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Detailed analysis of membrane adsorber pore structure and protein binding by advanced microscopy

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ABSTRACT

Commercial Sartobind® porous cation exchanger membranes, based on stabilized regenerated cellulose and with sulfonic acid (S) or carboxylic acid groups (C), were analysed with respect to their pore structure in dry, slightly swollen and wet state by three microscopic methods, conventional scanning electron microscopy (SEM), environmental SEM (ESEM), and confocal laser scanning microscopy (CLSM). The dehydration behaviour of the membranes was *in situ* observed at varied vapour pressure in the chamber of the ESEM, indicating some deformations of the macropore structure (largest pore diameters up to 20 μm) and significant changes in dimension and mobility of smaller cellulose fibers within these macropores, both as function of water content of the membrane. The binding of mono-Cy5-labelled lysozyme inside fluoresceine-labelled and unlabelled Sartobind® membranes was monitored by CLSM. The characteristic fluorescence intensity distributions in areas of (146 μm × 146 μm) indicated that protein binding takes place predominately in a layer which is anchored to a fine cellulose fiber network and, to a lower degree, directly to thick cellulose fibers. Due to the limited thickness of this binding layer, a significant fraction of the macropores remained free of protein. Protein binding as function of concentration and incubation times was also monitored by CLSM and discussed related to the binding isotherms for the membrane Sartobind® S and C. Further, a flow-through cell for the *in situ* monitoring with CLSM of protein binding during the binding step was built, and the results obtained for binding of lysozyme in membranes Sartobind® S indicate this experiment can give very important information on the dynamic behaviour of porous membrane adsorbers during separation: the lateral microscopic resolution in the x, y plane enables the identification of different breakthrough times as function of the location (pore structure), and this information can help to explain possible reasons for axial dispersion (in z-direction) observed in breakthrough analyses of the same separation in a chromatography system. The combination of advanced microscopy with detailed investigations of static and dynamic protein binding will provide a better understanding of the coupling between mass transfer and reversible binding in membrane adsorbers onto separation performance, and it will provide valuable guide-lines for the development of improved membrane adsorbers.

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1. Introduction

Separations with membrane adsorbers (membrane chromatography, solid phase extraction) are a very attractive and rapidly growing field of application for functional macroporous membranes [1]. The key advantages in comparison with conventional porous adsorbers (particles, typically having a diameter of ≥50 μm) result from the pore structure of the membrane which allows a directional convective flow through the majority of the pores. Thus, the characteristic distances (i.e. times) for pore diffusion will be drastically reduced [2–4]. The separation of substances is based on their reversible binding on the functionalized pore walls. The most frequently used interactions are ion-exchange and various types of affinity binding. Hence, the internal surface area of the membrane and its accessibility is most important for the (dynamic) binding capacity. Typical specific surface areas of macroporous membranes (otherwise used for microfiltration or conventional filtration, or as solid supports) are only moderate. Consequently, the development of high-performance membrane adsorbers should proceed via an independent optimization of pore structure and surface layer functionality, providing a maximum amount of binding sites with optimum accessibility [5].

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The most relevant commercial membrane adsorbers are indeed based on macroporous supports from established membrane formation process technology and subsequent chemical modification for the attachment of functional groups acting as binding sites. High binding capacities can be achieved by three-dimensional functional polymer layers on the pore wall. An example is the Sartobind® cation exchange membranes, consisting of a macroporous support from cross-linked regenerated cellulose and a grafted synthetic polymer containing sulfonic acid or carboxylic acid groups [6].

Typical characterization methods for membrane adsorbers are focussed on their functionality, i.e. the binding performance for various target solutes or nanoparticles, especially under flow-through conditions. Break-through curves give information about the influences of convective flow, pore diffusion and binding kinetics. Various mathematical models of membrane adsorption have also been developed [7–10]. However, even the most sophisticated state-of-the-art models use only average parameters (such as binding constant, binding capacity per membrane volume, flow rate and pore diameter) for the porous membrane with their functional pore surface.

In a real membrane adsorber, the pore structure can be quite heterogeneous (including pore shape and size distribution, tortuosity and dead-ended pores), binding can occur in relatively thick functional hydrogel layers extending into the pore space, and this structure may be dynamic due to swelling/shrinking of the base-material and/or the functional layer under separation conditions, or deformation by mobile phase flow.

The environmental scanning electron microscope (ESEM) allows investigation of wet and insulating samples without prior specimen preparation. It has been used to study the swelling behaviour of cellulose fibers *in situ* [11] and the wetting properties of macroporous polymer membranes [12]. By controlling sample temperature and the pressure of water vapour surrounding it, the water content of the sample can be adjusted *in situ*.

Confocal laser scanning microscopy (CLSM) provides the possibility of measuring the fluorescence emission of tracer fluorophores within three-dimensional objects with high local resolution and depth discrimination. The method had also been proposed for the characterization of porous membrane adsorbers [13–15]. The use of two different fluorescence dyes for labelled membrane and protein which can be detected independently enables simultaneous visualization of membrane pore structure and protein binding to the membrane functional layer by CLSM.

In this work, pore structure including morphology and dynamics in pore structure, as well as protein binding of commercial Sartobind® cation exchanger membranes were investigated by using conventional SEM and ESEM as well as CLSM. Further, the static binding patterns of protein in the membranes after various incubation conditions were investigated. As model protein, Cy5-labelled lysozyme was separated from unlabelled native protein to eliminate artefacts of non-specific interactions and ensure that all proteins are visible under the CLSM conditions [16,17]. *In situ* monitoring with CLSM of protein binding in the membranes was done with help of a special flow cell. In combination with the static and dynamic protein binding data, this work provides a better understanding of the coupling between mass transfer and reversible binding in membrane adsorbers onto separation performance.

2. Experimental materials and methods

2.1. Membranes and chemicals

The used membrane adsorbers were flat-sheet materials made from stabilized regenerated cellulose (Sartorius-Stedim Biotech, Göttingen, Germany): a strong cation-exchanger (Sartobind S; batch #2229) with sulfonic acid groups, a weak cation-exchanger (Sartobind C; batch #2231) with carboxylic acid groups and the unmodified base membrane as reference, all nominal pore size of 3–5 μm. Membrane thickness was 200–250 μm. The thickness of individual samples used for characterization was measured using digimatic micrometer (Mitutoyo Corporation, Japan). Static binding capacity for lysozyme according to the manufacturer are 0.8 mg/cm^2 (29 mg/ml) for Sartobind S and 0.6 mg/cm^2 (22 mg/ml) for Sartobind C [6]. The membranes were washed with Milli-Q water (from a system of Millipak®-40, Millipore) and equilibrated with buffer before the characterizations.

Lysozyme from hen egg (either “crystalline, powder, 85,400 units/mg” from Fluka, or “∼95% protein, ∼50,000 units/mg” from Sigma) was used as model protein for this work. The fluorescence dyes were 5-(4,6-dichlorotriazinyl) aminofluorescein (5-DTAF isoform, excitation/emission wavelengths of 492/516 nm) from Invitrogen and Cy5 mono-reactive NHS ester (excitation/emission wavelengths of 633/654 nm) from GE Healthcare. Sodium chloride (99.7%) was from VWR International, France. Potassium Hydrogenphosphate and potassium dihydrogenphosphate (98.0–100.5%) was from Applichem. Sodium carbonate (99%) and potassium chloride (≥99.0%) were from Fluka.

Buffers were prepared using Milli-Q water. 10 mM potassium phosphate buffer, pH 7.0 (buffer A) and 10 mM potassium phosphate buffer with 1 M sodium chloride, pH 7.0 (buffer B) were used for the measurements of binding isotherm and breakthrough curves. Sodium carbonate buffer (100 mM, pH 9.3) was used for labelling. Sodium phosphate buffer (variable ionic strength, pH 7.0) was used in the separation of labelled protein from native protein, and also in CLSM experiments.

2.2. Methods for SEM and measuring of wet samples using ESEM

Standard SEM images were recorded at high vacuum using an environmental scanning electron microscope (QUANTA FEG 400, FEI, Eindhoven, The Netherlands). The samples were pre-coated with silver DAG 1415 (Plano GmbH, Wettlar, Germany) for 1 min. The wet samples for ESEM images were equilibrated in 10 mM phosphate buffer for 24 h, after slightly drying with fine paper they were directly put into the ESEM chamber pre-set at a vapour pressure of 3.4 Torr, representing a relative humidity of 60%.

2.3. Membrane dehydration/hydration investigations

In situ study with ESEM was performed at 2 °C with help of a Peltier chip-controlled cooling stage (Table 1) using the same ESEM with modest humidity in chamber. Original Sartobind and gold-coated Sartobind membranes were used. The gold coating on the membrane resulted from step-wise current-less metallization using the solutions PD 11, SLOTONIP 61 and SLOTOGOLD 10 (Schlötter Galvanotechnik, Geislingen, Germany), respectively, for surface activation by deposition of catalytic amounts of palladium,

Table 1
ESEM relative humidity isobar chart at 2 °C [26]

Vapour pressure (Torr)	4.2	4.0	3.7	3.4	3.2	2.9	2.6	2.4	2.1	1.8	1.6	1.3
Rel. humidity (%)	80	75	70	65	60	55	50	45	40	35	30	25

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