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FACTORS CONTRIBUTING TO INTRINSIC LOADING CAPACITY IN SILICA-BASED PACKING MATERIALS FOR PREPARATIVE ANION-EXCHANGE PROTEIN CHROMATOGRAPHY*

M. A. ROUNDS, W. KOPACIEWICZ and F. E. REGNIER*
Department of Biochemistry, Purdue University, West Lafayette, IN 47907 (U.S.A.)
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SUMMARY

Properties of the matrix and stationary phase which affect the intrinsic loading capacity of silica-based packing materials for preparative anion-exchange chromatography of proteins were investigated.

Polyethyleneimine-coated controlled porosity glass beads ranging from 100 to 2000 Å in pore diameter were used to evaluate the effects of pore diameter and surface area. Protein binding was found to depend on accessible, rather than total, support surface area. Consequently, wide-pore, high surface area media provide maximum intrinsic loading capacity.

Increasing the number of positively charged sites on the stationary phase by increased coating or by quaternization of amines increases hemoglobin-binding capacity.

INTRODUCTION

In selecting a silica-based matrix for surface-mediated preparative chromatography of proteins, materials of high 'intrinsic loading capacity" are desirable. Intrinsic loading capacity has been defined as the maximum amount of solute that can be loaded onto a medium (per unit of mass or volume) without regard to chromatographic separation[1]. This inherent characteristic of a sorbent is useful both for investigating media contributions to protein adsorption and comparing operational loading capacity of columns. It is the purpose of this paper to identify some physical and chemical factors which contribute to intrinsic loading capacity in anion-exchange chromatographic packing materials.

Ion-exchange chromatography requires interaction between solutes and the support surface. Thus, the capacity of such a sorbent depends, in part, on its surface area. Change *et al.*[2] have shown that binding capacity is also influenced by support pore diameter. A diethylaminoethyl-containing stationary phase was bonded to con-

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trolled porosity glass beads of 100, 240 or 550 Å pore diameter. Hemoglobin (Hb) ion-exchange capacity (expressed as mg Hb/cm^3) was highest for the 240 Å support, even though its surface area (130 m^2/g) is less than that of the 100 Å pore diameter media (170 m^2/g). It was hypothesized that the relatively large Hb molecule could not reach all available ion-exchange groups. Lowest ion-exchange capacity was exhibited by the 550 Å beads. Although these larger pore diameter media should permit better access, lower surface area (70 m^2/g) means fewer ligands are available for binding protein.

These results suggested that ion-exchange fractionation on supports having pore diameters similar to solute molecular dimensions is actually a combination of ion-exchange and size-exclusion chromatography. Size discrimination by ion-exchange media can be overcome by the use of large pore diameter particles. Pore diameters of 1000 Å (or greater) should allow molecules over 10^5 daltons to have nearly complete access to the internal surface[3]. Unfortunately, since the surface area of these materials is low, they are of relatively low ion-exchange capacity. Techniques for overcoming this problem via polymeric coatings were examined by Vanacek and Regnier[3].

In the present work, pore diameter and surface area effects were further investigated using controlled porosity glass (CPG) beads ranging from 100 to 2000 Å in pore diameter. Controlled porosity glass was chosen because these media are of narrow pore-size distribution and constant geometry[4]. The beads were coated with polyethyleneimine (PEI)[5] and their binding capacities for three proteins of different molecular weight were determined by means of static assays. Four (PEI-coated) commercial silicas of widely varying surface area were also evaluated for Hb-binding capacity in order to examine general applicability of the CPG data.

Stationary phase contributions to intrinsic binding capacity were studied using the same PEI coating on silica. Several modifications of the basic coating method[6] produced anion-exchange stationary phases of varying ligand density and amine type. Comparisons were made using Hb.

MATERIALS AND METHODS

Support materials

CPG beads of 100, 240 and 550 Å pore diameter were purchased from Pierce (Rockford, IL, U.S.A.). Those of 1000 and 2000 Å pore diameter were purchased from Electro-Nucleonics (Fairfield, NJ, U.S.A.). All were 37–74 μm particles. Surface areas, as specified by the manufacturer, are listed in Table I.

Vydac 101TPB (5.5 or 15–20 μm particles) silica was a gift from The Separations Group (Hesperia, CA, U.S.A.). PQ 200 Å and 270 Å experimental silicas were gifts from The PQ Corporation (Valley Forge, PA, U.S.A.). Serva Si-200 silica was obtained from Serva Fine Biochemicals (Westbury, NY, U.S.A.). Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.) provided A-50-120 DEAE Sephadex (40–120-μm particles).

Reagents

Polyethyleneimine-6 and 1,3-diglycidylglycerol were supplied by Polysciences (Warrington, PA, U.S.A.). Methyl iodide and "glycidol" (GLY) (1,2-epoxy-3-hy-

droxypropane) were purchased from Aldrich (Milwaukee, WI, U.S.A.). Eastman-Kodak (Rochester, NY, U.S.A.) supplied 1,2-epoxy-3-(*p*-nitrophenoxy)propane (ENPP), 1,2,2,6,6-pentamethylpiperidine and Coomassie Brilliant Blue G-250 dye (CI 42655; for Bradford assay).

Inorganic reagents and solvents were AR grade or of comparable quality.

Biochemicals

Proteins emplyed were: horse heart myoglobin (MYO; 17 500; 7.3); bovine hemoglobin (Hb; 64 500; 6.8); equine ferritin (FER; 440 000; 4.3); bovine serum albumin (BSA; 69 000; 5.0) and egg white ovalbumin (OVA; 43 500; 4.7). Numerical values in parentheses refer to molecular weight and isoelectric point[7,8], respectively. All were purchased from Sigma (St. Louis, MO, U.S.A.) except FER which was obtained from Pharmacia Fine Chemicals. The proteins used to investigate pore diameter and surface area effects were twice crystallized or of equivalent purity. "Crude" bovine Hb was used for binding assays on coated silicas.

Intrumentation

A Perkin-Elmer Model 55 spectrophotometer (Perkin-Elmer, Norwalk, CT, U.S.A.) and a Spectronic 70 (Bausch and Lomb, Rochester, NY, U.S.A.) were used to measure concentrations in protein-binding assays.

Application and modification of stationary phases

All support materials were coated with polyethyleneimine (PEI)-6, a branched-chain polyamine of molecular weight *ca.* 600, based on the method of Alpert and Regnier[5]. CPG beads and silicas were coated with one percent PEI-6 (10% for high ligand lensity material) and cross-linked with five percent diglycidylglycerol (in methanol) as described by Kopaciewicz *et al.*[6]. Subsequent epoxide derivatization of this weak anion-exchange coating, with "glycidol" or 1,2-epoxy-3-(*p*-nitrophenoxy) propane[6], produced the GLY and ENPP stationary phases, respectively.

The quaternary amine strong anion-exchange packing material was prepared as follows: 0.8 g of PEI-coated silica was suspended in 4.5 ml of dry dimethylformamide (DMF) to which had been added 105 μl of 1,2,2,6,6-pentamethylpiperidine[9]. After brief sonication, 0.5 ml of methyl iodide was added. This suspension was heated in an oil bath at 60°C overnight. The support material was isolated in a sintered glass funnel and washed successively with water, methanol, triethylamine and methanol. After 0.5 h in the funnel, the methylated polyamine-coated silica was dried in a vacuum dessicator.

Static protein-binding capacity assay

Static assays with Hb and other proteins were performed as described by Kopaciewicz *et al.*[6] except that the ionic desorption solution was 1.0 *M* in sodium chloride (instead of 0.5 *M*). Data reported are total desorbed protein (ionic + non-ionic) in mg/g support material. For all assays on the controlled porosity glass beads, aliquots of the desorption solutions were analyzed by the Bradford method[10] using appropriate standard curves (absorbance at 595 nm *vs.* μg protein). Experimental error was within 5%.

RESULTS AND DISCUSSION

Identifying factors which contribute to the intrinsic loading capacity of a column packing material required the use of media in which particle size, pore diameter, surface area and the chemical nature of the stationary phase could be controlled. This was accomplished by application of an adsorbed polyamine coating[5] to either CPG beads or silica of known dimensions.

Pore diameter and surface area effects

As an extension of the work previously discussed[2], the effects of pore diameter and surface area on intrinsic loading capacity were investigated using 100–2000 Å CPG beads. Since seventy percent of the proteins reported in the literature are acidic[7], an anion-exchange stationary phase was prepared by applying a "thin" coating of PEI to the beads. The polyamine coating itself has been shown to consume pore volume[3]; thus, manufacturer's specified pore diameters and surface areas were corrected to account for the thickness of the PEI layer (see Table I).

Hemoglobin-binding capacity assays were performed on the coated beads. When these data (mg Hb/g support) were plotted (Fig. 1), the same trend noted earlier was seen. Highest Hb loading was achieved with supports of intermediate pore diameter and surface area. (The same was true for Hb bound to uncoated beads at pH 6.0; data not shown.) Since the initial study utilized a different type of ion-exchange stationary phase, Chang's[2] hypothesis appears to be generally applicable. A mathematical statement of this concept is developed below.

The total surface area of a chromatographic support (A_t, in m^2/g) is the sum of the internal (A_i) and external (A_e) surface areas. More than 95% of the surface area of a porous media is internal[2,11]; consequently, A_t is essentially equal to A_i. Before a protein can bind to the surface of this support material, it must diffuse into

TABLE I

PHYSICAL CHARACTERISTICS OF CONTROLLED POROSITY GLASS BEADS BEFORE AND AFTER COATING WITH POLYETHYLENEIMINE

CPG beads were coated with 1% PEI-6 (5% diglycidylglycerol to cross-link)[6] to produce a weak anion-exchange stationary phase.

*Support material**	*Pore diameter (Å)***		*Surface area (m^2/g)****	
	Mfr's	*Coated*	*Mfr's*	*Coated*
CPG-100	100	60	170	102
CPG-240	240	200	130	108
CPG-550	550	510	70	65
CPG-1000	1000	960	26.1	25
CPG-2000	2000	1960	8.2	8

* Particle size = 37–74 μm for all.

** Based on the data of Vanacek and Regnier[3], a thickness of 20 Å was assumed for the 1% PEI coating; thus, manufacturer's (Mfr's) pore diameters decreased by 40 Å upon application of the coating.

*** If the pore is treated as a cylinder, total surface area decreases linearly with pore diameter upon coating. Surface area of the coated media was estimated by multiplying Mfr values by the ratio for coated/uncoated pore diameter.

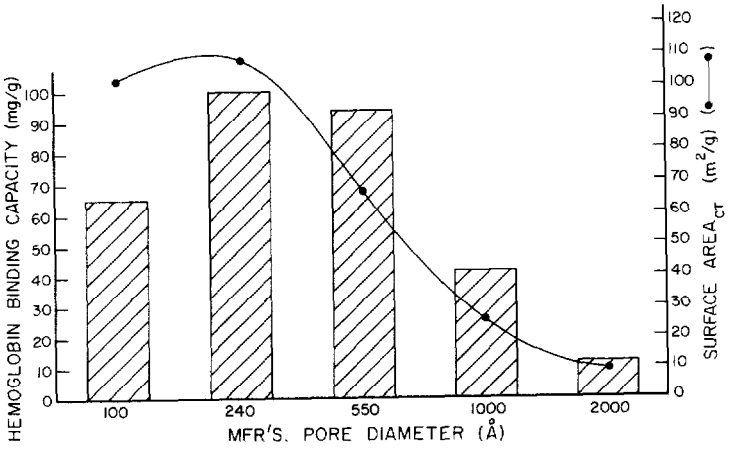


Fig. 1. Hemoglobin-binding capacities of PEI-coated controlled porosity glass beads of varying pore diameter. Surface areas (connected by solid line) of the coated (CT) supports were estimated as explained in Table I. Coating and assay procedures were as described in Materials and methods with Hb quantitated by the Bradford assay.

the matrix pore. If access to A_i is restricted, intrinsic loading capacity will be a function of the available or accessible surface area (A_{ac}) rather than A_t. As suggested by Regnier[11], this may be expressed as

$$A_{ac} = K_{ac}A_t \tag{1}$$

where A_{ac} is the accessible surface area of a support in m^2/g, A_t is the total surface area and K_{ac} is a protein-specific surface accessibility coefficient which varies from zero to one (complete access). A precedent for this concept is found in size-exclusion chromatography where the size-exclusion distribution coefficient is considered to be a measure of the differential permeability of a solute into the matrix pore volume[12]. Eltekov and co-workers[13,14] have studied polymer adsorption on various supports including porous silicas and have shown that this phenomenon is dependent on pore size.

In order to compare sorbents of different surface areas, the protein-binding capacity of a given medium may be divided by the total surface area of that sorbent to obtain a specific binding capacity (S). When S is the specific protein binding capacity in a non-size discriminating system (under saturating conditions), it is designated S'. The value of S' will not be the same for all proteins due to differences in equilibrium constants, molecular size and charge, etc.

If S' is known for a protein, then the relative accessibility of any medium to that protein may be expressed by K_{ac}, the accessibility coefficient, where

$$K_{ac} = S/S' \tag{2}$$

Assuming eqns. 1 and 2 are valid, the following relationship between intrinsic loading capacity (L_i) and accessible surface area is proposed

$$L_i = S' A_{ac} = S' K_{ac} A_t \tag{3}$$

TABLE II

SPECIFIC BINDING CAPACITIES AND ACCESSIBILITY COEFFICIENTS FOR THREE PROTEINS ON PEI-COATED CONTROLLED POROSITY GLASS BEADS OF VARYING PORE DIAMETER

Support material coated with 1% PEI-6. See Table I for pore diameters and estimated total surface areas of the coated media.

Support material	*Specific binding capacity, (S)**			*Accessibility coefficient, (K_{ac})***		
	MYO	*Hb*	*FER*	*MYO*	*Hb*	*FER*
CPG-100	8.0	9.7	0	0.6	0.4	0
CPG-240	6.0	14.4	0	0.4	0.6	0
CPG-550	10.0	22.5	16.0	0.7	1.0	0.4
CPG-1000	14.8	26.0	38.0	1.0	1.0	1.0
	$S' = 14.3$	23.6	39.6			
CPG-2000	13.8	21.2	41.2	1.0	1.0	1.0

* Specific binding capacity (S) in nmol/m^2 was calculated by dividing static binding capacity (expressed as nmol protein/g support) by surface area (m^2/g) of the coated support.

** $K_{ac} = S/S'$.

To test these relationships, additional static binding assays were performed, using proteins of different molecular weight. Specific binding capacities (see Table II) for MYO (MW = 17 500), Hb (MW = 64 500), and FER (MW = 440 000) are shown in Fig. 2. If each protein had equal access to the internal surface area, there would be no difference in S for a given protein on different pore diameter matrices. While S for the smallest protein, MYO, is reasonably independent of pore size, S for both Hb and FER varies directly with pore diameter. Similar S values were exhibited by the 1000 and 2000 Å beads for each of these as well as several other proteins (data not shown). This confirms that media having $\geqslant 1000$ Å pore diameters do not distinguish proteins on the basis of size in the range tested. Consequently, S values on

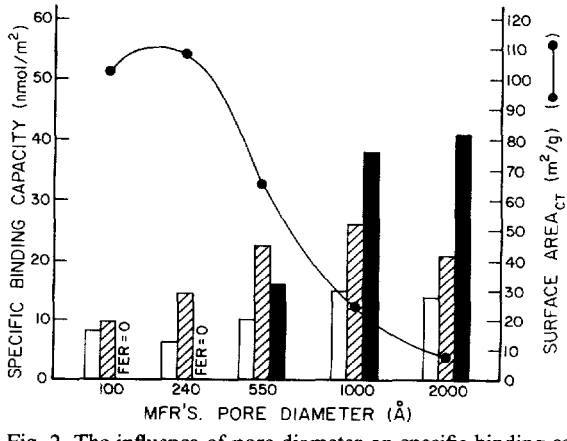


Fig. 2. The influence of pore diameter on specific binding capacity (calculated as described in Table II). Surface areas of the PEI-coated CPG beads (connected by solid line) were estimated as explained in Table I. □ = MYO; ▨ = Hb; ■ = FER. Binding assays were performed as described in Materials and methods with all proteins quantitated by the Bradford assay.

the 1000 and 2000 Å supports were averaged to provide an estimate of S' for each of the three proteins studied (see Table II). (On the basis of size, one might expect FER to occupy more surface, thereby decreasing the number of molecules bound per unit area; this would be true, however, only if all proteins adsorbed as a monolayer.) Accessibility coefficients, K_{ac}, calculated for MYO, Hb and FER on the 100, 240 and 550 Å pore diameter CPG beads (Table II) suggest that K_{ac} is inversely related to the ratio of molecular size to pore size.

Finally, protein accessibility coefficients (K_{ac}) and total surface areas (A_t, corrected for coating thickness as explained in Table I) were used to calculate (by eqn. 1) the surface area accessible (A_{ac}) to these three proteins. The results are shown in Fig. 3 where A_{ac} is plotted against pore diameter (corrected for coating thickness). According to eqn. 3, intrinsic loading capacity is directly proportional to accessible surface area. Thus, Fig. 3 suggests that maximum loading capacity for different proteins will be achieved on supports of varying pore diameter. Proteins of intermediate size such as Hb should exhibit maximum adsorption on 200–400 Å pore diameter supports. (Curves similar to that for Hb were also obtained for BSA, MW = 69 000 and OVA, MW = 43500; data not shown.) Large proteins such as FER will require large pore diameter materials and small proteins, small pore diameter media. Consequently, support materials of very high surface area and small pore diameter may actually have less intrinsic loading capacity than a medium of lower surface area but larger pore diameter.

Commercial silicas

The results discussed above may be used to explain differences in Hb-binding capacity observed with four (PEI-coated) commercial silicas (Table III). The Serva and PQ support materials bound considerably more Hb than Vydac silica. Vydac 101TPB (300 Å) silica is a very high-quality analytical support of relatively low surface area (80 m^2/g) which will withstand pressures in excess of 700 atm. By increasing pore volume, the manufacturers of Si-200 and the two PQ silicas have maintained macroporosity and enhanced protein binding capacity. The tradeoff, of course, is

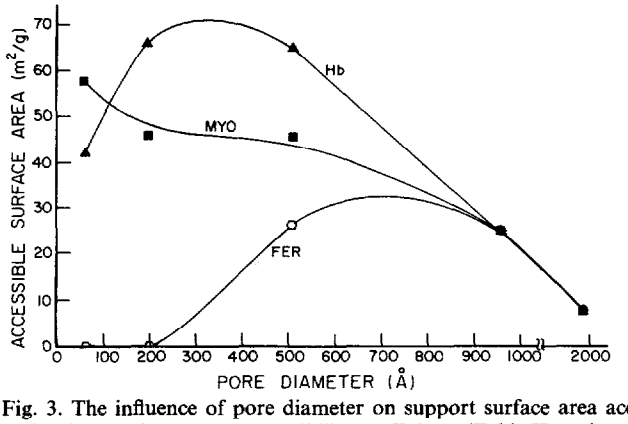


Fig. 3. The influence of pore diameter on support surface area accessible to three proteins of different molecular weight. Protein accessibility coefficients (Table II) and total surface areas (corrected for coating thickness) were used to calculate (eqn. 1) the surface areas accessible to MYO, Hb, and FER on PEI-coated CPG beads of varying pore diameter.

TABLE III

HEMOGLOBIN-BINDING CAPACITIES OF (PEI-COATED) COMMERCIAL SILICAS OF VARYING SURFACE AREA

All supports were coated with 1% PEI-6 (5% diglycidylglycerol to cross-link). The two PQ silicas were experimental materials.

Silica	*Particle size (μm)*	*Pore diameter** *(Å)*	*Surface area** *(m^2/g)*	*Hb-binding capacity** (mg Hb/g)*
Vydac 101TPB	15–20	300	80	27
PQ-270 Å	20	270	200	103
PQ-200 Å	20	200	272	134
Serva Si-200	30	250	290	123

* As specified by manufacturer (see Materials and methods).
** Ionically desorbed Hb quantitated directly at 410 nm.

decreased pressure stability (we have found these materials stable to 200 atm). Since 15–30 μm particles do not require high operating pressure, however, the lower pressure stability of these silicas should not preclude their use in large preparative columns. (When compared on a volumetric basis, the Hb-binding capacities of the Serva and PQ silicas were equal to or greater than that of Pharmacia A-50-120 DEAE Sephadex; data not included.)

Stationary phase contributions

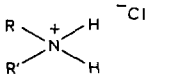
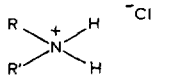
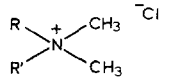
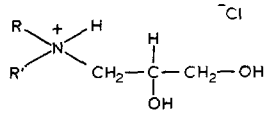
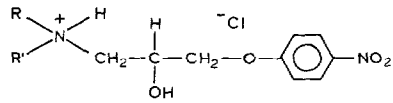
Previous work[6] has suggested that both the chemical nature and ligand density of an ionic stationary phase can influence protein adsorption. Several stationary phases which varied in charge density and amine type (Table IV) were generated by modification of the basic PEI coating as described in Materials and methods. These (silica-based) anion-exchange packing materials were analyzed for Hb-binding capacity. The influence of ligand density was clearly demonstrated by the low and high ligand density materials; the latter, with more charged amine, exhibited greater protein binding capacity. Chromatographic retention was also increased on this stationary phase (data not shown). A second route to high loading capacity with PEI-coated media appears to be quaternization of low ligand density stationary phases. When such a matrix was exhaustively methylated, Hb-binding capacity increased to that of the high ligand density material. In contrast, derivatization with GLY or ENPP reduced Hb-binding capacity.

These results may be explained in terms of both ionic and steric effects about the stationary phase ion-exchange moiety. Adsorption of a protein at a surface is an equilibrium process which depends on solute concentration, ligand density, fraction of the surface covered with solute molecules and the equilibrium constant. An increased number of amine groups shifts the equilibrium constant in favor of adsorption of protein. Alpert and Regnier[5] have shown that only a portion of the amines on the adsorbed PEI-6 coating is ionized at pH 8. Exhaustive methylation converts primary and secondary amines to quaternary amine salts. The resulting increase in surface charge promotes protein binding. In contrast, increasing the distance between charged centers on the support and the solute decreases the strength of adsorption,

TABLE IV

EFFECT OF STATIONARY PHASE COMPOSITION ON Hb-BINDING CAPACITY

Application of a polyamine coating produces an anion-exchange stationary phase which may vary in amine content, *i.e.* ligand density. Further reaction with electrophiles such as methyl iodide or monofunctional epoxides (*e.g.* GLY or ENPP) modifies amine nitrogens as shown below. R and R′ denote remainder of the cross-linked matrix.

Description	*Abbreviation*	*Hb-binding capacity***	*Structure*
"Low" ligand density* (250 μmol amine/g support)	LLD	47	
"High" ligand density (550 μmol amine/g support)	HLD	62	
Quaternary amine (250 μmol amine/g support)	QME	69	
Glycidol-modified LLD (250 μmol amine/g support)	GLY	29	
Nitrophenyl-modified LLD (250 μmol amine/g support)	ENPP	37	

* Ligand density of the base coating was estimated using the picric acid assay[5].
** Total desorbed Hb (quantitated directly at 410 nm) in mg Hb/g support.

reducing the amount of protein bound. The introduction of bulky groups around charged centers on the ion-exchange matrix by derivatization with GLY or ENPP reduces loading capacity, possibly due to steric effects[6]. It should be noted that although such derivatives may exhibit lower loading capacities, they can provide unique chromatographic selectivity[6].

CONCLUSIONS

The intrinsic loading capacity of an anion-exchange chromatographic medium is determined by both porosity of the matrix and the chemical nature of the stationary phase. The contributions of pore diameter and surface area were investigated by means of static protein binding assays using PEI-coated controlled porosity glass beads of 100–2000 Å pore diameter. In agreement with earlier findings[2], maximum Hb adsorption was exhibited by media of intermediate pore diameter and surface area. Apparently, the internal surface area of the smallest pore diameter support was not completely accessible to this protein. To investigate this hypothesis, the binding of a small (MYO) and a large (FER) protein by these media was also measured. FER (MW = 440 000) did not bind to either the 100 Å or 240 Å CPG, while MYO (MW = 17 500) exhibited maximum adsorption on the 100 Å material. Specific binding capacities (nmol protein/m^2 surface area) were determined and used to calculate accessibility coefficients (K_{ac}) for each protein with respect to various pore

diameter supports. The product of K_{ac} and support surface area provided an estimate of accessible surface area (A_{ac}). The maximum surface area accessible to Hb (MW = 64 500) was available from supports of 200–400 Å pore diameter. Larger and smaller pore diameter media provided maximum A_{ac} for the binding of larger and smaller proteins, respectively. Obviously, both pore diameter and protein size are important considerations with regard to maximizing the intrinsic loading capacity of ion-exchange supports. Pores must be big enough to permit complete access of the protein(s) of interest, but not so large as to unnecessarily sacrifice the surface upon which binding takes place.

In addition to physical properties of the matrix, stationary phase composition also plays a role in protein binding. High ligand density coatings adsorbed more protein than low ligand density surfaces as a result of the increased availability of charged sites. Exhaustive methylation of amines also increased Hb binding by increasing surface charge. In contrast, the addition of monofunctional epoxides to stationary phase amines decreased protein loading capacity. It has been postulated that the addition of such moieties sterically inhibits protein binding[6].

The above findings imply that pore diameter, surface area and stationary phase composition must all be considered relative to the protein of interest in order to maximize intrinsic loading capacity. Unfortunately, this is not always possible due to the proprietary nature of many commercial packing materials. However, an understanding of the basic concepts discussed may facilitate the selection of media for preparative anion-exchange chromatography of proteins.

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