were relocating after only 5 min of sodium acetate buffer recirculation. This was the only

time that movement was observed at this relatively low velocity. Thus, it can firmly be concluded that the magnetic force is crucial to maintain an entire membrane covered by the biocatalytic layer. Yet, compared to the small particles, bigger aggregates were resistant to the wash-out possibly due to strong adhesive interactions with the M^{SP} .

Figure 10. Influence of the magnetic field (0.1 T):(a) a magnetic field of 0.1 T and (b) no magnetic field. 2.4 mg NP^{SP} dispersed on 13.3 cm² of membrane by a magnetic field (0.1 T). Sodium acetate buffer of pH 4.5 was used as the feed at v^{xf} 0.3 cm/s. No pressure was applied and observation were made through the red CAL filter i.e. emission at 630/60 and excitation 580/20.

3.1.6 Influence of a pulsating crossflow

To increase the v^{xf} , a peristaltic pump was used without a pulse dampener. This experiment accidentally resulted in an interesting observation. The pulsating behaviour of the pump resulted in an impulse flow, with a possibly higher velocity than the applied v^{xf} . After this impulse passed, the fluid smoothly flew in the direction of the pump. This impulse-relaxation process created an extra shear on the aggregates in different directions, resulting in slow crumbling of the aggregates. Hence, the NP^{SP} started drifting even at a v^{xf} of 0.1 cm/s, which

is substantially smaller than the v^{xf} (0.9 cm/s) observed earlier. Although it is not as clear as what was visually inspected, the difference in the membrane surface coverage can be detected from the picture taken at the start of the experiment (Figure 11-a) and after 20 min (Figure 11-f). It is therefore highly imperative to keep a smooth velocity profile.

In addition to a fluctuating fluid velocity, the biocatalytic bed was also sensitive to air bubbles. Air scouring is a common way to diminish fouling. In the XF-BMR^{SP}, intrusion of an air bubble seriously affected the stability of the biocatalytic layer as the height of the flow cell was too small to allow movement of the air bubble (Figure S4).

Figure 11. Influence of a pulsating crossflow velocity on the stability of the NP^{SP} layer when 2.4 mg NP^{SP} was magnetically dispersed on 13.3 cm² while recirculating sodium acetate buffer of pH 4.5 at v^{xf} 0.1 cm/s applied by the peristaltic pump without permeation, observed through the green FITC filter i.e. emission at 535/40 and excitation 480/30.

3.1.7. Influence of feed concentration

The stability of the NP^{SP}/Enz^{SP} in the previous section were evaluated while recirculating a sodium acetate buffer without permeation. However, evaluating the stability of the layer while recirculating a foulant and application of TMP is highly imperative.

First, the flow cell was filled with buffer and 2.4 mg of Enz^{SP} was magnetically dispersed over the surface of the membrane. As a control, the stability of the layer was evaluated by recirculating the buffer at 0.6 cm/s for 1 h. Subsequently, the feed was changed to 0.5 wt% CMC. The viscosity of a 0.5 wt% CMC solution was 0.0113 Pa*s and of the buffer was 0.00095 Pa*s. Change in the feed viscosity will obviously have an effect on the Reynolds number, and subsequently on the laminar-turbulent transition v^{xf} . Both change in the feed viscosity and application of permeation to mimic real XF-BMR^{SP} will obviously change the force balance on the NP^{SP}. A minor change in biocatalytic bed structure can be observed right after switching the feed from buffer to CMC (Figure 12-b&c). This minor movement of NP^{SP} can be ascribed to a velocity fluctuation due to pump restarting. Nonetheless, this change is very small and was only observed at the beginning of the experiment. Although a propagation in turbulence due to switching to a high viscosity feed was expected, the applied TMP might have a counter effect in keeping the NP^{SP} on the surface of the membrane. This could be the reason why the change observed after 1 h of CMC filtration was only slight. Therefore, it can be concluded that a v^{xf} of 0.6 cm/s or lower, in combination with a desired TMP, can be suitable conditions to be applied in the combined *in-situ* foulant degradation and cellulose valorisation into

bioethanol using a XF-BMR^{SP}.

Figure 12. Effect of CMC on the stability of NP^{SP} layer when 2.4 mg NP^{SP} was magnetically dispersed on 13.3 cm² of membrane, while circulating sodium acetate buffer and a 0.5 wt% CMC at v^{xf} of 0.6 cm/s at a TMP of 1 bar, observed through the red CAL filter i.e. emission at 630/60 and excitation 580/20.

3.2. Crossflow BMR^{SP}

In order to evaluate the *in-situ* fouling degradation and cellulose bio-valorization capacity of Enz^{SP} in the XF-BMR^{SP}, a smooth v^{xf} of 0.6 cm/s i.e. conditions in which a stable biocatalytic bed was obtained, were selected. To allow permeation, a constant TMP of 1 bar was applied. The feed tank was immersed inside a thermo-regulated bath at 50°C and it was fed with 0.5 wt% CMC.

The steady-state clean water flux of the M^{SP} in the XF-BMR^{SP} at 2 bar was 83 ± 17 L/m² h.

Subsequently, 14.4 mg Enz^{SP} was magnetically dispersed on the M^{SP} . As a control, 14.4 mg of NP^{SP} instead of the Enz^{SP} were magnetically dispersed on the M^{SP} in a parallel experiment. The flux over time was monitored in both the control and the biocatalytic system in order to evaluate the *in-situ* fouling degrading capacity of the Enz^{SP} .

For the control system, unlike in the dead-end BMR^{SP} [2, 14], where a continuous increase in the filtration resistance was observed, the flux reached quasi steady state 90 min after the onset of filtration. This is due to limitation of fouling formation by the shearing effect of the v^{xf} and a decreasing permeate drag force on the foulants at the membrane-solution interface [15]. For the $\text{XF-BMR}^{\text{SP}}$, steady-state was achieved after 75 min and the value was ~42% higher than the control system (Figure 13-a). This difference can be attributed to the *in-situ* degradation of the foulants by the magnetically immobilized Enz^{SP} .

This improved performance can be further tuned through optimization of the various operating parameters and amount of enzyme in order to operate a system with a negligible pumping cost. For instance, due to the different stringent design criteria, the filtration cell has a very small fluid volume. This created a practical limitation in the homogenous dispersion of the Enz^{SP} on the M^{SP} , which was also exacerbated by the presence of air bubbles. These are features that can be resolved in the future by allowing relatively large fluid volumes that potentially promotes both the homogeneous dispersion of the Enz^{SP} and easy floating of the air bubble.

Figure 13. (a) Flux and (b) total product concentration in the XF-BMR^{SP} over time at TMP of 1 bar v^{xf} of 0.6 cm/s at 50°C using 0.5 wt% CMC as feed. The membrane was covered with 14.4 mg (Cel^{NP} or NP^{SP} on 13.3 cm² under 0.1 T Magnetic field.

In addition to monitoring flux, HPLC analyses were performed on the permeate samples collected over a period.

For the control system, there was not product formation detected in all the streams. Whereas for the biocatalytic system, the Enz^{SP} were able to produce small sugar molecules. The specific hydrolysis of CMC by the enzymatic cocktail results in three main products i.e. glucose, cellobiose and cellotriose.

Despite the initial disturbance of the biocatalytic bed due the air bubbles, the XF-BMR^{SP} gave a relatively constant productivity over time (Figure 13-b). This constant productivity can be attributed to stable covalent immobilization of enzyme on the NP^{SP}, stable magnetic immobilization of the Enz^{SP} on the M^{SP} and limited enzyme product inhibition.

However, compared to the theoretically estimated reactor productivity at the steady-state mass transfer (40 g/m² h), the obtained γ (3 g/m² h) was significantly lower.

Since the reaction is expected to occur both on the surface and along the cross-section of the biocatalytic bed, part of the products formed due to surface biocatalysis might be recycled back to the feed tank along with the retentate. In addition, part of the product, especially the smallest portion may diffuse-back and end-up in the feed tank. In order to consider these, samples of the feed tank at the beginning and end of the experiment were also analysed.

In order to evaluate back-diffusion of glucose, the Peclet (Pe) number was calculated as in [13]:

$$Pe = \frac{lv}{D} \quad 1$$

where D is the diffusivity of glucose (m²/s), l is the effective diffusion length (m) and v the perpendicular fluid velocity (m/s).

In literature, the diffusivity value of glucose is around 6*10⁻⁶ cm²/s [20]. Since the BMR^{SP} was operated at 50°C and diffusivity increases with temperature, the highest value of the two was used in the Pe number calculation. The superficial velocity was 4.6*10⁻⁶ m/s and the effective diffusion length was the thickness of the filtration cell (1*10⁻⁵ m) [13]. Based on equation 1, the Pe number (6.9*10⁻²) was significantly lower than one. Thus, back-diffusion exceeds convective transport of glucose within the boundary layer of the system and needs to be integrated in the mass balance in Equation 2 [13]:

$$\frac{d(CV)}{dt} = JC_pA + QC_o - (Q - JA)C_i + A\gamma \quad 2$$

where dC/dt is the concentration gradient, Q is the cross-flow rate in the system [L/h], J is the flux [L/m² h] and γ is the productivity [g/m² h], C_p, C_o and C_i are total product concentrations in the permeate, initial feed stream and final feed stream (g/L) and A is the

membrane area (m^2).

The total product concentrations in the feed tank at the beginning and end of the experiment were 18.1 mg/L and 106.0 mg/L respectively. The initial feed volume was 0.5 L while the final was 0.47 L, hence the volume concentration factor could be negligible. Thus, the total product concentration measured at the end of the filtration was mainly due to accumulation of product in the feed tank. This results in an extra γ of $16.5 \text{ g/m}^2 \text{ h}$ over the $\sim 3 \text{ g/m}^2 \text{ h}$ productivity obtained from the analysis of the permeate samples. Thus, the overall productivity of the XF-BMR^{SP} was $19.5 \text{ g/m}^2 \text{ h}$ and a considerable amount ended up in the retentate stream. From the total reactor productivity, the specific enzyme activity was $5.4 \text{ g/g}_{\text{enz}} \text{ h}$.

4. Conclusions

The force balance in a crossflow magnetic-responsive biocatalytic membrane reactor containing magnetically immobilized Enz^{SP} was qualitatively investigated. Time-sequenced and detailed images of the dynamic layer of the Enz^{SP} layer were obtained using fluorescence microscopy. The initial deposition of the Enz^{SP} and its dispersion over the membrane surface were significantly dominated by the magnetic field, whereas the size of the Enz^{SP} aggregate was mainly dominated by its degree of dispersion in the bulk prior to its magnetic immobilization on the membrane. Due to the strong external magnetic force, the Enz^{SP} layer stayed stable over a broad range of crossflow velocities, pH-values and feed concentrations. There was no significant particle washout observed even at very high crossflow velocities corresponding to turbulent flow regime.

The hydrolytic efficiency of the XF-BMR^{SP} was evaluated using cellulose feed. Although the XF-BMR^{SP} was operated under non-optimal conditions due to loss of Enz^{SP} and inhomogeneous distribution over the surface of the membrane, it gave a 40% higher flux and 20 g/m² h sugar productivity.

Of the 20 g/m² h total reactor productivity, only 3 g/m² h ended up in the permeate. Hence, in addition to optimization of the XF-BMR^{SP}, sequential integration of the XF-BMR^{SP} with another membrane operation in order to recover the products from the feed tank is highly imperative.

The detailed, time resolved images can provide solid foundation for the development of theoretical models that can describe the membrane fouling and the force balance on such system.

References

- [1] C.R. Soccol, S.K. Brar, C. Faulds, L.P. Ramos, *Green Fuels Technology*, Springer, 2016.
- [2] A.Y. Gebreyohannes, M. Dharmjeet, T. Swusten, M. Mertens, J. Verspreet, T. Verbiest, C.M. Courtin, I.F.J. Vankelecom, Simultaneous glucose production from cellulose and fouling reduction using a magnetic responsive membrane reactor with superparamagnetic nanoparticles carrying cellulolytic enzymes, *Bioresource Technology*, 263 (2018) 532-540.
- [3] A. Deneyer, T. Renders, J. Van Aelst, S. Van den Bosch, D. Gabriëls, B.F. Sels, Alkane production from biomass: chemo-, bio- and integrated catalytic approaches, *Current Opinion in Chemical Biology*, 29 (2015) 40-48.
- [4] A. Berlin, V. Maximenko, N. Gilkes, J. Saddler, Optimization of enzyme complexes for lignocellulose hydrolysis, *Biotechnol Bioeng*, 97 (2007) 287-296.

- [5] P. Andrić, A.S. Meyer, P.A. Jensen, K. Dam-Johansen, Reactor design for minimizing product inhibition during enzymatic lignocellulose hydrolysis: II. Quantification of inhibition and suitability of membrane reactors, *Biotechnology Advances*, 28 (2010) 407-425.
- [6] A.Y. Gebreyohannes, R. Mazzei, E. Curcio, T. Poerio, E. Drioli, L. Giorno, Study on the in Situ Enzymatic Self-Cleansing of Microfiltration Membrane for Valorization of Olive Mill Wastewater, *Industrial & Engineering Chemistry Research*, 52 (2013) 10396-10405.
- [7] A. Mahboubi, P. Ylittero, W. Doyen, H. De Wever, B. Molenberghs, M.J. Taherzadeh, Continuous bioethanol fermentation from wheat straw hydrolysate with high suspended solid content using an immersed flat sheet membrane bioreactor, *Bioresource Technology*, 241 (2017) 296-308.
- [8] L.T. Nguyen, K.R.S. Neo, K.-L. Yang, Continuous hydrolysis of carboxymethyl cellulose with cellulase aggregates trapped inside membranes, *Enzyme and Microbial Technology*, 78 (2015) 34-39.
- [9] A.Y. Gebreyohannes, M.R. Bilad, T. Verbiest, C.M. Courtin, E. Dornez, L. Giorno, E. Curcio, I.F.J. Vankelecom, Nanoscale tuning of enzyme localization for enhanced reactor performance in a novel magnetic-responsive biocatalytic membrane reactor, *Journal of Membrane Science*, 487 (2015) 209-220.
- [10] R. Mazzei, L. Giorno, E. Piacentini, S. Mazzuca, E. Drioli, Kinetic study of a biocatalytic membrane reactor containing immobilized β -glucosidase for the hydrolysis of oleuropein, *Journal of Membrane Science*, 339 (2009) 215-223.
- [11] L. Wu, X. Yuan, J. Sheng, Immobilization of cellulase in nanofibrous PVA membranes by electrospinning, *Journal of Membrane Science*, 250 (2005) 167-173.
- [12] S. Judd, The status of membrane bioreactor technology, *Trends in Biotechnology*, 26 (2008) 109-116.
- [13] A.Y. Gebreyohannes, L. Giorno, I.F.J. Vankelecom, T. Verbiest, P. Aimar, Effect of operational parameters on the performance of a magnetic responsive biocatalytic membrane reactor, *Chemical Engineering Journal*, 308 (2017) 853-862.

- [14] A.Y. Gebreyohannes, R. Mazzei, T. Poerio, P. Aimar, I.F.J. Vankelecom, L. Giorno, Pectinases immobilization on magnetic nanoparticles and their anti-fouling performance in a biocatalytic membrane reactor, *RSC Advances*, 6 (2016) 98737-98747.
- [15] M. Mulder, *Basic Principles of Membrane Technology*, 1996.
- [16] A.K. Hołda, I.F.J. Vankelecom, Understanding and guiding the phase inversion process for synthesis of solvent resistant nanofiltration membranes, *Journal of Applied Polymer Science*, 132 (2015) n/a-n/a.
- [17] J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, Fiji: an open-source platform for biological-image analysis, *Nature methods*, 9 (2012) 676.
- [18] C. Tsouris, T.C. Scott, Flocculation of Paramagnetic Particles in a Magnetic Field, *Journal of Colloid and Interface Science*, 171 (1995) 319-330.
- [19] J.F. Schenck, The role of magnetic susceptibility in magnetic resonance imaging: MRI magnetic compatibility of the first and second kinds, *Medical Physics*, 23 (1996) 815-850.
- [20] T. Zhang, H.H. Fang, Effective diffusion coefficients of glucose in artificial biofilms, *Environmental technology*, 26 (2005) 155-160.
- [21] A. Broeckmann, J. Busch, T. Wintgens, W. Marquardt, Modeling of pore blocking and cake layer formation in membrane filtration for wastewater treatment, *Desalination*, 189 (2006) 97-109.

Supporting information

1. CFD simulation of the XF-BMR^{SP}

In order to check one of the design criteria of the XF module, i.e. achieving a laminar flow regime, CFD modelling was used to check at which flow rates the transition towards a turbulent regime occur. This cell was designed on scaled-down version (factor 10) of a prior filtration cell that was well known to work under laminar flow regime [21].

The flow profile stays laminar and the flow is mainly in the longitudinal direction with 2 minor stagnant zones at the beginning corners of the flow cell. The flow on entry is pushed a little to the sides, but leaving small dead zones in the corners (Figure S1c). At the outlet, the fluid is only compressed at the very wall of the cell. Figure S1a&b illustrates the minor dead zones marked in blue where very low speed velocities prevail in comparison to the centre. Although they look like severe vortices, the liquid is rather stagnant there, which is not expected to lead to problems.

A conservative velocity of 1.4 cm/s is selected and is deemed at the edge of turbulence. This is at a flow rate of 21.6 mL/min. Simulations with higher velocity (2 cm/s or 32.4 mL/min) lead to turbulent behaviour at the inlet which propagated throughout the geometry. These calculations are in line with the calculations of the Reynolds number (Table S1). The velocity seems to be higher at the outlet than the inlet. However, the velocities are depicted at the middle of the height of the filtration unit. Due to the parabolic flow profile at the outlet, the velocities are indeed higher than the inlet but closer to the walls this is not the case (read: slices at different heights).

There is one very important difference between the practical XF module and the CFD simulation reported above. The inlet and outlet are positioned differently, this influences the estimations listed above. In the real XF module the inlet and outlet were mounted on top of the module and not on the side. One can expect that this will lead to a transition to a turbulent regime at lower XF velocities (lower than 1.4 cm/s). However, the fluid velocity in the middle zone should be more or less the same as the results in the CFD simulation show.

Figure S1. Velocity profile in the crossflow module in different directions: (a) Velocity magnitude, (b) Velocity in the z-direction and (c) Velocity in the x-direction.

Table S1. Estimations of the Reynolds number.

Flow rate (mL/min)	Crossflow velocity (cm/s)	Reynolds number
1	0.1	52
3	0.2	156
5	0.3	260
10	0.6	520
15	0.9	780
20	1.2	1040
25	1.5	1301
50	3.1	2601
100	6.4	5202

2. Effect of crossflow velocity

Figure S2. Biocatalytic bed containing 14.4 mg of Enz^{SP}, during recirculation of a buffer solution under different crossflow velocities over time.

3. Influence of amount Enz^{SP}

The dispersed amount of Enz^{SP} was varied from 0 mg (Figure S3a), 0.5 mg (Figure S3b) to 14.4 mg (Figure S3c). When the conversion of CMC is kept in mind in the XF-BMR^{SP}, the visibility of Fluo^{NP} mixed with a large amount of Enz^{SP} is highly important. Full coverage of the membrane surface with Enz^{SP} is also important. Thus, observing the movement of the biocatalytic layer in an entirely Enz^{SP} covered membrane is much more relevant. 2.6 μ g of Fluo^{NP} mixed with 14.4 mg Enz^{SP} was found to be ideal for the observation of a fully covered membrane surface.

Figure S3. Influence of amount Enz^{SP} magnetically dispersed on 13.3 cm² M^{SP} membrane without permeation: (a) 2.6 μ g Fluo^{NP}, (b) 2.6 μ g Fluo^{NP} mixed with 0.5 mg Enz^{SP} (c) 2.6 μ g Fluo^{NP} mixed with 14.4 mg Enz^{SP}. The observation was made through the green FITC filter.

Figure S4. Effect of air bubbles: the top view of the XF-BMR^{SP} module (a) before and (b) after air scouring on a 14.4 mg Enz^{SP} magnetically dispersed on 13.3 cm² of membrane. A 0.5 wt% CMC feed was used at v^{xf} 0.3 cm/s at a TMP of 1 bar.