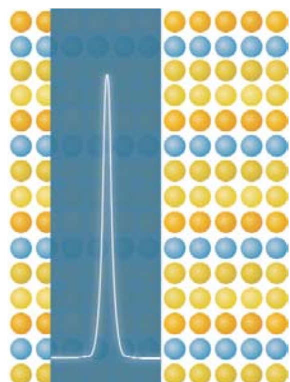

Validated Alternatives to Protein A Sorbents for Antibody Production.

Hydrophobic Charge-Induction Chromatography and novel IgG selective ligands replace Protein A sorbents in manufacturing.

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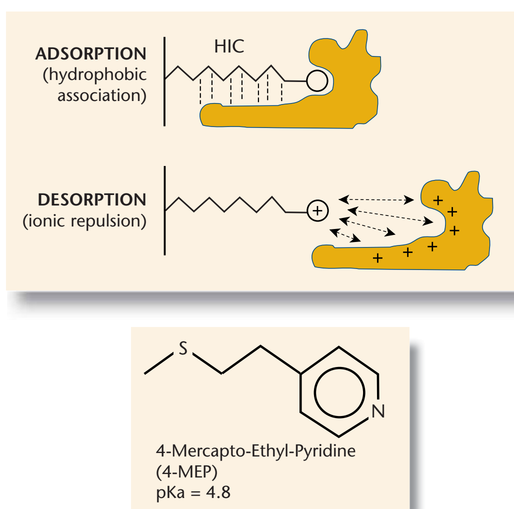
Today's Antibody Production Challenge.

Monoclonal antibodies constitute the largest number of protein-based therapeutic and diagnostic molecules currently in clinical trials. The broad applicability and proven commercial success for this class of molecules suggest the future market potential to be significant. Current manufacturing capacity for biopharmaceutical, in general, is fast becoming a rate-limiting factor in the growth of the biotech sector. As antibody therapeutics represent such a large part of this market, there is an immediate need for novel antibody manufacturing approaches that deliver greater productivity.

Affinity chromatography on protein A-based chromatography sorbents has been successfully employed in the purification of antibodies for more than 25 years. Used in combination with ion exchange and gel filtration chromatography steps, protein A-based methods have become the "classic" antibody isolation method for many biopharmaceutical companies. Despite their common usage, however, there is a growing need and demand for effective alternatives that address the many known problems associated with protein A-based sorbents; chief among these are sorbent cost, lifetime in usage and protein A leaching issues. At large-scale, the contribution to total production cost made by protein A-based can be significant. This problem is compounded by the restricted cleaning tolerance and limited lifetime in use of these sorbents as compared with traditional chemical-ligand (e.g. ion exchange) sorbents. In addition, despite numerous advances in sorbent engineering, the leaching of protein A fragments during antibody purification, exacerbated by proteases present in cell culture media, is still a major issue.

Figure 1. Adsorption and desorption mechanism.



HCIC sorbents are manufactured in large batches similar to ion exchange sorbents to meet industrial demand.

CIPHERGEN®

Hydrophobic Charge Induction Chromatography (HCIC) represents an effective, new approach to antibody purification that employs a low cost, dual-mode (chemical) ligand to provide a high degree of selectivity for antibodies. Introduced in 1999 (Genetic Engineering News, Vol. 20, No. 13, July 2000), the first HCIC-based sorbent (MEP HyperCel®) has gained widespread industrial acceptance as a successful alternative to protein A-based sorbents for the production of monoclonal and polyclonal antibodies. To complement HCIC, novel mixed-mode ligands have been engineered (MBI HyperCel®), which offer different selectivity for antibodies. The choice of sorbent depends largely on the isoelectric point and pH stability of the antibody to be purified. Used alone or in combination, MEP HyperCel® and MBI HyperCel® provide a powerful alternative to protein A-based sorbents for the capture and purification of therapeutic and diagnostic antibodies at process-scale.

Hydrophobic Charge Induction Chromatography: Straightforward antibody capture without adjustment to pH or ionic strength.

The binding and elution of proteins on HCIC-based sorbents is based on the pH-dependent behavior of certain dual-mode ligands. Incorporating HCIC technology, MEP HyperCel® carries an antibody-selective ligand, 4-Mercapto-Ethyl-Pyridine (4-MEP). As illustrated in Figure 1, protein adsorption is achieved through mild hydrophobic interaction; antibody selectivity is the result of the cooperative influence of a sulfur atom proximal to the aromatic ring. No addition of lyotropic salt is needed for binding, in contrast with conventional hydrophobic interaction chromatography. Antibody desorption occurs by charge repulsion, induced by a reduction in eluant pH (to pH 4.0-4.5).

MEP HyperCel® can be used for the purification of antibodies from a wide variety of biological sources (see Table 1). HCIC is particularly well suited for direct capture of IgG from mammalian cell culture supernatants at physiological pH values. For example, when used for the capture of antibodies from protein-free supernatants, capture efficiency and purification results typically exceed 95%; MEP HyperCel® serves very much like an antibody concentration device (Table 1 and Figure 2.A.). HCIC can also be effectively used with more challenging (i.e. protein-containing) feedstock as well. When used in the purification of antibodies from serum-containing feedstock, overall yield and purity are reduced. Lower purity and recovery is the result of the presence of other hydrophobic proteins in the feedstock such as albumin that can also bind to the hydrophobic HCIC ligand (Table 1 and Figure 2.B.). Antibody purity can be improved, however, through the use of specific wash steps or orthogonal chromatography steps such as ion exchange. The use of our newest ligand MBI which does not bind albumin at adsorption pH, could be the right alternative in the case of serum-containing feedstock.

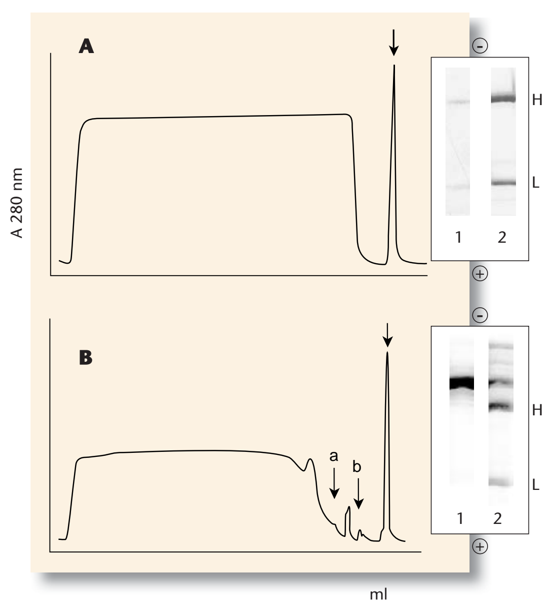
MEP HyperCel® has been applied to the purification of IgG from serum (Dr. Hanne Bak, Biocentrum and DTU, Denmark). Data presented at GAB'2002 Conference, Porto). Crude filtered serum from hyperimmunized animals was diluted 1:1 with PBS and loaded directly onto a column of MEP HyperCel® to the point of sorbent saturation. IgG purities of 88-90% and recoveries as high as 87% were achieved in a single pass.

Feedstock	Total proteins (mg)	IgG content (mg)	IgG initial purity (%)	Final IgG purity (%)	Recovered IgG (mg)	Overall IgG yield (%)	Purification factor
CCS* with FBS	1740	30	1.7	69	23	76	40
Protein-free CCS*	330**	17	5**	99	17	99	19**
Bovine serum	440	121	28	75	105	87	3
Ascites fluid	55	9	16	83	7	79	5

Table 1. Examples of Purifications on MEP HyperCel.

* CCS = Cell culture supernatant ** BCA methodology

Figure 2. Monoclonal antibodies purification on MEP HyperCel from cell culture supernatants.



Sample A = 300 ml protein-free cell culture supernatant.
Sample B = 300 ml cell culture supernatant containing 5% fetal bovine serum; Equilibration: 50 mM Tris-HCl, pH 8; Elution: 50 mM acetate, pH 4; Flow rate: 70 cm/h. In curve B, (a) and (b) are respectively water and 25 mM sodium caprylate washings; SDS-PAGE (reduced conditions): (1) = crude sample, (2) = purified IgG. H = Heavy chain; L = Light chain.

	Binding capacity
Human polyclonal IgG	32 mg/ml
Murine monoclonal IgG_1 (from ascites fluid)	37 mg/ml
Murine monoclonal IgG_{2a} (from cell culture)	34 mg/ml

Table 2. MEP HyperCel Capacities.

Dynamic capacities equivalent to protein A and less subclass sensitivity.

In contrast with protein A affinity, MEP HyperCel® has a broad selectivity to bind various IgG variants from different species. Human, Rat, Goat, Sheep, Mouse antibodies have been successfully purified. Interestingly, even for Murine IgG_1, capacities of >30 mg/ml at 10% breakthrough are routinely achieved with MEP HyperCel®, without the addition of salts or special buffers. Other "difficult" antibody families or subclasses such as $rIgG_{2a}$, or IgM, IgE or IgA have also been reported. IgM can be also purified on a case-by-case basis, but reduced capacity is generally observed, attributed to steric/exclusion effects.

Dynamic binding capacity is an important parameter to achieve good productivity. The capacity of HCIC is equivalent or greater (Murine IgG_1) to that of reference protein A based sorbents. At binding pH values from 7-9, the capacity of MEP HyperCel® typically ranges from 25-35 mg of IgG/ml (see Table 2).

No significant variation in capacity is observed with IgG concentrations of 50 µg to 5 mg/ml, thus no concentration is needed, even with very dilute material.

In practical operation, process columns can be operated at less than 0.5 bar at linear velocities of 200 cm/h with standard chromatography equipment.

Purification of antibody fragments.

Although not specifically developed for IgG fragments, HCIC can be successfully applied in some cases. As with protein A sorbents, the interaction between MEP HyperCel® and an antibody involves mainly the Fc fragment; with Fab, the capacity of MEP for fragments will be lower than for the complete IgG.

In a recent paper *(J.Chromatogr., 786 (2003) 161-176),* Dr. Nico Mertens et al. from the department of Molecular Biomedical Research, VIB, at the University of Gent, Belgium, have reported the successful purification of single chain variable fragments (scFv) coupled to C-terminus of Fab-chains. Their study compared different methods, including protein L affinity, IMAC and hydroxyapatite. HCIC was judged to be a suitable method for the capture of Fab and scFv fragments, giving good product recovery, while the bulk of either serum albumins derived from cell culture media or bacterial proteins from *E.coli* expression was removed in the MEP HyperCel® column flowthrough and wash.

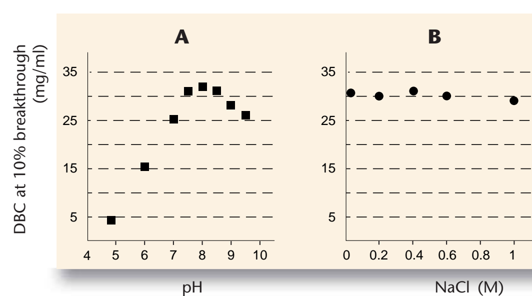
In BioSepra® laboratories, it has been found that HCIC can separate Fc and Fab fragments from enzymatically hydrolyzed IgG, by a sequential desorption based on pH (Guerrier et al.).

"No salt" is the hallmark of HCIC: Protein capture at high conductivity, no dilution, no environment unfriendly waste disposal.

In contrast with traditional hydrophobic interaction chromatography, protein binding to HCIC sorbent is controlled on the basis of pH rather than salt concentration. The influence of pH and ionic strength is illustrated in Figure 3.

The data suggest that typical feedstock can be loaded without adjustment of ionic strength; neither diafiltration nor lyotropic salt addition is required. This "quasi-independence" of protein binding to salt makes HCIC an attractive alternative to conventional

Figure 3. Influence of pH and ionic strength on the antibody binding capacity of MEP HyperCel.



IgG capacities obtained at 10% breakthrough on MEP HyperCel vs. pH (**A**) and ionic strength (**B**) of the binding buffer. Experimental conditions: Column 1.1 cm ID x 9 cm; Sample: IgG (2 mg/ml); Flow rate: 90 cm/h.

HIC (i.e. phenyl-based sorbents), where high concentrations of salt are needed for protein binding (sometimes in excess of 3 M NaCl !). Besides the cost of some lyotropic salts, the high-salt dependence of conventional HIC creates heavy waste-disposal constraints and additional investment in salt-resistant equipment. For this reason, MEP HyperCel® is now used as an alternative to traditional HIC, for "non-antibody" molecules in clinical trials. The protocols need to be optimized case by case for such applications.

Benefits in large-scale production of therapeutic-grade monoclonal antibodies: lower cost, cleaning stability and no protein A leakage.

MEP HyperCel® can be used as an alternative to expensive protein A-based sorbents for the capture and concentration of antibodies from cell culture media. In this role, MEP HyperCel® can serve as an effective first element in a multi-step purification protocol (involving other orthogonal chromatography technologies such as ion exchange) to achieve the overall purity levels (>99.9 %) required for therapeutic applications. The economic benefits to substituting MEP HyperCel® for protein A-based sorbents have been significantly great to justify a switch by several large-scale manufacturers even as late as Phase III of clinical trials.

Even as progress is being made to improve the linkage chemistry of protein A sorbents, the fundamental nature of these materials limits their stability when exposed to protease-containing feedstock and caustic cleaning agents. These limitations reduce the useful lifetime of protein A sorbents, place extra burdens on cleaning validation protocols and introduce the need for analytical tests to track and quantify leaching protein A fragments and purification methods to remove them when detected. MEP HyperCel® is a chemical ligand, not unlike the ligands on ion exchange sorbents. It is stable at pH values up to 14 and can be cleaned regularly with sodium hydroxide solutions of 1 M concentrations. (A full regulatory Support File with data illustrating stability over 200 CIP cycles of 1 M NaOH, 1 h contact time, is available on request). This outstanding base resistance is attractive to industrial users, like Dr. Estzer Birck-Wilson at Genzyme Transgenics Corp. (Framingham MA), who reported the importance of base stability for long-service sorbent life for the purification of IgG from transgenic goat milk (GEN, July 2000). The increased stability to biological feedstock and NaOH CIP of MEP HyperCel® typically translate into 300-500% greater service lifetime over protein A sorbents. When this advantage is coupled with the significantly reduced initial cost of MEP HyperCel®, the effective cost (cost per g of antibody produced) can be dramatically lower. And there is no risk – by definition – of protein A leakage !

Validation, virus clearance and large-scale production.

Viral-clearance studies were conducted using MVM (Minute Virus of Mice) and other models in the presence of IgG and are shown in Table 3. Data suggest a level of viral clearance (4 log reduction) equivalent to that reported for protein A sorbent. Combined with other orthogonal steps (ion exchange), virus and host cell protein removal levels compatible with therapeutic-grade IgG purity can be achieved.

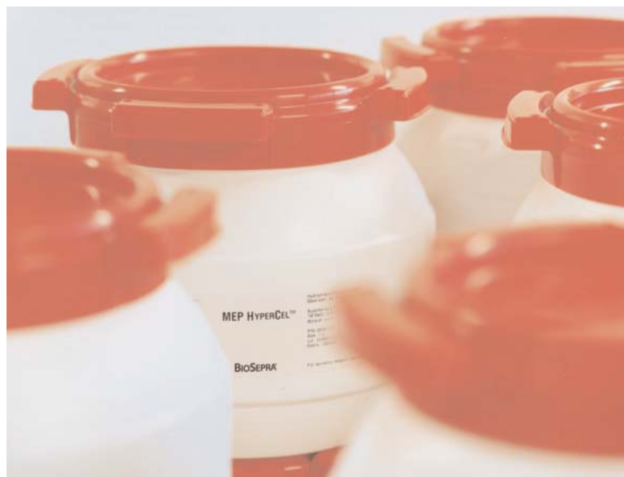
In contrast with other "protein A mimics" described, HCIC is a truly scaleable method. Since the commercial introduction of the method in 1999, production has been scaled-up to meet industrial needs. Batch sizes exceeding 100 liter are manufactured in BioSepra® Process Division facility of Ciphergen, and have been delivered for production purposes. Validation packages are available to assist industrial users in their own validation. These packages include full relevant information about sorbent manufacturing, ligand stability, and toxicology studies.

Table 3. Viral Clearance Studies on MEP HyperCel.

	MVM *(parvoviridae)*	MuLV *(retroviridae)*
Nucleic acid	ssDNA	dsRNA
External structure	Non enveloped	Enveloped
Size	18-26 nm	100 nm
Load	1.58×10^8	5.14×10^5
Flowthrough	2.33×10^7	5.14×10^5
Wash, pH 8.0	1.02×10^6	2.89×10^4
Elute, pH 4.0	1.17×10^4	2.14×10^2
Reduction	~ 4.3 logs	~ 3.38 logs

Novel complementary methods taylored to antibody stability and biological activity: MEP and MBI HyperCel®.

With MEP HyperCel®, desorption of antibody is achieved by decreasing the pH (typically pH 4.0, using sodium citrate or sodium acetate buffer). This relatively mild pH reduces aggregate formation and preserves activity of most antibodies. However, some antibodies or impurities present in the feedstreams will still aggregate at acidic pH.

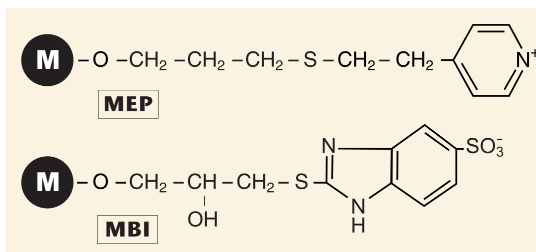


For this purpose, BioSepra® has developed a novel sorbent, which exploits the specific binding/elution properties of a new ligand: Mercapto-Benzimidazole-Sulfonic acid, MBI.

This ligand, which is complementary to MEP, (Figure 4) has been engineered to include the presence of an heterocycle, a sulfur atom and an acidic moiety, which have been proven to be effective for selective antibody adsorption. At the working pH, major contaminants such as albumin are not retained by MBI HyperCel®. Compared to MEP HyperCel®, MBI HyperCel® adsorbs antibodies at slightly acidic pH (pH 5.5), and elutes at alkaline pH (pH 9), without adjustment of the ionic strength.

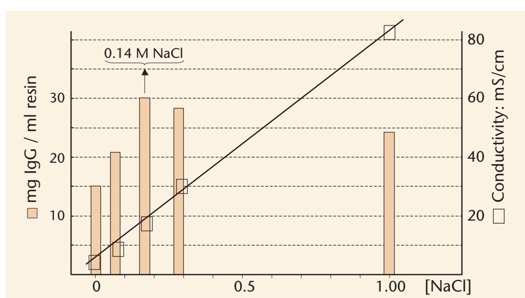
As shown in Figure 5, like MEP, the MBI ligand binds antibodies with high capacity (25 mg/ml for human IgG) in the absence of salt, allowing its use at physiological ionic strength. Only the pH needs to be adjusted (pH 5.5) for optimal binding.

Figure 4. MEP-MBI antibody selective ligands complementary structures.



Heterocycles contribute for mild ionic net charges. Thiophilicity by "S" enhances ability to capture antibodies. "M" represent the sorbent matrix.

Figure 5. MBI HyperCel: Capacity vs. ionic strength.



5 mg/ml human IgG in 50 mM sodium acetate, pH 5.5; Flow rate 100 cm/h; Capacity determinations at 10% breakthrough.

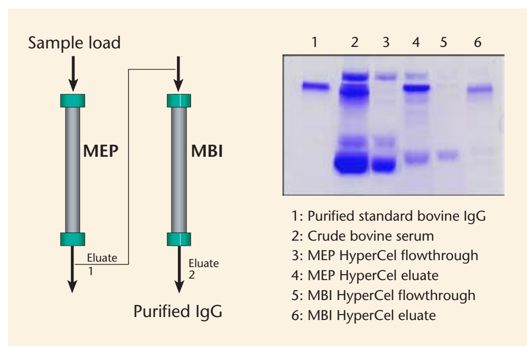
The alkaline pH used for desorption could be of interest to preserve the biological activity of acidic-sensitive IgGs.

As shown in Figure 6, the respective properties of MEP HyperCel® and MBI HyperCel® are complementary and allow a tandem use for 2 step high-purity purification of antibodies.

Replacing Protein A for clinical-grade antibody purity.

Like MEP, MBI HyperCel® represents another option to replace standard protein A sorbents used as capture step in a three-column purification sequence. In a study carried out in a biopharmaceutical company, MBI HyperCel® was compared to Protein A in such a sequence, and the final purity of the antibody assessed. Results summarized in Table 3 suggest that MBI HyperCel®, used in combination with ion exchangers, can achieve final purity and recovery results comparable to the reference protein A-based process for this specific antibody. Like for MEP HyperCel®, it is anticipated that a longer service-life and smaller sorbent cost could result in significant economic benefits at larger scale.

Figure 6. Tandem purification of IgG from serum MEP/MBI HyperCel.



1. MEP HyperCel Capture step: direct capture of the IgG by loading the feedstream on a MEP HyperCel column, without adjustment of pH or ionic strength; Elution of the antibody achieved by decreasing the pH to 4.0 - 4.5.
2. Direct loading of the eluate on the MBI HyperCel without adjustment of pH. Elution by increasing the pH to pH 9.0, in a 50 mM Tris buffer containing 0.15 - 1.0 M NaCl.
3. Sanitization cycle using 0.5 - 1 M NaOH.

Table 4. Use of MBI HyperCel in a three step sequence as alternative to protein A for clinical-grade IgG purification.			
Process	IgG purity (%)	HCP (ng/mg IgG)	Protein A (ng/mg IgG)
MBI HyperCel + IEX1 + IEX2	99.3	3.52	NR*
Protein A sorbent + IEX1 + IEX2	99.9	<0.64	<0.15

Data courtesy of Dr. Philippe Marschal, Novartis Pharma AG.
IEX = Ion Exchange step, HCP = Host Cell Proteins, NR = Non Relevant.

Conclusions: The end of the "protein A monopoly" for IgG purification ?

Hydrophobic Charge Induction Chromatography (HCIC) and novel ligands are robust tools available to industrial users. Though less specific than protein A for IgG in a single step, when combined to other standard chromatography, these easy to use products consistently produce IgG with similar levels of purity, recovery and safety, with more favourable process economics. Finally, in contrast with a number of protein A mimics described in the literature, HCIC sorbents have been available on large-scale since 1999 and are increasingly used at manufacturing scale for antibodies and other recombinant proteins in clinical trials.

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Over the past 25 years, BioSepra® chromatography products & services have earned an outstanding reputation for product innovation and technical support. Our expanded R&D sorbent program, new ISO 9001 manufacturing plant and recently launched MEP HyperCel™ and CM HyperZ™ sorbents represent our latest commitment to the biopharmaceutical industry.

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