Chapter Five: The Effects of Orientation, Local Spatial Density and Ligand Architecture on the Performance of Peptide-Based Affinity Sorbents

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Abstract

This report studies the effect of the orientation, local spatial density and ligand architecture of affinity peptide ligands immobilized within a low-solids content, large-particle diameter cellulose support for use as an affinity sorbent. Two linear peptides are studied here, a fibrinogen affinity peptide (FAP) ligand and a protein A mimetic (PAM) peptide ligand, which binds immunoglobulins. The use of the linear peptide recognition sequence is compared with the use of tetrameric and octameric branched-chain assemblies of the same linear peptide motif. \( N \)-terminal and \( C \)-terminal oriented immobilization of these peptide ligands is facilitated through the use of epoxide and carbodiimide coupling chemistries, respectively. A novel epoxide-gradient activation method (epoxy-GAM) is utilized for creating a gradient of support activation to control the spatial distribution of these peptide ligands within the support. In conjunction with the epoxy-GAM, classical bulk one-step and two-step coupling techniques are compared with regards to affinity sorbent performance. The two-step coupling (TSC) sequence comprised of peptide permeation under slow coupling conditions followed by conditions having fast coupling reaction kinetics to further assist in distributing the peptide ligand within the support matrix. Affinity peptide sorbents containing volume-averaged peptide densities of 0.5 – 4.0 mg/ml of support were evaluated. The static binding efficiency (\( \eta_{\text{eff}} \)) of an affinity sorbent is defined in this study on two bases, with regards to the amount of peptide ligand immobilized per ml of support and on the amount of peptide recognition sequence immobilized per ml of support. The static binding efficiency (\( \eta_{\text{eff}} \)) is defined as the experimentally observed binding capacity of the target protein (mg target protein/ml support) divided by the maximum theoretical target protein binding capacity based upon either a 1:1, target protein: peptide ligand stoichiometric ratio, or a 1:1, target protein: recognition sequence stoichiometric ratio, and is expressed as a percentage. The optimal affinity sorbents obtained using epoxy-GAM/TSC methods in conjunction with oriented carbodiimide-mediated coupling chemistry were deemed to contain a more uniform spatial distribution of immobilized peptide ligand and were considerably more active relative to those affinity sorbents made using epoxy-GAM/one-step methods that contained locally high peptide densities near the bead particle edge. For example, using oriented carbodiimide-mediated
coupling, the PAM affinity sorbents demonstrated a 3.3-fold increase in binding efficiency based on the amount of PAM ligand immobilized utilizing the epoxy-GAM/TSC methods relative to the classical bulk one-step method using the same octameric ligand structural format. Utilizing the same techniques and ligand architecture, the FAP affinity sorbents demonstrated increases in binding efficiencies of 1.7-fold based on the amount of FAP ligand immobilized. Although the peptide ligand distribution for each case was deemed well within the interior of the large-particle diameter cellulose beads, no limitation in mass transport was observed using HETP analysis. The utility of these FAP and PAM affinity sorbents were also demonstrated in the purification of recombinant human fibrinogen from transgenic swine milk and 7D7B10 monoclonal antibodies from hybridoma cell culture supernatant, respectively. The optimal engineering of a peptide-based affinity sorbent is demonstrated by this study to be considered a synergistic orchestration of several important design factors, which include the effects associated with immobilized ligand orientation, local spatial density and ligand architecture.
Introduction

Affinity chromatography uses covalently immobilized ligands to selectively adsorb their respective target protein from a complex mixture into a support matrix. These peptides can form high affinity complexes with protein targets and are one of the newest technologies arising from combinatorial chemistry and phage display technology with applications to the difficult problem of purifying high molecular weight proteins from complex biological mixtures. Peptide-based affinity sorbents are being developed in order to surmount the inherent limitations, e.g., expensive cost, contamination issues and stability, posed by monoclonal antibodies that are used as ligands in immunosorbents (Baumbach et al., 1992). This study identifies factors analogous to those factors manifested in immunosorbtent performance that may also be important in the optimal design of peptide-based affinity sorbents. In general, previous research with the design of immunosorbents have found that immunosorbent performance, i.e., antigen-binding efficiency, is substantially dependent upon several factors, which include effects associated with ligand orientation, local spatial density as related to steric encumbrance of target protein binding sites, and transport phenomena as related to the underutilization of intramatrix support volume (Eveleigh et al., 1977; Orthner et al., 1991; Velander et al., 1991; Subramanian et al., 1994, 1996) (see Figure 1). Two structural motifs, which are common to small peptide affinity ligands, are studied here: linear peptide versus tetrameric and octameric branched chain assemblies of the same linear amino acid recognition sequence motif. One linear peptide studied here forms a high affinity complex with fibrinogen (Buettner et al., 1998) (see Figure 2). Fibrinogen is an important therapeutic plasma protein used in the treatment of inherited blood clotting disorders and in the formulation of hemostatic preparations (fibrin glues or sealants) (Brennan, 1991; Greco et al., 1991; Alving et al., 1995; Cottingham et al., 1996; Singh et al., 1996). The fibrinogen affinity peptide (FAP) studied here contains an additional glutamate residue to facilitate the use of carboxy-terminus oriented coupling chemistry (see Figure 3). Another linear peptide recognition sequence binds the Fc region of immunoglobulins (Fassina et al., 1997) (see Figure 4). Immunoglobulins are valuable plasma proteins used in therapeutic applications, such as in imparting passive immunity. The static binding efficiency ($\eta_{\text{eff}}$) of an affinity sorbent is defined in this
study on two bases, with regards to the amount of peptide ligand immobilized per ml of support and on the amount of peptide recognition sequence immobilized per ml of support. The static binding efficiency ($\eta_{\text{eff}}$) is defined as the experimentally observed binding capacity of the target protein (mg target protein/ml support) divided by the maximum theoretical target protein binding capacity based upon either a 1:1, target protein: peptide ligand stoichiometric ratio, or a 1:1, target protein: recognition sequence stoichiometric ratio, and is expressed as a percentage. These same effects appear to determine the performance of small ligand, peptide-based affinity matrices. Hence, while combinatorial chemistry and phage-display technology have assisted in developing well-characterized peptide ligands, which afford enhanced stability, selectivity, and efficient process costs, methods of ligand installation, which afford increased binding efficiency, as well as support platforms, which afford enhanced mass transfer characteristics and pressure-flow performance, can significantly impact the cost effectiveness of laboratory, preparative and commercial scale affinity chromatography (Kaster et al., 1993).

Our previous studies with immunosorbents have found that the local spatial ligand density more directly correlates with binding efficiency ($\eta_{\text{eff}}$) than does volume-averaged ligand density (Subramanian et al., 1994). Here we refer to the volume-averaged ligand density ($\langle \rho \rangle$) as the total amount of immobilized ligand on a bulk average basis. Whereas, the local ligand density ($\rho_{\text{loc}}$) refers to the local spatial distribution of ligand within the support particle. Pragmatically, both $\langle \rho \rangle$ and $\rho_{\text{loc}}$ are expressed as mg ligand/ml support. As detailed in the previous chapter, the high local densities of immobilized mAb derived from conventional bulk activation (BA) and bulk coupling (BC) methods decreased immunosorbent performance relative to those immunosorbents prepared using the epoxy-GAM and TSC techniques for the spatial manipulation of mAb within the support. In addition, the binding activity of an affinity ligand is inherently affected by its immobilized orientation. An improperly oriented immobilized ligand may be rendered completely inactive or exhibit a significant reduction in binding activity due to conformational restrictions, multipoint attachment and ligand-matrix interactions (see Figure 1). The oriented immobilization of mAb
through the Fc domain, rather than the Fab binding domain, was found to increase substantially immunosorbent binding efficiency (Subramanian et al., 1996). Similarly, peptide ligands typically require complete accessibility of their N-terminal α-amino groups for the necessary stabilizing interactions involved in the formation of the ligand-target complex. The use of a branched-chain structural format in peptide ligand design affords multiple sites on the ligand for covalent immobilization, thus increasing the probability of retaining recognition properties upon immobilization (Fassina, 1992). Therefore, the optimal engineering of a peptide-based affinity sorbent would be considered a synergistic orchestration of several important design factors, which include the effects associated with immobilized ligand orientation, local spatial density and ligand architecture.

The effects of affinity ligand orientation, local spatial density and ligand architecture on peptide-based affinity sorbent performance are quantitatively assessed in the study presented herein. The use of the linear peptide recognition sequence is compared with the use of tetrameric and octameric branched-chain peptide assemblies of the same linear peptide motif (see Figures 7 and 8). N-terminal and C-terminal oriented immobilization of these peptide ligands into the cellulose supports is facilitated through the use of conventional epoxide and carbodiimide (EDAC) coupling chemistries. The reaction chemistries utilized in this study are illustrated in Figures 10 – 14 (Sundberg et al., 1974; Matsumoto et al., 1979; Hermanson et al., 1992; Nakajima et al., 1995). The base cellulose structure comprising the low-solids content, large-particle diameter cellulose support is shown in Figure 9. Figure 10 shows the epoxy-activation of the cellulose support using epichlorohydrin. Figure 11 shows the amination of the epoxy-activated cellulose support using ammonium hydroxide. Figure 12 shows the EDAC-mediated coupling of peptide ligands onto the aminated cellulose supports. Figure 13 shows the concomitant crosslinking of the peptide ligands during EDAC-mediated support coupling. Figure 14 shows the epoxy-mediated immobilization of the peptide ligands onto the epoxy-activated cellulose support. A novel epoxide-gradient activation method (epoxy-GAM) is utilized for creating a gradient of support activation to control the spatial distribution of these peptide ligands within the support. In conjunction
with the epoxy-GAM, classical bulk one-step and two-step coupling techniques are compared with regards to affinity sorbent performance. The two-step coupling (TSC) sequence comprised of peptide permeation under slow coupling conditions followed by conditions having fast coupling reaction kinetics to further assist in distributing the peptide ligand within the support matrix. Here we specifically manipulate the affect of local density on binding efficiency using a novel epoxide-gradient activation method (epoxy-GAM) for creating a gradient of support activation. We compare the results from affinity sorbents made with the epoxy-GAM using different coupling chemistries on a low-solids content, large-particle diameter (500 – 900 \( \mu \text{m} \)) cellulose support to effect changes in local density (Kaster et al. 1993; Velander et al. 1994, 1999). These large-particle diameter, uncross-linked, beaded cellulose hydrogel supports of only a solids content of 2.0 - 3.5 wt. % were designed to more optimally afford high rates of intraparticle mass transfer while maintaining mechanical stability (Kaster et al., 1993). Finally, the intraparticle transport properties are evaluated by comparing the theoretical and experimental height-equivalent-to-a-theoretical plate (HETP) values calculated according to the methods of Mikes et al. (1975) (see Figure 15) and Snyder et al. (1979) (see Figure 16), respectively.

**Fibrinogen Affinity Peptide (FAP)**

Fibrinogen is a plasma protein involved in the blood clotting cascade. An ample, pathogen-free supply of fibrinogen is required for the treatment of blood disorders (Butler et al., 1997). Therefore, efficient and cost-effective methods for fibrinogen isolation involving peptide affinity ligands have been developed. Various peptide sequences have been developed which exhibit fibrinogen binding (Table 1) (Buettner et al., 1998; Mondorf et al., 1998; Kuyas et al., 1990). Buettner et al. (1996) developed a linear affinity peptide ligand for the isolation of human fibrinogen from blood plasma fraction I paste (Figure 2). The fibrinogen binding peptide was found by Baumbach et al. (1997) to exhibit cooperative binding properties. Cooperative binding is the phenomenon in which multiple ligand interactions facilitate the effective binding of the target. Therefore, this peptide-based affinity sorbent for fibrinogen isolation would be
dependent upon an optimum peptide density. An optimum affinity binding profile would be expected for a peptide ligand exhibiting binding cooperativity as shown in Figures 5 and 6. Steric hindrance effects would inhibit target binding at high local spatial ligand densities. A low local spatial ligand density would provide too few of the multiple ligand interactions to provide an effective ligand-target affinity interaction. As shown in Table 2, although the performance results of Baumbach et al. (1997) indicate an increase in static binding capacity with an increase in peptide ligand surface density, static binding efficiency is greatly reduced with an increase in peptide ligand surface density due to steric hindrance effects. For example, with a peptide density of 3.8 mg/ml support, a static binding capacity of 6.50 mg hFib/ml support is obtained, yielding a static binding efficiency of 0.40 %. In comparison with a higher peptide density of 22.00 mg/ml support, a static binding capacity of 20.60 mg hFib/ml support is attained, yielding a static binding efficiency of 0.22 %.

*Protein A Mimetic (PAM)*

Monoclonal antibodies account for the largest class of pharmaceutical product derived from the biotechnology industry. Monoclonal antibodies can be used as therapeutic and imaging agents, in addition to *in vivo* and *in vitro* diagnostic agents. In the United States, the preponderance of monoclonal antibodies in clinical trials are developed as imaging and diagnostic agents. These monoclonal antibodies must have precisely defined avidity, potency, specificity and stability. The presence of contaminants or other antibodies can diminish the accuracy of monoclonal antibodies used as *in vitro* diagnostic agents. In addition, monoclonal antibodies used in immunoaffinity chromatography must be highly purified and characterized. Conventional isolation processes for monoclonal antibodies typically involve precipitation, ion exchange chromatography and affinity chromatography using immobilized *Staphylococcal* or recombinant protein A, protein G, or protein A/G hybrids (Coleman et al., 1990; Dancette et al., 1999).
Protein A exhibits high and specific binding affinity for the Fc domain of immunoglobulins. This specificity has been utilized in the development of immobilized Protein A matrices for affinity chromatography. However, the isolation of monoclonal immunoglobulins has posed problematic issues for pharmaceutical applications. These issues include the high cost, and low stability of the affinity sorbent, and the difficulty in the elimination of biological contaminants. In addition, protein A cannot recognize all immunoglobulin subclasses and does exhibit variable affinity for immunoglobulins derived from different species. Affinity media, such as thiophilic, histidyl, and boronic acid supports have been prepared for the isolation of antibodies. However, these affinity supports lack adequate specificity for antibody isolation. This factor has been an impetus for the design for highly specific and well characterized synthetic protein A biomimetics. In order to surmount these constraining issues, several researchers have pursued different strategies in the development of a protein A peptide mimetic. For example, Li et al. (1996) developed a nonpeptidyl biomimetic ligand for staphylococcus protein A using computer-aided molecular modeling. Lihme et al. (1997) developed an expanded bed adsorption support based on a low molecular weight protein A mimetic ligand, which has an extensive binding specificity for mouse, rat, and human immunoglobulin, including IgM. In particular, Fassina et al. (1997) developed a protein A mimetic (PAM) peptide ligand. PAM, which was derived from synthesizing and screening a combinatorial peptide library, has the ability to recognize the Fc domain of immunoglobulin G from a range of species, including human, rabbit, goat, sheep, and mouse. The PAM ligand has also been found to exhibit IgA and IgM binding affinity. The PAM ligand has a multimeric structure consisting of recognition sequences of tripeptide tetramers (Figure 6).

Commercially available synthetic polymeric supports, which bear active groups for the covalent coupling of peptides, have been previously evaluated for PAM immobilization. These supports include: CH-Sepharose 4B® (Pharmacia), CH-Sepharose 6B®, Eupergit C30N®, Eupergit C150N®, and Protein-Pak®. A density of 20 mg PAM/ml support is reported to have been obtained using these supports. Eupergit C is a matrix composed of a cross-linked polymer of methylacrylamide, N-
methylenebis-acrylamide, and monomers containing reactive oxirane groups, which react with amino, thiol, and hydroxyl groups of peptides. Eupergit C150N®, and C30N® consists of porous 150 µm diameter, and 30 µm diameter beaded particles, respectively (Fleminger et al. 1990). As shown in Table 7, most of these PAM affinity supports developed using commercially available supports and conventional amine-reactive coupling chemistries have yielded low immunoglobulin binding efficiencies most likely due to a high surface density of immobilized PAM. For example, PAM immobilized at a density of 5.5 mg PAM/ml support [Protein-Pak™ (Waters)] yielded only a 3.6 % binding efficiency (12.5 mg IgG/ml support or 2.3 mg IgG/mg PAM) for rabbit IgG isolation and a 1.84 % binding efficiency (6.5 mg IgG/ml support or 1.2 mg IgG/mg PAM) for swine IgG isolation. For the Waters support, the dynamic binding efficiency was 0.46 % calculated according to the ratio of mol swine IgG to immobilized PAM recognition sequence. Using CH-Sepharose-4B with PAM immobilized at a density of 6.6 mg/ml support, the dynamic binding capacity was 0.7 mg swine IgG/ml support, yielding dynamic binding efficiencies of 0.04 % (mol swine IgG/mol recognition sequence) and 0.17 % (mol swine IgG/mol PAM ligand). Using the Eupergit C30N support with PAM immobilized at a density of 1.74 mg/ml support, the dynamic binding capacity was 2.0 mg swine IgG/ml support, yielding higher dynamic binding efficiencies of 0.45 % (mol swine IgG/mol recognition sequence) and 1.79 % (mol swine IgG/mol PAM ligand).

Several strategies have been employed in the optimal design of immunosorbents, which are applicable to the development of peptide-based affinity sorbents under consideration here. In order to minimize multipoint attachment, immobilization parameters such as coupling reaction pH and duration, including the density of reactive functional groups available for covalent coupling, have been manipulated (Velander et al. 1991). A uniform spatial distribution of reactive functional groups within the support matrix would aid in developing an affinity sorbent with optimal binding characteristics. The GAM was developed in order to facilitate the uniform spatial distribution of immobilized affinity ligand within a support matrix, such as a cellulose hydrogel. Under study here is the use of a chromatographic support in which the interior volume of the support particles could be more effectively utilized for peptide
immobilization. The use of a large-particle diameter, low-solids content support with enhanced intraparticle transport characteristics for large protein targets in conjunction with these ligand installation techniques would allow for an increase in binding efficiency due to improved spatial distribution of immobilized peptide ligands and therefore more effective utilization of immobilized peptide ligand.

**Materials and Methods**

*Materials and chemical reagents*

Fibrinogen affinity peptide (FAP) and protein A mimetic (PAM) ligand derivatives were kindly provided by C.G. Russell (Research Genetics, Inc., Huntsville, AL) and G. Fassina (Tecnogen, Inc., Naples, Italy). Standard human fibrinogen (hFib) was kindly provided by the American Red Cross (Rockville, MD). Transgenic swine milk containing recombinant human fibrinogen (rhFib), including nontransgenic control swine milk, was supplied by our laboratory. Large-diameter (~500 μm), low-solids content (2.0 - 3.5 wt. %) beaded cellulose supports were prepared in our laboratory (Velander et al., 1994, 1999). Affinity purified, whole-molecule goat anti-mouse, sheep anti-mouse, rabbit anti-goat and including goat anti-mouse and anti-rabbit antibodies conjugated to horse radish peroxidase (HRP), swine IgG, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) and 1-chloro-2,3-epoxypropane (epichlorohydrin) were purchased from Sigma Chemical Co. (St. Louis, MO). PVDF Immobilon P membranes were purchased from Millipore (Bedford, MA). Polyclonal rabbit anti-human fibrinogen antibodies were purchased from American Diagnostica (Greenwich, CT). The metal enhanced diaminobenzidine kit was purchased from Pierce Chemical Co. (Rockford, IL). Immulon II microtiter plates were purchased from Fisher Scientific (Pittsburgh, PA). The JB4 histological embedding kit was purchased from Polysciences, Inc. (Warrington, PA, USA). O-Phenylenediamine-2HCl (OPD) reagent was purchased from Abbott Laboratories (Chicago, IL). Column chromatography experiments were conducted using C10 columns (column dimensions: 15 × 1 cm) from Pharmacia Biotech (Piscataway, NJ) and a Masterflex peristaltic pump. Chromatographic separations were monitored using a Rainin data acquisition system with a Knauer inline variable
wavelength spectrophotometer. All other reagents were purchased at the finest grade available from Sigma Chemical Co. (St. Louis, MO).

**Gradient activation method (GAM)**

The epoxy-activation of cellulose supports was performed by using the gradient activation method (GAM). Cellulose support (10 ml) was slurry packed into a 15 ml Pharmacia C\textsubscript{10} chromatography column. For the washing steps, flow was performed from the bottom of the column to the top at 2 ml/min. The support was washed with three column volumes (CV) each of water, then with 100 % ethanol. The support was then washed with a solution of 50 % (v/v) epichlorohydrin prepared in 100 % ethanol. The support was then incubated in the epichlorohydrin/ethanol solution for 30 minutes at room temperature (25 °C). The flow was then reversed to drain the support of interstitial fluid to yield a support-cake. The support-cake was then transferred into a round-bottomed flask. 1 M NaOH (3 CV) containing 2 mg/ml sodium borohydride was then added to the flask. The activation reaction was allowed to proceed for 24 hours at room temperature (25 °C) with gentle stirring. After the activation reaction, the support was then washed on a sintered-glass filter-funnel with 10 CV each of water, 100 % ethanol, then water again at 4 °C. The epoxy-activated cellulose support was then used immediately for epoxy-mediated peptide immobilization. For the EDAC-mediated peptide immobilization studies, the epoxy-activated support was aminated by further reacting the support with 1 M ammonium hydroxide (2 CV) for at least three hours at 45 °C on a rotator. The support was then washed with ten column volumes each of 1 M NaCl, then water on a sintered-glass filter-funnel. The aminated supports were stored in 20 % ethanol until further use.

**Peptide ligand immobilization**

*Classical one-step bulk coupling procedure for epoxy-mediated (N-terminal) peptide immobilization*

Epoxy-activated cellulose support (1 ml) prepared for each of the case studies as described above was allowed to react with each peptide (5 – 10 mg) at a constant
pH 9.5 – 10.0 using 0.1 M sodium carbonate/DMSO (50 – 80 (v/v) %) (2 ml) overnight (24 hrs) at 45 – 50 °C on a rotator. The column was then washed in column mode (1 ml/min) with at least three column volumes each of coupling buffer, 1 M NaCl, then water. Residual amine-reactive epoxy groups on the support were then blocked with 2 M Tris-Base, pH 9.5 or with 10 (v/v) % ethanolamine, pH 9.5 by incubating overnight (24 hrs) at room temperature (25 °C) on a rotator. The affinity supports were then stored in 20 % ethanol. The initial and final supernatant fractions were then analyzed for peptide concentration and a material balance performed to determine peptide coupling efficiency (ψ).

Two-step coupling (TSC) procedure for epoxy-mediated (N-terminal) peptide immobilization

This coupling technique utilized a two-step permeation and coupling sequence in the immobilization of peptide in order to manipulate local ligand density (Velander et al. 1994). Activated column support (1 ml) prepared for each of the case studies as described above was allowed to incubate with peptide (5 – 10 mg) at a constant pH 6.0 using 0.1 M sodium carbonate/DMSO (50 – 80 (v/v) %) (2 ml) at room temperature (25 °C) for one hour on a rotator. The reaction mixture was then adjusted to pH 9.5 – 10.0 and allowed to proceed overnight (24 hrs) at 45 – 50 °C on a rotator. The column was then washed in column mode (1 ml/min) with at least three column volumes of each of coupling buffer, 1 M NaCl, then water. Residual amine-reactive groups on the epoxide support were then blocked with 2 M Tris-Base, pH 9.5 or with 10 (v/v) % ethanolamine, pH 9.5 by incubating overnight (24 hrs) at room temperature (25 °C) on a rotator. The affinity supports were then stored in 20 % ethanol. The initial and final supernatant fractions were then analyzed for peptide concentration and a material balance performed to determine peptide coupling efficiency (ψ).

Classical one-step bulk coupling procedure for EDAC-mediated (C-terminal) peptide immobilization

Aminated cellulose support (1 ml) prepared for each of the case studies as described above was allowed to react with each peptide (5 – 10 mg) at a constant pH 6.5 – 7.0 using 0.1 M MES/DMSO (50 – 80 (v/v) %) (2 ml) containing 100 – 200-fold
molar excess of EDAC to peptide overnight (24 hrs) at room temperature (25 °C) on a rotator. The column was then washed in column mode (1 ml/min) with at least three column volumes each of coupling buffer, 1 M NaCl, then water. The affinity supports were then stored in 20 % ethanol. The initial and final supernatant fractions were then analyzed for peptide concentration and a material balance performed to determine peptide coupling efficiency (ψ).

**Two-step coupling (TSC) procedure for EDAC-mediated (C-terminal) peptide immobilization**

Aminated cellulose support (1 ml) prepared for each of the case studies as described above was allowed to incubate with each peptide (5 – 10 mg) at a constant pH 6.5 – 7.0 using 0.1 M MES/DMSO (50 – 80 (v/v) %) (2 ml) for one hour at room temperature (25 °C) on rotator. EDAC (100 – 200-fold molar excess of EDAC to peptide) was added and then the mixture was allowed to react overnight (24 hrs) at room temperature (25 °C) on a rotator. The column was then washed in column mode (1 ml/min) with at least three column volumes each of coupling buffer, 1 M NaCl, then water. The affinity supports were then stored in 20 % ethanol. The initial and final supernatant fractions were then analyzed for peptide concentration and a material balance performed to determine peptide coupling efficiency (ψ).

**Assays**

**Total protein determination**

A colorimetric dye-binding assay kit was used to determine total protein concentration (Pierce Chemical Co.). Bovine serum albumin (BSA) was used as the standard (Sigma Chemical Co., St. Louis, Mo).

**Determination of peptide by thin-layer chromatography**

Peptide concentration was determined by thin-layer chromatography. Briefly, 3 – 5 µl of prepared known standard and unknown samples were applied in triplicate onto silica gel plates (10 × 20 cm: 200 µm layer thickness) (Whatman, Inc.). Plates can be washed with 45 – 50 % ethanol prior to detection. The plate was then sprayed
uniformly with ninhydrin solution and allowed to dry at room temperature (25 °C). The ninhydrin solution consisted of 95 parts of 0.2 (w/v) % ninhydrin in n-butanol and 5 parts of 10 (v/v) % glacial acetic acid and mixed thoroughly before use. The plate was heated to 120 °C and baked for about 10 – 20 minutes. The plate was then subjected to densitometry by scanning the plate at 570 nm.

**Determination of human fibrinogen by ELISA**

The concentration of human fibrinogen (hFib) was determined by polyclonal ELISA. Anti-hFib IgG (whole molecule; 1:1,000 dilution; 5 µg/ml) prepared in 0.1 M NaHCO₃ pH 9.6 was added to Immunlon II microtiter plates (100 µl/well) and incubated overnight (24 hrs) at 4 °C. Wells were washed three times with 12.5 mM Tris-HCl/50 mM NaCl/0.05% (w/v) Tween 20, pH 7.2 (TBST). Residual reactive sites were blocked with TBST containing 0.1 (w/v) % BSA (TBST-BSA) and incubated at room temperature (25 °C) for 20 minutes. Wells were then washed three times with TBST. Dilutions of hFib standard and unknown samples were prepared in TBST-BSA, added in triplicate (100 µl/well) and incubated for 20 minutes at 37 °C. Wells were then washed three times with TBST. Horse-radish peroxidase-conjugated anti-hFib IgG (1:1,000 dilution) was added to the wells (100 µl/well) and incubated for 20 minutes at 37 °C. Wells were then washed three times with TBST. The bound chromophore complex was detected and developed with OPD substrate. The absorbance of each well was read at 490 nm using an EL-308 Bio-Tek microplate reader.

**Determination of swine IgG by ELISA**

The concentration of swine IgG was determined by polyclonal ELISA. Anti-swine IgG (whole molecule; 1:200 dilution; 5 µg/ml) prepared in 0.1 M NaHCO₃ pH 9.6 was added to Immunlon II microtiter plates (100 µl/well) and incubated overnight at 4 °C. Wells were washed three times with 12.5 mM Tris-HCl/50 mM NaCl/0.05% (w/v) Tween 20, pH 7.2 (TBST). Residual reactive sites were blocked with TBST containing 0.1 (w/v) % BSA (TBST-BSA) and incubated at room temperature (25 °C) for 20 minutes. Wells were then washed three times with TBST. Dilutions of swine IgG
standard and unknown samples were prepared in TBST-BSA, added in triplicate (100 µl/well) and incubated for 20 minutes at 37 °C. Wells were then washed three times with TBST. Horseradish peroxidase-conjugated anti-swine IgG (1:1,000 dilution) was added to the wells (100 µl/well) and incubated for 20 minutes at 37 °C. Wells were then washed three times with TBST. The bound chromophore complex was detected and developed with OPD substrate. The absorbance of each well was read at 490 nm using an EL-308 Bio-Tek microplate reader.

**Determination of 7D7B10 mAb by ELISA**

The concentration of 7D7B10 mAb was determined by polyclonal ELISA. Anti-mouse IgG (whole molecule; 1:200 dilution; 5 µg/ml) prepared in 0.1 M NaHCO₃ pH 9.6 was added to Immunlon II microtiter plates (100 µl/well) and incubated overnight at 4 °C. Wells were washed three times with 12.5 mM Tris-HCl/50 mM NaCl/0.05% (w/v) Tween 20, pH 7.2 (TBST). Residual reactive sites were blocked with TBST containing 0.1 (w/v) % BSA (TBST-BSA) and incubated at room temperature (25 °C) for 20 minutes. Wells were then washed three times with TBST. Dilutions of swine IgG standard and unknown samples were prepared in TBST-BSA, added in triplicate (100 µl/well) and incubated for 20 minutes at 37 °C. Wells were then washed three times with TBST. Horseradish peroxidase-conjugated anti-mouse IgG (1:1,000 dilution) was added to the wells (100 µl/well) and incubated for 20 minutes at 37 °C. Wells were then washed three times with TBST. The bound chromophore complex was detected and developed with OPD substrate. The absorbance of each well was read at 490 nm using an EL-308 Bio-Tek microplate reader.

**Evaluation of peptide affinity sorbents by column chromatography**

**FAP column chromatography**

**Evaluation of static hFib binding efficiency**

FAP affinity sorbents were evaluated under static binding conditions using pure human fibrinogen (hFib). FAP affinity sorbents (column bed volume: 1 ml) were conditioned with three column volumes of each of the following buffers in sequence at 1
ml/min: 10 mM sodium acetate/1 M NaCl, pH 5.1; 10 mM sodium acetate, pH 3.9; 0.1 M glycine/2 % acetic acid, pH 2.0; 6 M urea/25 mM sodium citrate, pH 7.3; and 70 % ethanol/2 % acetic acid. The columns were then equilibrated with binding buffer (75 mM Arginine/75 mM ε-aminocaproic acid/5 mM imidazole/0.1 M NaCl, pH 6.5) (2 ml) in a polypropylene test tube. Pure hFib (10 mg) was allowed to incubate with the FAP affinity sorbents for at least one hour at room temperature (25 °C) on a rotator. After incubation, the affinity sorbents were then packed into a Pharmacia C10 column. The columns were then eluted at 1 ml/min using the following sequence of buffers: 10 mM sodium acetate/1 M NaCl, pH 5.1; 10 mM sodium acetate, pH 3.9; 0.1 M glycine/2 % acetic acid, pH 2.0; 6 M urea/25 mM sodium citrate, pH 7.3; and 70 % ethanol/2 % acetic acid. FAP columns were stored in 20 % ethanol. Static binding experiments were done in triplicate. Chromatographic elution fractions were subjected to hFib determination by ELISA.

*Milk processing*

Control and transgenic pig milk was diluted 1:1 volumetric ratio with 50 mM Tris/200 mM EDTA, pH 8.5 and frozen at -90 °C. Milk was defatted by centrifugation at 15,000 × g for 20 minutes at 4 °C. The top fat layer was removed and the resulting whey fraction was then stored at -90 °C until further use.

*Purification of hFib from doped control swine milk*

The FAP affinity sorbent utilizing the octameric form of the FAP ligand in conjunction with the EDAC-mediated coupling chemistry using the GAM/TSC method was evaluated in its ability to capture human fibrinogen (hFib) from nontransgenic control swine milk. The column bed volume was 5 ml. Human fibrinogen was doped into nontransgenic control swine whey (10 ml) at a final concentration of 50 µg/ml. Briefly, the swine whey was diluted in a 1:1 volumetric ratio with the binding buffer and loaded onto the column at 0.5 ml/min until a steady baseline was attained. The FAP column was then eluted and conditioned using the same procedure as described in the preceding section on the evaluation of static hFib binding efficiency. All column
fractions for each experiment were collected and subjected to hFib determination by ELISA, and including SDS-PAGE and western blotting analysis. These experiments were performed in triplicate. A set of identical control chromatographic experiments utilizing nontransgenic control swine milk in the absence of either recombinant or standard human fibrinogen was also performed.

**Purification of rhFib from transgenic swine milk**

The FAP affinity sorbent utilizing the octameric form of the FAP ligand in conjunction with the EDAC-mediated coupling chemistry using the GAM/TSC method was evaluated in its ability to capture recombinant human fibrinogen (rhFib) from transgenic swine milk. The column bed volume was 5 ml. rhFib was estimated in the transgenic swine whey (10 ml) at a concentration of approximately 100 µg/µl. Briefly, the transgenic swine whey was diluted in 1:1 volumetric ratio with the binding buffer and loaded onto the column at 0.5 ml/min until a steady baseline was attained. The FAP column was then eluted and conditioned using the same procedure as described in the preceding section on the evaluation of static hFib binding efficiency. All column elution fractions for each experiment were collected and subjected to rhFib determination by ELISA, and including SDS-PAGE and western blotting analysis. These experiments were performed in triplicate.

**PAM column chromatography**

**Evaluation of static swine IgG binding efficiency**

PAM affinity sorbents were evaluated under static binding conditions using pure swine IgG. PAM affinity sorbents (column bed volume: 1ml) were conditioned with three column volumes of each of the following buffers at 1 ml/min: 0.1 M glycine/2 % acetic acid, pH 2.0; 2 M NaSCN; 6 M Urea; and 4 M NaCl. The columns were then equilibrated with binding buffer (25 mM Bis-Tris, pH 6.5) (2 ml) in a polypropylene test tube. Pure swine IgG (10 mg) was allowed to incubate with the PAM affinity sorbents for at least one hour at room temperature (25 °C) on a rotator. After incubation, the affinity sorbents were then packed into a Pharmacia C10 column. The columns were then eluted at 1 ml/min using the following sequence of buffers: 0.1 M glycine/2 % acetic
acid, pH 2.0; 2 M NaSCN; 6 M Urea; and 4 M NaCl. PAM columns were stored in 20 % ethanol. Chromatographic elution fractions were subjected to swine IgG determination by ELISA. Static binding experiments were done in triplicate.

**GAM and PAM ligand density management**

The GAM was employed using varying concentrations of epichlorohydrin to effect subsequent changes in the level of support activation in order to facilitate the management of the local spatial density of immobilized PAM ligand. Epichlorohydrin concentrations of 5, 10, 25 and 50 (v/v) % were evaluated with the GAM using a 2 wt. % cellulose support. PAM affinity sorbents were prepared utilizing the tetrameric branched form immobilized through the classical bulk coupling technique. Epoxy-mediated and EDAC-mediated ligand coupling chemistries were employed to effect an approximate volume-averaged density of 1 mg/ml support. These PAM affinity sorbents (column bed volume: 1 ml) were then evaluated for static swine IgG binding efficiency as described in the preceding section.

**Purification of 7D7B10 mAb from hybridoma cell culture supernatant**

The PAM affinity sorbent utilizing the octameric form of the PAM ligand in conjunction with the EDAC-mediated coupling chemistry using the GAM/TSC method was evaluated in its ability to capture 7D7B10 mAb from hybridoma cell culture supernatant. The column bed volume was 1 ml. Briefly, the hybridoma cell culture supernatant was diluted in a 1:1 volumetric ratio with the binding buffer and loaded onto the column at 0.5 ml/min until a steady baseline was attained. The PAM column was then eluted and conditioned using the same procedure as described in the preceding section on the evaluation of static swine IgG binding efficiency. All column fractions for each experiment were collected and subjected to mAb determination by ELISA, and including SDS-PAGE and western blotting analysis. These experiments were performed in triplicate.
SDS-PAGE analysis

SDS-PAGE analysis was performed using the procedure of Laemmli (1970). SDS-PAGE analysis was conducted using a Pharmacia PhastSystem™ PhastGel™ Unit with 4 – 15 % gradient gels following the manufacturers instructions (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) and polyacrylamide gradient gels from Novex (San Diego, CA). Gradient gels (8 – 16 % and 4 – 12 %) were used for reduced and nonreduced conditions, respectively. Tris-glycine buffer (20 mM Tris/0.1 % SDS/200 mM glycine, pH 7.2) was used as the running buffer. Prestained molecular weight markers were used for the molecular weight determination of proteins (BioRad Laboratories). Samples (1 µl) were loaded and electrophoresed until the 7.0-kDa molecular weight marker reached the end of the gel. Silver-stained SDS-PAGE was used to examine the proteins in the chromatographic fractions.

Western blotting analysis

After the proteins were resolved using SDS-PAGE analysis, the proteins were transferred to PVDF Immobilon P membranes (Millipore, Bedford, MA) and subjected directly to immunoanalysis. The western blotting procedure was a modification of the technique used by Matsudaira (1987). Briefly, gels were transferred in Towbin buffer (10 % methanol/200 mM glycine/25 mM Tris/0.1 % SDS, pH 7.2) using the Novex X-Cell II blot module following the manufacturers instructions. Membranes were blocked using 20 mM Tris/50 mM NaCl/0.05 % Tween 20/0.5 % bovine casein, pH 7.2 (TBSTC) for at least three hours with gentle agitation at room temperature (25 °C). Blots were probed with the primary antibody for 1 – 3 hours at 37 °C with gentle agitation. The primary antibody used for the detection of fibrinogen was polyclonal rabbit anti-human fibrinogen IgG (American Diagnostica, Greenwich, CT) (1:1,000 dilution). The secondary antibody was goat anti-rabbit IgG, horseradish peroxidase conjugate (Sigma Cat. No. A9169). The primary and secondary antibodies used for the detection of 7D7B10 mAb were goat anti-mouse IgG (Fc specific, Sigma Cat. No. M2650) and rabbit anti-goat IgG (whole molecule; horseradish peroxidase conjugate, Sigma Cat. No. A4174), respectively. After incubation, the blots were washed with water and developed using a metal
enhanced diaminobenzidine kit according to the manufacturers instructions (Pierce Chemical Co., Rockford, IL).

**HETP analysis of affinity sorbents**

The performance efficiency of various peptide affinity sorbents are compared using HETP analysis. The HETP value is a measure of the resolving efficiency of a chromatography column. The intraparticle transport properties of each of the affinity sorbents are evaluated by comparing the theoretical and experimental height-to-a-theoretical-plate (HETP) values calculated according to the methods of Mikes et al. (1975) and Snyder et al. (1979), respectively. The experimental HETP correlation is provided as:

$$HETP = \frac{L}{N} = \frac{L}{5.54 \left( \frac{t_R}{W_h} \right)^2}$$

Where $L$ is the column bed length (cm), $N$ is the number of theoretical plates, $t_R$ is the retention of the sample within the column and $W_h$ is the peak width at half peak height. The theoretical HETP correlation is provided as:

$$HETP = H_{\text{particle size}} + H_{\text{particle diffusion}} + H_{\text{film diffusion}}$$

$$H_{\text{particle size}} = 1.64r$$

$$H_{\text{particle diffusion}} = \frac{D_v}{(D_v + \epsilon)^2} \frac{0.142r^2F}{D_s}$$

$$H_{\text{film diffusion}} = \left( \frac{D_v}{D_v + \epsilon} \right)^2 \frac{0.266r^2F}{D_L \left(1 + 70rF\right)}$$
Where $r$ is the particle radius, $D_v$ is the mg solute bound per ml column bed/mg solute per ml solution, $F$ is the superficial velocity, $D_s$ is the diffusion coefficient within the support particle, $D_L$ is the diffusion coefficient in the solvent and $\varepsilon$ is the volume void fraction of the particle.

**Results**

**FAP affinity sorbent performance**

Tables 3 – 6 present performance results for the FAP affinity sorbents. The FAP affinity sorbent utilizing the linear ligand with the epoxy coupling chemistry in conjunction with the one-step coupling method yielded a coupling efficiency of $29.0 \pm 0.5\%$ with a ligand density of $0.89 \pm 0.06$ mg ligand/ml support ($0.95 \pm 0.07 \mu$mol ligand/ml support; $0.95 \pm 0.07 \mu$mol recognition sequence/ml support). The static binding capacity was $0.02 \pm 0.08$ mg hFib/ml support ($16,147.28 \pm 13.87$ mol ligand/mol hFib; $16,147.28 \pm 13.87$ mol recognition sequence/mol hFib). The static binding efficiencies were $0.01 \pm 0.02\%$ (mol hFib/mol recognition sequence) and $0.01 \pm 0.02\%$ (mol hFib/mol ligand). The FAP affinity sorbent utilizing the linear ligand with the epoxy coupling chemistry in conjunction with the two-step coupling (TSC) method yielded a coupling efficiency of $38.0 \pm 0.5\%$ with a ligand density of $1.42 \pm 0.11$ mg ligand/ml support ($1.52 \pm 0.18 \mu$mol ligand/ml support; $1.52 \pm 0.18 \mu$mol recognition sequence/ml support). The static binding capacity was $0.06 \pm 0.09$ mg hFib/ml support ($8,5877.69 \pm 11.05$ mol ligand/mol hFib; $8,5877.69 \pm 11.05$ mol recognition sequence/mol hFib). The static binding efficiencies were $0.01 \pm 0.02\%$ (mol hFib/mol recognition sequence) and $0.01 \pm 0.02\%$ (mol hFib/mol ligand). The FAP affinity sorbent utilizing the linear ligand with the EDAC coupling chemistry in conjunction with the one-step coupling method yielded a coupling efficiency of $35.0 \pm 0.5\%$ with a ligand density of $1.75 \pm 0.12$ mg ligand/ml support ($1.87 \pm 0.22 \mu$mol ligand/ml support; $1.87 \pm 0.22 \mu$mol recognition sequence/ml support). The static binding capacity was $2.71 \pm 0.88$ mg hFib/ml support ($234.32 \pm 1.28$ mol ligand/mol hFib; $234.32 \pm 1.28$ mol recognition sequence/mol hFib). The static binding efficiencies were $0.43 \pm 0.12\%$ (mol hFib/mol recognition sequence)
and $0.43 \pm 0.12\ %$ (mol hFib/mol ligand). The FAP affinity sorbent utilizing the linear ligand with the EDAC coupling chemistry in conjunction with the TSC method yielded a coupling efficiency of $41.0 \pm 0.5\ %$ with a ligand density of $2.05 \pm 0.15$ mg ligand/ml support ($2.19 \pm 0.19$ µmol ligand/ml support; $2.19 \pm 0.19$ µmol recognition sequence/ml support). The static binding capacity was $4.69 \pm 0.43$ mg hFib/ml support ($158.61 \pm 1.53$ mol ligand/mol hFib; $158.61 \pm 1.53$ mol recognition sequence/mol hFib). The static binding efficiencies were $0.63 \pm 0.05\ %$ (mol hfib/mol recognition sequence) and $0.63 \pm 0.05\ %$ (mol hFib/mol ligand). The FAP affinity sorbent utilizing the tetrameric ligand with the epoxy coupling chemistry in conjunction with the one-step coupling method yielded a coupling efficiency of $68.0 \pm 0.5\ %$ with a ligand density of $3.40 \pm 0.20$ mg ligand/ml support ($0.80 \pm 0.14$ µmol ligand/ml support; $3.19 \pm 0.42$ µmol recognition sequence/ml support). The static binding capacity was $1.48 \pm 0.40$ mg hFib/ml support ($183.31 \pm 1.43$ mol ligand/mol hFib; $733.24 \pm 1.17$ mol recognition sequence/mol hFib). The static binding efficiencies were $0.14 \pm 0.04\ %$ (mol hfib/mol recognition sequence) and $0.55 \pm 0.15\ %$ (mol hFib/mol ligand). The FAP affinity sorbent utilizing the tetrameric ligand with the epoxy coupling chemistry in conjunction with the TSC method yielded a coupling efficiency of $76.0 \pm 0.5\ %$ with a ligand density of $3.80 \pm 0.20$ mg ligand/ml support ($0.89 \pm 0.05$ µmol ligand/ml support; $3.57 \pm 0.17$ µmol recognition sequence/ml support). The static binding capacity was $2.64 \pm 0.31$ mg hFib/ml support ($114.85 \pm 1.34$ mol ligand/mol hFib; $459.42 \pm 1.14$ mol recognition sequence/mol hFib). The static binding efficiencies were $0.22 \pm 0.03\ %$ (mol hfib/mol recognition sequence) and $0.87 \pm 0.10\ %$ (mol hFib/mol ligand). The FAP affinity sorbent utilizing the tetrameric ligand with the EDAC coupling chemistry in conjunction with the one-step coupling method yielded a coupling efficiency of $74.0 \pm 0.5\ %$ with a ligand density of $3.70 \pm 0.25$ mg ligand/ml support ($0.87 \pm 0.07$ µmol ligand/ml support; $3.47 \pm 0.16$ µmol recognition sequence/ml support). The static binding capacity was $3.52 \pm 0.57$ mg hFib/ml support ($83.87 \pm 1.51$ mol ligand/mol hFib; $335.50 \pm 1.16$ mol recognition sequence/mol hFib). The static binding efficiencies were $0.30 \pm 0.05\ %$ (mol hfib/mol recognition sequence) and $1.19 \pm 0.19\ %$ (mol hFib/mol ligand). The FAP affinity sorbent utilizing the tetrameric ligand with the EDAC coupling chemistry in conjunction with the TSC method yielded a
coupling efficiency of 77.0 ± 0.5 % with a ligand density of 3.85 ± 0.15 mg ligand/ml support (0.90 ± 0.06 µmol ligand/ml support; 3.61 ± 0.18 µmol recognition sequence/ml support). The static binding capacity was 5.81 ± 0.88 mg hFib/ml support (52.88 ± 1.83 mol ligand/mol hFib; 211.50 ± 1.22 mol recognition sequence/mol hFib). The static binding efficiencies were 0.47 ± 0.07 % (mol hfib/mol recognition sequence) and 1.89 ± 0.29 % (mol hFib/mol ligand). The FAP affinity sorbent utilizing the octameric ligand with the epoxy coupling chemistry in conjunction with the one-step coupling method yielded a coupling efficiency of 55.0 ± 0.5 % with a ligand density of 2.75 ± 0.20 mg ligand/ml support (0.32 ± 0.02 µmol ligand/ml support; 2.56 ± 0.16 µmol recognition sequence/ml support). The static binding capacity was 2.23 ± 0.22 mg hFib/ml support (48.79 ± 1.98 mol ligand/mol hFib; 390.35 ± 1.32 mol recognition sequence/mol hFib). The static binding efficiencies were 0.26 ± 0.02 % (mol hfib/mol recognition sequence) and 2.05 ± 0.20 % (mol hFib/mol ligand). The FAP affinity sorbent utilizing the octameric ligand with the epoxy coupling chemistry in conjunction with the TSC method yielded a coupling efficiency of 67.0 ± 0.5 % with a ligand density of 3.35 ± 0.20 mg ligand/ml support (0.39 ± 0.03 µmol ligand/ml support; 3.12 ± 0.24 µmol recognition sequence/ml support). The static binding capacity was 3.49 ± 0.35 mg hFib/ml support (37.98 ± 1.24 mol ligand/mol hFib; 303.84 ± 1.46 mol recognition sequence/mol hFib). The static binding efficiencies were 0.33 ± 0.03 % (mol hfib/mol recognition sequence) and 2.64 ± 0.27 % (mol hFib/mol ligand). The FAP affinity sorbent utilizing the octameric ligand with the EDAC coupling chemistry in conjunction with the one-step coupling method yielded a coupling efficiency of 60.0 ± 0.5 % with a ligand density of 3.00 ± 0.15 mg ligand/ml support (0.35 ± 0.05 µmol ligand/ml support; 2.79 ± 0.39 µmol recognition sequence/ml support). The static binding capacity was 3.58 ± 0.51 mg hFib/ml support (33.16 ± 1.77 mol ligand/mol hFib; 265.25 ± 1.17 mol recognition sequence/mol hFib). The static binding efficiencies were 0.38 ± 0.05 % (mol hfib/mol recognition sequence) and 3.02 ± 0.43 % (mol hFib/mol ligand). The FAP affinity sorbent utilizing the octameric ligand with the EDAC coupling chemistry in conjunction with the TSC method yielded a coupling efficiency of 64.0 ± 0.5 % with a ligand density of 3.20 ± 0.15 mg ligand/ml support (0.37 ± 0.06 µmol ligand/ml support; 2.98 ± 0.48 µmol recognition sequence/ml support).
support). The static binding capacity was $6.43 \pm 0.50$ mg hFib/ml support ($19.69 \pm 1.56$ mol ligand/mol hFib; $157.53 \pm 1.25$ mol recognition sequence/mol hFib). The static binding efficiencies were $0.63 \pm 0.05$ % (mol hfib/mol recognition sequence) and $5.08 \pm 0.40$ % (mol hFib/mol ligand).

**Purification of hFib from doped control swine milk**

The FAP affinity sorbent utilizing the octameric form of the FAP ligand in conjunction with the EDAC-mediated coupling chemistry using the GAM/TSC method was evaluated in its ability to capture human fibrinogen (hFib) from nontransgenic swine whey. The western blotting analysis of the column fractions obtained from the purification study was performed using an 8 – 16 % SDS-PAGE as shown in Figure 22. Nonreduced conditions were used to eliminate the crossreactivity with the swine fibrinogen present. Lane 1 contains the human fibrinogen standard reference (130 ng). Lane 2 is a blank sample lane. Lane 3 is the starting material containing approximately 50 µg/ml of human fibrinogen standard. Lane 4 is the column flowthrough fraction. Lane 5 contains the elution fraction using 0.1 M glycine/2 (v/v) % acetic acid, pH 2. Lane 6 contains the 4 M NaCl elution fraction. Lane 7 contains the 2 M NaSCN elution fraction. The total amount of fibrinogen bound was determined from the summation of fibrinogen contained in the 0.1 M glycine/2 (v/v) % acetic acid, 2 M NaSCN and 4 M NaCl elution fractions. The majority of the bound fibrinogen (> 98 %) was eluted from the column with the glycine elution buffer. The average dynamic binding capacity for human fibrinogen standard determined for the three identical experiments using this column was approximately $95.06 \pm 3.56$ µg. The dynamic binding efficiency was determined to be $0.10 \pm 0.06$ %.

**Purification of rhFib from transgenic swine milk**

The FAP affinity sorbent utilizing the octameric form of the FAP ligand in conjunction with the EDAC-mediated coupling chemistry using the GAM/TSC method was evaluated in its ability to capture recombinant human fibrinogen (rhFib) from transgenic swine whey. The western blotting analysis of the column fractions obtained
from the purification study was performed using an 8 – 16 % SDS-PAGE as shown in Figure 23. Nonreduced conditions were used to eliminate the crossreactivity with the swine fibrinogen present. Lane 1 contains the human fibrinogen standard reference (130 ng). Lane 2 is a blank sample lane. Lane 3 is the starting material containing approximately 50 µg/ml of human fibrinogen standard. Lane 4 is the column flowthrough fraction. Lane 5 contains the elution fraction using 0.1 M glycine/2 (v/v) % acetic acid, pH 2. Lane 6 contains the 4 M NaCl elution fraction. Lane 7 contains the 2 M NaSCN elution fraction. The total amount of fibrinogen bound was determined from the summation of fibrinogen contained in the 0.1 M glycine/2 (v/v) % acetic acid, 2 M NaSCN and 4 M NaCl elution fractions. The majority of the bound fibrinogen (> 98 %) was eluted from the column with the glycine elution buffer. The average dynamic binding capacity for human fibrinogen standard determined for the three identical experiments using this column was approximately 59.94 ± 5.50 µg. The dynamic binding efficiency was determined to be 0.05 ± 0.03 %.

PAM affinity sorbent performance

Tables 8 – 11 present performance results for the PAM affinity sorbents. The PAM affinity sorbent utilizing the linear ligand with the epoxy coupling chemistry in conjunction with the one-step coupling method yielded a coupling efficiency of 24.0 ± 0.5 % with a ligand density of 0.65 ± 0.15 mg ligand/ml support. The static binding capacity was 0.06 ± 0.02 mg IgG/ml support (3,538.74 ± 23.72 mol ligand/mol IgG; 3,538.74 ± 23.72 mol recognition sequence/mol IgG). The static binding efficiencies were 0.03 ± 0.02 % (mol IgG/mol recognition sequence) and 0.03 ± 0.02 % (mol IgG/mol ligand). The PAM affinity sorbent utilizing the linear ligand with the epoxy coupling chemistry in conjunction with the TSC method yielded a coupling efficiency of 38.0 ± 0.5 % with a ligand density of 0.97 ± 0.15 mg ligand/ml support. The static binding capacity was 0.08 ± 0.03 mg IgG/ml support (3,960.66 ± 9.50 mol ligand/mol IgG; 3,960.66 ± 9.50 mol recognition sequence/mol IgG). The static binding efficiencies were 0.03 ± 0.01 % (mol IgG/mol recognition sequence) and 0.03 ± 0.01 % (mol IgG/mol ligand). The PAM affinity sorbent utilizing the linear ligand with the EDAC
coupling chemistry in conjunction with the one-step coupling method yielded a coupling efficiency of \(12.0 \pm 0.5\) % with a ligand density of \(0.60 \pm 0.15\) mg ligand/ml support. The static binding capacity was \(0.65 \pm 0.08\) mg IgG/ml support (301.53 \(\pm\) 2.56 mol ligand/mol IgG; 301.53 \(\pm\) 2.56 mol recognition sequence/mol IgG). The static binding efficiencies were \(0.33 \pm 0.04\) % (mol IgG/mol recognition sequence) and \(0.33 \pm 0.04\) % (mol IgG/mol ligand). The PAM affinity sorbent utilizing the linear ligand with the EDAC coupling chemistry in conjunction with the TSC method yielded a coupling efficiency of \(19.0 \pm 0.5\) % with a ligand density of \(0.95 \pm 0.15\) mg ligand/ml support. The static binding capacity was \(1.22 \pm 0.28\) mg IgG/ml support (254.36 \(\pm\) 2.10 mol ligand/mol IgG; 254.36 \(\pm\) 2.10 mol recognition sequence/mol IgG). The static binding efficiencies were \(0.39 \pm 0.09\) % (mol IgG/mol recognition sequence) and \(0.39 \pm 0.09\) % (mol IgG/mol ligand). The PAM affinity sorbent utilizing the tetrameric ligand with the epoxy coupling chemistry in conjunction with the one-step coupling method yielded a coupling efficiency of \(22.0 \pm 0.5\) % with a ligand density of \(1.10 \pm 0.15\) mg ligand/ml support. The static binding capacity was \(0.72 \pm 0.10\) mg IgG/ml support (98.19 \(\pm\) 1.12 mol ligand/mol IgG; 392.76 \(\pm\) 1.30 mol recognition sequence/mol IgG). The static binding efficiencies were \(0.25 \pm 0.03\) % (mol IgG/mol recognition sequence) and \(1.02 \pm 0.15\) % (mol IgG/mol ligand). The PAM affinity sorbent utilizing the tetrameric ligand with the epoxy coupling chemistry in conjunction with the TSC method yielded a coupling efficiency of \(23.0 \pm 0.5\) % with a ligand density of \(1.15 \pm 0.15\) mg ligand/ml support. The static binding capacity was \(1.34 \pm 0.08\) mg IgG/ml support (55.16 \(\pm\) 1.92 mol ligand/mol IgG; 220.63 \(\pm\) 2.33 mol recognition sequence/mol IgG). The static binding efficiencies were \(0.45 \pm 0.02\) % (mol IgG/mol recognition sequence) and \(1.81 \pm 0.10\) % (mol IgG/mol ligand). The PAM affinity sorbent utilizing the tetrameric ligand with the EDAC coupling chemistry in conjunction with the one-step coupling method yielded a coupling efficiency of \(21.0 \pm 0.5\) % with a ligand density of \(1.05 \pm 0.15\) mg ligand/ml support. The static binding capacity was \(1.63 \pm 0.38\) mg IgG/ml support (41.40 \(\pm\) 1.09 mol ligand/mol IgG; 165.61 \(\pm\) 3.26 mol recognition sequence/mol IgG). The static binding efficiencies were \(0.60 \pm 0.03\) % (mol IgG/mol recognition sequence) and \(2.41 \pm 0.19\) % (mol IgG/mol ligand). The PAM affinity sorbent utilizing the tetrameric ligand with the EDAC coupling
chemistry in conjunction with the TSC method yielded a coupling efficiency of 30.0 ± 0.5 % with a ligand density of 1.50 ± 0.15 mg ligand/ml support. The static binding capacity was 7.70 ± 0.75 mg IgG/ml support (12.52 ± 1.83 mol ligand/mol IgG; 50.08 ± 2.45 mol recognition sequence/mol IgG). The static binding efficiencies were 1.99 ± 0.09 % (mol IgG/mol recognition sequence) and 7.98 ± 0.29 % (mol IgG/mol ligand). The PAM affinity sorbent utilizing the octameric ligand with the epoxy coupling chemistry in conjunction with the one-step coupling method yielded a coupling efficiency of 15.0 ± 0.5 % with a ligand density of 0.75 ± 0.15 mg ligand/ml support. The static binding capacity was 0.78 ± 0.03 mg IgG/ml support (30.45 ± 2.38 mol ligand/mol IgG; 243.60 ± 1.00 mol recognition sequence/mol IgG). The static binding efficiencies were 0.41 ± 0.06 % (mol IgG/mol recognition sequence) and 3.28 ± 0.20 % (mol IgG/mol ligand). The PAM affinity sorbent utilizing the octameric ligand with the epoxy coupling chemistry in conjunction with the TSC method yielded a coupling efficiency of 24.0 ± 0.5 % with a ligand density of 1.20 ± 0.15 mg ligand/ml support. The static binding capacity was 2.13 ± 0.17 mg IgG/ml support (17.84 ± 1.25 mol ligand/mol IgG; 142.73 ± 2.89 mol recognition sequence/mol IgG). The static binding efficiencies were 0.70 ± 0.04 % (mol IgG/mol recognition sequence) and 5.61 ± 0.27 % (mol IgG/mol ligand). The PAM affinity sorbent utilizing the octameric ligand with the EDAC coupling chemistry in conjunction with the one-step coupling method yielded a coupling efficiency of 18.0 ± 0.5 % with a ligand density of 0.90 ± 0.15 mg ligand/ml support. The static binding capacity was 1.97 ± 0.34 mg IgG/ml support (14.47 ± 1.54 mol ligand/mol IgG; 115.74 ± 3.65 mol recognition sequence/mol IgG). The static binding efficiencies were 0.86 ± 0.08 % (mol IgG/mol recognition sequence) and 6.91 ± 0.43 % (mol IgG/mol ligand). The PAM affinity sorbent utilizing the octameric ligand with the EDAC coupling chemistry in conjunction with the TSC method yielded a coupling efficiency of 22.0 ± 0.5 % with a ligand density of 1.10 ± 0.15 mg ligand/ml support. The static binding capacity was 8.10 ± 0.61 mg IgG/ml support (4.30 ± 2.88 mol ligand/mol IgG; 34.41 ± 2.90 mol recognition sequence/mol IgG). The static binding efficiencies were 2.91 ± 0.02 % (mol IgG/mol recognition sequence) and 23.25 ± 0.77 % (mol IgG/mol ligand).
PAM Affinity Sorbent Performance: Effect of Ligand Orientation and Density

As shown in Table 8, the column support activated using 5.0 % (v/v) epichlorohydrin with the PAM ligand immobilized through EDAC-mediated coupling chemistry yielded a static binding capacity of $5.20 \pm 0.11$ mg IgG/ml support ($8.05 \pm 0.17$ % binding efficiency). Identical columns prepared using epoxy-mediated PAM immobilization yielded a static binding capacity of $2.20 \pm 0.14$ mg IgG/ml support ($3.41 \pm 0.22$ % binding efficiency). The column support activated using 10.0 % (v/v) epichlorohydrin with PAM immobilized through EDAC-mediated coupling chemistry yielded a static binding capacity of $3.10 \pm 0.16$ mg IgG/ml support ($4.80 \pm 0.25$ % binding efficiency). Identical columns prepared using epoxy-mediated PAM immobilization yielded a static binding capacity of $1.11 \pm 0.01$ mg IgG/ml support ($1.72 \pm 0.02$ % binding efficiency). The column support activated using 25.0 % (v/v) epichlorohydrin with PAM immobilized through EDAC-mediated coupling chemistry yielded a static binding capacity of $1.10 \pm 0.01$ mg IgG/ml support ($1.70 \pm 0.01$ % binding efficiency). Identical columns prepared using epoxy-mediated PAM immobilization yielded a static binding capacity of $0.80 \pm 0.03$ mg IgG/ml support ($1.24 \pm 0.04$ % binding efficiency). The column support activated using 50.0 % (v/v) epichlorohydrin with PAM immobilized through EDAC-mediated coupling chemistry yielded a static binding capacity of $0.82 \pm 0.02$ mg IgG/ml support ($1.27 \pm 0.03$ % binding efficiency). Identical columns prepared using epoxy-mediated PAM immobilization yielded a static binding capacity of $0.78 \pm 0.02$ mg IgG/ml support ($1.21 \pm 0.04$ % binding efficiency).

Purification of 7D7B10 mAb from hybridoma cell culture supernatant

The PAM affinity sorbent utilizing the octameric form of the PAM ligand in conjunction with the EDAC-mediated coupling chemistry using the GAM/TSC method was evaluated in its ability to capture 7D7B10 mAb from hybridoma cell culture supernatant. The column fractions obtained from the purification study were subjected
to gel electrophoresis using an 8 – 12 % SDS-PAGE as shown in Figure 30. The corresponding western blot is shown in Figure 31. The volume-averaged FAP ligand density was $1.10 \pm 0.15$ mg/ml support. The column bed volume was 1 ml. Lane 1 contains the 7D7B10 mAb standard reference (100 µg/ml). Lane 2 contains the hybridoma cell culture starting material. Lane 3 contains the column flowthrough fraction. Lane 4 contains the product elution fraction using 0.1 M glycine/2 (v/v) % acetic acid, pH 2. The majority of the bound swine IgG (> 99 %) was eluted from the column with the glycine elution buffer. The average dynamic binding capacity for the swine IgG standard determined for the three identical experiments using this column was approximately $2.20 \pm 0.25$ mg. The average dynamic binding efficiency was determined to be $5.31 \pm 0.33$ %.

**HETP analysis of affinity sorbents**

The results of the theoretical and experimental HETP analysis of the FAP and PAM affinity sorbents are shown in Table 12. The octameric forms of each peptide ligand were immobilized onto 3.0 wt. % cellulose support with an average particle diameter of 500 µm. Peptides were immobilized via epoxy-GAM using EDAC-mediated coupling chemistry. The experimental HETP values were calculated using the method described in Snyder et al. 1979. The theoretical HETP values were calculated according to the method of Mikes et al. (1975). The one-step and the TSC methods for the octameric FAP sorbent demonstrated experimental HETP values of 0.003 cm and 0.002 cm, respectively. Whereas, the one-step and the TSC methods for the octameric PAM sorbent demonstrated theoretical HETP values of 624 cm and 666 cm, respectively. Similarly, the one-step and the TSC methods for the octameric PAM affinity sorbent demonstrated experimental HETP values of 0.005 cm and 0.003 cm, respectively. Whereas, the one-step and the TSC methods for the octameric PAM affinity sorbent demonstrated theoretical HETP values of 720 cm and 762 cm, respectively.
Discussion

Classical methods used to immobilize affinity ligands onto chromatographic supports are typically performed in a single step resulting in a high coupling yield, which is attributed to a very high level of support activation. As shown in the preparation of immunosorbents, these conventional methods are characterized by the simultaneous occurrence of diffusional transport and covalent coupling of the ligand where the rate of covalent coupling is much more rapid than the diffusional transport of the ligand within the support (Subramanian et al., 1994). Therefore, to facilitate the uniform distribution of ligand, the GAM was developed as described in the preceding chapter to facilitate the management of the distribution of reactive epoxy-sites within the support. The epoxy-GAM in conjunction with the TSC method affords the management of local ligand density near the particle surface and including the support interior, therefore allowing for optimal target binding. In addition, the EDAC-mediated coupling method used for the immobilization of peptide affinity ligands affords a more oriented immobilization with concomitant crosslinking of the peptide ligand, thereby offering an improved configuration for the capturing of target proteins. In addition to the covalent coupling of the C-terminal carboxyl groups present on the peptide ligand with the primary amine groups within the support, the EDAC-mediated coupling chemistry utilizes (1-ethyl-3-dimethylaminopropyl) carbodiimide (EDAC) to facilitate the conjoining of N-terminal α-amine groups and C-terminal carboxyl residues to form a stable, covalent amide linkage, thereby affording ligand crosslinking (Hermanson et al., 1992; Nakajima et al., 1995). The use of the branched-chain peptide ligand assemblies have also been found to increase binding efficiency ($\eta_{\text{eff}}$) in relation to the linear peptide ligand forms by increasing the opportunity for covalent ligand attachment/crosslinking and in the introduction of multiple recognition sequence peptides inherent in each branched ligand.

The general performance characteristics of the FAP and PAM affinity sorbents were established using the large-particle diameter, low-solids content cellulose supports. The epoxy-GAM was employed in conjunction with one-step bulk coupling (BC) and two-step coupling (TSC) techniques for ligand immobilization to prepare affinity sorbents with volume-averaged FAP ligand densities ($<\rho>$) of approximately 1 –
4 mg FAP ligand/ml support and similarly with PAM ligand densities of approximately 1 mg PAM ligand/ml support. Linear, and tetrameric and octameric branched-chain assemblies of the peptide ligands were evaluated with regards immobilization chemistry using amine-reactive (N-terminal), epoxy-mediated coupling and C-terminal, carboxyl-reactive EDAC-mediated coupling. For each coupling chemistry case study, BC and TSC techniques were compared. The performance results for the FAP and PAM affinity sorbents are presented in Tables 3 – 6 and Tables 8 – 11, respectively. The performance results are shown graphically in Figures 17 – 30.

**FAP affinity sorbent performance**

FAP affinity sorbent performance was found to be dependent upon a variety of factors including the total amount of ligand immobilized, ligand spatial density and immobilization orientation. As shown in Table 3, the tetrameric and octameric branched-chain FAP assemblies consistently provided coupling efficiencies of greater than 50 % with initial ligand loadings of approximately 5 mg, providing final \( \rho \) of about 3 – 4 mg FAP/ml. Whereas, the linear FAP ligand provided coupling efficiencies of only approximately 30 – 40 %, with an equivalent initial ligand loading of 5 mg, yielding lower final \( \rho \) of 1 – 2 mg FAP/ml support. The increase in coupling efficiency of the branched-chain peptide ligands in comparison to the linear FAP ligands may be attributed to the increase in the number of reactive sites (N-terminal \( \alpha \)-amino groups and C-terminal carboxyl groups) on the branched-chain peptide ligands available for covalent attachment and, in particular, crosslinking with the use of the EDAC-mediated coupling chemistry. As shown in Table 3, the increase in the total amount of FAP ligand immobilized was greater than in comparison to the amount of linear FAP immobilized. FAP affinity sorbents prepared using the GAM/BC method using both the epoxy and EDAC-mediated coupling chemistry indicate that the FAP ligand is preferentially coupled at high \( \rho_{\text{loc}} \) on the surface and within the outer strata of the bead particle as demonstrated by subsequent decreases in \( \eta_{\text{eff}} \). Those affinity sorbents prepared using the GAM/TSC method demonstrated a higher \( \eta_{\text{eff}} \) with approximately equivalent \( \rho \) to those affinity supports prepared using the GAM/BC method indicating a decrease in
$\rho_{loc}$ for both EDAC and epoxy-mediated coupling chemistries. Therefore, the TSC method favored the initial transport of ligand within the support particle by decreasing the rate of covalent ligand coupling by reducing the local concentration of ligand within the support. Increases in $\eta_{\text{eff}}$ were more pronounced with increasing $<\rho>$ for the affinity sorbents utilizing the tetrameric and octameric branched-chain ligand assemblies in conjunction with the EDAC-mediated coupling chemistry which afforded additional ligand coupling through ligand crosslinking. For example, using oriented EDAC-mediated coupling, the FAP affinity sorbents demonstrated a 1.7-fold increase in binding efficiency based on the amount of FAP ligand immobilized utilizing the epoxy-GAM/TSC methods relative to the BC method using the same octameric ligand structural format. As shown in Figure 18, the use of the large-particle diameter, low-solids content cellulose supports in conjunction with the combination of the GAM/TSC method with the EDAC-mediated coupling of the branched-chain peptide assemblies afford an increase in $\eta_{\text{eff}}$ in relation to the conventional affinity sorbent configurations (Baumbach et al., 1997). As demonstrated by the results in Table 2, the conventional linear FAP affinity sorbents exhibit high $\rho_{loc}$ on the surface and within the outer strata of the particle support, thereby significantly decreasing $\eta_{\text{eff}}$ for a concomitant increase in $<\rho>$ (Baumbach et al., 1997). As shown in figure 18 and 19, FAP sorbents prepared using the multibranched peptide chain assemblies immobilized onto the large-particle diameter, low-solids content cellulose supports using the EDAC-mediated TSC chemistry consistently demonstrated higher performance in terms of binding efficiency in comparison to the conventional affinity sorbents with higher volume-averaged FAP densities. As shown in figure 19, FAP ligand efficiency was increased markedly with the use of the TSC method with the EDAC-mediated coupling chemistry in comparison to the performance of the conventional supports. In combination with the EDAC-mediated coupling chemistry, the use of the octameric FAP ligand afforded an increase in binding efficiency due to the multiple number of peptide recognition sequences available for crosslinking and therefore extension of the crosslinked FAP ligands from the surface of the support. These results demonstrate that through the combined use of the octameric peptide branched-chain assembly, with the use of the oriented EDAC-mediated TSC chemistry in conjunction with the large-particle diameter, low-solids
content cellulose supports yielded an optimal configuration for an increase in binding efficiency.

_Purification of hFib from doped control swine milk_

The FAP affinity sorbent utilizing the octameric form of the FAP ligand in conjunction with the EDAC-mediated coupling chemistry using the GAM/TSC method was evaluated in its ability to capture human fibrinogen (hFib) from nontransgenic swine whey. As was similarly found in the pure system studies, the majority of the bound human fibrinogen was eluted with the glycine buffer. The binding efficiency demonstrated by the column utilized for this study was much lower in comparison to the same column support used in the pure system studies. Due to the significant hydrophobic character of the FAP ligands, nonspecific adsorption with milk proteins was found to be a major factor in the decrease in binding efficiency. Significant precipitation occurred with the doped whey studies and to a lesser extent with the pure fibrinogen binding case studies at higher concentrations. The presence of recombinant human fibrinogen in the flowthrough fractions indicates that the column capacity was exceeded under these operating conditions.

_Purification of rhFib from transgenic swine milk_

The FAP affinity sorbent utilizing the octameric form of the FAP ligand in conjunction with the EDAC-mediated coupling chemistry using the GAM/TSC method was evaluated in its ability to capture recombinant human fibrinogen (rhFib) from transgenic swine milk. The majority of the recombinant human fibrinogen bound was eluted with the 4 M NaCl and 2 M NaSCN buffers. The lower intensity of the recombinant fibrinogen band is probably due to the slight differences in the glycosylation patterns between the native human fibrinogen standard as found in the doped studies and the recombinant human fibrinogen. This difference in posttranslational modification, primarily of the beta and gamma chains, between the two fibrinogen forms resulted in a loss of the affinity of the antibody for these recombinant
forms, therefore the reduced signal intensity. The recombinant form appeared to bind much more tightly in comparison to the native form since the recombinant form is not eluted until the application of the 4 M NaCl buffer, while the native form is eluted entirely by the preceding glycine buffer in the elution scheme. The binding efficiency demonstrated by the column utilized for this study was much lower in comparison to the same column support used in the pure system studies. Due to the significant hydrophobic character of the FAP ligands, nonspecific adsorption of milk proteins onto the affinity sorbent was concluded to be the major factor in the decrease in binding efficiency. Significant precipitation occurred with the doped whey studies and with the pure fibrinogen binding case studies at higher concentrations. The presence of recombinant fibrinogen in the flowthrough fractions indicates that the column capacity was exceeded under these operating conditions.

**PAM affinity sorbent performance**

As similarly as found in the evaluation of the FAP affinity sorbents, PAM affinity sorbent performance was found to be dependent upon a variety of factors including the total amount of ligand immobilized, ligand spatial density and immobilization orientation. As shown in Table 9, the tetrameric and octameric branched-chain peptide assemblies consistently provided coupling efficiencies of typically between 10 – 30 % with initial ligand loadings of approximately 5 mg, providing final $\rho$ of about 1.0 mg PAM/ml support. The coupling efficiencies and ligand densities obtained were generally within the same range (≈ 1 mg PAM/ml support) for each structural ligand configuration. The multibranched peptide chain format appeared to provide no advantage in enhancing coupling efficiency for the PAM affinity sorbents. However, the increase in the probability of peptide ligand crosslinking yielded an increase in binding efficiency for the octameric and tetrameric branched-chain peptide assemblies. As similarly found in the preparation of the FAP sorbents, PAM affinity sorbents prepared using the GAM/BC method using both the epoxy and EDAC-mediated coupling chemistry indicate that the PAM ligand is preferentially coupled at high $\rho$ on the surface and within the outer strata of the bead particle as demonstrated by subsequent decreases in $\eta_{\text{eff}}$. Those affinity sorbents prepared using the GAM/TSC method demonstrated a higher $\eta_{\text{eff}}$ with
approximately equivalent \(<p>\) to those affinity supports prepared using the GAM/BC method indicating a decrease in \(\rho_{loc}\) for both EDAC and epoxy-mediated coupling chemistries. Therefore, the TSC method favored the initial transport of ligand within the support particle by decreasing the rate of covalent ligand coupling by reducing the local concentration of ligand within the support. Increases in \(\eta_{eff}\) were more pronounced with increasing \(<p>\) for the affinity sorbents utilizing the tetrameric and octameric branched-chain ligand assemblies in conjunction with the EDAC-mediated coupling chemistry which afforded additional ligand coupling through ligand crosslinking. For example, using oriented EDAC-mediated coupling, the PAM affinity sorbents demonstrated a 3.3-fold increase in binding efficiency based on the amount of PAM ligand immobilized utilizing the epoxy-GAM/TSC methods relative to the BC method using the same octameric ligand structural format. As shown in Figure 18, the use of the large-particle diameter, low-solids content cellulose supports in conjunction with the combination of the GAM/TSC method with the EDAC-mediated coupling of the branched-chain peptide assemblies afford an increase in \(\eta_{eff}\) in relation to the conventional affinity sorbent configurations. As shown by the results in Tables 9 – 11, the conventional linear PAM affinity sorbents exhibit high \(\rho_{loc}\) on the surface and within the outer strata of the particle support, thereby significantly decreasing \(\eta_{eff}\) for a concomitant increase in \(<p>\). As shown in Figures 25 and 26, PAM affinity sorbents prepared using the multibranched peptide chain assemblies immobilized onto the large-particle diameter, low-solids content cellulose supports using the EDAC-mediated TSC chemistry consistently demonstrated higher performance in terms of binding efficiency in comparison to the conventional affinity sorbents with higher volume-averaged FAP densities. As shown in Figure 26, PAM ligand efficiency was increased markedly with the use of the TSC method with the EDAC-mediated coupling chemistry in comparison to the performance of the conventional supports. In combination with the EDAC-mediated coupling chemistry, the use of the octameric PAM ligand afforded an increase in binding efficiency due to the multiple number of peptide recognition sequences available for crosslinking and therefore extension of multiple crosslinked PAM ligands from the surface of the support. It is hypothesized that this extensive crosslinking of the PAM ligands would act as a pseudo spacer arm and therefore aid target binding due to
increased ligand accessibility. These results demonstrate that through the combined use of the octameric peptide branched-chain assembly, with the use of the oriented EDAC-mediated TSC chemistry in conjunction with the large-particle diameter, low-solids content cellulose supports, an optimal configuration for an increase in binding efficiency can be obtained.

**GAM and PAM ligand density management**

As shown in Table 8 and illustrated in Figure 29, using the 2 wt. % cellulose support, immobilized PAM ligand orientation and local density had a marked effect on swine IgG binding efficiency. Using 5.0 % (v/v) epichlorohydrin with the GAM and utilizing oriented EDAC-mediated coupling of the tertrameric PAM ligand provided the most active support. This affinity support yielded a binding capacity of 5.20 ± 0.11 mg IgG/ml support (8.05 ± 0.17 % binding efficiency). Through the use of the GAM to install a uniform distribution of activated groups throughout the interior of the support matrix, in addition to a low concentration of epichlorohydrin, this support provided a uniform, sparse distribution of activated epoxy groups available for PAM ligand immobilization. For the EDAC-mediated, oriented and the epoxy-mediated, nonoriented immobilization of PAM ligand, the functional efficiency decreased considerably with an increase in the level of activation. The EDAC-mediated (oriented) coupling chemistry provided the most active support, starting with an initial binding efficiency of 8.05 ± 0.17 %, and decreasing to 4.80 ± 0.25 %, 1.70 ± 0.01 %, and 1.27 ± 0.03 % for activation levels 2, 5, and 10 × that of the initial activation level, respectively. The epoxy-mediated (nonoriented or random) coupling chemistry provided a less active support, starting with an initial binding efficiency of 3.41 ± 0.22 %, and decreasing to 1.72 ± 0.02 %, 1.24 ± 0.04 %, and 1.21 ± 0.04 % for activation levels 2, 5, and 10 × that of the initial activation level, respectively. This decrease in functional efficiency can be attributed to an increase in the local spatial density of immobilized PAM ligand within the support matrix. With an increase in the level of activation, which provides a higher density of reactive epoxy groups available for covalent coupling, the immobilized PAM ligand would be more likely to be distributed within the outer-strata of the cellulose hydrogel particle. The higher local density of PAM ligand within the outer-strata of the support
contributes markedly to a decrease in functional efficiency due to steric hindrance, which affects IgG accessibility, and therefore binding efficiency. Although the PAM ligand was immobilized via a one-step coupling method in this study, the implementation of a two-step coupling method may afford an increase in binding efficiency due to a decrease in local density effects. In addition to the effect of local spatial PAM ligand density, the effect of oriented coupling in the immobilization of PAM ligand had a pronounced effect on binding efficiency as well. For example, the functional efficiency decreased by over one-half for PAM ligand immobilized via nonoriented, epoxy-mediated coupling chemistry using identical supports with regards to activation level (5 % (v/v) epichlorohydrin). This support yielded a binding capacity of 2.20 ± 0.14 mg IgG/ml support (3.41 ± 0.22 % binding efficiency) in comparison to the support prepared using the oriented, EDAC-mediated PAM ligand immobilization (5.20 ± 0.11 mg IgG/ml support). The differences in the functional efficiency between the two coupling chemistries can be attributed to orientation effects. Retention of the IgG recognition and affinity binding properties of the immobilized PAM ligand would be expected with the use of the EDAC-mediated coupling chemistry. The EDAC-coupling chemistry forms a covalent linkage between the primary amine groups on the support matrix and the carboxyl group of the C-terminal glutamate residue of the PAM ligand, which is not involved in the formation of the affinity complex. However, through the use of the epoxy-mediated coupling chemistry, which forms a covalent linkage between the epoxy groups on the support matrix and the amine groups within the N-terminal arginine residues of the PAM ligand, molecular recognition properties would be expected to be diminished, if not completely lost.

Peptide ligands typically require complete accessibility of their N-terminal α-amine groups, which are involved in the stabilizing interactions necessary for formation of the affinity complex (Fassina, 1992). Obstruction of these amine groups through covalent immobilization would subsequently have a detrimental impact by reducing binding efficiency as is shown in the results in Table 8. In addition, since the tetrameric PAM ligand contains four N-terminal tripeptide recognition sequences for affinity binding, multipoint attachment, and conformational restrictions would be contributing
factors in the decrease in binding efficiency demonstrated by the affinity supports prepared using the nonoriented, epoxy-mediated coupling chemistry. These results indicate that local spatial ligand density and ligand orientation can adversely affect affinity sorbent performance if these factors are not considered in the immobilization of peptide affinity ligands. In addition, since the primary amine groups of the PAM ligand were not protected during the immobilization using the EDAC-mediated coupling chemistry, crosslinking of the peptide ligand probably readily occurred. Since the multimeric, PAM peptide ligand composed of recognition sequences of tripeptide tetramers, crosslinking of the peptide may aid in providing a pseudo-spacer arm which would improve ligand accessibility and potentially increase binding efficiency. The use of a two-step immobilization procedure would be expected to provide for a more uniform distribution of PAM ligand, and thereby resulting in an expected increase in binding efficiency.

Purification of 7D7B10 mAb from hybridoma cell culture supernatant

The PAM affinity sorbent utilizing the octameric form of the PAM ligand in conjunction with the EDAC-mediated coupling chemistry using the GAM/TSC method was evaluated in its ability to capture 7D7B10 mAb from hybridoma cell culture supernatant. As similarly found with the pure system studies, the majority of the bound swine IgG (> 99 %) was eluted from the column with the glycine elution buffer. The average dynamic binding capacity for this column was in close agreement with the pure system studies thereby indicating that nonspecific adsorption of other proteins was not significant.

HETP analysis of FAP and PAM affinity sorbents

As similarly shown in the use of immunoaffinity chromatography, the use of low solids content matrices in the development of the peptide-based affinity sorbents were shown to demonstrate convective and diffusive modes of intraparticle transport for large proteins. As shown in Table 12, the low-solids content, large-particle diameter cellulose matrices indicate an increase in effective intraparticle mass transport, which
could not be attributed to an unhindered mode of diffusion alone as predicted by the theoretical correlation (Mikes et al., 1975).

Conclusions

Important design factors in the optimal engineering of a peptide-based affinity sorbent include immobilized ligand orientation, local spatial density and ligand architecture. The local spatial density of immobilized peptide ligand ($\rho_{\text{loc}}$), as opposed to volume-averaged peptide ligand density ($<\rho>$) is of more critical importance in determining the optimal performance in the engineering of peptide-based affinity sorbents. As similarly demonstrated in the preparation of immunosorbents in the preceding chapter, these results appear to be a salient feature of using low-solids content, large-particle diameter cellulose supports. In conjunction with the octameric branching architecture in peptide ligand design, oriented ligand immobilization through using EDAC-mediated coupling/crosslinking chemistry affords the optimal configuration for increasing affinity sorbent binding efficiency. In addition, the installation of epoxy-sites through the epoxy-GAM in addition to the TSC method allows for improved management of $\rho_{\text{loc}}$ near the particle surface as well as within the support interior resulting in an increased affinity sorbent binding efficiency as well.

Acknowledgements

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Nomenclature & Symbols

- $\eta_{\text{eff}}$ binding efficiency (%)
- BA bulk activation
- BC bulk coupling
- CV column volume (ml)
- $\psi$ covalent coupling efficiency (%)
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tr>
<td>$\rho_{\text{vol}}$</td>
<td>volume-averaged peptide density (mg peptide/ml support)</td>
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<tr>
<td>$\rho_{\text{loc}}$</td>
<td>local spatial peptide density (mg peptide/ml support)</td>
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<tr>
<td>EACA</td>
<td>$\varepsilon$-aminocaproic acid</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>FAP</td>
<td>fibrinogen affinity peptide</td>
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<tr>
<td>GAM</td>
<td>gradient activation method</td>
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<td>HETP</td>
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<td>human fibrinogen</td>
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**References**


