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An Overview of Biofunctional Membranes for Tunable Separations, Metal-Ion Capture, and Enzyme Catalysis Based on Research from the Laboratories of Allan Butterfield and Dibakar Bhattacharyya

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An Overview of Biofunctional Membranes for Tunable Separations, Metal-Ion Capture, and Enzyme Catalysis Based on Research from the Laboratories of Allan Butterfield and Dibakar Bhattacharyya

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Abstract: At the 2007 North American Membrane Sciences (NAMS) National Meeting in Orlando, a session largely devoted to functionalized membranes was held in honor of Professor Dibakar Bhattacharyya of the University of Kentucky Department of Chemical and Materials Engineering and the Center of Membrane Sciences. This review will highlight some of the newest developments in biofunctional membranes conducted collaboratively by the Butterfield and Bhattacharyya laboratories. Specific details or raw data can be accessed in the original papers; this review will highlight the functionalization processes, major results, and applications. Emphasis is placed on: preparation of polypeptide-functionalized polycarbonate, track-etched membranes that are used in tunable separations; polythiol-functionalized alumina membranes that are used for metal-ion capture; and layer-by-layer assembled biofunctional membranes that are used for enzyme catalysis.

Keywords: biofunctional membranes, layer-by-layer assembly, metal-ion capture, tunable membranes

INTRODUCTION

Biofunctional membranes are entities in which biomolecules or cells are immobilized onto and into polymeric or inorganic membranes to give

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these relatively chemically inert matrices biological properties (1,2). Biofunctional membranes are used in catalysis (immobilized enzymes), analysis (biosensors), selective separations (affinity membranes), and as artificial organs (immobilized specialized cells, e.g., neurons; hepatocytes; islet cells) (1,2).

The Butterfield and Bhattacharyya laboratories have employed biofunctional membranes in most of the applications listed above. For example, we earlier collaboratively studied randomly immobilized enzymes on both hydrophobic and hydrophilic membranes to show:

a. Different membrane functionalization strategies can be used for maximum benefit depending on circumstances and applications desired (1,2).
b. Immobilized enzymes are more resistant to changes in conformation due to temperature, pH, ionic strength, etc., than are the enzymes themselves in free solution (3–6).
c. Using electron paramagnetic resonance in conjunction with active site-specific spin labels that the active site structure of immobilized enzymes could reflect the accessibility of the active site to substrates and the activity of the enzymes (3,7).
d. That combining the powerful methods of molecular biology with enzyme immobilization on membranes led to a state in which essentially all enzyme molecules were oriented away from the polymer surface and had activities equal to the enzymes in free solution and possessed the stability associated with immobilization (8,9).

As informative as these studies were in the principles of biofunctional membranes, these and other (10–12) studies suffered from not taking advantage of the much larger surface area of membrane pores compared to membrane surfaces. Some recent collaborative studies between the Butterfield and Bhattacharyya laboratories have addressed this shortcoming, and illustrations of applications of biofunctional membranes to tunable separations, metal ion capture, and enzyme catalysis conducted under convective flow in a variety of membrane settings are presented in this brief review.

POLYPEPTIDE-FUNCTIONALIZED POLYCARBONATE TRACK-ETCHED MEMBRANES: A STUDY IN TUNABLE SEPARATION

Polycarbonate track-etched membranes are characterized by their uniform pore geometry and narrow pore size distribution. We modified extant methods for electroless gold plating (13,14) using conditions of

convective flow in a pressurized filtrations cell rather than the usual diffusive conditions (15). When poly(vinylpyrrolidone) (PVP)-coated polycarbonate track-etched (PCTE) membranes were treated this way, pores were relatively uniformly coated with Au particles, which were highly distinct from the clumps of Au particles observed when the electroless gold plating was conducted under diffusive conditions (15).

Since Au has a high affinity for sulfur, thiol-gold chemistry was used to immobilize different ligands to the pores of PCTE membranes. For example, treatment of the Au-coated PCTE membranes with 3-mercapto-1,2-propanediol (MPD) permitted covalent attachment of vicinyl OH groups that were subsequently reacted with $NaIO_4$, producing a terminal aldehyde by well-known chemistry. The aldehyde functionality was reacted with poly(L-glutamic acid) (PLGA) to form the imine, that was subsequently reduced to the secondary amine by the reducing agent, $NaBH_4$. PLGA of greater than 17 kDa (116 repeat units) was immobilized on 30 nm pore size PCTE membranes, while PLGA of greater than 54 kDa (356 repeat units) was immobilized on 100 nm pore size PCTE membranes (15). PLGA ($pKa = 4.4$) undergoes conformational changes as a function of pH. For example, at low pH (<5.5) PLGA forms a helix with consequent lower charge and shortened length; in contrast, at higher pH PLGA is anionic and the repulsive charges cause an extended conformation of PLGA chains (15–17), i.e., the membrane is tunable.

At pH >6, at which the PLGA chains are in the extended conformation, the relatively uniform pore sizes of the respective 30 nm and 100 nm PCTE membranes modified with PLGA by using conditions of convective flow in a pressurized cell were significantly reduced, but still were sufficiently large to permit rapid water permeation. Use of the Hagen-Poisseuille relationship for capillary flow for the assumed cylindrical pores led to calculated pore radii as shown in Table 1. That is, PLGA modification of Au-coated, thiol-modified PCTE membranes

Table 1. Pore diameters calculated by the Hagen-Poisseuille law[a]

Membrane	30 nm Pore size	100 nm Pore size
Bare Membrane	100	100
Following Electroless Au Coating	71.7	65.5
Functionalized with PLGA (116 repeat units)	40.0	—
Functionalized with PLGA (356 repeat units)	—	56.6

[a]Percent respective diameter of the Bare Membrane.

under convective conditions led to relatively uniform pores that were highly functional (15). To validate the functional utility of these membranes, rejection studies of charged solutes (salts) were conducted. Since Donnan exclusion dictates separation of ions, the rejection of salts will depend highly on the membrane charge, which, again, because of the PLGA, is tunable by pH. In agreement with theory, rejection of Na_2SO_4 was two-fold greater than that of NaCl for equivalent concentration of anions. However, rejection decreased with increased salt concentration due to partial neutralization of membrane charge by the Na^+ counter ion.

Many other characterizations of these PLGA-linked PCTE membranes were reported (15). However, the key innovation was the development of a new method of polypeptide immoblization resulting in high chain density and a membrane performance that could be reversibly tuned by variation of pH. An additional value-added outcome of this research is the possibility of producing other stimuli-responsive materials using similar chemistry.

POLYTHIOL-FUNCTIONALIZED ALUMINA MEMBRANES FOR SELECTIVE METAL-ION CAPTURE

A different thiol modification method on an inorganic membrane than described above was used to carry out selective capture of Hg^{2+} (18). Inorganic chemistry principles of interactions of hard acids-hard bases and soft acids-soft bases underlie the observed strong interaction of Hg^{2+} with sulfur. Based on these interactions, prior methods to remove Hg^{2+} from wastewater or other matrices utilized thiol-based materials (19). However, generally such approaches, though better than materials that relied simply on physical adsorption, had drawbacks mostly due to the small fraction of immobilized ligands available for Hg^{2+} capture. In this work between the Butterfield and Bhattacharyya laboratories (18), we again used convective flow to maximize the amount and availability of Hg^{2+} -binding thiol ligands.

Inorganic alumina membranes are characterized by microfiltration pore sizes (in this case, 0.2 μm), are stable to a large number of solvents, and, because of their rich hydroxyl content, are amenable to functionalization (20). To maximize sorbent capacity for Hg^{2+}, the number of ligands needs to be large. Attempts to increase sorbent capacity by increasing the surface area of sorption materials invariably increase the diffusion resistance to ion transport. Hence, it is important to maximize the number of Hg^{2+} -binding ligands to achieve optimal Hg^{2+} capture. This paper outlined several approaches and tested their performance.

The alumina membranes were functionalized by a single thiol moiety or by polythiols. In the former case, we used two approaches. In one, 3-mercaptopropyl trimethoxysilane (MPS) was immobilized directly in the pores and surface of the membranes under convective flow by taking advantage of the known ability of silanes to form Si–O covalent bonds on the alumina membrane. In the second approach of single thiol modification, cysteine was added. This method required reaction of the OH groups of the alumina membranes with 3-glycidoxypropyl trimethoxysilane (GOPS), resulting in a terminal epoxide on the membrane ligand. Since epoxides easily react with primary amines, the amine-containing cysteine was coupled to the membrane as a single-point covalent attachment. Noting the pKa of amino groups, we performed this reaction at pH >10 in order to keep the amine unprotonated.

For polythiol coupling, poly(L-cysteine) was attached to the membrane using the same GOPS-mediated binding chemistry. This procedure was modified to extend the length away from the membrane surface of cysteine groups by first coupling PLGA to the membrane using the GOPS approach, followed by activation of each γ-carboxylic acid group of glutamic acid with dihexylcarbodiimide (DCC), and lastly followed by amide formation of cysteine on the activated carboxylic acid groups. In each case the pores of the alumina membranes were bifurcated: from the outside circumference chains of mono- or multiple cysteine residues extended into the pore, while a variable sized channel region in the interior of the pore remained (Fig. 1). Table 2 summarizes the thiol loading efficiency of these various approaches.

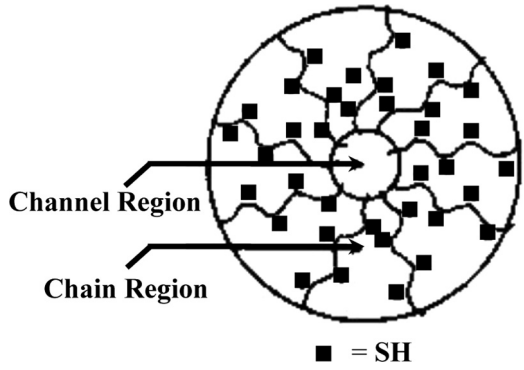

Figure 1. Schematic diagram of pore-resident polythiol chains in an alumina membrane. See text.

Table 2. Thiol attachment on alumina membranes and Hg^{2+} capture capability

Ligand	mmol SH per volume of the pore (2.5×10^{-2} cm^3)	Hg^{2+} Capture capacity (mm Hg^{2+}/g membrane)	Relative water flux in the presence of Hg^{2+} to the flux in the absence of Hg^{2+}
MPS	2.2	0.7	0.9
Cysteine	1.6	1.2	N.D.
Poly(L-cysteine)	3.9	1.2	0.25
PLGA-Cysteine (Polythiol A)	3.2	2.1	0.5
PLGA-Cysteine synthesized in the presence of 0.5 M NaCl (Polythiol B)	5.6	2.3	0.5

Studies of metal-ion capture were carried out using Hg^{2+} or Ca^{2+} nitrates under convective flow (Table 2), thereby minimizing mass transfer as a factor. The Hg^{2+} sorption efficiency is subject to the residency time of this metal ion in the pore, which in turn is related directly to the membrane porosity, surface area, and thickness and inversely to the flux through the membrane pores. Single cysteine binding from use of MPS, as expected, was not as efficient in Hg^{2+} capture as polythiol ligands due to the relatively large channel region resulting from a small amino acid attachment to the outside rim of the pore. Polythiol ligands led to greater Hg^{2+} capture than with cysteine alone. Moreover, polythiol A and polythiol B contain a carboxylic acid group per repeat unit, thereby leading to greater Hg^{2+} capture than poly(L-cysteine). Water flux decreased in the presence of Hg^{2+}, presumably because the channel volume is lessened by capture of this divalent cation. For the MPS-bound ligand, the flux drop was the least, and for poly(L-cysteine) the flux drop was the most, even greater than that of Polythiol A or Polythiol B ligands, which as shown in Table 2 have more SH groups per g membrane than those of poly(L-cysteine) (18). Even though poly(L-cysteine) has shorter chains (about 40 repeat units) compared to Polythiol A and Polythiol B (with about 240 repeat units), we suspect that this decreased flux in the presence of Hg^{2+} in the former case results from two considerations:

a. the chain density of poly(L-cysteine) is greater than that of Polythiol A and Polythiol B, resulting in less void volume between chains.
b. Polythiol A and Polythiol B were synthesized with PLGA, which in the presence of divalent cations is known to form helices (16).

Hence, the channel region of the pore would be increased by the helical conformation of the PLGA, thereby increasing flux compared to the case with poly(L-cysteine). That the flux drop primarily was due to Hg^{2+} binding to the ligands was verified by repeating the studies with another divalent cation, Ca^{2+}. In contrast to the nearly 50 percent drop in flux of Polythiol B-modified alumina membranes in the presence of Hg^{2+}, only a 20 percent drop in flux was observed in the presence of Ca^{2+}. Since Ca^{2+} is a hard acid, covalent bond formation with cysteine, a soft base, is unlikely, but binding to the carboxylic acid functionality of Polythiol B would be expected.

The key innovations of this study were the use of an inorganic membrane of good chemical and thermal resistance and abundant OH groups required for ligand attachment coupled with polythiol functionalities bound to the pores under convective conditions led to significant, high capacity Hg^{2+} capture from water streams. The presence of COOH groups on Polythiol A and Polythiol B led to decreased resistance to ion transport, which then requires lower operating pressures. Fast sorption kinetics and high accessibility of thiol groups are additional factors that make this system ideal for removing Hg^{2+} from wastewater.

Given that Hg^{2+} contamination in the environment represents a serious health risk and economic consideration of industrial processes in which Hg^{2+} is a by-product, measures to capture this toxic metal ion from solution are necessary. The process developed as a collaboration between the Bhattacharyya and Butterfield laboratory reviewed above represents a potentially powerful approach for mitigation of these serious problems of Hg^{2+} contamination.

LAYER-BY-LAYER ASSEMBLED BIOFUNCTIONAL MEMBRANES FOR ENZYME CATALYSIS

Several methods for immobilizing enzymes onto membranes exist, among which are random and site-specific covalent attachment, encapsulation, sol-gel solubilization, and such (1,2). Normally, enzyme-containing layer-by-layer assemblies are produced in membranes by alternating polyanion/polycation deposition on the external surface, thereby yielding thin films of thickness in the range of 10-several hundred nm. However, as stated above, due to the relatively small external membrane surface area only a small amount of enzyme can be immobilized. Using convective conditions, one can grow the multilayer assemblies inside the membrane pores that have a larger surface area compared to the outside surface area of membranes, and these can be used for traditional nanofiltration separations (21), or to produce biofunctional membranes (22).

Assuming the membrane pores are parallel and cylindrical, the ratio of the internal surface area to the external surface area of a membrane is given by

$$R = 2N\pi r_P L/[N\pi r_P^2(1-\varepsilon)\varepsilon^{-1}] = [L/r_P][\varepsilon/(1-\varepsilon)],$$

where r_P is the membrane pore radius, L is the membrane thickness, N is the number of pores, and ε is the membrane porosity. For example, for the Immunodyne ABC nylon membranes used in this study, assuming $L = 50\,\mu m$, $r_P = 450\,nm$, and $\varepsilon = 0.5$, R would equal 111, or an approximate 100-fold increase in accessible surface area in the pores relative to the membrane surface.

In this collaborative study between the Butterfield and Bhattacharyya laboratories using the layer-by-layer approach to form biofunctional membranes (22), the goal was to determine if a highly efficient, high capacity biofunctional membrane system could be produced. Different polyelectrolytes were used in this study as shown in Fig. 2. Immunodyne ABC nylon membranes (average pore size of 450 nm) were obtained from Pall Corporation with prefunctionalized anhydride groups on the membrane surfaces and coating the inside walls of the membrane pores. Since anhydrides readily react with amines, the N-terminal amine of poly (L-lysine) (PLL) or poly(L-glutamic acid) (PLGA) was first covalently

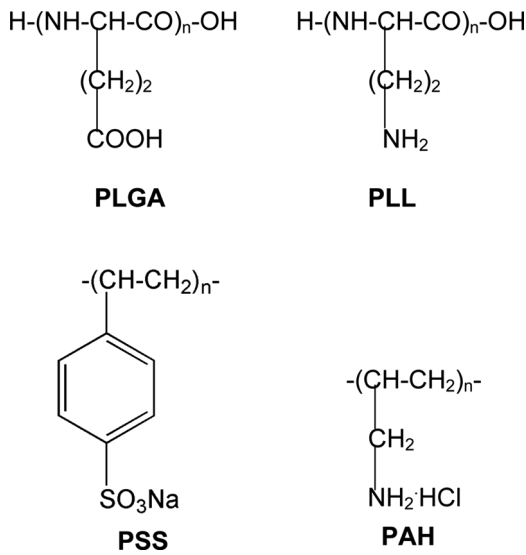

Figure 2. Structures of the polyelectrolytes used in formation of multibilayer assemblies. See text.

coupled to the membrane under convective conditions, thereby making a covalent link within the pores. The choice of initiating the multilayer assembly with PLL or PLGA was made on the basis of the first layer being positively charged or negatively charged, respectively. Since PLL has both the backbone and side chain amines, the PLL coupling was conducted at pH > 9.4 to ensure that only the alpha-amino group was deprotonated, and thereby being the only amino group used for coupling to the membrane.

Multilayers were constructed under convective flow either starting with PLL followed by PSS then by PAH to form a three-layer structure of positive-negative-positive charges or by initiating with PLGA followed by PAH then PSS to form a three-layer structure of negative-positive-negative charges. If stoichiometric interactions among ionizable groups is assumed following adsorption of each layer, the overall charge on PLL-PSS-PAH nylon membranes was positive, while that on PLGA-PAH-PSS nylon membranes was negative. The overall "brush" thickness of both the PLL-PSS-PAH membrane the PLGA-PAH-PSS membrane was estimated to be approximately 120 nm, and an estimated 22 percent of the pore volume was occupied by the multilayer (22). There was less of the positively charged multilayer assembly formed due to the steric effects of polymer size of PLL (MW 74.9 kDa) compared to PLGA (MW 21.5 kDa), making the initial covalent attachment of the amine more difficult. Of course, membrane permeability decreased after forming the multilayers.

Attachment of proteins to the multilayer assembly was investigated. Avidin (pI 10.5) was attached to the negatively charged PLGA-PAH-PSS membrane under convection at pH 6–8. When biotinylated amidobenzoic acid (BABA) was added to this system under convection approximately 85% of the four biotin binding sites per avidin were accessible, compared to about 50% of biotin binding sites of avidin randomly immobilized on membranes (23). Clearly, protein immobilization via convective flow into polyelectrolyte membranes formed by a layer-by-layer approach is of higher capacity than that of random immobilization.

To investigate whether the activity of enzymes would be similarly enhanced compared to random immobilization of enzymes to membranes, two enzymes were studied, galactose oxidase (GOX, pI 4.7) and alkaline phosphatase (AP, pI 4.5) at pH 6.0–8.0. In Table 3, K_M and V_{MAX} and relative activity from Lineweaver-Burk kinetics analyses are presented for both these enzymes in solution, attached electrostatically to the PLL-PSS-PAH membrane subjected to substrate permeation, and randomly immobilized on membranes, and the relative activity is given. As indicated, the K_M values remain nearly unchanged in immobilized enzymes compared to enzymes in solution. Consequently, the results

Table 3. Kinetic parameters of GOX and AP in homogeneous solution and immobilized onto PLL-PSS-PAH membranes

State of enzyme	K_M (mM)	V_{MAX} (mmol/mg-min)	Relative activity (%)
A. GOX			
Homogenous	33.3	0.244	100
Electrostatic immobilization	36.7	0.189	76
Random immobilization	58.3	0.006	2
B. AP			
Homogenous	0.33	26.2	100
Electrostatic immobilization	0.44	18.5	71
Random immobilization	0.67	0.21	1

are consistent with the notion that operation under convection conditions can eliminate diffusion resistance, and that the overall rate is dependent on the turnover rate of the enzyme. Moreover, the V_{MAX} values suggest that high apparent activity of the enzymes is achieved, in marked contrast to random immobilization of these enzymes (Table 3). These findings were consistent with the accessibility of binding sites of avidin as noted above.

Consequently, this simple and versatile method of protein immobilization can be used to form catalytic or affinity-based biofunctional membranes. Clearly, this system has weaknesses as well, most notably the instability of the electrostatically based molecular interactions under high ionic strength conditions. Nevertheless, this layer-by-layer assembly of polyelectrolytes is well suited for highly functional protein immobilization and conceivably could be of significant utility in medical, pharmaceutical, agricultural, and other industrial applications.

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This invited review arose from the author's presentation at the 2007 North American Membrane Society (NAMS) National Meeting in Orlando, Florida in which Professor Dibakar Bhattacharyya was honored by NAMS for his long and distinguished career in membrane research and for producing many rising stars in the next generation of excellence in membrane research. Many years ago now, D.B. Bhattacharyya and I formed the University of Kentucky Center of Membrane Sciences. We made a strategic decision at that time (1986) to pursue research on all aspects of membrane science, but especially focus on biofunctional

membranes. The University of Kentucky Center of Membrane Sciences remains one of the leading academic centers in this area of research, due in no small part to the contributions of Professor Bhattacharyya. Consequently, it is a high honor and distinct pleasure for me to dedicate this review paper for this special issue of *Separation Science and Technology* on Functionalized Membranes as a personal tribute to D.B. Bhattacharyya, who I regard as an extraordinarily gifted membrane scientist and a dear friend.

The recent studies outlined in this review article were the result of the dissertation research of one of the author's Ph.D. students, Vasile Smuleac. I greatly thank him for his excellent research. Professor Bhattacharyya served as Co-Preceptor for Dr. Smuleac, who is one of more than 5 such students who received advanced degrees in either Chemistry (with Butterfield the Preceptor and Bhattacharyya the Co-Preceptor) or Chemical and Materials Engineering (with Bhattacharyya the Preceptor and Butterfield the Co-Preceptor). This cross-college interaction and graduate student education within the University of Kentucky illustrates in a different way the collegial and collaborative relationship enjoyed by Professors Bhattacharyya and Butterfield.

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