Biomembranes

International Edition: DOI: 10.1002/anie.201411263
German Edition: DOI: 10.1002/ange.201411263

Enzymatically Active Ultrathin Pepsin Membranes**

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Abstract: *Enzymatically active proteins enable efficient and specific cleavage reactions of peptide bonds. Covalent coupling of the enzymes permits immobilization, which in turn reduces autolysis-induced deactivation. Ultrathin pepsin membranes were prepared by facile interfacial polycondensation of pepsin and trimesoyl chloride. The pepsin membrane allows for simultaneous enzymatic conversion and selective removal of digestion products. The large water fluxes through the membrane expedite the transport of large molecules through the pepsin layers. The presented method enables the large-scale production of ultrathin, cross-linked, enzymatically active membranes.*

The unique architecture of enzymatic proteins allows for the hydrolysis of peptide bonds under mild conditions.[1] The high enzyme specificity and activity are attractive for the isolation of peptides and in food upgrading processes. However, enzyme re-usage is complicated by self-cleavage-induced deactivation and difficulties associated with the recovery of dilute enzyme solutions used in relevant processes.[2] Immobilized enzymes can be used instead, potentially reducing deactivation and increasing conversion specificity and activity.[3] Enzyme mobility can be restricted by covalent coupling to a substrate or by self-cross-linking of the enzyme. Enzyme immobilization allows for simultaneous enzymatic conversion and removal of the converted products while maintaining the enzyme activity of the immobilized proteins.[4] The most common approach to achieve enzyme immobilization is the formation of a polymer–protein bioconjugate.[5] Current methods to make self-cross-linked freestanding or supported protein layers are based on template- or self-assembly-assisted crosslinking, generally using glutaraldehyde as the cross-linking agent. Examples include freestanding protein films by assembly of proteins on sacrificial cadmium hydroxide templates for controlled drug release or nanofiltration purposes.[6] More recently, enzymatically active protein films have been prepared based on hierarchical self-assembly of protein–polymer conjugates cross-linked in glutaraldehyde vapor, confirming that the native protein function can be maintained despite the high degree of covalent bonding between proteins.[7] Nonetheless, the production of freestanding protein films in this manner is not easily scalable, and a large amount of precious protein is required for small membrane surface areas.

Herein, we propose to use a facile interfacial polycondensation reaction for the production of ultrathin enzymatically active pepsin membranes.[8] Pepsin is a nonspecific acidic endopeptidase that preferentially cleaves proteins at the carboxylic groups of aromatic amino acids, such as phenylalanine, tryptophan, leucine, and tyrosine.[9] The ultrathin cross-linked pepsin membranes with a thickness of 50–150 nm show molecular retention of polyethylene glycol (PEG) with a molecular weight above 10 kDa and exhibit enzymatic activity comparable to that of pristine pepsin. The pepsin membrane on top of a porous PAN support is shown in Scheme 1 (left). The ultrathin pepsin layer allows for the simultaneous retention and selective cleavage of large molecules. The high water permeability enables the expeditious removal of digestion products.

Pepsin film formation by interfacial polymerization is achieved using a 0.46 wt% pepsin solution in phosphate-buffered saline (PBS, pH 7) and a trimesoyl chloride (TMC) solution in hexane. The pH of the buffer solution, the reaction time, and the reactant concentrations were identified as critical parameters for effective film formation. The optimal pepsin and TMC concentrations were found to be higher than for conventional interfacial polymerization processes. This can be attributed to the large size of the pepsin, the low reactivity of the functional groups, and the nature of the formed film. In acidic environment, the protein reactivity is limited by the large number of protonated, unreactive amino acid groups. The neutral pH value of the PBS buffer is critical for ensuring a sufficient availability of reactive amines while preventing permanent pepsin deactivation. Pepsin film formation atop a flat polyacrylonitrile ultrafiltration membrane (PAN, SolSep, Netherlands) was achieved by prewetting the support with the aqueous pepsin solution and subsequently contacting the support with a solution of TMC in hexane. Film formation was confirmed by X-ray photoelectron spectroscopy (XPS) and attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR). The ATR-FTIR absorbance spectra of pepsin powder and PAN-supported ultrathin

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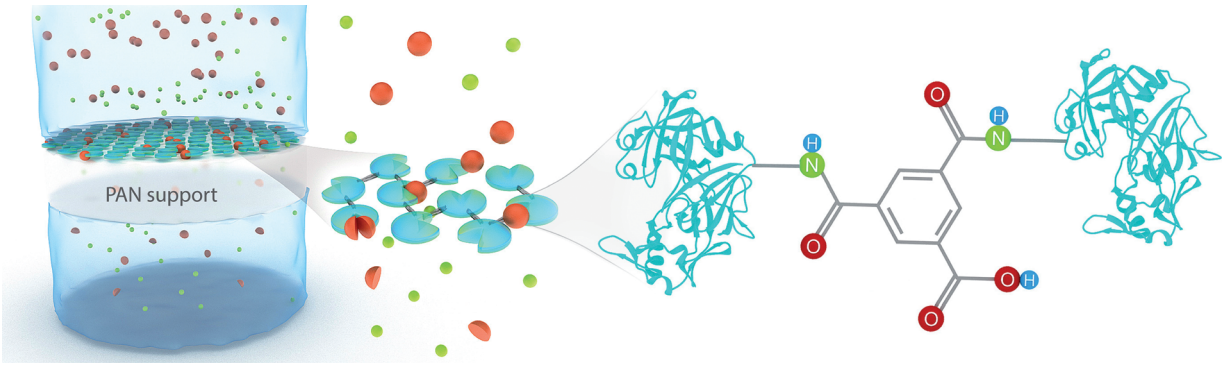
[**] M.W., M.B., and M.T. acknowledge support through the Alexander von Humboldt Foundation. This work was performed in part at the Center for Chemical Polymer Technology (CPT), which is supported by the EU and the federal state of North Rhine-Westphalia (EFRE 30 00 883 02).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201411263.



Scheme 1. Pepsin membrane formation. The pepsin membrane is positioned atop a porous PAN support, which is represented by the white area. The pepsin membrane consists of pepsin molecules that are randomly cross-linked by TMC, forming amide bridges between the pepsin molecules. The pepsin layer simultaneously acts as the enzymatic surface and the membrane sieve. The larger molecules are retained by the membrane and selectively cleaved by the pepsin. The large water flux through the membrane enhances the transport of solute molecules to the membrane surface area and the removal of digestion products by membrane permeation.

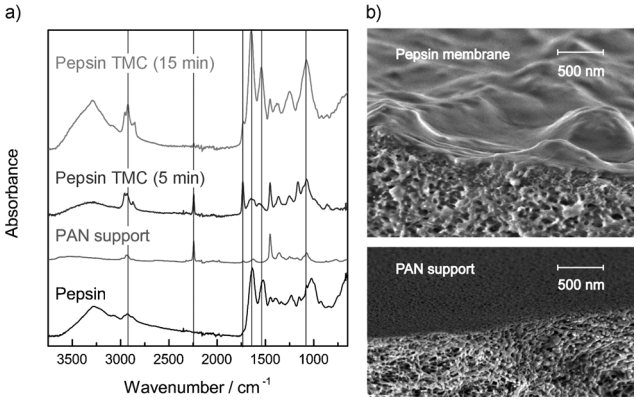


Figure 1. a) ATR-FTIR absorbance spectra of pepsin powder and PAN-PO supported ultrathin pepsin membranes prepared with reactions times of 5 and 15 minutes. The absorbance peaks at 3000–3500 cm^{-1} represent the different C—C, C—H, and O—H bonds present in the pepsin. Amino acid bonds are located at 1650 (N—H bending) and 1540 cm^{-1} (C=O stretching). b) Scanning electron micrographs of a pepsin membrane atop a PAN support (top) and a bare PAN support (bottom). The pores present on the top of the PAN support are not visible for the pepsin membrane PAN support.

pepsin membranes prepared with reaction times of 5 and 15 minutes are shown in Figure 1 a. The absorbance spectra of the pepsin membranes show peaks corresponding to the infrared absorbance signature of the pepsin powder, indicating that the nature of the pepsin does not change upon crosslinking. As film formation advances, absorbance bands associated with the phenyl rings and amide bonds emerge at 1240 and 1400 cm^{-1}. The intensity of the pepsin-related peaks increased with the reaction time for interfacial polymerization, which is due to the continued progress of the pepsin film growth. Film growth is sustained by the open character of the formed film, which allows for diffusion of the monomer reactant. This is different from conventional film formation by interfacial polymerization, where inhibition of the reactant diffusion limits film growth.[10] The scanning electron micrographs shown in Figure 1 b substantiate film formation atop the porous PAN support. Whereas the top-view micrograph of the PAN support clearly reveals the presence of pores, the pepsin membranes appear as dense layers with a distinct morphology that is different from those of conventional interfacial polymerization membranes. The layer thickness was determined to be in the range of 50–150 nm from cross-sectional micrographs (see the Supporting Information).

A reaction time of 15 minutes was used to obtain sufficiently thick membranes for further characterization. The degree of pepsin crosslinking by the TMC groups was determined by XPS analysis of the supported films. The deconvoluted C 1s and O 1s binding energy spectra of the pepsin powder and pepsin membranes prepared by reacting presoaked PAN supports with TMC in hexane solution (0.2 or 0.5 wt %) are shown in Figure 2. The C 1s and O 1s binding energy spectra of the PAN support are given as a reference. The C 1s spectra confirm the formation of a covalent bond between the pepsin and TMC atop the PAN support. The C 1s binding energies of the pepsin membranes at 285, 286.5, and 288 eV, which are due to saturated hydrocarbon, amine, and amide groups, respectively, correspond to the binding energy peaks of the pepsin powder. The increase in the binding energy peak area at 285 eV (A) with respect to the peaks at 286.5 (B) and 288 eV can be rationalized by an increase in the aromatic carbon atom content. The degree of the reaction between pepsin and TMC can be calculated from the change in the A/B ratio of the C 1s peak areas. Moreover, the carbon to nitrogen elemental ratio gives an indication of the relative increase in the number of aliphatic carbon atoms in the pepsin membranes. The number of reacted TMC groups per pepsin molecule was estimated to be 40 for the pepsin membrane prepared using 0.2 wt % TMC in hexane. The high degree of crosslinking results in effective immobilization of the pepsin, although it is unlikely that all TMC molecules connect two different pepsin molecules. This is reflected by the C 1s binding energy peak at 285.8 eV in the pepsin membrane spectra, which is associated with carbonyl groups that are formed by the reaction of excess acyl chloride groups with water. The membranes prepared using 0.5 wt % TMC in hexane were estimated to feature five reacted TMC groups per pepsin, which is a significantly lower value than for the

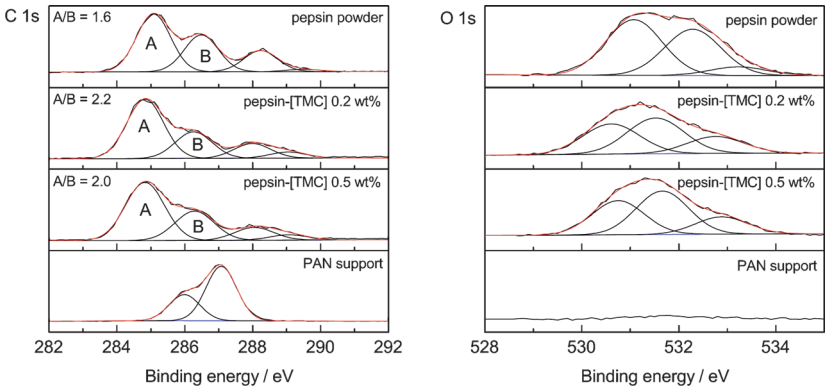


Figure 2. Deconvoluted C 1s and O 1s spectra of the PAN support, the pepsin powder, and PAN-supported pepsin membranes prepared using the 0.2 and 0.5 wt% TMC solutions in hexane with a reaction time of 15 minutes. The spectra were fitted using Gaussian functions with similar full width at half maximum (FWHM) values. A complete analysis of the relative areas of the C 1s, N 1s, and O 1s binding energy peaks for these systems is given in the Supporting Information.

membranes prepared with lower TMC concentrations. This finding is likely due to the acidification of the aqueous phase upon contact with excess TMC monomers, hampering the amine group reactivity that is required for effective pepsin polycondensation. The absence of a peak in the pepsin membrane spectra at 287.1 eV, associated with the PAN C 1s binding energy, indicates that the membrane layer is thicker than the X-ray beam penetration depth of approximately 10 nm. This observation is in accordance with the thickness observed by SEM analysis. The O 1s spectra shown in Figure 2 (right) underline the partial conversion of acyl chloride groups into carboxylic acid groups upon interfacial polymerization. The peaks associated with carbonyl (531.5) and hydroxy groups (532.9 eV) increase in intensity with respect to the amide peaks upon pepsin membrane formation, suggesting that a relatively large fraction of the reactive groups on the TMC are converted into carboxylic acids. Nonetheless, the determination of the fraction of unconnected TMC groups is complicated by the pronounced shift in the fitted binding energy peak maxima and overlaps in the binding energy peaks of the different functional groups.

The pepsin activity was tested in two consecutive digestion runs using hemoglobin and bovine serum albumin (BSA, fraction V) as the substrates at a temperature of 37 °C and pH 2 (HCl adjusted). Multi-angle laser light scattering (MALLS), ultraviolet (UV) absorption, and refractive index (RI) measurements were used to determine the molecular weight distributions of the protein and the digestion product. The hemoglobin and BSA digestion kinetics for the TMC cross-linked pepsin membranes on PAN support were compared with those of pepsin in solution. The molecular weight distributions of hemoglobin in solution after different contact times with the pepsin membranes prepared using the 0.2 and 0.5 wt% TMC solutions are shown in Figure 3. The digestion kinetics of pepsin in solution were determined for comparison (Figure S3). To determine the degree of pepsin deactivation, two consecutive assay digestion runs were performed using the same membrane sample. The initial hemoglobin molecular weight distribution, represented by the dotted line at $t = 0$, is given by a single peak with a maximum at 36 kDa. After one hour, there is a significant decrease in the hemoglobin peak while a second peak at 8 kDa emerges. Further evolution of the molecular weight distribution with time shows a continued decrease in hemoglobin concentration and an increase in the concentration of the digestion product. The membranes prepared with the 0.2 wt% TMC solution showed the highest apparent activity. The low activity of the membranes prepared with the 0.5 wt% TMC solution is likely a result of the lower pepsin reactivity, which is due to acidification of the aqueous solution during interfacial polymerization. As it was not possible to quantify the amount of pepsin in the layer, an exact determination of the degree of deactivation was not possible. However, the hemoglobin digestion was slightly higher in the second run than in the first run for both layers, indicating that little deactivation occurred over the course of each run (ca. 30 h each). The formation of the digestion product, on the other hand, is more pronounced in the first runs (●) than in the second runs (○). Moreover, the formation of the digestion product does not correspond to the continuous hemoglobin removal. The undervalued product formation is likely caused by the absorption of digestion products by the membranes (Figure S6). The digestion kinetics of BSA are similar to those measured for hemoglobin (Figure S4 and S5).

Molecular weight cut-off (MWCO) measurements for the pepsin membranes on PAN support were performed using a stirred dead-end permeation cell. The feed and permeate concentrations were sampled to determine the degree of retention for PEG molecules with a range of molar masses. The retention was calculated from the ratio of the concentrations in the permeate and in the feed. A more detailed description of this setup is given in the Supporting Information. Permeation experiments were performed at a pressure difference of 2 bar using aqueous feed solutions of poly(ethylene glycol) with mean molar masses of 2, 6, 8, 10, 20, or 40 kDa (each fraction: 1 $g L^{-1}$). The permeate composition was sampled after 30, 60, 90, and 120 minutes. Gel permeation chromatography (GPC) was used to analyze the feed and permeate compositions. Figure 4 displays the molar mass distribution of the feed and the permeate of the two membranes prepared with 0.2 and 0.5 wt% TMC solutions.

The flux was determined to be approximately 50 $L m^{-2} h^{-1}$ for all pepsin membranes and about 400 $L m^{-2} h^{-1}$ for the PAN support layer. The concentration of the PEG molecules in the feed after filtration for 120 min increased with respect to the initial PEG concentration of the feed. The permeate concentration was also lower than the feed concentration. The enrichment of PEG molecules in the feed implicates that the pepsin membranes retain large molecules. The PAN support without a pepsin layer does not show any retention for the same range of PEG molar masses (Figure S7). The retention data, calculated for a permeation time of 120 minutes, reveal that the membranes prepared with

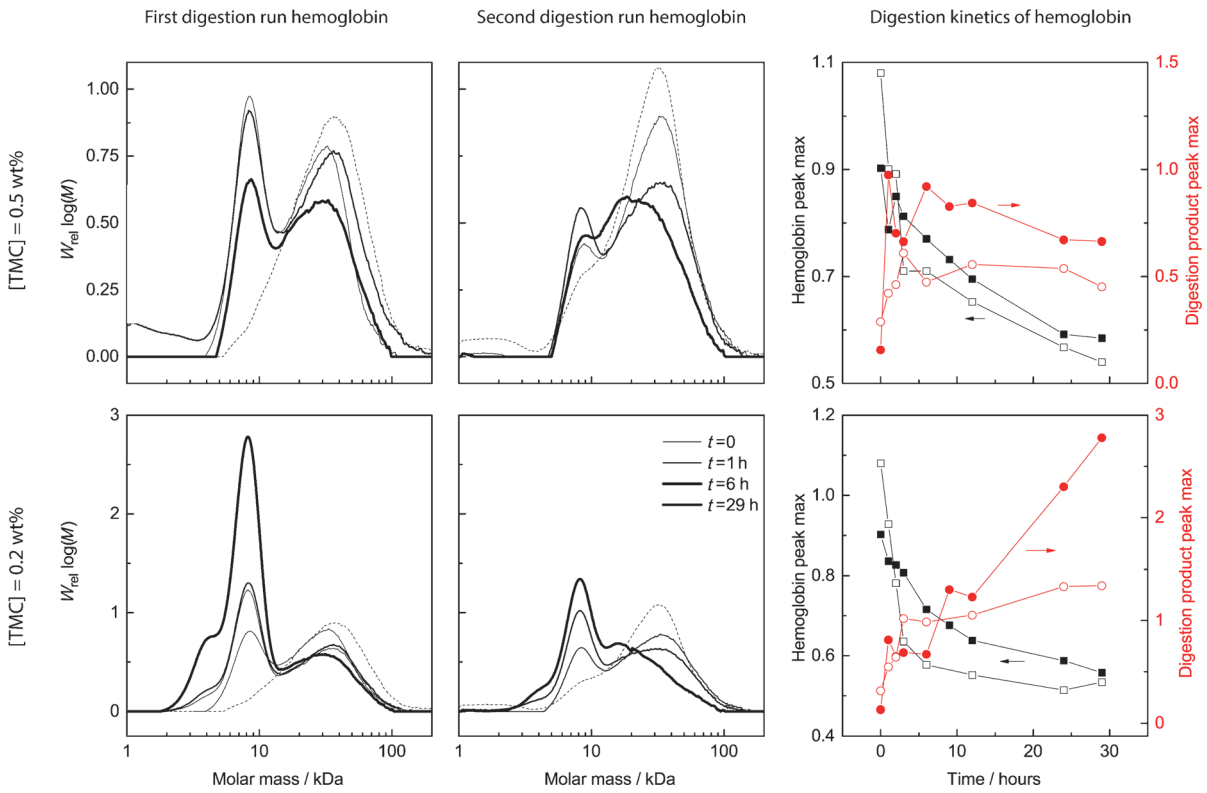


Figure 3. Molar mass distributions of the hydrolysate products after a hemoglobin solution (1 g L^{-1}, pH 2, HCl adjusted) had been in contact with a pepsin membrane at a temperature of 37 °C for 1, 6, or 29 hours. Two consecutive runs were performed with membranes prepared using the 0.5 wt% TMC (top) and 0.2 wt% TMC (bottom) solutions on PAN support. To determine the extent of pepsin deactivation, the second runs (middle) were performed with the same membrane samples that were used in the first run (left). Hemoglobin digestion kinetics as a function of time (right), derived from the evolution of the peak maxima of hemoglobin (■) and the digestion product (●).

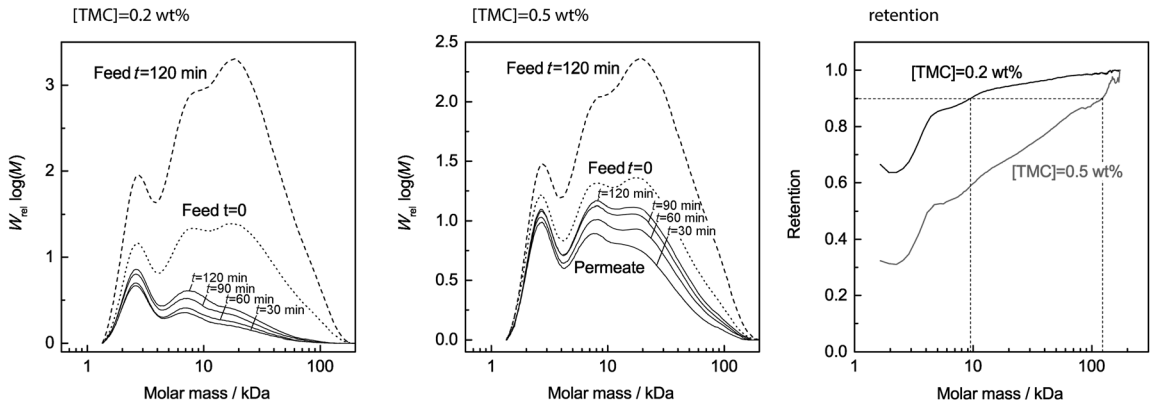


Figure 4. Molar mass distribution of the feed at $t = 0$ and 120 min and of permeate samples taken every 30 minutes during dead-end filtration of aqueous PEG solutions with mean molar masses of 2–40 kDa. Permeate compositions of the samples from membranes prepared with the 0.2 (left) and 0.5 wt% (middle) TMC solutions. Right panel: Retention as a function of the PEG molar mass in the permeate fraction calculated from the molar mass distribution. The MWCO values of the membranes prepared with the 0.2 and 0.5 wt% TMC solutions are 9.5 kDa and 120 kDa, respectively.

a lower TMC concentration (0.2 wt%) show a stronger retention of large molecules than membranes prepared with higher concentrations (0.5 wt%). The membranes prepared with the 0.2 wt% TMC solution retained 90% of the PEG molecules with a molar mass above 9.5 kDa. The feed and permeate compositions for the PAN support were the same, implicating that the retention of PEG molecules can be ascribed to the ultrathin cross-linked pepsin layer. This finding is in agreement with the XPS data, which suggested a higher cross-linking density for the membranes produced using lower TMC concentrations. The molecular weights for which the membranes show retention is in the same range as the molecular weight of the proteins used for the digestion experiments. Integration of the protein thin film on a mem-

brane support would allow for the selective removal of enzymatic conversion products while retaining larger molecules, such as proteins.

In conclusion, we have presented a method for the production of ultrathin pepsin membranes that show persistent enzymatic activity. Further studies of the membrane formation process should be considered to determine the number of active enzymes in the layer and the nanoscale enzyme distribution in the film. The interfacial polymerization technique can easily be applied to other proteins and other covalent organic linkers with different sizes, various numbers of reactive groups, and diverse reactivity.

Keywords: biomembranes · interfacial polymerization · membranes · pepsin · proteins

How to cite: *Angew. Chem. Int. Ed.* **2015**, *54*, 5910–5914
Angew. Chem. **2015**, *127*, 6008–6012

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Received: November 20, 2014
Revised: January 6, 2015
Published online: March 16, 2015

