Membranes

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Reversible Supramolecular Surface Attachment of Enzyme–Polymer Conjugates for the Design of Biocatalytic Filtration Membranes

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Abstract: *To be used successfully in continuous reactor systems, enzymes must either be retained using filtration membranes or immobilized on a solid component of the reactor. Whereas the first approach requires large amounts of energy, the second approach is limited by the low temporal stability of enzymes under operational conditions. To circumvent these major stumbling blocks, we have developed a strategy that enables the reversible supramolecular immobilization of bioactive enzyme–polymer conjugates at the surface of filtration membranes. The polymer is produced through a reversible addition–fragmentation transfer method; it contains multiple adamantyl moieties that are used to bind the resulting conjugate at the surface of the membrane which has surface-immobilized cyclodextrin macrocycles. This supramolecular modification is stable under operational conditions and allows for efficient biocatalysis, and can be reversed by competitive host–guest interactions.*

The world faces tremendous environmental challenges in terms of resource scarcity and energy needs. Industrial production processes may offer a solution by manufacturing on a large scale,[1] but they require the right catalysts to be economically viable and sustainable. Enzymes are of particular interest because they function under aqueous conditions and do not need high operating temperatures.[1b,2] However, most enzymes are fairly fragile proteins, and their stability often needs to be improved to allow for their industrial use. Protein engineering[3] and immobilization on solid supports[4] are efficient strategies for improving enzyme stability, thereby allowing their use under harsh operational conditions.[4d,5]

Another limitation to the use of enzymes in continuous processes is their soluble nature, as they must be retained in the reactor system.[4a] Enzymes may be retained using appropriate filtration devices with relatively low cut-off values but higher amounts of energy are often needed to pump the permeate through these filters and the filters themselves are more easily fouled. One possibility for circumventing these problems is to immobilize the enzyme on the filtration membrane.

Numerous strategies have been developed to stably immobilize enzymes on filtration membranes.[6] In addition to simple adsorption processes, covalent bio-functionalization of filtration membranes can be achieved either by chemical activation of the membrane surface using wet chemistry or by exposing the membrane to some form of radiation (e.g., UV,[6a] plasma,[6b] or γ-rays[6c]). However, these approaches are limited by the low temporal stability of enzymes. Indeed, when an enzyme's activity decays below the threshold required for the biocatalytic process, the membranes must be replaced. To our knowledge, none of these approaches allows for a controlled and reversible modification of the membrane material to regenerate it in situ with fresh biocatalysts. Such a system would undeniably open up new possibilities for the use of enzymes in continuous biocatalytic processes.

Reversible surface modification may be achieved by exploiting supramolecular interactions at solid–liquid interfaces. Reinhoudt and co-workers successfully demonstrated that self-assembled monolayers of densely packed macrocyclic molecules serving as molecular hosts could act as "molecular printboards" to immobilize a series of target molecules containing multiple guest moieties.[7] These surfaces were used for precise positioning of multivalent guest molecules and fabrication of 3D nanostructures by microcontact printing and nanoimprint lithography.[7c,d] In 2012, Yang et al. demonstrated that this approach could be adapted to attach tagged proteins to gold surfaces.[8] This strategy was also used to fabricate enzymatically functionalized microfluidic systems.[9] Recently, Harada and co-workers demonstrated that multivalent inclusion phenomena could also be exploited at the macroscopic range.[10] However, most of these applications required a high density of host molecules, hence limiting the range of surfaces to that where a high density of chemical modification can be reached.

Herein, we demonstrate that multiple-point interactions can be used to stably but reversibly immobilize enzyme–polymer conjugates at the surface of polymeric filtration membranes that have a low density of macrocylic host (cyclodextrin, CD) entities on their surface. We demonstrate that this approach can be used to design a membrane bioreactor in which an enzyme, namely β-galactosidase, is immobilized. This immobilization was stable under operational conditions but could be competitively reversed. We confirmed the efficiency of this membrane bioreactor by using it to hydrolyze lactose in milk serum, a complex and rich medium.

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We used polyethersulfone (PES) as a model membrane system. PES is one of the most widely used organic membrane materials owing to its low chemical reactivity, low cost, its processability, and good chemical, thermal, and mechanical resistance. The first step of the attachment process consisted of introducing reactive functions to the PES surface that can be further coupled to a CD macrocycle.

We developed a UV photografting strategy that enables carboxylic acid functions to be introduced to the membrane surface. UV irradiation causes homolytic bond cleavage of the PES chain, predominantly at the surface of the material.[11] The resulting radical species can react with alkenes through a homolytic addition mechanism. In our procedure, we soaked the PES membrane in a diluted aqueous solution of acrylic acid before UV irradiation. UV irradiation of the entire surface of the membrane caused the covalent grafting of the acrylic acid monomer to the polymeric material, thereby resulting in the introduction of carboxylic acid functions to the surface (AA-PES). In parallel, we produced a photo-patterned membrane using a photomask (100 × 100 μm squares). The non-exposed surface of this membrane represented a reference sample, which facilitated the microscopic assessment of the supramolecular binding.

Fourier transform infrared spectroscopy (FTIR) analysis of the fully irradiated membrane revealed the carboxylate carbonyl stretching vibration band at 1708 cm^{-1} (Figure 1), thus confirming the successful grafting of acrylic acid to the surface. Field-emission scanning electron microscopy (FE-SEM) analysis did not show any relevant difference between reference and irradiated membranes (Figure 1). Additionally, the surface roughness (root mean square) measured by atomic force microscopy remained relatively unchanged by UV irradiation, with a value of 191 nm compared to that of the starting material of 202 nm. This parameter is important because membrane surface roughness has a significant influence on membrane fouling properties.[12]

The carboxylic functional groups introduced at the surface of PES were activated using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) to form active *O*-acyl-isourea intermediates. The activated leaving groups were displaced by a nucleophilic attack from the primary amino groups of heptakis(6-deoxy-6-amino)-β-cyclodextrin (NH-CD) to form the desired amide (NH-CD-PES). The successful coupling of NH-CD was confirmed by a significant decrease in intensity of the carbonyl stretching band measured at 1708 cm^{-1} for the carboxylic acid and the appearance of the stretching band of the amide carbonyl (which resulted from the coupling to the amine functions of NH-CD) at 1641 cm^{-1}. FE-SEM micrographs did not show any alteration of the membrane surface morphology and the surface roughness was measured to be 206 nm.

In addition to water solubility, the multivalent enzyme–polymer (MEP) conjugate should have multiple chemical functions capable of forming inclusion complexes with the

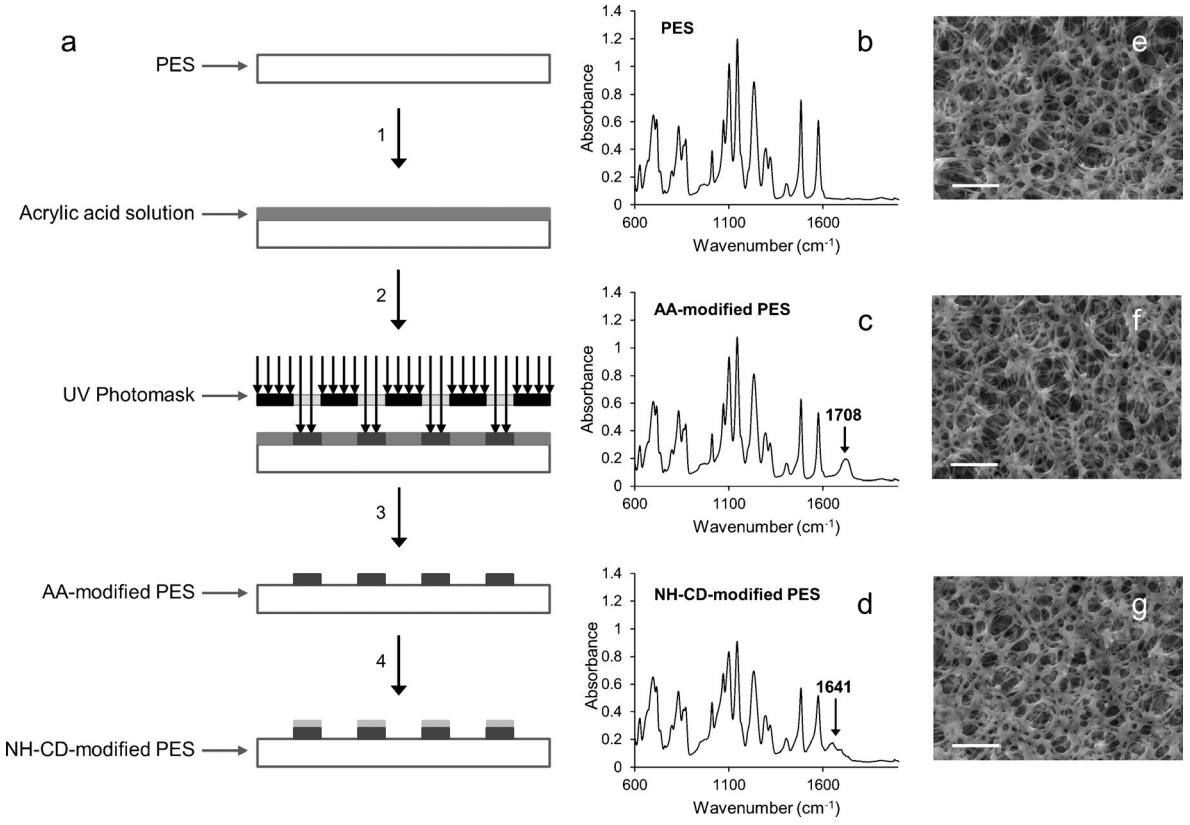

Figure 1. a) Schematic representation of the synthetic route to covalently bind β-CD derivatives at the surface of PES membranes in a micro-patterned fashion. b–d) FTIR spectra of b) bare PES membrane, c) PES membranes modified with acrylic acid, and d) with NH-CD. e–g) FE-SEM micrographs of e) PES, f) AA-PES, and g) NH-CD-PES membranes at a magnification of x10^4. Scale bars: 5 μm.

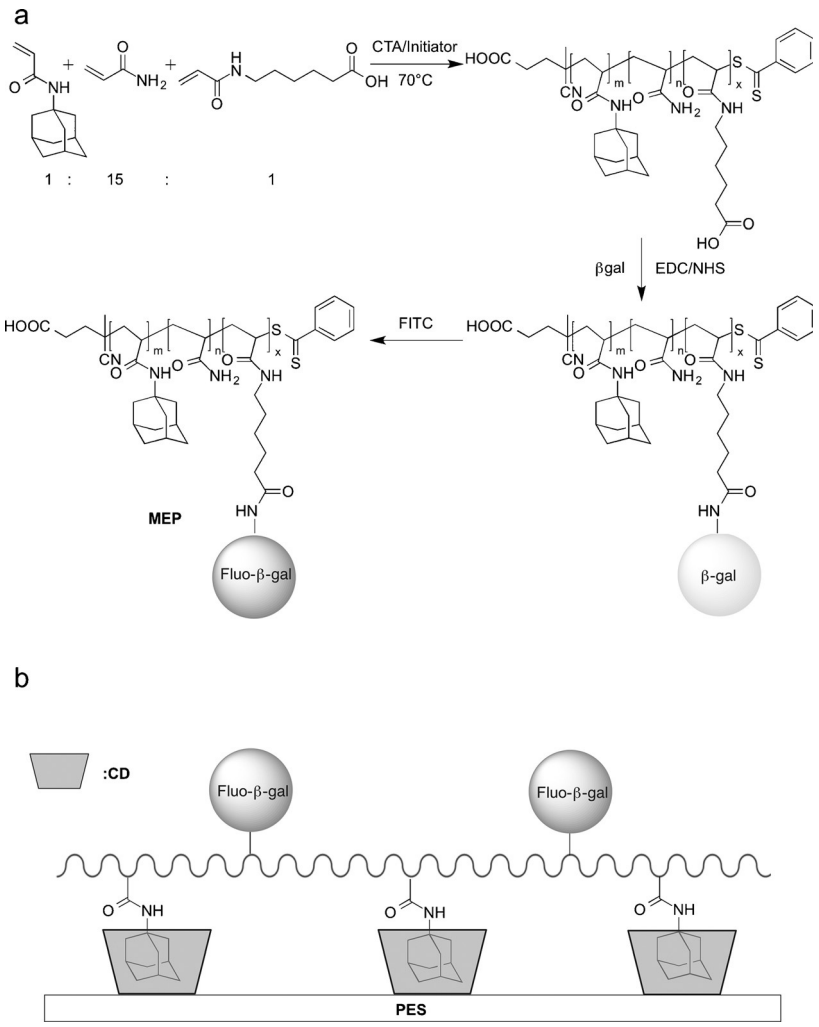

Figure 2. a) Synthetic route to fluorescently labeled MEP, The acrylamide-based polymer was synthesized following a RAFT strategy, using 4,4′-azobis(4-cyanopentanoic acid) as a radical initiator and 4-cyanopentanoic acid dithiobenzoate as a chain transfer agent. The synthesis was followed by covalent attachment of β-gal and subsequent labeling with a FITC. b) Schematic representation of the multivalent supramolecular binding of MEP to covalently immobilized CDs on the membrane surface.

CD hosts immobilized on the membrane surface. We synthesized an acrylamide-based polymer using adamantyl acrylamide, acryloyl-6-aminocaproic acid, and acrylamide as monomers (Figure 2). Polymerization was carried out using a reversible addition–fragmentation transfer (RAFT); the resulting had a molecular weight of 11 600 (polydispersity index 2.2) corresponding to 8.1 adamantyl functions per polymer molecule.

The covalent attachment of a β-galactosidase (β-gal; EC 3.2.1.23) was achieved by first activating the carboxylate functions of the polymer using EDC and *N*-hydroxysulfosuccinimide, and then treating them with β-gal. The produced MEP was subsequently labeled with a fluorescent dye (fluorescein isothiocyanate, FITC) to allow us to assess the polymer binding on the filtration membranes. Fluorescently labelled β-gal (fluo-β-gal) was also produced to serve as a control. A reference MEP conjugate (MEP_{ref}) was produced using the same method but omitting the adamantyl acrylamide monomer (Supporting Information).

We used fluorescence microscopy to investigate the reversible supramolecular binding of MEP to covalently immobilized CDs on PES membrane (CD-PES) surfaces (Figure 3). After incubation with MEP, the NH-CD-PES membrane showed fluorescent square patterns with a size identical to that of the photomask we used for patterning the surface, thus confirming the attachment of the fluorescent MEP on CD-modified areas of the membrane. No other reference showed any relevant fluorescent signal, which confirmed the selectivity of the adsorption of the MEP on the surface (Supporting Information). The reversible nature of the surface grafting was confirmed by the absence of fluorescent signal after washing the membrane with soluble CD.

We studied the enzymatic activities of MEP-modified membranes in a batch process first, using a model reaction with *ortho*-nitrophenyl-β-galactoside (ONPG) as a synthetic substrate. NH-CD-PES membranes were incubated in a solution of MEP (0.01 $g\,L^{-1}$; Figure 4 and Supporting Information). The ONPG conversion rates at the surfaces of all control membranes were less than 0.002 μmol $min^{-1}\,cm^{-2}$. A significantly higher ONPG conversion rate of 0.043 μmol $min^{-1}\,cm^{-2}$ was measured for the MEP-modified membrane. This suggests that the catalytic properties of the enzyme were preserved after specific immobilization of MEP at the surface of filtration membranes.

The ONPG conversion rate at the surface of β-CD-incubated filtration membranes dropped to 0.0018 μmol $min^{-1}\,cm^{-2}$ (Figure 4). This shows a successful removal of 96% of immobilized MEP from the membrane surface. We repeated the MEP

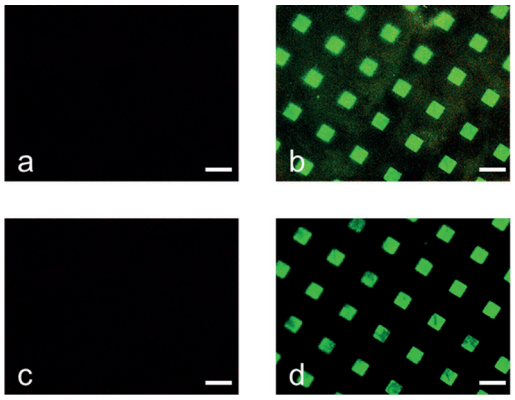

Figure 3. Fluorescence micrographs of the NH-CD-PES membrane, a) before (NH-CD-PES) and b) after incubation in MEP solution; c) after incubation in a solution of β-CD and d) after a second incubation in MEP solution. Scale bars: 200 μm.

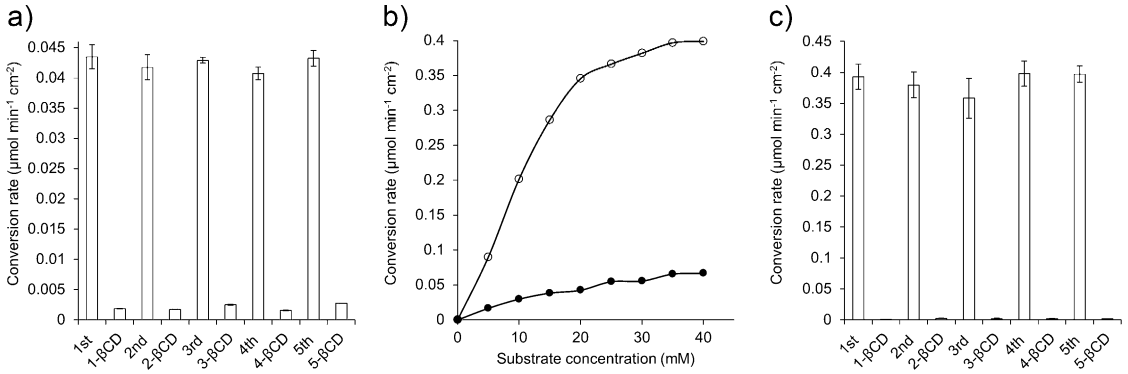

Figure 4. Measurements of the enzymatic activities of MEP-modified membranes, a) Measurements of ONPG conversion rates at the surface of membranes in a batch process after incubation in a MEP solution, and subsequent incubation in a β-CD solution as a competitor host. The procedure was repeated five times (1-βCD–5-βCD). b) Measurements of ONPG conversion rates at the surface of membranes modified in batch (black circles) and continuous (white circles) processes using increasing concentrations of ONPG. c) Measurements of lactose conversion rates at the surface of membranes after filtration of milk serum solution through the MEP-modified membranes, followed by filtration of a β-CD solution as a competitor host.

binding assay five times and measured a recovery of 95–100% of the initial ONPG conversion rate after each cycle. These results confirm the possibility of regenerating MEP-modified PES membranes without significant loss of enzymatic activity.

We also performed the supramolecular immobilization of MEP at the surface of PES filtration membranes in a continuous filtration process. Each membrane was fixed using a syringe filter while the fluid flow rate through the membrane was controlled using a syringe pump. The solution of MEP ($0.01\ g\,L^{-1}$) was filtered through the NH-CD-PES membrane at a rate of $4\ mL\,h^{-1}$. Subsequently, an ONPG solution was filtered ($4\ mL\,h^{-1}$) through the membrane at 40 °C. The ONPG conversion rate at the surface of filtration membranes was $0.39\ \mu mol\,min^{-1}\,cm^{-2}$, which is substantially higher than the corresponding batch process ($0.043\ \mu mol\ min^{-1}\,cm^{-2}$).[13] Two hypotheses could explain the higher ONPG conversion rate at the membrane surface in the continuous filtration process: the substrate diffusion is limiting in the batch process; or the density of immobilized MEPs at the membrane surface is higher when the immobilization is done in a flow-through fashion.

To better understand substrate diffusion at the surface of filtration membranes in a batch process, we modified membranes with MEPs in batches and subsequently incubated them in solutions of ONPG at concentrations ranging from 5 to 40 mM at 40 °C. All references showed an activity of less than $0.002\ \mu mol\,min^{-1}\,cm^{-2}$ (Supporting Information). For the MEP-modified membranes, as ONPG concentration increased, the biocatalytic conversion rate increased steadily from 0.017 to $0.07\ \mu mol\,min^{-1}\,cm^{-2}$ before reaching a plateau (Figure 4). This confirmed that the batch process was limited by the diffusion of the substrate. However, the highest biocatalytic conversion rate in the batch process ($0.07\ \mu mol\ min^{-1}\,cm^{-2}$) was still lower than in the filtration process ($0.39\ \mu mol\,min^{-1}\,cm^{-2}$). We modified the membranes with MEPs in a continuous filtration process and studied their enzymatic activities in batches using different concentrations of the substrate ranging from 5 to 40 mM. By increasing the ONPG concentration, the biocatalytic conversion rate increased from 0.09 to $0.39\ \mu mol\,min^{-1}\,cm^{-2}$ before reaching a plateau (Figure 4). The maximum biocatalytic conversion rate reached the same value obtained in the original continuous filtration process. These results confirmed that the batch process is limited by both substrate diffusion and the quantity of immobilized MEPs, and therefore the continuous filtration process resulted in higher biocatalytic conversion rates.

To assess the stability and efficiency of the MEP-modified membranes under operational conditions in a complex matrix, we studied the hydrolysis of lactose in milk serum. We performed an assay using MEP-modified membranes alongside a control experiment in which β-gal was filtered through the membrane as a free enzyme. Milk serum was then filtered ($4\ mL\,h^{-1}$) through the MEP-modified and reference membranes at 40 °C. The immobilized enzymatic activities on the membranes were measured (Figure 4c). The lactose conversion rate at the surface of the reference membrane (using free enzyme, β-gal) was only $0.003\ \mu mol\,min^{-1}\,cm^{-2}$, whereas the conversion rate at the surface of the MEP-modified membrane was $0.39\ \mu mol\,min^{-1}\,cm^{-2}$. MEP-modified membranes have similar conversion rates for ONPG and lactose as substrates, because β-gal has a similar maximum velocity for ONPG and lactose as substrates ($600\ \mu mol\ min^{-1}\,mg^{-1}$).[14]

To regenerate the membrane surfaces, β-CD solution was filtered ($4\ mL\,h^{-1}$) through the MEP-modified membrane. The lactose conversion rate dropped to $0.0004\ \mu mol\ min^{-1}\,cm^{-2}$, which confirmed the removal of more than 99.9% of immobilized MEP from the membrane surface. We repeated the MEP binding assay and assessed the hydrolysis of lactose in milk serum five times and recovered 92–102% of the initial lactose conversion rate after each cycle.

In summary, we have developed a supramolecular strategy to immobilize enzyme–polymer conjugates at the surface of PES filtration membranes in a stable yet reversible manner. Our method relies on multiple-point host–guest inclusion interactions between the enzyme–polymer conjugates and the

surface of the filtration membrane. We show that this approach enables the stable immobilization of β-galactosidase at the surface of the filtration membranes. The membrane bioreactor efficiently hydrolyzed lactose in milk serum. This versatile method can function on a large variety of surfaces with theoretically any type of enzyme. It overcomes several limitations of existing methods and is thus expected to be valuable for a wide range of industrial bio-based applications.

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