A New One-Media Monoclonal Antibody Purification Method Using Preparative Porous Ethylenediamine-N, N'-Tetra(methylphosphonic) Acid (EDTPA) Modified Porous Zirconia

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Abstract

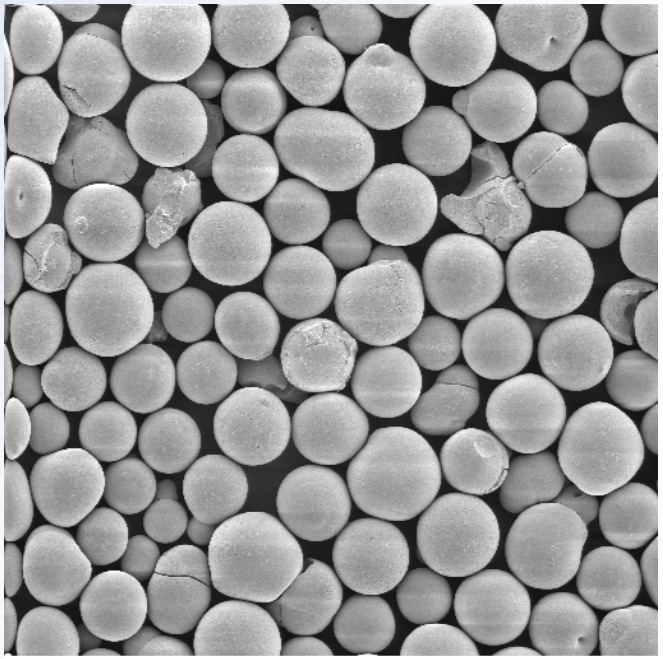
The research presented here describes the manufacture of a new class of *porous zirconia micro-spheres*, by spray drying, for large-scale preparative liquid chromatography of bio-molecules. Porous zirconia particles with an average diameter of 25 microns are coated with ethylenediamine-N, N'-tetra(methylphosphonic) acid (EDTPA) to produce a bio-compatible cation-exchange stationary phase for the purification of proteins. The coated zirconia particles can be packed into preparative liquid chromatographic columns and used for *rapid large-scale purification of monoclonal antibodies*. These mechanically stable zirconia columns can be run at very high mobile phase linear velocities compared to soft affinity gels functionalized with Protein A or Protein G. Thus *dramatic increases in purification throughput* are possible with the new zirconia phase. Most importantly, EDTPA modified zirconia (Rhinophase®-AB) can purify a wide range of IgG subclasses, as well as IgA and IgM, providing a *robust alternative to affinity chromatographic media*.

Outline

- Physical characteristics of 25 micron porous zirconia
- State-of-the-art Mab purification method
- Small scale Mab purification on 3 micron Rhinophase®-AB
- Preparative Mab purification on 25 micron Rhinophase®-AB
- Direct Comparison of Mab purified with Rhinophase®-AB versus affinity gel Protein G media
- Binding Capacity of Different Subclasses of Mab on Rhinophase®-AB
- Binding Capacity of IgGs derived from different animal sources on Rhinophase®-AB
- Binding Capacity of IgG, IgA and IgM on Rhinophase®-AB
- Conclusions

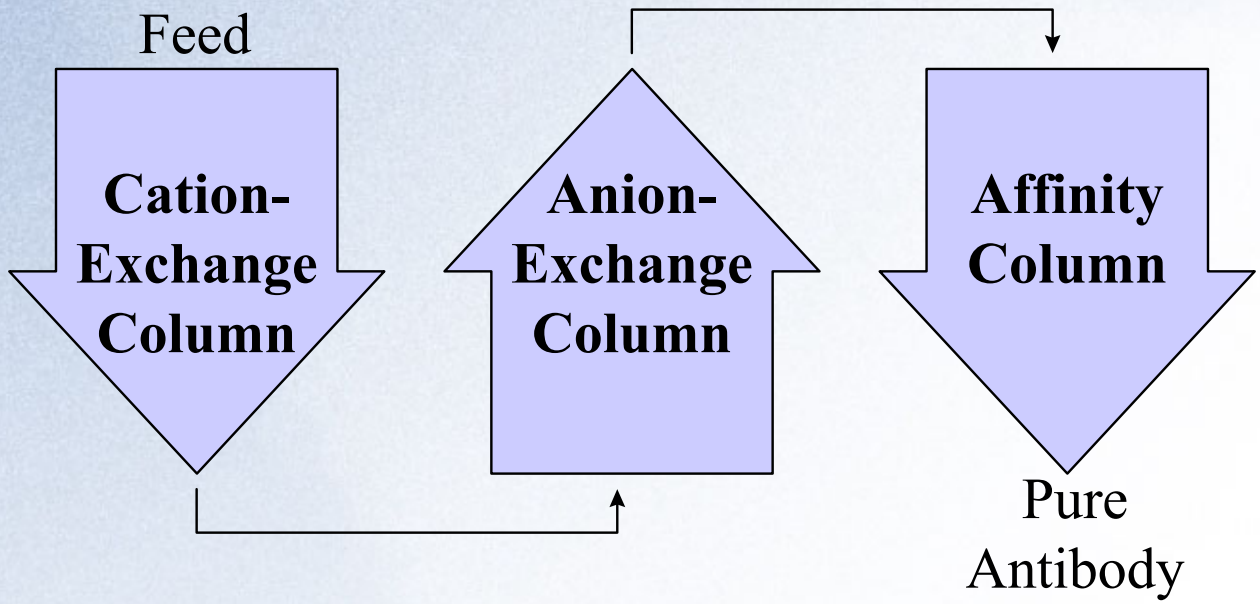
SEM and Nitrogen Porosimetry Data for Rhinophase®-AB

The spray dried particles are easily size classified using standard screens. The final material has large pores so that large bio-molecules can diffuse into the porous beads.



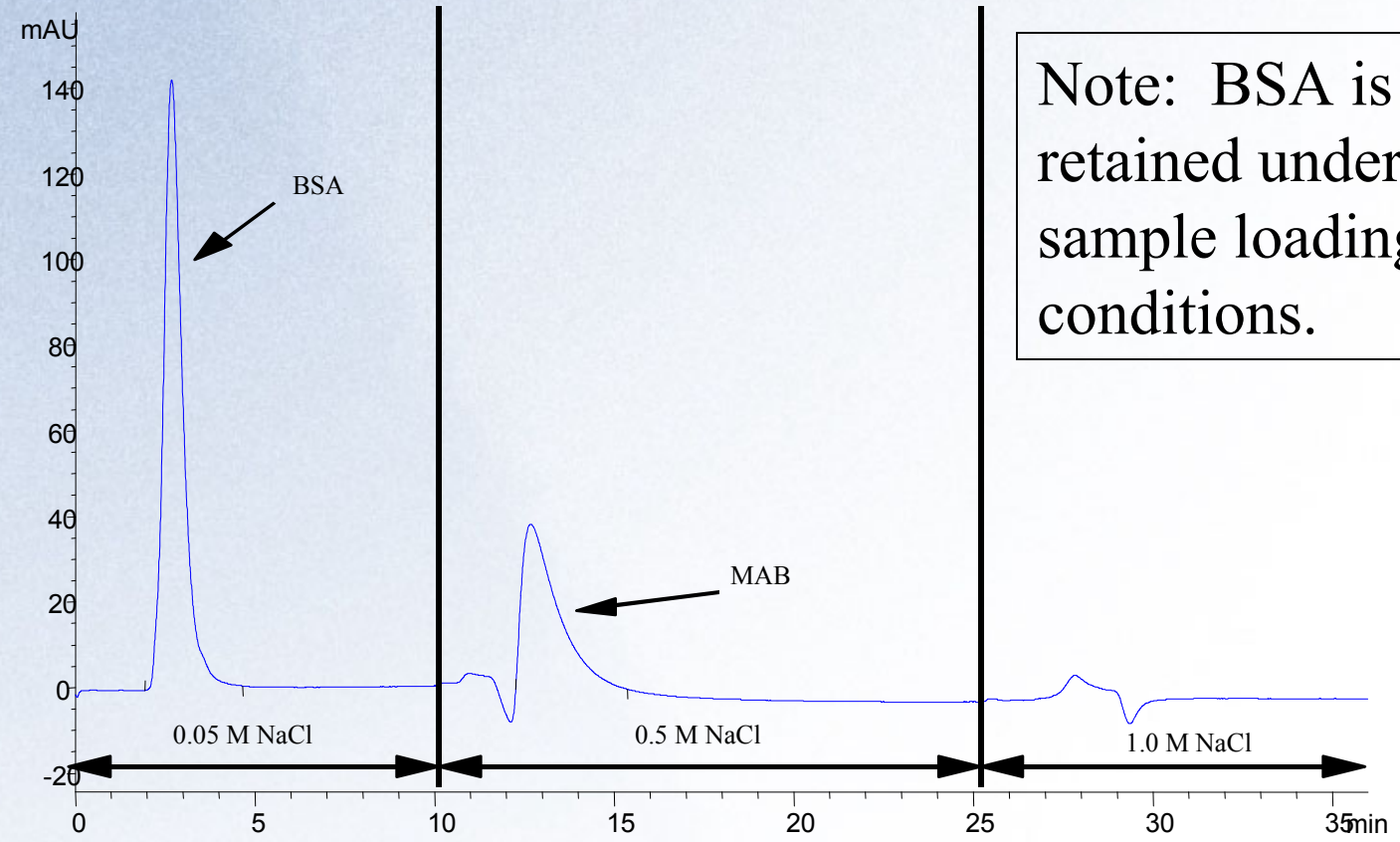
Sample	Surface Area (m^2/g)	Pore Volume (ml/g)	Average Pore Diameter (Å)
Rhinophase®-AB	14	0.100	300

State of the Art Mab Purification Method



A widely used method for the preparative purification of antibodies involves the use of three LC columns, first a cation-exchange column, followed by an anion-exchange column and finally an affinity column as the final purification media. The method is outlined above. This purification protocol is extremely time-consuming, but the final product is adequately pure (>95%). The first two columns in the purification protocol are an anion-exchange and a cation-exchange column. These two columns are used as initial clean-up columns and are followed by an affinity column that specifically binds and then releases the pure monoclonal antibody. (Reference: M. Fitchum, et. al., Bio-Rad Laboratories, bulletin 1917 US/EG Rev. B Hercules CA USA, 1994.)

Small-Scale Purification of Mab IgG_{2a} Contaminated with BSA



Note: BSA is not retained under sample loading conditions.

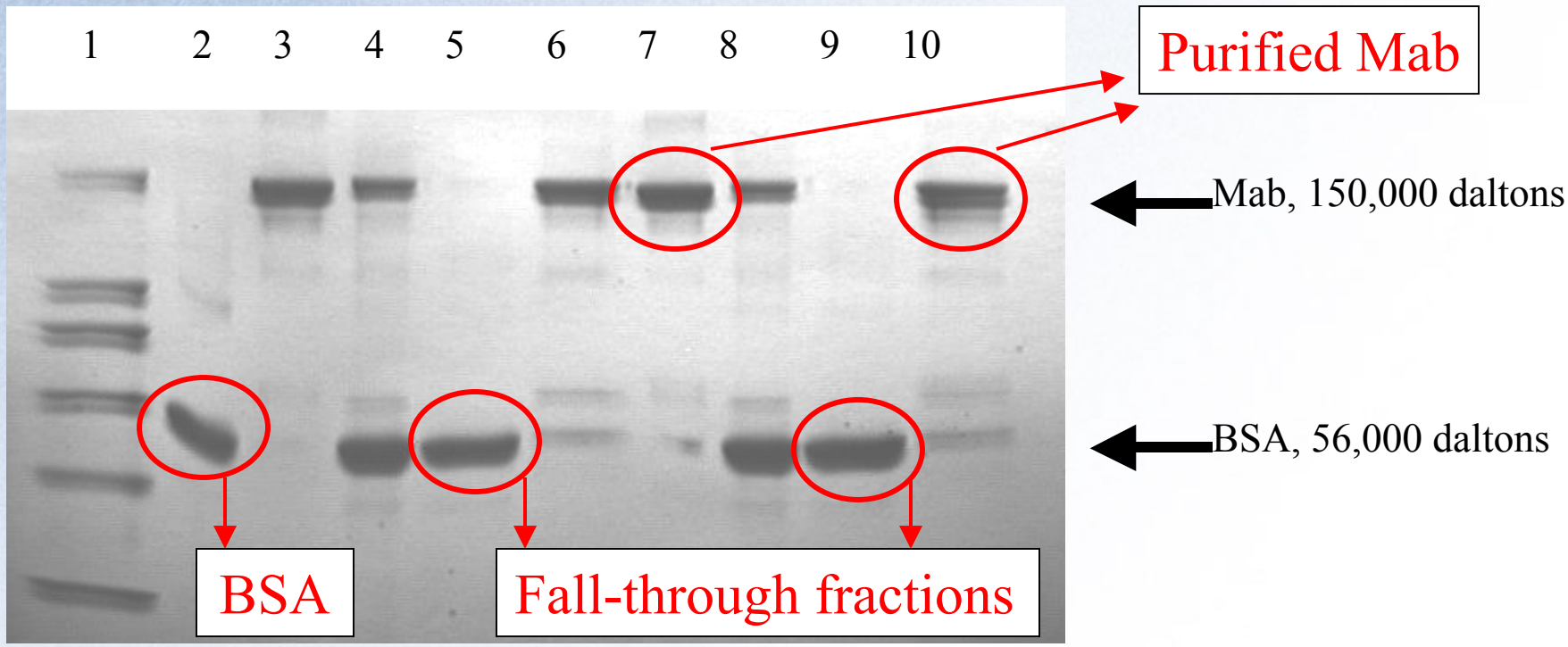
LC Conditions: 100 *u*l injection of BSA (6.0 mg/ml) contaminated MAB (1.0 mg/ml) eluted by salt step gradient. Mobile phase: 20 mM MES, 4 mM EDTPA, 0.05 M-to-1.0 M NaCl pH=5.5. Flow rate: 2.0 ml/min. Temperature: 30°C. Detection: 280 nm.

ELISA Results for Small Scale Cell Culture Supernatant IgG_{2a} Purification

	Amount of MAB (ug)					
	Feed	Unretained (Fraction 1)	Eluate (Fraction 2)	Wash (Fraction 3)	**% Yield**	**%Overall Detected**
Run #1	588.9	18.41	564.16	5.61	**95.8%**	**99.9%**
Run #2	605.4	18.32	544.06	4.89	**89.9%**	**93.7%**

- The amount of MAB in each fraction was estimated by ELISA.
- Cell culture supernatant was used as feed to the columns. Sample volumes of cell culture supernatant were lyophilized and reconstituted to give the desired MAB concentrations in feed. In each feed application the ratio of BSA to MAB remained relatively constant at 3.5 to 1.
- The % yield of the MAB was determined as a ratio of the total MAB in the eluate fraction (Fraction #2) to the total MAB present in the feed.
- The % overall detected amount of the MAB as determined by the ratio of the total MAB in the eluate fraction (Fraction #2) plus the MAB in fraction 1 and 3 to the total MAB present in the feed.

Electrophoresis of Small Scale IgG_{2a} Purification Fractions

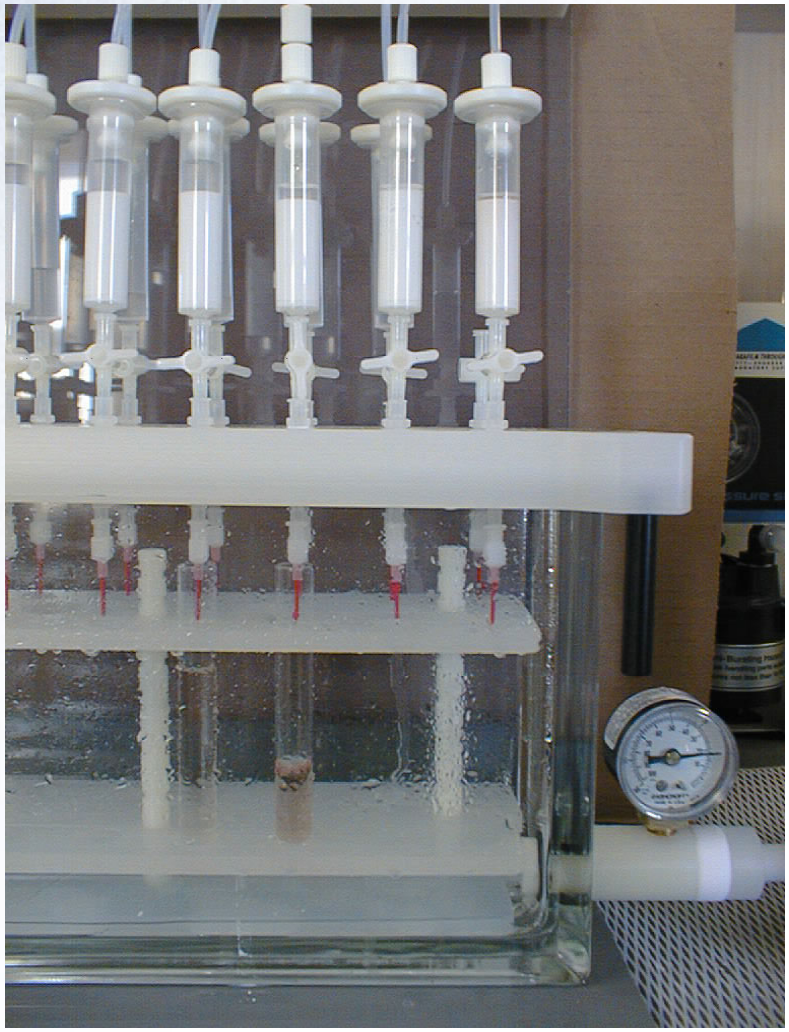


Lane 1 shows a molecular weight ladder. Lane 2 shows a 3 *u*g application of pure BSA. Lanes 3 and 7 show application of pure BSA and MAB at a total protein level of 2 *u*g, respectively. Lanes 4 and 8 show an application of cell culture supernatant at a total protein level of 4 *u*g. The cell culture supernatant has two distinct protein bands corresponding to BSA with a molecular weight of 56 KDa and MAB (IgG) with a molecular weight of 150 KDa with some additional minor bands. Lane 5 shows the unretained fraction from Run #1 at a total protein level of 3 *u*g. Lane 9 shows the unretained fraction from Run #2 at a total protein level of 3 *u*g. The fall through fraction gave a band around 56 kDa that is similar to the pure BSA in Lane 2. Lane 10 shows the elution fraction from Run #2 at a total protein level of 3 *u*g. The eluted fraction gave a band around 150 kDa that is similar to the pure MAB in Lane 3. The purity of the MAB in the eluate fraction (Lanes 6 and 10) is estimated to be greater than 96% by digital image processing.

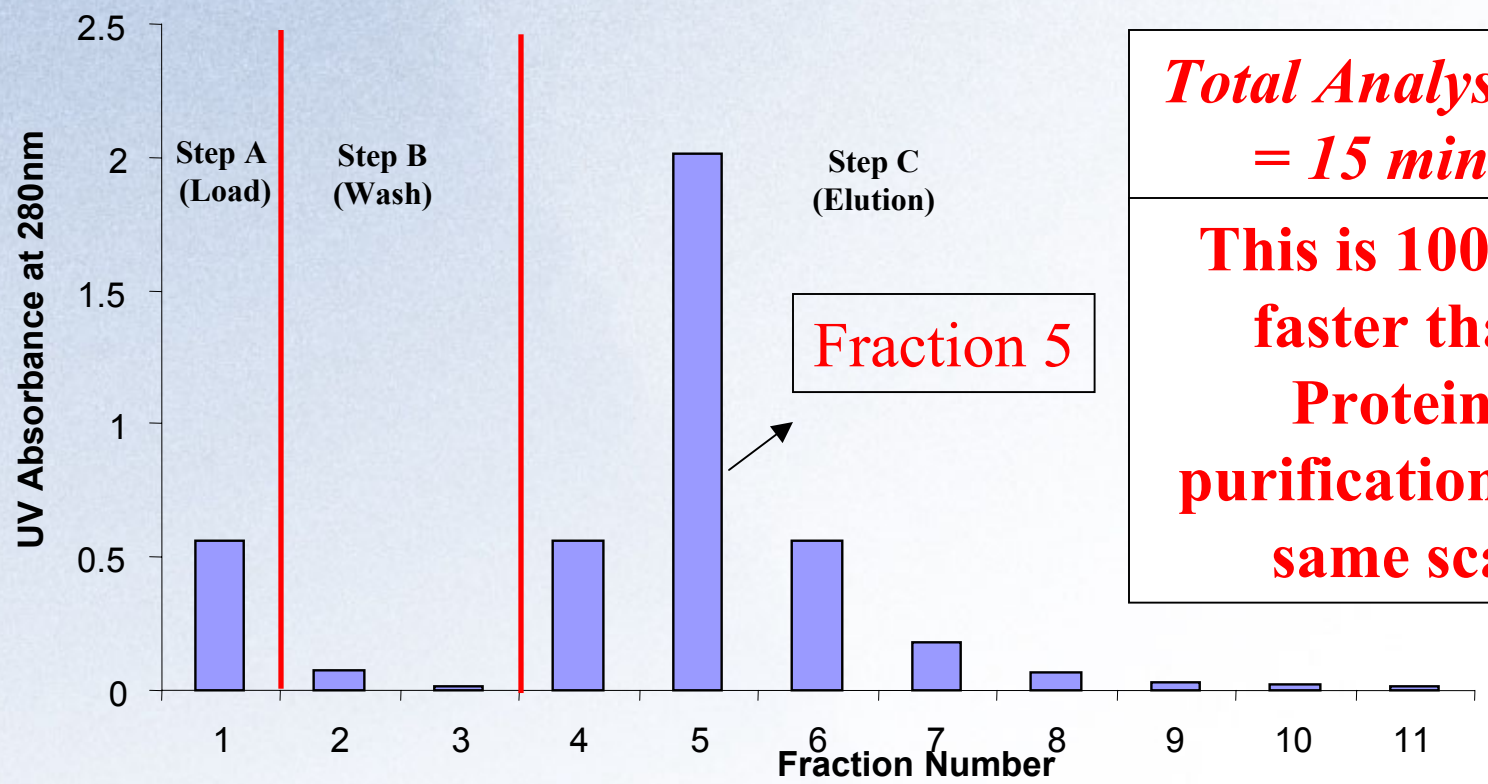
A New Ultrafast Preparative Purification Method Using Rhinophase®-AB

ZirChrom®

Due to zirconia's very high mechanical strength, Mab purifications can be performed at high mobile phase linear velocities. A simple vacuum filtration apparatus can be use to achieve very high flow rates through a packed bed (90 mL/min). This approach is not possible using soft affinity gels such as Protein A and Protein G media.



Ultrafast Preparative Purification of IgG_1 Using Rhinophase®-AB



Total Analysis Time = 15 minutes

This is 100 times faster than a Protein G purification of the same scale!

Step A = 20 mM MES buffer, 4 mM EDTPA, 50 mM NaCl @ pH 4.0, Step B = 20 mM MES buffer, 4 mM EDTPA, 50 mM NaCl @ pH 4.0, Step C = 20 mM MES buffer, 4 mM EDTPA, 2.0 M NaCl @ pH 4.0. Flow Rate = 60 mL/min, Injection size = 31.6 mL serum-free cell culture supernatant diluted 4-times with loading buffer, (3.98 mg of Mab), Amount of Rhinophase®-AB in tube = 10 grams.

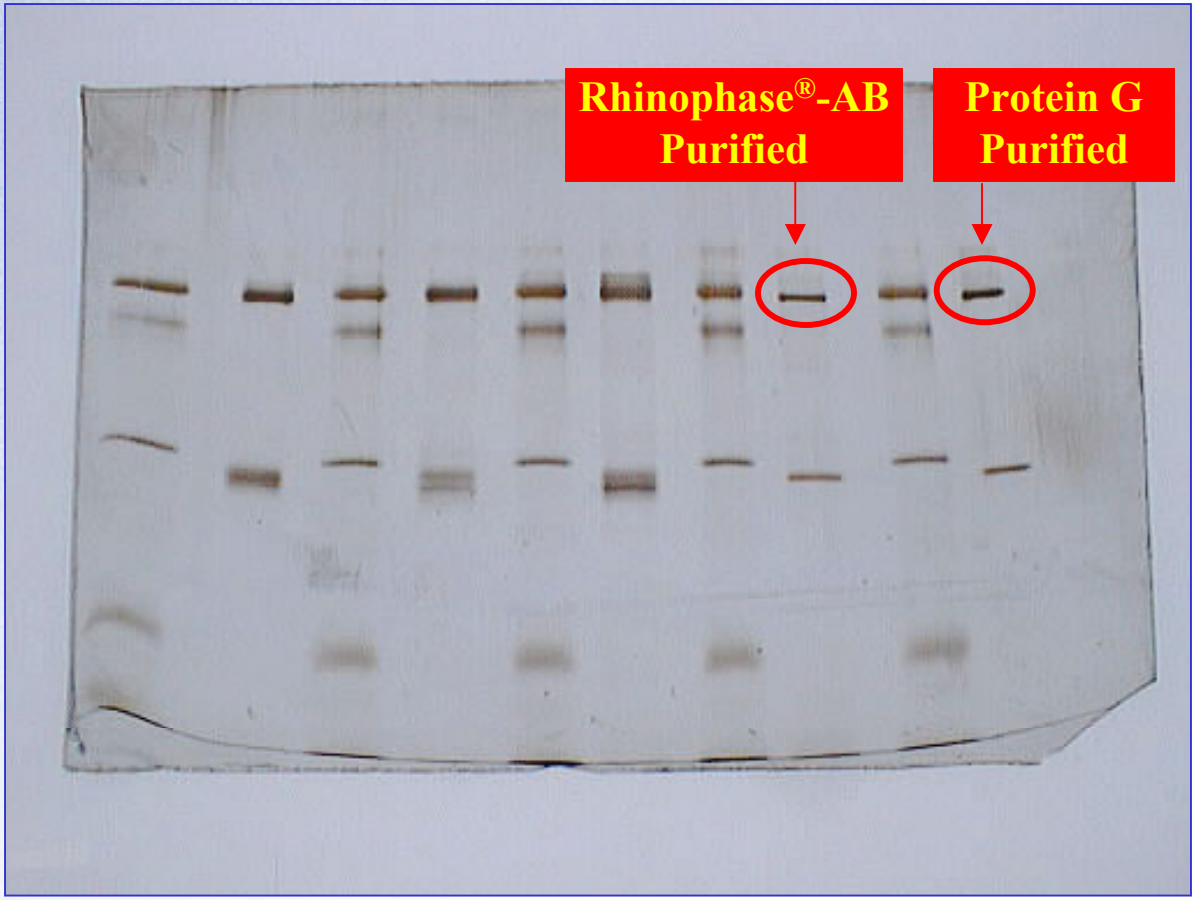
ELISA Plate Comparison of Protein G and the Ultrafast Purification on Rhinophase®-AB

	OD 280 nm		
Protein Offered	Protein G Purified IgG	Rhinophase®-AB Fraction 5	Fraction 5
15.6	0.0531	0.0502	94.5%
31.3	0.0946	0.0992	104.9%
62.5	0.1676	0.1892	112.9%
125	0.3176	0.3632	114.4%
250	0.5596	0.6362	113.7%
500	1.0166	1.1507	113.2%
1000	1.8151	1.8632	102.6%
		Average %	108.0%

An ELISA plate analysis using the same amount of Mab from Protein G and Rhinophase®-AB purifications showed an increased signal for the Rhinophase®-AB purified Mab.

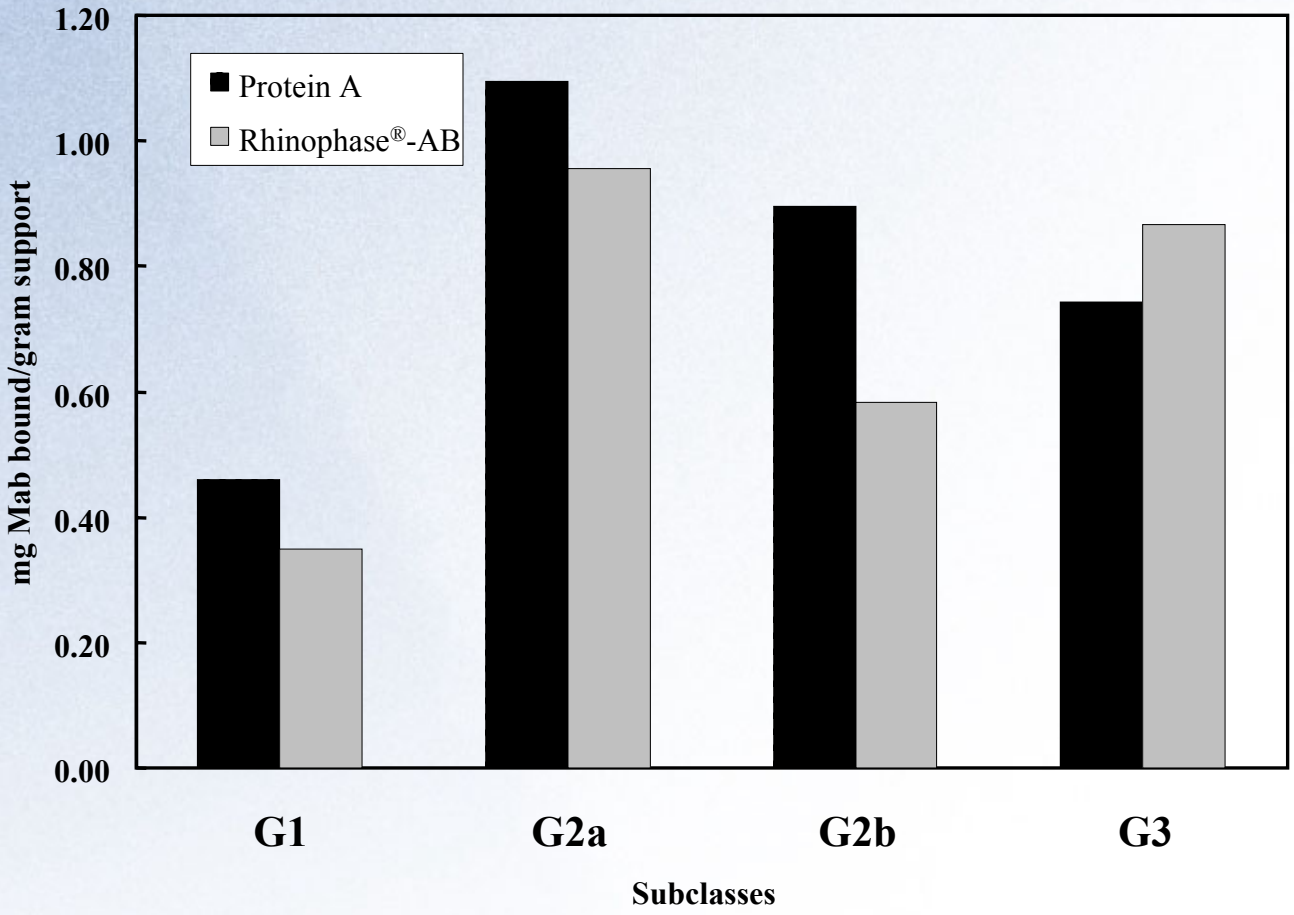
Mab Purity Comparison from Semi-Preparative Run

Purified Mab was equally pure using Protein G and Rhinophase®-AB.



Silver-stained, SDS-PAGE gel Comparing IgG_1 purified by Protein G (row 1 from right) and Rhinophase®-AB (row 3 from right). Electrophoresis was run under reducing conditions. Sample loading at 1 *u*g per lane. All other lanes are standards.

Relative Binding Strength of Different Subclasses of Mab



Rhinophase®-AB had high binding capacity for a variety of different Mab subclasses and is comparable to Protein A media.

Binding Capacity of Other Immunoproteins on Rhinophase®-AB

	Rhinophase®-AB
Sample	Capacity (mg antibody/ml particles)*
hIgG	28
hIgA	9
hIgM	2

*All values are reported as an average of 3-independent replicate experiments. The standard deviation is less than 5%. All capacities are reported as mg Ig bound per ml of beads.

Binding Studies of IgGs Derived from Different Sources

Rhinophase®-AB		
Sample	Offered	Bound
	mg/ml beads*	mg/ml beads*
Porcine IgG	37	28
	18	11
	9	8
	5	4
	1	1
Bovine IgG	22	19
	11	10
	6	5
	3	3
	1	1
Human IgG	22	19
	11	11
	6	5
	3	3
	1	1

*All values are reported as an average of 2-independent replicate experiments. The standard deviation is less than 5%. All capacities are reported as mg Ig bound per ml of beads.

Conclusions

- Rhinophase®-AB provides a widely applicable alternative to currently used Protein A and Protein G antibody purification media.
- Typical yields of Mab purifications greater than 95%, with purity levels equal to or greater than affinity gel-type media.
- Due to Rhinophase®-AB's excellent mechanical stability, purifications can be performed 100-fold faster with equivalent results.
- Rhinophase®-AB is chemically durable over the entire pH range, which allows for cleaning and depyrogenation (data not shown).
- ELISA plates produced with Rhinophase®-AB purified Mab showed greater signal than those produced with Protein G purified Mab.
- Rhinophase®-AB has affinity for a wide range of immunoprotein classes and subclasses including monoclonal and polyclonal IgG, IgA, and IgM.
- Acknowledgement: National Institutes of Health Grant # 5 R44 GM58354-03