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(54) Title: CHARGED FILTRATION MEMBRANES AND USES THEREFOR

(57) Abrégé/Abstract:
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CHARGED FILTRATION MEMBRANES AND USES THEREFOR

FIELD OF THE INVENTION

The invention relates to charged filtration membranes and their use for separation of a protein from solvent, low molecular weight solutes or a mixture of proteins. Modification of the membranes to generate charge includes modification of membrane pores to alter charge within a pore and alter the size of a pore. Consequently, the protein is separated from other solutes in a mixture based on size as well as net protein charge and membrane charge.

BACKGROUND OF THE INVENTION

A filtration membrane useful for protein separations is a synthetic (frequently polymeric) selective barrier for industrial or lab-scale microfiltration (MF) or ultrafitration (UF) (*see* Leos J. Zeman and Andrew L. Zydney, "Microfiltration and Ultrafiltration: Principles and Applications," 1996, Marcel Dekker, Inc., p. 3). In these processes, certain feed stream components, such as proteins, pass through pores of the membrane into a filtrate, while other, usually larger, proteins or components are retained by the membrane in the retentate (*see* Zeman and Zydney, *supra*, p. 3).

Protein ultrafiltration is a pressure-driven membrane process used for the concentration or purification of protein solutions (Robert van Reis and Andrew L. Zydney, "Protein Ultrafiltration" *in* Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis, and Bioseparation, M.C. Flickinger and S.W. Drew, eds., John Wiley & Sons, Inc. (1999), p. 2197). UF membranes typically have a mean pore size between 10 and 500 Angstroms, which is between the mean pore size of reverse osmosis and microfiltration membranes. Ultrafiltration separates solutes based on differences in the rate of filtration of different components across the membrane in response to a given pressure driving force (R. van Reis and A.L. Zydney, *supra*, p. 2197). Solute filtration rates, and thus membrane selectivity, are determined by both thermodynamic and hydrodynamic interactions (R. van Reis and A.L. Zydney, *supra*, p. 2197). Ultrafiltration is frequently used in downstream processing for protein concentration, buffer exchange and desalting, protein purification, virus clearance, and clarification (R. van Reis and A.L. Zydney, *supra*, p. 2197).

Protein purification is also accomplished using high-performance tangential flow filtration (HPTFF), with the desired protein collected in either the retentate or filtrate depending on the relative filtration rates (R. van Reis and A.L. Zydney, *supra*, p. 2197). HPTFF is useful for separating proteins of similar size using semipermeable membranes (See, for example, R. van Reis, et al., Biotech. Bioeng. 56:71-82 (1997) and R. van Reis et al., J. Memb. Sci. 159:133-142 (1999)). HPTFF achieves high selectivity by controlling filtrate flux and device fluid mechanics in order to minimize fouling and exploit the effects of concentration polarization (R. van Reis et al., J. Memb. Sci. 159:133-142 (1999)).

Despite the value of these advanced filtration methods, there is a need for improved filtration membrane characteristics such that separation speed may be increased without sacrificing membrane selectivity. Such improvements would reduce cost of separation and increase yield of valuable proteins.

SUMMARY OF THE INVENTION

The invention relates to filtration membranes possessing a net charge, either positive or negative, and further relates to methods of making the charged membranes and using them in the separation of a protein from a solute or mixture of solutes, such as salts, buffer solutes, or proteins. The proteins are separated based, in part, on the size of the proteins and, in part, on the net charge of the proteins. The charged membranes repel proteins having the same charge polarity as the membrane, thereby retaining such proteins on the upstream side of the membrane. Proteins pass through the membrane pores if they have a net neutral charge or a polarity opposite that of the membrane and are smaller than the average pore diameter. The sieving property of the charged membranes of the invention, measured

as the sieving coefficient, is dramatically improved relative to an uncharged membrane, while permeability is not sacrificed.

Conventional protein filtration balances membrane permeability with solute sieving. Ideally permeability is high to allow rapid separation, while sieving is low for selective retention of the desired protein. Using conventional filtration membranes, however, one of ordinary skill in the art sacrifices permeability to gain selectivity, thereby limiting separation speed and protein recovery, respectfully. The charged membranes of the invention, on the other hand, provide high permeability, thereby speeding separation, while lowering sieving for more selective separation and higher yield of the desired protein.

In one aspect, the invention involves a filtration membrane covalently modified with a charged compound or a compound capable of being chemically modified to possess a charge. The filtration membrane may be any membrane including, but not limited to, cellulose, cellulose diacetate and triacetate, cellulose nitrate, and cellulose diacetate/cellulose nitrate blends. Filtration membranes are commercially available from various sources including, but not limited to, Millipore Corp., Bedford, MA, and Pall Filtron Corp., Northborough, MA. Preferably the filtration membrane has hydroxyl groups available on the membrane surface for reaction with a derivatizing compound. Preferably the hydroxyl groups are primary alcohol moieties, such as those of a cellulose matrix. Preferably, the membrane is cellulose, more preferably the membrane is composite regenerated cellulose (CRC) (See, for example, U.S. Patent No. 5,522,991 for a description of cellulosic membranes.) A cellulose membrane has the advantages of inherent low fouling characteristics, hydrophilicity, the availability of primary alcohol groups (from the glucose moieties of cellulose) for reaction with a charged compound, and stability under alkaline conditions. A CRC membrane has the additional advantage of mechanical strength.

In one embodiment, the membrane is a cellulose membrane, preferably a CRC membrane, modified to have a net charge (positive or negative), wherein the permeability versus sieving performance is enhanced. For example, for a given membrane permeability, the sieving coefficient is decreased by at least 1.5 fold for a protein retained on the upstream side of the membrane when the membrane and retained protein have like charge polarity (positive or negative).

In another embodiment, the invention involves a membrane, preferably a CRC membrane, in which a plurality of the primary alcohol groups are covalently linked to a charged compound. Preferably, the charged compound retains its charge under the conditions used to separate the protein from solutes or a mixture of proteins.

In another embodiment, the charged compound is positively charged. The positive charge may be generated from any compound that retains its charge under the conditions of protein separation. For example, the charge may be generated by, but not limited to, an amine, a quaternary amine, and the like. Where the compound is an amine, preferably the amine has two or three lower alkyl groups covalently attached to achieve an amine that is capable of maintaining its charge polarity under the conditions of protein separation. Preferably, the lower alkyl groups have from one to 8 carbon atoms. More preferably, the alkyl groups are methyl, ethyl, and propyl groups. For example, the amine moiety of the positively charged compound may be a trialkyl or dialkyl amine, such as trimethyl amine, triethyl amine, diethylamino ethyl, and the like.

In another embodiment, the charged compound is negatively charged. The negative charge may be generated from any compound that retains its charge under the conditions of protein separation. For example, the charge may be generated by, but not limited to, an acid, such as a carboxylic acid, a sulfonic acid, carboxymethyl sulfonate, methyl sulfonate, and the like.

In still another embodiment, the charged compound comprises a linker arm between the charged moiety and the moiety covalently linked to a reactive group of the membrane. Where the membrane is a CRC membrane, the

linker arm separates the charged moiety and a primary alcohol group of the cellulosic matrix with which the linker reacts. The linker arm allows the charged moiety to project away from the surface of the membrane. Where the charged compound modifies the surface of a membrane pore, the linker arm allows the compound to project into the lumen of the pore, thereby modifying the size of the pore. Larger membrane pores will be reduced in size, smaller pores are filled, and still smaller pores fail to be penetrated by the compound due to steric hinderance and/or electrostatic repulsion. Consequently, membrane pore size distribution is narrowed, providing improved protein separation.

In another embodiment, the linker arm comprises an alkyl chain of from one to and including twenty carbon atoms. The alkyl chain may be branched and the branch may link a first charged moiety to a second (or additional) charged moiety. The linker arm may be any chain of atoms or molecular moieties that are themselves inert to the reaction conditions used to link the charge to the membrane, and inert to the conditions of protein separation. The length of the linker arm is chosen according to the desired pore size modification. Preferably the linker arm length allows the reactive charged compound to penetrate and react within some of the pores thereby narrowing the pore size distribution. Linker arms may include, but are not limited to, carbohydrates, dextrans, saccharides, peptides (having charged or uncharged amino acid side chains), polymers (such as polyvinyl derivatives, polyether derivatives, and the like), and like chains. Where the filtration membrane is hydrophilic and aqueous reaction conditions are used to link the charged compound to the membrane, the linker arm is preferably hydrophilic and the charged compound in its reactive form (for reaction with the membrane) is soluble in the aqueous reaction solution.

Accordingly, the invention may comprise a linker arm that is an alkyl chain of between one and twenty carbon atoms in length, preferably one to ten, more preferably one to seven carbon atoms. Alternatively, the linker arm may be a carbohydrate or dextran chain of from one to, and including, fifteen saccharide moieties, preferably one to ten, more preferably one to five saccharide moieties. The linker arm may be a peptide of one to twenty five amino acids, preferably one to fifteen, more preferably one to ten amino acids. In addition, the linker arm may be any polymer of from one to twenty five repeat units that is inert to the reaction and separation conditions. The linker arm may be branched, wherein each branch is shorter than the length of the main branch (the linker arm) linked to the reactive group. Each branch may end in a charged moiety and the charge of each charged moiety is the same polarity (either positive or negative).

In an embodiment, the reactive charged compound has the general formula, X-L-Y, where X is a reactive group that reacts with a reactive group on the membrane, L is the linker arm, and Y is the charged group. Accordingly, where the membrane is a CRC membrane and the reactive groups on the membrane are primary alcohol groups, X of the reactive charged compound is a moiety that promotes reaction with the primary alcohol groups under aqueous conditions. As such, X is preferably a leaving group susceptible to nucleophilic attack by a primary alcohol group to form an ether linkage between the cellulosic carbohydrate matrix and the linker arm of the charged compound. Thus, useful reactive charged compounds are alkyl halides, and the reactive group is a halide including, but not limited to, chloride, bromide, or iodide.

In yet another aspect, the invention involves a method of making a charged membrane of the invention, the method comprising reacting a reactive charged compound with a plurality of reactive sites on the membrane such that the charged portion of the charged compound is covalently linked to the membrane. After reaction, the net charge of the membrane is positive or negative. Preferably, the reactive charged compound penetrates a plurality of membrane pores and modifies the pore size distribution. Preferably, the pore size distribution is narrowed.

In an embodiment, the method of the invention comprises a reactive charged compound that is uncharged during the covalent attachment to the membrane and is later reacted to create the charge (either positive or negative)

that generates the net charge for the membrane. The reactive charged compound may be added to the end of the linker arm (or branches) after the linker arm is covalently attached to the membrane. Where the charged group is positive, the charged group may be an amine, a quaternary amine, such as a dialkyl or trialkyl, and the like. Where the charged group is negative, the charged group is an acid, such as a sulfonic acid, a carboxylic acid, a carboxymethyl sulfonyl group, and the like. The charged group is chosen as a moiety that will maintain its charge under the conditions of the protein separation method of the invention.

In another aspect, the invention involves a membrane filtration method for separating a protein from a solute or mixture of solutes (such as salts, buffer solutes, or proteins) using a charged membrane of the invention. Preferably the method involves (1) contacting a protein within a solute mixture with a charged cellulosic membrane, more preferably a CRC membrane that has been reacted with a reactive charged compound to generate a link, via a linker arm, to a charged group, wherein the protein to be retained has a net charge that is the same as the charge polarity of the membrane under the separation conditions, and (2) separating the protein from at least one other solute or protein in the mixture, wherein the other protein has a different net charge or is neutral.

In an embodiment of the invention, prior to contacting a protein within a mixture with the membrane, the pH of the mixture is altered causing the net charge of the desired protein to be the same as the charge polarity as the membrane. At the same time, the pH change renders at least one solute to be separated from the desired protein neutral or opposite the membrane charge. During the contacting and separating steps, the neutral solute passes through the charged membrane into the filtrate, while the desired charged protein is retained on the upstream side of the membrane. This embodiment of the invention may be repeated to successively remove solutes from the protein mixture.

In an embodiment of the invention, the filtration method is ultrafiltration (see generally, van Reis and Zydney, "Protein Ultrafiltration," *supra*, p. 2197). In another embodiment of the invention, the filtration method is high-performance tangential flow filtration (see generally, R. van Reis, et al., J. Memb. Sci. 159:133-142 (1999)).

These and other objects, advantages and features of the present invention will become apparent to those persons skilled in the art upon reading the details of the compositions, methods, and uses as more fully set forth below. Each reference cited herein is herein incorporated by reference in its entirety with particular attention to the description of subject matter associated with the context of the citation.

DESCRIPTION OF THE DRAWINGS

Fig. 1 is a diagram showing charged spheres representing proteins having a net charge based on the pKa values of surface amino acids at a given pH of the separation solutions (retentate and filtrate). A filtration membrane is diagrammed having a net positive charge. Flow of the separation solution is in the downward direction through the membrane, retaining proteins having the same charge as the membrane (positive) in the retentate, while net neutral and negative proteins flow through the membrane with the filtrate.

Fig. 2A is a diagram of a cellulose membrane reacting with a reactive charged compound, 3-bromopropyl trimethyl ammonium bromide. The -OH moieties represent the primary alcohols of a CRC matrix. The primary alcohols react with the alkyl halide in this diagram to form a covalent ether linkage connecting the charged moiety to the cellulose matrix. Fig. 2B is a similar diagram of a cellulose membrane in which the primary alcohols reacted with a reactive charged compound, 3-bromopropyl sulfonate, to form a covalent ether linkage. In these examples, the reactions are base catalyzed.

Fig. 3 is a diagram showing a pore of a filtration membrane reacted with 3-bromopropyl trimethyl ammonium bromide. One pore is enlarged to show the charged compound linked to the surface of the pore and projecting into the lumen of the pore. As a result of the surface modification, the pore size is effectively decreased. In addition, the

positive charge of the pore repels a protein of like charge, increasing the likelihood that the protein will be retained relative to neutral proteins in a mixture.

Fig. 4 is a graph showing the relationship between membrane permeability (x axis) and protein sieving (y axis) for ten commercially available uncharged filtration membranes. The graph indicates that increasing permeability corresponds to an increase in sieving. The test solute for the commercial membranes, having a mean size of 10 kD, was uncharged dextran. "F" and "M" refer to membrane manufacturers Filtron and Millipore, respectively.

Fig. 5 is a graph showing the relationship between filtrate flux (ml/min, x axis) and sieving (y axis) for uncharged and charged membranes having 300 kD or 1000 kD MW cutoffs. Flux is proportional to membrane permeability (*see*, Zeman and Zydney, *supra*, p. 16). As flux increases, sieving increases. Unlike in conventional membranes, however, when charge is added to the membrane, the sieving coefficient of a like-charged protein decreases for a given flux value.

Fig. 6 is a graph showing the relationship between membrane permeability (y axis) and sieving (x axis) for an uncharged membrane having a 300 kD average pore size (white diamond) and an uncharged membrane having a 1000 kD average pore size (white circle) defining the solid line. A positively charged membrane having a 300 kD average pore size (black diamond) and a positively charged membrane having a 1000kD average pore size (black circle) define the dashed line. The solute protein is positively charged recombinant human anti-HER2 monoclonal antibody (rhuMAb HER2). The data indicate that for a given permeability, sieving is dramatically decreased (in this example, by approximately two orders of magnitude) when the membrane has the same charge as the protein being tested.

Before the present filtration membranes and methods of making and using them are described, it is to be understood that this invention is not limited to the particular compositions of matter and processes described, as such compositions and methods may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting since the scope of the present invention will be limited only by the appended claims.

DESCRIPTION OF THE EMBODIMENTS

Definitions

"Cellulose membrane" refers to a cellulose polymer on a microporous membrane, where the cellulose is repeating units of D-glucose. The primary alcohol group of a glucose monomer provides the reactive species on the membrane to which the charged compound is covalently attached.

"CRC membrane" refers to a composite regenerated cellulose membrane prepared by casting cellulose on a microporous substrate to control the average pore size and limit the number of defects in the cellulose sheet. CRC membranes are preferred in the practice of the invention because they have greater mechanical strength than cellulose membranes while retaining the hydrophilicity and low fouling characteristics of cellulose useful in protein separations.

"Charged compound" refers to the compound linked to the filtration membrane, wherein the compound comprises a moiety having a positive or negative charge under the conditions used to separate a protein from a mixture of proteins. According to the invention, the charged compound may further comprise a linker arm between the membrane and the charged moiety such that the charged compound projects from the surface of the membrane. Where the charged compound projects from the surface of a pore into the lumen of the pore, the charged compound modifies the effective size of the pore and modifies the pore size distribution of the membrane.

"Reactive charged compound" refers to the charged compound prior to linkage to the membrane, such that the reactive charged compound still retains the reactive moiety that promotes the membrane-reactive charged compound reaction. For example, where the charged compound is a propyl trimethyl ammonium ion covalently

attached to a CRC membrane, the reactive charged compound may be bromopropyl trimethyl ammonium bromide. The covalent attachment involves nucleophilic displacement of the alkyl bromine by a primary alcohol of the cellulose matrix (see Fig. 2A, for example).

"Linker arm" refers to the portion of the charged compound molecule between the portion that reacts or has reacted with a reactive group on the surface of a filtration membrane and the charged moiety. Preferably, the linker arm is a chain of atoms or molecular subunits, which chain is inert to the reaction conditions used to covalently link the charged compound to the membrane, and is further inert to the aqueous conditions used during protein separation. A linker arm may comprise, but is not limited to, an alkyl chain of from one to twenty carbon atoms, a carbohydrate chain of from one to fifteen saccharide moities (including, for example, ribose and deoxyribose), a dextran chain of from one to fifteen saccharide moities, an amino acid chain of from one to twenty five amino acids, and other polymers (such as those used to manufacture the membrane itself) of from one to twenty five repeat units. Where a charged compound comprises an amino acid chain as a linker arm and the charged moiety is the terminal amino acid of the chain, the side chain of the terminal amino acid is preferably a charged side chain.

"Sieving" refers to the ratio of the concentration of a particular solute in the filtrate (downstream of the membrane) to the concentration of the same solute in the feed solution (upsteam of the membrane) (*see* Zeman and Zydney, *supra*, p. 308). Generally a high sieving value suggests that the solute readily passes through the membrane, while a low sieving value suggests that the solute is largely retained by the membrane. Where it is desired to retain a solute upstream of the membrane, a reduced sieving coefficient is preferred.

"Permeability" refers to the filtration rate divided by the net pressure drop across the membrane. Permeability is therefore the inverse of membrane resistance. Membrane permeability is primarily determined by pore size distribution, porosity (pore density), membrane thickness, and solvent viscosity. Generally, as permeability increases, sieving increases. When sieving is improved due to the addition of a charged compound to the membrane, the sieving improvement is an improvement relative to a membrane having substantially the same permeability (within 50%, preferably 30%, more preferably within 10% the same permeability) as the charged membrane, but lacking the charged compound. Thus, where the improvement is a reduction in sieving because a charged solute, such as a protein, is retained by a like-charged membrane, the sieving is a reduction at comparable or substantially the same permeability. Consequently, the rate of filtration is maintained. while the selectivity of the membrane is improved.

"Net charge" when referring to a membrane or protein charge is meant a charge that is predominately positive or negative, but does not refer to a specific value for the number of positive charges versus the number of negative charges on the membrane or protein, unless otherwise noted. Similarly, "like charge" and "same charge" refer to the situation in which a protein having a given charge, positive or negative, is related to a membrane or other protein having a given charge, either positive or negative.

"Protein mixture" refers to various proteins of different sizes and net charges under given separation conditions of pH and ionic strength. According to the separation method of the invention, when the net charge of a protein of interest is known or is manipulated by alteration of pH conditions to have a net positive charge or net negative charge, the protein may be separated from differently charged or neutral proteins by passing the protein mixture through a membrane of the invention having the same charge as the protein of interest (i.e., either a net positive charge or a net negative charge). For example, the invention contemplates a method of separating a desired protein from at least one protein of a mixture of proteins in an aqueous buffered solution, altering the pH of the solution such that the desired protein has a net charge and a protein to be separated from it is neutral or has a net charge that is opposite the net charge of the desired protein. Next the protein mixture is contacted with a charged cellulose filtration membrane of the invention wherein the desired protein and the membrane have like net charges.

The desired protein is separated from the neutral protein and the oppositely charged protein by retaining the desired protein upstream of the membrane and filtering the neutral or oppositely charged protein through the membrane. This process is repeated until the desired protein is separated from a chosen number of proteins of the mixture.

Alternatively, the invention contemplates a method of separating a desired protein from at least one protein of a mixture of proteins in an aqueous buffered solution by altering the pH of the solution such that the desired protein is neutral and the protein to be separated from it has a net charge that is the same as the charged membrane. Next the protein mixture is contacted with the charged cellulose filtration membrane of the invention. The desired protein is separated from the charged protein by retaining the charged protein upstream of the membrane and filtering the desired protein through the membrane. This process is repeated until the desired protein is separated from a chosen number of proteins of the mixture.

"Pore size distribution" refers, basically, to the number of pores having an actual radius, R, near some theoretical radius, r, expressed as the probability density function (*see*, Zeman, L.J. and Zydney, A.L., *supra*, p. 299-301). As the standard deviation of actual pore radii increases, the pore size distribution increases. Narrowed pore size distribution results from a reduction in the standard deviation of the pores from the theoretical value. This is achieved, for example, when the sizes of some of the larger pores are reduced by addition of charged compound into the larger pores of a charged membrane. Fig. 3 diagrams such a pore size reduction. The principle of liquid-liquid pore intrusion is useful for measuring pore size distribution (*see* R. van Reis and A.L. Zydney, *supra*, p. 2201). According to this principle, two highly immiscible liquids, such as solutions of a sulfate salt and a poly(ethylene glycol) are contacted through mixing to reach equilibrium partitioning. The membrane to be tested is primed with one of the liquids so that all pores are filled. After draining the feed channels, the second fluid is introduced into the system. The first fluid is then displaced out of the pores by the second fluid, and the flow rate is measured as a function of trans-membrane pressure. The resulting data provide information on pore size distribution and can be correlated with the nominal molecular weight cutoff (*see* R. van Reis and A.L. Zydney, *supra*, p. 2201).

EXAMPLES

The following examples are provided so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make the compositions of the invention and how to practice the methods of the invention and are not intended to limit the scope of what the inventor regards as his invention. Efforts have been made to insure accuracy with respect to numbers used (e.g. amounts, temperature, etc.), but some experimental errors and deviation should be accounted for. Unless indicated otherwise, temperature is in degrees C and chemical reactions were performed at atmospheric pressure.

Example 1: Preparation of Charged Filtration Membranes

For illustrative purposes, this example discloses charged CRC membranes and methods of making them. It is understood that charge may be added to a membrane by chemistries known to one of ordinary skill in the art such that the product membrane is charged and selectively retains a desired protein in the retentate, and passes uncharged or oppositely charged proteins with the filtrate. The product charged membrane has the characteristic of a lower sieving coefficient for a given permeability relative to an uncharged membrane, or a higher permeability for a given sieving coefficient.

CRC membranes are preferred due to their hydrophilicity as well as their mechanical stability to reverse pressure. Stability under basic conditions allows for complete, rapid cleaning and storage. CRC membrane hydrophilicity minimizes protein fouling and simplifies membrane cleaning. The membranes are preferably reacted with the reactive charged compound in an aqueous solution. Aqueous reaction conditions are advantageous because flammable, organic compounds are avoided and the aqueous-soluble compounds are more readily and completely

removed from a charged CRC membrane intended for use in pharmaceutical applications. CRC membranes were obtained from Millipore, Corp. (Bedford MA.The 300 kD MW membranes were designated PLCMK. The 1000 kD MW membranes were designated PLCXK).

The primary alcohol groups of the D-glucose moieties of cellulose are targeted for derivatization because they can react without undermining the integrity of the cellulose matrix. Primary alcohols (R-OH) react with alkyl halides (R'-X, where X is Br, for example) to produce ethers (R-O-R'). In this example, a CRC membrane was reacted with 2 molar 3-bromopropyl trimethyl ammonium bromide (as diagrammed in Fig. 2A) in 0.1 N NaOH at room temperature overnight. The reacted membranes were washed in distilled water and used for protein filtration or stored in 0.1 N NaOH at ambient temperature. The membranes are stable at these conditions for at least six months.

The quaternary ammonium ion has a positive charge in aqueous solution from approximately pH 2 to pH 10, inclusive. This pH range corresponds to the pH range in which most proteins are structurally intact or recoverable to an active state, making a quaternary amine useful as the charged moiety of the charged compound. Halides such as bromide, chloride, and iodide are useful reactive moieties because they are good leaving groups for nucleophilic attack by the oxygen of the primary alcohol. It is understood, however, that other anionic groups well known to those of ordinary skill in the art may be used to facilitate the reaction.

Reacting the alkyl halide directly with the primary alcohol of the cellulose is advantageous because it is a one-step reaction. This method also has the advantage of avoiding the need to first derivatize the membrane with a strongly nucleophilic moiety that may not be fully reacted during subsequent steps or may require additional reactions to remove or deactivate the added nucleophile. Another advantage of the method is that many alkyl halides are commercially available. Thus, the method of preparing the charged membranes of the invention is rapid and convenient, thereby saving time and cost.

Negatively charged membranes were generated using a similar method. The reactive compound was 2 molar 3-bromopropane sulfonic acid in 0.1 N NaOH. The reaction is diagrammed in Fig. 2B, and the reaction conditions were as disclosed above for 3-bromopropyl trimethyl ammonium bromide. The sulfonic acid moiety remains negatively charged in a pH range useful for many protein separations.

To verify that a positively or negatively charged CRC membrane was uniformly derivatized, the membranes were stained with a dye having a charge opposite that of the tested membrane. CRC-O-propyl trimethyl ammonium bromide membranes were rinsed extensively in distilled water and dipped in aqueous ponceau red solution. The negatively charged dye uniformly stained the positively charged membrane, but did not stain a control membrane that had been exposed to 0.1 N NaOH without the 3-bromopropyl trimethyl ammonium bromide. Similarly, CRC-O-propane sulfonic acid membranes were extensively rinsed in distilled water and dipped in aqueous methylene blue solution. The positively charged dye stained the negatively charged membrane, but did not stain the control membrane that had been exposed to 0.1 N NaOH in the absence of 3-bromopropane sulfonic acid. Thus, the membranes were uniformly and extensively derivatized by the alkyl halides in this example.

Example 2: Membrane Pore Size Distribution is Modified

Membrane pores are infiltrated by reactive charged compounds when steric factors, such as size and linker arm length, and electrostatic factors, such as charge repulsion, allow it. Thus, according to the invention, membrane pores large enough to allow infiltration of a given reactive charged compound are derivatized by the charged compound such that the size of the pore lumen is reduced. Fig. 3 shows a diagram of a membrane pore having propyl trimethyl ammonium ions covalently attached to the wall of the pore and projecting into the lumen.

The length of the linker arm controls the degree to which the charged compound projects into the lumen and reduces its effective diameter. The charge generates a positively charged region from which a similarly charged

protein will be excluded. Thus, a desired protein having an overall charge that is the same as that of the derivatized membrane is repelled by the surface of the membrane as well as by the pores of the membrane, thereby improving retention of the desired protein. Neutral proteins will tend to pass through the charged membrane pores with the filtrate, being neither attracted to nor repelled from the surface or pores of the membrane. Example 3: Methods of **Using a Charged CRC Membrane to Separate Proteins**

Using conventional membranes, permeability is sacrificed for improved sieving, or sieving is sacrificed for improved permeability. In general, as permeability of a membrane increases, the sieving coefficient increases. This reflects the fact that more of the protein to be retained passes through a highly permeable membrane, making the filtration process faster, but less selective (high sieving coefficient), resulting in lower yield of desired protein and less complete separation. Fig. 4 is a graph demonstrating that commercial ultrafiltration (UF) membranes sacrificed sieving for improved permeabililty. The UF membranes of Fig. 4 were tested using a standardized mixed dextran test (*see*, for example, Zeman and Zydney, *supra*, p. 183-88). The tested membranes were from Pall Filtron (Cellulose, Omega™ (polysulfone with hydrophilic modification), Alpha™ (polyethersulfone modified to lower fouling), and Nova™ (polyethersulfone)) and Millipore (Regenerated Cellulose (RC), Biomax™ with screen A, Biomax™ with screen B, and PES (polyethersulfone)).

The charged membranes of the invention decrease the sieving coefficient for a given permeability when compared to an uncharged membrane. By charge-modifying an uncharged membrane, the problem of sacrificing sieving for permeability (or vice versa) is solved. The membranes of the invention have dramatically improved sieving for a given permeability when compared to the uncharged control membrane.

Fig. 5 shows the relationship between filtrate flux (mL/min, x axis) and sieving (y axis) for uncharged and charged membranes having 300 kD or 1000 kD MW cutoffs. As flux, a factor that is proportional to membrane permeability (*see*, Zeman and Zydney, *supra*, p. 16), increases, sieving increases. Unlike in conventional membranes, however, when charge is added, the sieving coefficient decreases for a given flux value. At a relatively high flux of 8 mL/min (see "a" on Fig. 5), a 1000 kD CRC charged membrane (CRC 1000+) or a 300 kD CRC charged membrane (CRC 300+) has an approximately 10 fold lower sieving coefficient than the uncharged control membrane. At a lower flux of 5 mL/min (see "b" on Fig. 5), the sieving coefficient is reduced approximately 100 fold for both the 1000 kD and the 300 kD CRC charged membranes. In this example, the charged compound added onto the surface and pores of the CRC membrane was propyl trimethyl ammonium ion. The membranes were prepared as disclosed in Example 1. The protein was rhuMAb HER2 under conditions that rendered it positively charged, like the CRC+ membrane. The sieving coefficient was calculated using the rhuMAb HER2 concentration (ng/mL) in the feed solution (known) divided by the rhuMAb HER2 concentration in the filtrate (determined by ELISA).

The data at point "b" of Fig. 5 were recalculated and plotted to show the relationship between permeability and sieving in Fig. 6. These data also show that for a given permeability, the sieving is reduced 100 fold for both 300 kD and 1000 kD MW cutoff CRC membranes when charge is added. Thus, an optimum permeability may be chosen to allow rapid separation, while greatly improving (rather than harming) the sieving coefficient for the membrane and allowing improved separation and yield of the desired protein. These dramatic and unexpected improvements in membrane properties result from the addition of charged compounds to the surface and pores of the membrane.

Separation of a mixture of proteins according to the invention. A mixture of proteins, each having a different pI were separated using the charged membranes of the invention. In a first example, two proteins were separated. RhuMAb HER2, the desired protein, has pI 8.9. Bovine serum albumin (BSA), an impurity, has pI 4.8. The proteins were mixed in a pH 4.5 buffer causing rhuMAb HER2 to be positively charged, and BSA to be neutral. A positively charged membrane of the invention, such as a CRC-O-propyl trimethyl ammonium membrane was used for the

separation. The proteins and buffer were contacted with the positively charged membrane. Of the two proteins, only BSA passed through the membrane into the filtrate because it was not repelled by the positive surface or pores of the membrane. The desired protein, rhuMAb HER2 was retained upstream of the positively charged membrane.

A related strategy is useful for retaining a protein, such as BSA in this example, which has pI 4.8, while the desired product protein, for example, Fab (e.g. anti-VEGF Fab), having a pI of 8.1, is allowed to pass through a membrane to effect separation of the two similarly sized proteins, in this example 68 kD and 45 kD, respectively. The separation is performed using a pH 8 buffer, thereby causing Fab to be neutral. The BSA and Fab protein mixture in pH 8 buffer is contacted with a CRC-O-propane sulfonic acid membrane of the invention (negatively charged at pH 8) to allow the higher pI protein (Fab, pI 8.1) to pass through the membrane into the filtrate and retain the lower pI, negatively charged protein (BSA, pI 4.8) upstream of the negatively charged membrane.

A pH gradient may be preferred over the step-wise pH change described above, although a similar strategy for selective protein retention and removal is followed.

The foregoing written specification is considered sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the examples provided, since the examples are illustrative of certain aspects of the invention and any compositions or methods that are functionally equivalent are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

What is claimed is:

CLAIMS

1. A filtration membrane comprising a covalently attached charged compound,

wherein membrane sieving for a solute is improved by at least 1.5 fold relative to the same membrane lacking the charged compound,

wherein membrane permeability is substantially the same for the membrane lacking the charged compound, and

wherein the filtration membrane comprises a cellulose polymer.

2. The filtration membrane of claim 1, wherein membrane pore size distribution is reduced relative to the membrane lacking the charged compound.

3. The filtration membrane of claim 1, the compound comprising a linker arm.

4. The filtration membrane of claim 3, wherein the linker arm comprises a heteroatom selected from the group consisting essentially of N, O, S, and P.

5. The filtration membrane of claim 3, wherein the linker arm is selected from the group consisting essentially of an alkyl chain of from 1 to 20 carbon atoms, a branched alkyl chain of from 1 to 20 carbon atoms, a ring structure, a carbohydrate, a saccharide, a dextran, and an amino acid.

6. The filtration membrane of claim 1, wherein the charge is positive.

7. The filtration membrane of claim 6, wherein the compound comprises a charged moiety selected from the group consisting essentially of an amine and a quaternary ammonium ion.

8. The filtration membrane of claim 1, wherein the charge is negative.

9. The filtration membrane of claim 1, wherein the compound comprises a charged moiety selected from the group consisting essentially of an acid, a sulfonic acid, and a carboxylic acid.

10. The filtration membrane of claim 1, wherein the membrane is selected from the group consisting essentially of cellulose, composite regenerated cellulose (CRC), cellulose diacetate and triacetate, cellulose nitrate, and cellulose diacetate/cellulose nitrate blends.

11. The filtration membrane of claim 10, wherein the covalently attached charged compound is attached by an ether linkage.

12. A method of preparing a charged filtration membrane of claim 1, the method comprising:

contacting the membrane with a reactive charged compound; and

reacting surface reactive groups on the membrane with a reactive group of the charged compound such that the charged compound is covalently attached to the membrane.

13. A method of preparing a charged filtration membrane of claim 1, the method comprising:

contacting the membrane with a first compound comprising a reactive group;

reacting surface reactive groups on the membrane with the reactive group of the first compound such that the first compound is covalently attached to the membrane;

reacting the attached first compound with a second compound, the second compound comprising a charged group, such that the charged group is covalently attached to the first compound.

14. A method of separating a desired protein from at least one protein of a mixture of proteins in an aqueous buffered solution, the method comprising:

altering the pH of the solution such that the desired protein has a net charge and a protein to be separated from it is neutral or has a net charge that is opposite the net charge of the desired protein;

contacting the protein mixture with the charged filtration membrane of claim 1, wherein the desired protein and the membrane have like net charges;

separating the desired protein from the neutral protein and the oppositely charged protein by retaining the desired protein upstream of the membrane and filtering the neutral or oppositely charged protein through the membrane;

repeating the altering, contacting, and separating steps until the desired protein is separated from a chosen number of proteins of the mixture.

15. A method of separating a desired protein from at least one protein of a mixture of proteins in an aqueous buffered solution, the method comprising:

altering the pH of the solution such that the desired protein is neutral and a protein to be separated from it has a net charge that is the same as the net charge of the filtration membrane;

contacting the protein mixture with the charged filtration membrane of claim 1;

separating the desired protein from the charged protein by retaining the charged protein upstream of the membrane and filtering the desired protein through the membrane;

repeating the altering, contacting, and separating steps until the desired protein is separated from a chosen number of proteins of the mixture.

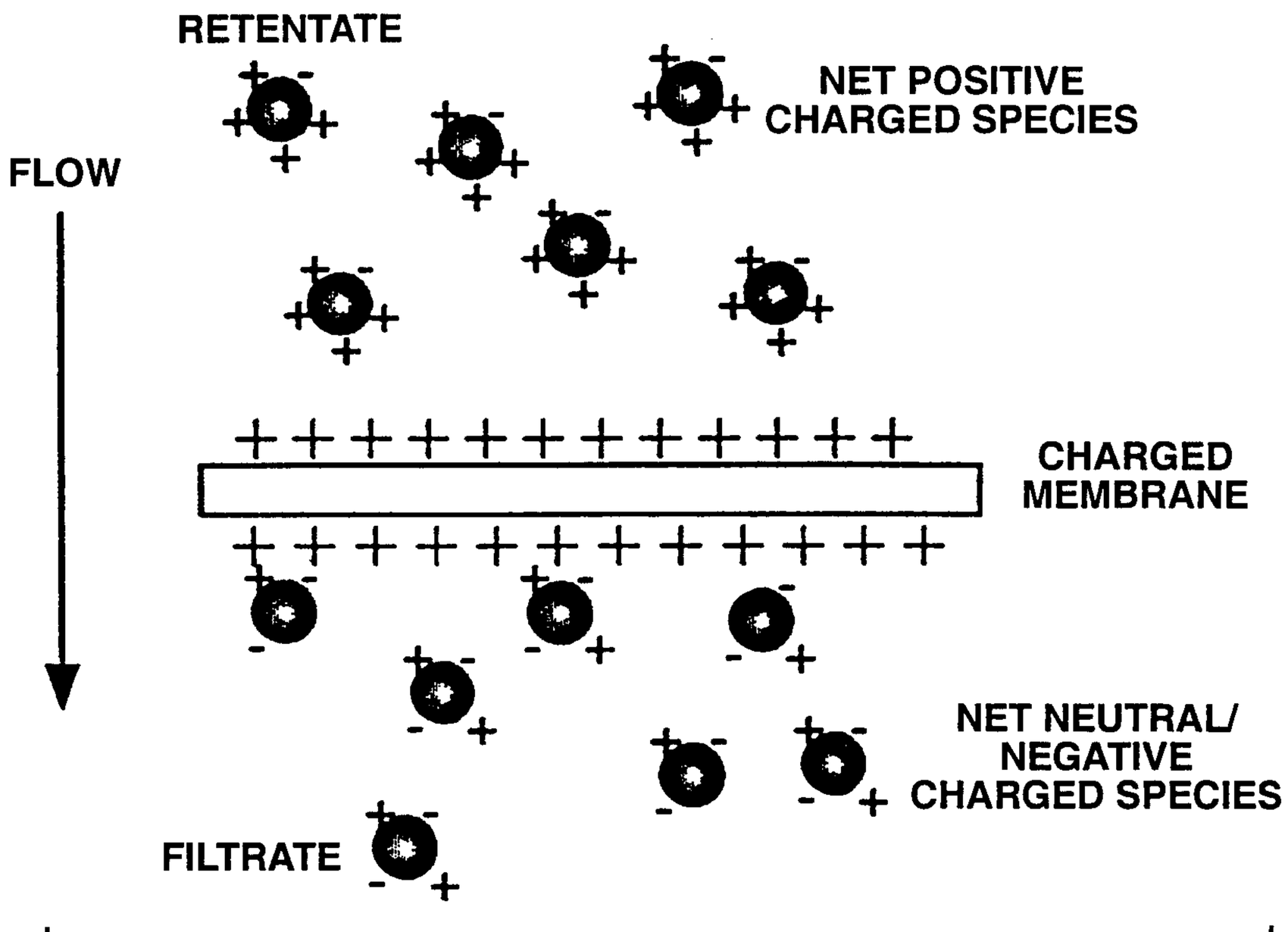
RETENTATE
NET POSITIVE CHARGED SPECIES
FLOW
CHARGED MEMBRANE
NET NEUTRAL/ NEGATIVE CHARGED SPECIES
FILTRATE

FIG._1

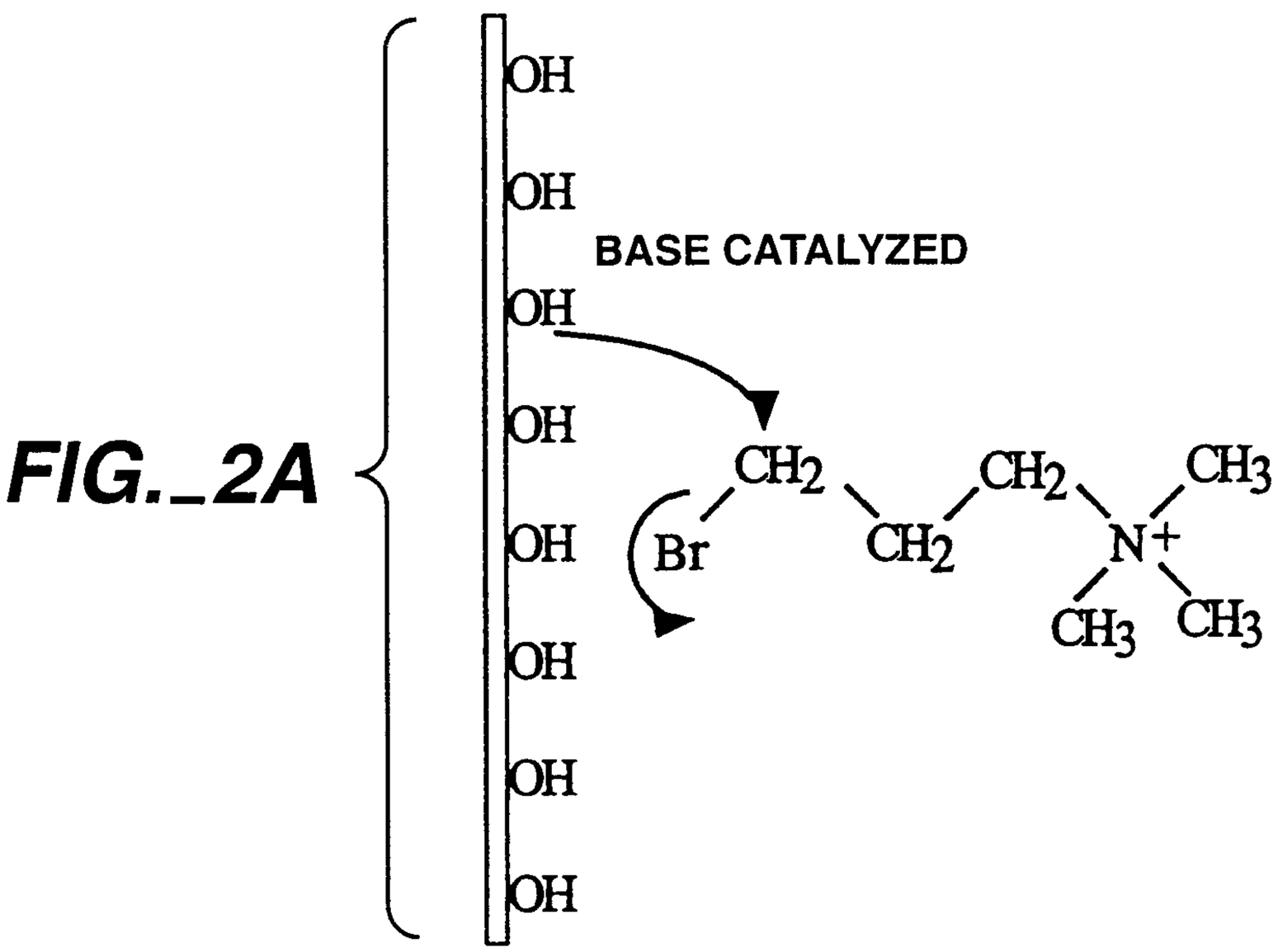
OH
OH
OH
OH
OH
OH
OH
BASE CATALYZED
CH2
CH2
CH2
CH3
Br
N+
CH3
CH3

FIG._2A

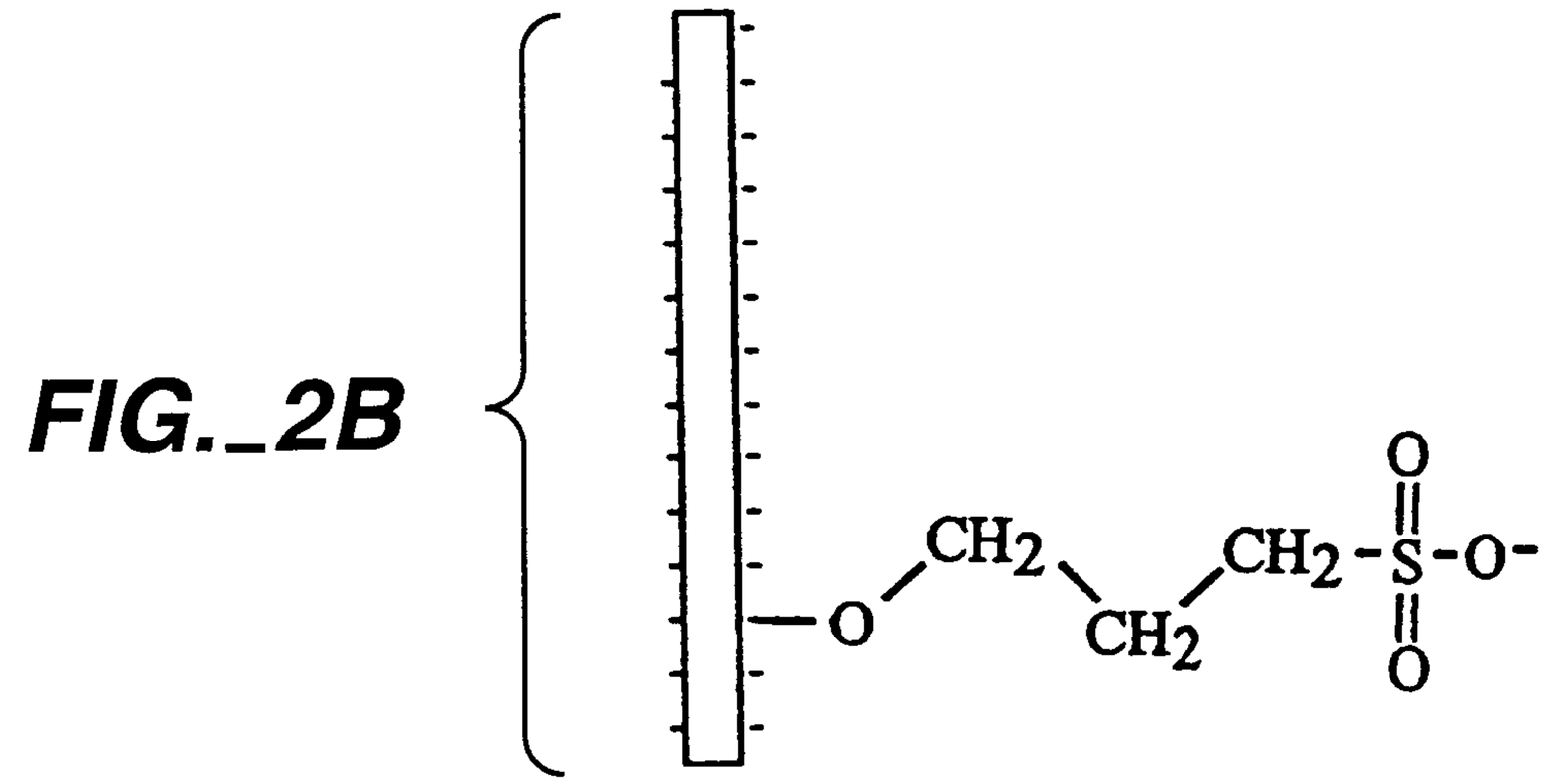

FIG._2B

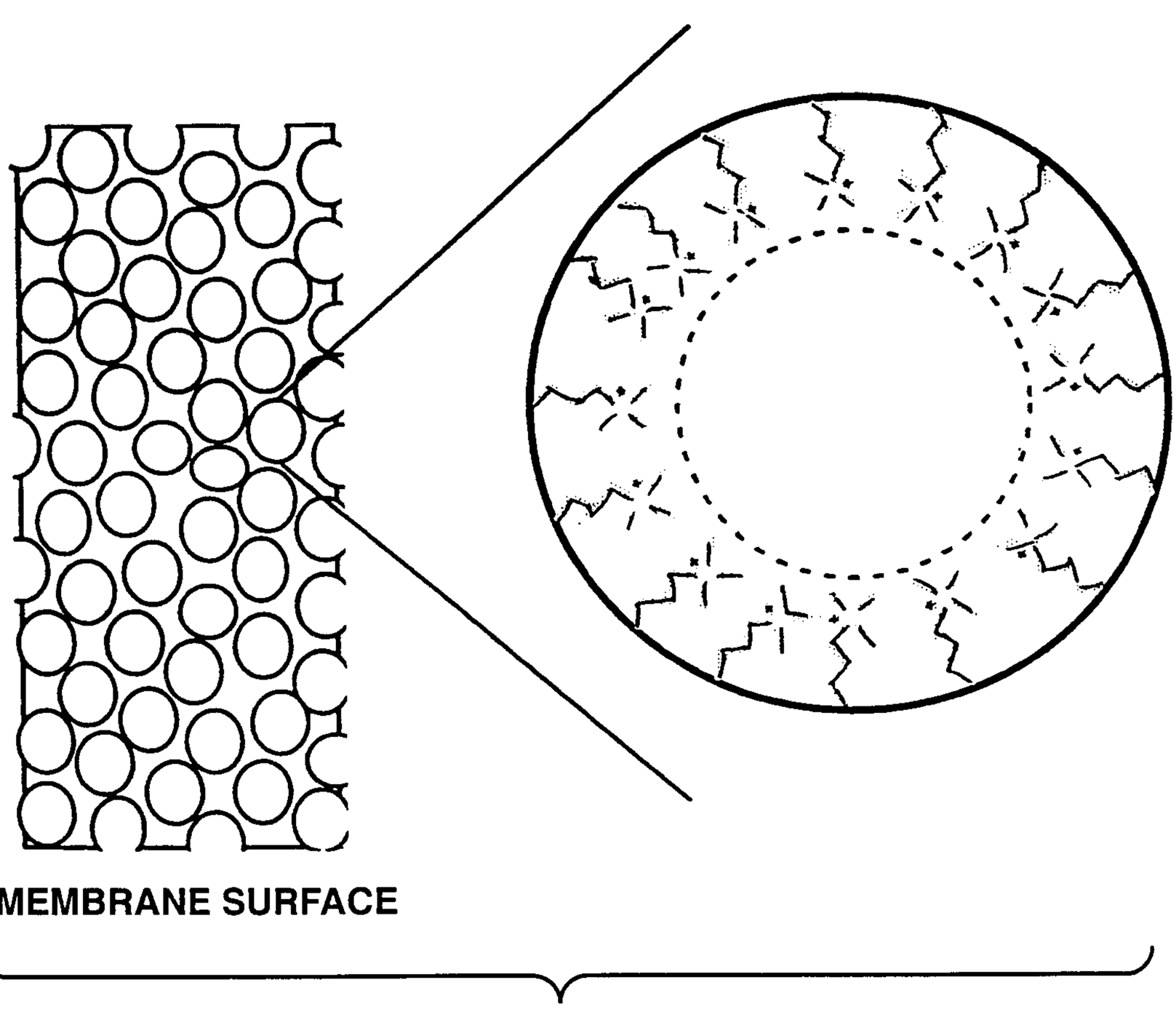

FIG._3

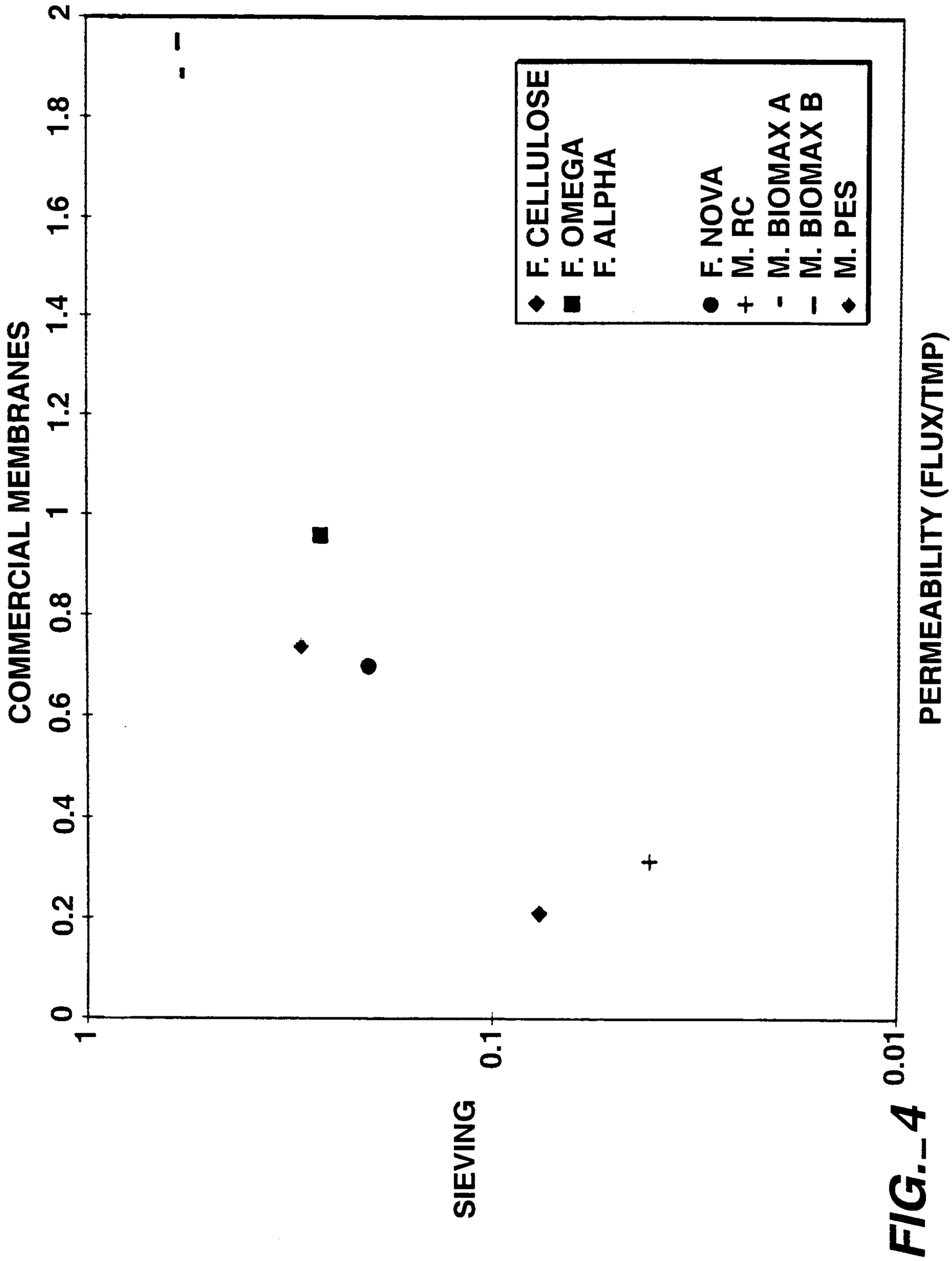
COMMERCIAL MEMBRANES
PERMEABILITY (FLUX/TMP)
SIEVING
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
0.1
0.01
F. CELLULOSE
F. OMEGA
F. ALPHA
F. NOVA
M. RC
M. BIOMAX A
M. BIOMAX B
M. PES

FIG._4

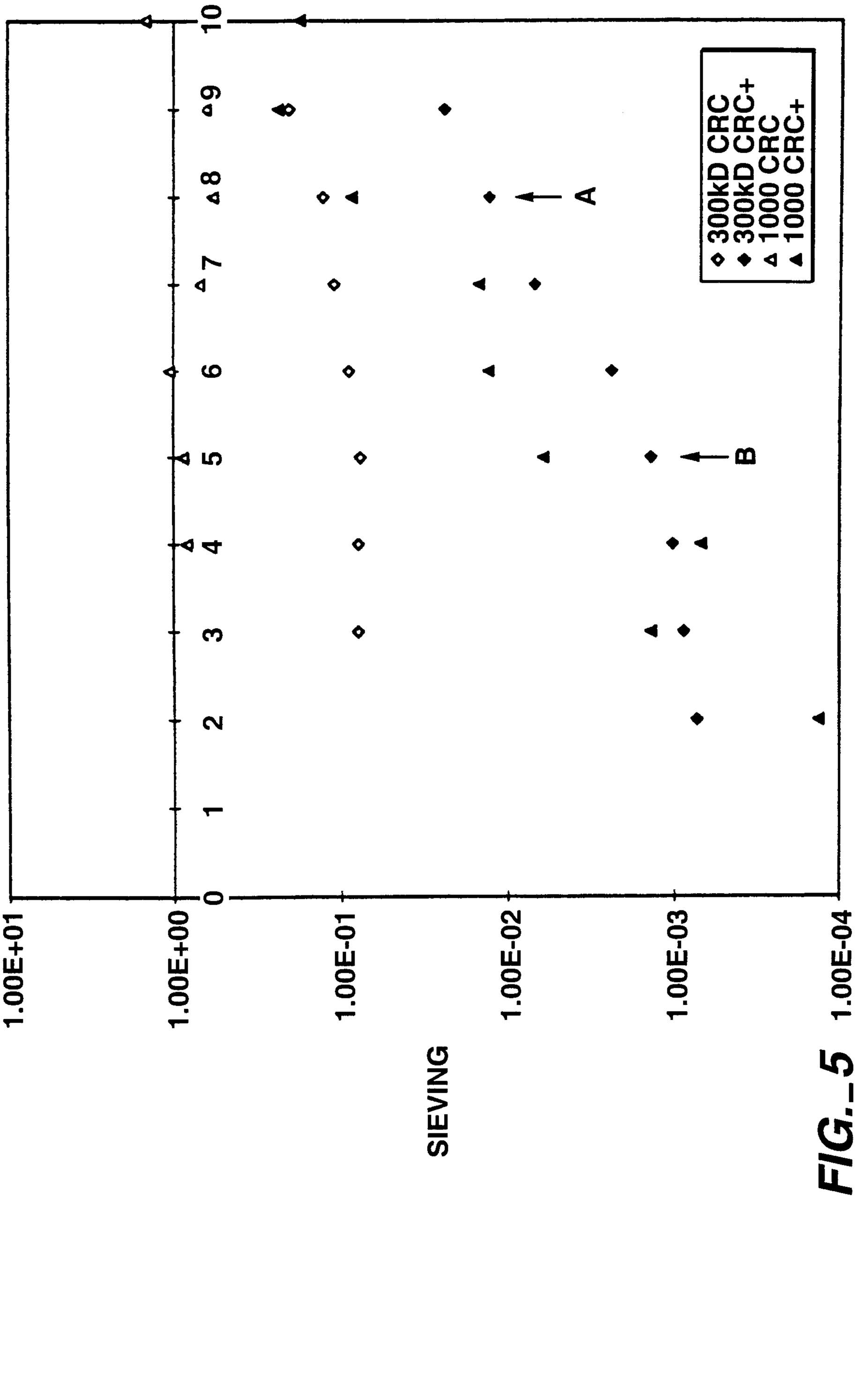
10
9
8
7
6
5
4
3
2
1
0
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
FILTRATE FLUX (mL/MIN)
SIEVING
A
B
300kD CRC
300kD CRC+
1000 CRC
1000 CRC+

FIG._5

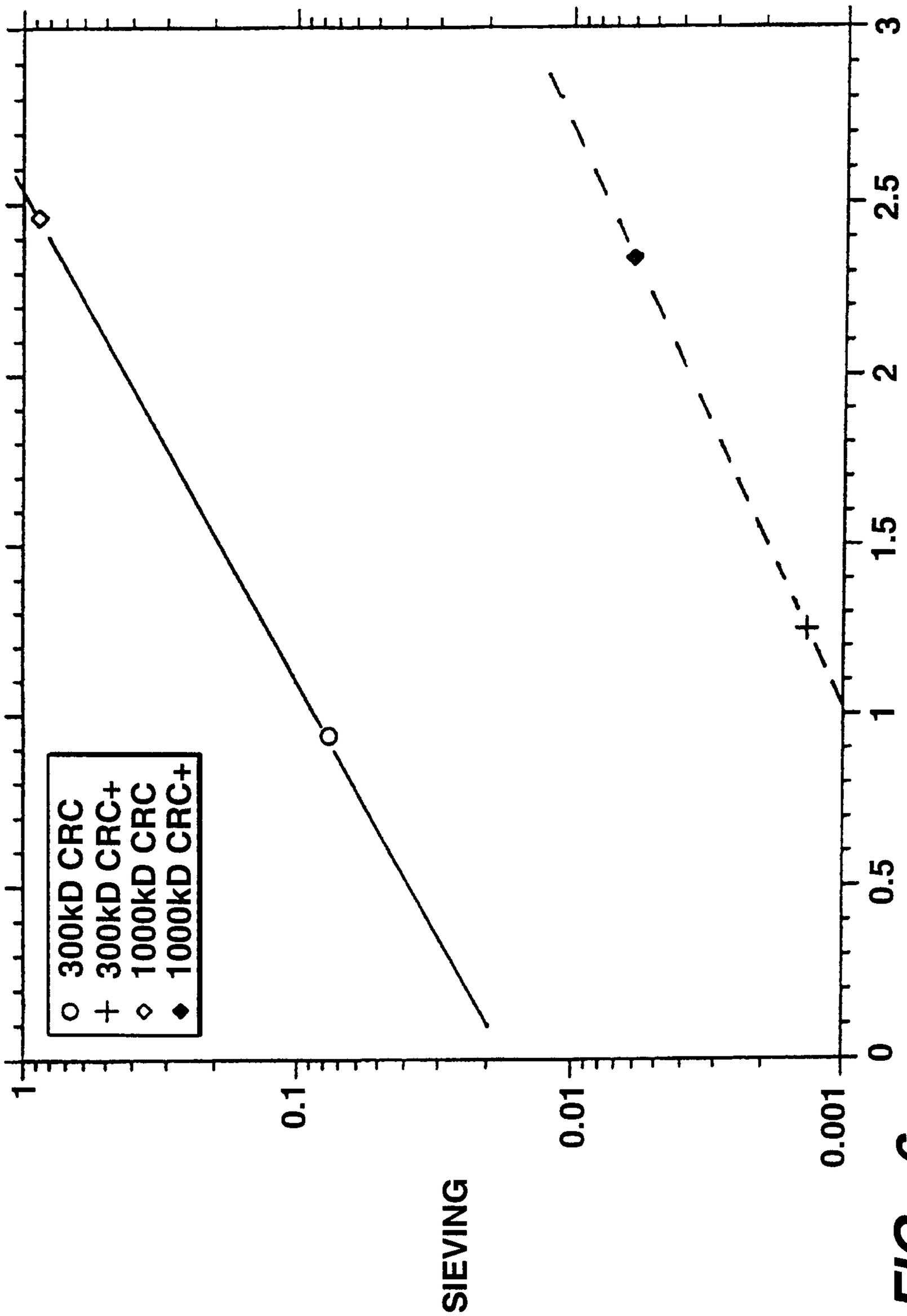
SIEVING
PERMEABILITY (flux/TMP)
0.00
0.01
0.1
1
0
0.5
1
1.5
2
2.5
3
300kD CRC
300kD CRC+
1000kD CRC
1000kD CRC+

FIG._6