Weak Affinity Chromatography

Magnus Strandh, Håkan S. Andersson, and Sten Ohlson

1. Introduction

Since the inception of affinity chromatography 30 years ago (1), it has developed into a powerful tool mainly for the purification of proteins. It is based on the reversible formation of a tight binding complex between a ligand, immobilized on an insoluble matrix and a substance, the ligate, to be isolated from the solution. Typically the ligate is adsorbed by a column with the immobilized ligand, whereas noninteracting substances are washed off. By changing the elution conditions, the ligate can be released in a highly purified form. Some researchers argue that this procedure is based on specific extraction rather than by chromatography, which should rely on the differential migration of various substances. Regardless of the definitions, it is clear that traditional affinity chromatography exploits high affinity or avidity (binding constant (Kₐ) > 10⁵/M) between the interacting molecules, which will result in an effective adsorption of the ligate. In this context the distinction between affinity and avidity is important: Whereas affinity describes the interaction in an individual binding site, avidity describes the multivalent binding between multiple binding sites of the ligand and ligate, respectively. High binding strength is required to achieve efficient adsorption, whereas weaker interactions will not produce adequate binding and therefore insufficient specificity will be acquired. This statement that strong specific binding is a prerequisite for the successful isolation of an interacting molecule has been in a nutshell the consensus of affinity chromatography.

Let us examine in more detail the validity of this statement by considering some theoretical aspects of affinity chromatography. It has been shown (2) that the retention of interacting substances in affinity chromatography principally depends on three distinctive factors: the amount of ligand and ligate, the affin-
ity or avidity between the ligand and ligate, and the physical characteristics of the matrix. A simple mathematical expression can be derived (3) that relates the retention (defined as the capacity factor, \( k' = (V_r - V_o)/V_o \); \( V_r \) is the retention volume of the ligate and \( V_o \) is the retention volume of a noninteracting substance) with the affinity (\( K_a \)), the amount of active ligand (\( Q_{max} \)) and the support characteristics (\( C \)):

\[
k' = C Q_{max} K_a \tag{1}
\]

Equation 1 is only valid when \( K_a c \) is much less than 1 (\( c \) is the concentration of ligate at equilibrium). The theory is more complex at higher ligate concentrations (4), but in general it can be stated that \( k' \) is then much less than is postulated by Eq. 1 and the chromatographic peaks are significantly distorted. A basic conclusion when considering Eq. 1 is that retention can be achieved in essentially two different ways: either by working at high \( K_a (> 10^5 / M) / low Q_{max} \) (traditional affinity chromatography) or by low \( K_a (< 10^5 / M) / high Q_{max} \). In other words, the theory states that by implementing weak affinities under high ligand load in chromatography—weak affinity chromatography (WAC)—we can produce significant retention of weakly interacting ligates. Furthermore, the performance of affinity chromatography systems can be greatly improved when utilizing weaker interactions as the basis for separation. Computer simulation of WAC (2) illustrates this, where peaks are sharpened by weaker affinities (Fig. 1). In conclusion, based on the above theoretical reasoning, it appears obvious that affinity chromatography not only can be run in the weak affinity mode but that it also can offer competitive advantages over traditional affinity chromatography discussed as follows.

During recent years, we have experienced a growing awareness of the importance of weak and rapid binding events governing many biological interactions. Here are just a few examples from various areas: protein–peptide interactions (5), virus-cell interactions (6), cell adhesion, and cell–cell interactions (7–9). A most intriguing question is how specificity can be accomplished in biological systems despite the fact that individual interactions are in the range of \( 10^2–10^3/M \) of \( K_a \). The overall view is that recognition is achieved by multiple binding either in a form of repeated binding events or by multivalent binding involving several simultaneous weak binding events. We feel certain that WAC can provide a tool for the researcher to study weak biological interactions not only for characterization of the biological event per se, but also for the purposes of analyzing and isolating the molecules taking part in the binding event.

Extensive experimental data are available today from us as well as from other laboratories demonstrating that chromatography in the weak affinity mode can be performed in a favorable manner. In addition, several of these studies have confirmed the theoretical predictions as discussed above. Since
the conception of WAC some 10 years ago (10), the potential to use weak monoclonal antibodies both of immunoglobulin G and M (IgG and IgM) for affinity chromatography has thoroughly been examined (11–14). Moreover, several other applications of weak affinity systems have been demonstrated, including the self-association of proteins (15), the use of peptides and antisense peptides as ligands for separating peptides and proteins (16–19), the separation of inhibitors with enzymes (20), carbohydrate recognition by lectins (21,22), and immobilized proteoliposome affinity chromatography (23). It is noteworthy that weak affinity interactions play a major role also as the mechanism for separation in related systems such as the chiral stationary phases (CSPs) based on cyclodextrins (24), and proteins (25,26), as well as the brush-type CSPs (27), and to some extent, molecularly imprinted polymers (28,29).

An important contributing factor for the realization of WAC has been the invention of high-performance liquid affinity chromatography (HPLAC) (30,31), and moreover, easy access to multimilligram amounts of ligands produced from chemical or biological libraries (32) as well as efficient coupling procedures for attaching ligands to supports (33).

Fig. 1. Computer-simulated chromatogram showing the effects of affinity on peak broadening at the same sample load. $K_a = (A) 10^3 \text{ M}^{-1}$, (B) $10^4 \text{ M}^{-1}$, and (C) $10^5 \text{ M}^{-1}$. The capacity factor ($k'$) was held constant, while $Q_{\text{max}}$ was increased with lower affinities (Eq. 1). From ref. 2. Used with permission.
This chapter introduces the novice researcher to the practical procedures of WAC. We have opted to describe the use of weak monoclonal antibodies, as these are a generous source of generic ligands for most molecular entities. These fuzzy monoclonal antibodies can be obtained from different classes such as IgG and IgM and examples of both are discussed as follows. It is important to note that the interest in using the antigen-binding site of the antibody for weak biomolecular recognition will be enhanced even further with the introduction of molecular cloning techniques for generating repertoires of antibody derived binding sites (34,35). We anticipate that these genetically engineered antibody fragments will give us a tremendous supply of potential ligands for weak affinity chromatography. Furthermore, we will briefly comment on the use of weak monoclonal antibodies in other related areas such as biosensors and capillary electrophoresis (Notes 1–3).

2. Materials

2.1. Chemicals

Deuterium oxide (Merck, Darmstadt, Germany), p-nitrophenyl (PNP) tagged and nontagged carbohydrates: glucose (Glc), isomaltose (Glcα1-6Glc), maltose (Glcα1-4Glc), and panose (Glcα1-6Glcα1-4Glc) (all in D-configuration), and steroids: digoxin and ouabain (all from Sigma, St. Louis, MO). Tetraglucose ((Glc)₄, (Glcα1-6Glcα1-4Glcα1-4Glc)) was kindly provided by Prof. Arne Lundblad, Linköping University, Linköping, Sweden. (Glc)₄ was conjugated to bovine serum albumin (BSA) according to ref. 36 and digoxin was conjugated to BSA and human transferrin (37). All other chemicals were of analytical grade and used as received.

2.2. Ligand Preparation

BALB/cJ female mice were obtained from Jackson Laboratories (Bar Harbor, ME). The hybridoma cell medium consisted of Dulbecco’s modified Eagle’s medium with 7–10% bovine serum (Fetalclone I), nonessential amino acids (all HyClone Labs, Logan, UT), L-Glutamine (Biological Industries, Haemek, Israel), and penicillin-streptomycin (Biochrom, Berlin, Germany).

Chromatography gels for Protein A (protein A-Sepharose CL-4B), ion-exchange (Q Sepharose FF), and size exclusion (Sephacryl S-300HR) were all purchased from Amersham Pharmacia Biotech (Uppsala, Sweden).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with equipment from Bio-Rad (Mini-Protean® II Electrophoresis Cell, Hercules, CA). Secondary antibodies and serum calibrator for the enzyme-linked immunosorbent assay (ELISA) were obtained from Dakopatts (Glostrup, Denmark).
2.3. **WAC Column Preparation**

Microparticulate silica (diameter 10 µm and pore size 300 Å) was obtained from Macherey-Nagel (Düren, Germany) and glycidoxypropyltrimethoxysilane from Hüls (Marl, Germany). An air-driven fluid pump (Haskel, Burbank, CA) was used for packing the HPLC-columns. The reference IgG and IgM antibodies were obtained from Dakopatts.

2.4. **Use and Maintenance of the WAC Column**

The HPLC system included a three-channel pump, a UV–Vis detector (Varian 9012 and 9050, Varian Associates, Walnut Creek, CA), as well as a pulsed amperometric detector (PAD) (ED40, Dionex, Sunnyvale, CA), and a column oven (C.I.L., Sainte Foy La Grande, France). Chromatography data handling software was purchased from Scientific Software (EZchrom version 6.5, San Ramon, CA).

The HPLC mobile phases consisted of 0.02 M sodium phosphate; 0.1 M sodium sulfate, pH 6.0 (IgG) and 0.1 M sodium phosphate pH 6.8 (IgM). The injection loop volumes were 20, 100, and 5000 µL (frontal chromatography).

3. **Methods**

3.1. **Ligand Preparation**

A number of techniques for obtaining an antibody ligand with the desired qualities are available. These include several immunization techniques and in vitro approaches making use of cloning and expression systems such as phage display. The screening of libraries for weak affinity antibody ligands is discussed in Note 4. Here, we describe the development of a murine hybridoma producing monoclonal IgG antibodies, as well as a human–mouse hybridoma producing monoclonal IgM antibodies.

1. IgG. Immunize BALB/cJ mice with (Glc)$_4$ coupled to keyhole limpet hemocyanin or BSA as the immunogen (38). The resulting hybridoma cell line producing monoclonal IgG2b against (Glc)$_4$ is designated 39.5.
2. IgM. Develop hybridomas producing monoclonal IgM antibodies against digoxin derivatives by in vitro immunization of human peripheral blood lymphocytes using a digoxin-transferrin conjugate. One such cell line is designated LH114 (κ light chain) (39).

2. Culture both IgG and IgM producing cells in stir flasks (1 L) in hybridoma cell medium at 37°C until the viability is <10%, usually 12–14 d.
3. Clarify the cell culture supernatants from cells and debris by centrifugation (10,000g, 4°C, 20 min) prior to further antibody purification.
4. Perform preparative chromatography at +8°C. Purify the IgG antibodies by affinity chromatography using immobilized protein A (13) and the IgM by using anion exchange (repeated for higher purity) followed by size exclusion chromatography (14); all steps according to the manufacturer’s instructions (Note 5).
5. Test the antigen-binding abilities of the IgG and IgM antibodies with ELISA where the microtiter wells are coated with (Glc)_4-BSA (13) and digoxin-BSA (39), respectively.

6. Analyze the purified antibodies with SDS-PAGE (40) to confirm the molecular weights and purities (should be at least 95%).

A high recovery of the binding activity after the purification steps is achieved, at least 90% for IgG. The purification of IgM may suffer from low yield, mainly in the anion–exchange chromatography step, and the overall recovery of active LH114 from the hybridoma cell supernatant has been 34% (as determined with antigen-specific ELISA).

3.2. Preparation of the WAC Column

1. Silanize silica with glycidoxypropyltrimethoxysilane (454 µmol diol groups/g silica) (30) (Note 6).

2. Place 1.1 g diol silica in a screw-cap test tube (1 g of silica equals approx 2 mL in column volume). Suspend the diol silica in 11 mL distilled H_2O, sonicate 1 min and add 1.1 g H_5IO_6.

3. Rotate the tube gently for 2 h at 22°C.

4. Wash the aldehyde silica by centrifugation (5 min at 200g) and resuspend the pellet in 10 mL H_2O (four times) and 0.1 M sodium phosphate buffer, pH 7.0 (twice). Centrifuge and discard the supernatant.

5. Dissolve the antibody in 0.1 M sodium phosphate buffer, pH 7.0 (or another suitable buffer) to at least 5–10 mg/mL. The buffer is chosen with respect to a pH optimum of coupling at pH 5.0–7.0. Transfer the antibody solution to the aldehyde silica pellet. Mix gently. A reaction volume of 5–10 mL is recommended. Add a protective ligand if applicable (Note 6).

6. Estimate the volume of the silica-antibody suspension. Add 5 mg sodium cyanoborhydride/mL from a bulk solution (100 mg/mL in H_2O, freshly made) immediately to the suspension. Work in a well-ventilated area.

7. Let the coupling reaction continue at 22°C for 40 h. Rotate the tube continuously to ensure a uniform suspension. This type of reaction is often at 90% yield after 5–6 h but is prolonged to ensure completion.

8. Wash the silica as described above with H_2O (twice), 0.5 M NaCl (twice), and 0.1 M sodium phosphate buffer, pH 7.0 (twice). Collect the supernatant from the washing fractions and measure the absorbance at 280 nm to obtain an estimate of the amount of nonimmobilized antibodies.

9. Perform an additional estimation of the coupling efficiency by direct UV measurement at 280 nm on a small aliquot of the antibody silica mixed with 3 M sucrose, which ensures the transparency of the silica particles. At least 80 mg antibody/g silica can be immobilized with > 50% of the antigen-binding capacity retained (Q_max, as determined by frontal chromatography [Note 7]). Prepare reference supports analogously using an irrelevant mouse/human polyclonal IgG/IgM as the immobilized ligand or by omitting the antibodies (Note 8).

10. Pack the antibody silica into an HPLC column (5.0 mm ID × 100 mm) using an
air-driven fluid pump at 300 bar in a 0.1 M sodium phosphate buffer (pH 6.8) both as the slurry and packing solvent (Note 9). Prepare the reference columns with either IgG or IgM, and a reference column with only diol-silica. Store the columns in packing solvent containing sodium azide (0.01%) at +4–6°C (up to 6 mo without any significant loss in activity).

3.3. Use of the WAC Column

1. Choose the detector to meet the analyte properties. When separating nontagged carbohydrates use a PAD, but in the case of the steroids UV absorption measurements at 230 nm is sufficient.
2. Perform all WAC experiments under thermostatic conditions to enhance reproducibility. Ideally, both the injection loop, the column and major parts of the inlet and outlet tubing should be included in the temperature-controlled environment. Prepare fresh solutions on a daily basis and filter (0.45 µm) and degas the mobile phases prior to use.
3. Set the flow rate at 1 mL/min. Use a variety of analytes for each system to evaluate the feasibility of WAC.

3.3.1. 39.5 Column

1. Dissolve all carbohydrates in the mobile phase or in a simulated crude extract containing 4% fetal bovine serum (FBS).
2. Enable detection of the nontagged carbohydrates by adding 0.2 M sodium hydroxide to the eluate to increase pH > 12.0 prior to the PAD inlet. Detect the tagged carbohydrates (p-nitrophenyl derivatives) at 300 nm.
3. Inject the samples fully into a 20 µL injection loop. The 39.5 column is able to completely separate a mixture of isomaltose, α-maltose, β-maltose, and α-panose within 14 min under isocratic conditions (Note 10). The contaminants in the crude mixture are not retarded and appear in the void volume where several reference saccharides such as glucose and lactose also elute.

The temperature dependence (4–40°C) of the system can also be studied. In Fig. 2 the separation of α- and β-maltose at four different temperatures is presented. The results suggest that the 39.5–carbohydrate interaction relies mainly on electrostatic forces and that alteration in temperature can be used as an elution procedure. Chemical parameters of the mobile phase such as the pH, ionic strength, and organic solvents also influenced the retention (13).

3.3.2. LH114 Column

1. Use the column to separate ouabain and digoxin dissolved in the mobile phase.
2. Inject 0.05–2 mg of the analytes into a 100-µL sample loop.
3. Monitor the chromatography by UV detection at 230 nm. Figure 3 shows a typical profile where digoxin and ouabain are separated from the void volume. A 5% ethanol supplementation of the mobile phase (in order to facilitate the dissolution of the steroids) has a minor effect on k’ (less than 10% decrease) and the introduction of contaminants in the samples (0.5% FBS) does not impair the retention.
Fig. 2. WAC on the 39.5 column. The anomers of maltose are separated at four different temperatures. Injected amount = 0.1–0.2 μg. From ref. 13. Used with permission.

Fig. 3. WAC on the LH114 column illustrating the separation of ouabain, digoxin and acetone (void marker). From ref. 14. Used with permission.
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4. Use frontal chromatography (Note 7) to estimate the $K_a$ values for the systems. The 39.5 column shows an affinity of $3.0 \times 10^4$ M$^{-1}$ for PNP-$\alpha$-maltose at 30°C, and similar values are obtained on the LH114 column ($1.7$ and $4.0 \times 10^4$ M$^{-1}$ at 22°C for digoxin and ouabain, respectively). Data from frontal chromatography are used to determine $Q_{\text{max}}$ for the 39.5 column. By assuming $Q_{\text{max}}$ to be constant for the analytes studied, the $K_a$ values of $\alpha$- and $\beta$-maltose ($2.8 \times 10^3$ M$^{-1}$ and $1.4 \times 10^4$ M$^{-1}$, respectively at 30°C) are determined by applying Eq. 1 to the zonal separation (Fig. 2).

5. Check the long term stability or the columns by repeating the different separations during 6 mo. Compare the $k'$ values for the different runs. The stability of affinity columns is a major concern and our results indicate that it favors the IgG system over the IgM. After a period of 3 mo, when the columns were stored at 4–6°C between usage, the 39.5 column exhibited a deterioration of retention of less than 10% after 150 runs, whereas the LH114 column showed a 24% decrease in performance (with regard to $k'$ for digoxin) after 60 runs under various conditions including substitution of the mobile phase with a 5% ethanol solution.

4. Notes

1. Weak monoclonal antibodies can also be used as ligands in affinity electrophoretic procedures. In Fig. 4 we show a capillary affinity gel electrophoretic separation (41) using the same weak monoclonal antibody (39.5) as was applied in the WAC experiments. An antibody gel was produced by polymerization of the antibody with 50% glutaraldehyde. Prior to antibody gel formation, the mixture was filled into a fused-silica capillary tubing by the aid of a peristaltic pump. Electrophoresis was carried out with a P/ACE 2050 (Beckman, Palo Alto, CA). As seen from Fig. 4, the 39.5 monoclonal antibody was able to separate tagged and structurally related carbohydrate antigens similar to what has been achieved with WAC (13). The tag (a p-nitrophenyl group) was introduced to allow convenient detection of the carbohydrates. To verify that the binding of the carbohydrate antigens to the 39.5 antibody was specific, a polyclonal mouse IgG capillary was used in a control experiment. The reference system indicated no significant binding of the carbohydrate antigens, as they were unretarded in the gel capillary. This preliminary study suggests that highly selective weak affinity separation can be performed in a capillary electrophoresis system.

2. One of the drawbacks of the current use of analytical columns (5 mm ID ¥ 50–250 mm) for WAC with immobilized monoclonal antibodies, is the considerable amounts of antibody (10–100 mg) required to study weak affinities as discussed above. However, as the theory suggests Eq. 1, the retention is proportional to the concentration and not to the absolute amount of ligand. This means that we should be able to perform the separation in a miniaturized format providing that we can maintain the concentration level of active ligand. Obviously, miniaturization places demands on the chromatography equipment (e.g., in terms of injection volumes, system dead volumes, and detector design). However, far less ligand (<1 mg antibody) is consumed, which is a significant advantage especially when the sup-
ply of antibody is limiting. Preliminary studies with immobilized 39.5 in μ-bore columns (column volume: 50–100 μL) have clearly demonstrated that equivalent separations can be obtained as with analytical columns (Bousios and Ohlson, unpublished data). We consider this to be an important technical improvement of WAC, which hopefully will make the technology available for a much wider audience.

3. The recently introduced biosensor instruments based on surface plasmon resonance (42), provide a way to further investigate the nature of weak affinity antibody–antigen interactions. On BIACore X™ (Biacore AB, Uppsala, Sweden), the weak monoclonal antibody 39.5 was immobilized on the sensor chip (CM5, Biacore AB) and various concentrations of the carbohydrate antigens were injected (43,44). The results show good correlation with the WAC experiments; the affinity (K_a) ranged from 1.4 × 10^4 M⁻¹ (maltose, 25 °C) to 1.0 × 10^3 M⁻¹ ([Glc]₄, 40°C), which is comparable to 5.0 × 10^3 M⁻¹ at 25°C for tetraglucitol (which has the same affinity as (Glc)₄ (38)) as calculated from frontal chromatography. Kinetic data (association and dissociation rate constants, k_a and k_d) were impossible to measure since the equilibrium states were almost momentarily set (<1 s) resulting in a square pulsed appearance of the

Fig. 4. Capillary affinity gel electrophoresis using a 3.5%, 39.5 monoclonal antibody. A mixture of (A) PNP-α-D-glucopyranoside and ONP(o-nitrophenyl)-β-D-glucopyranoside (both unretarded), (B) PNP-α-D-maltoside, and (C) PNP-β-D-maltoside is separated within 15 min. Conditions: gel length 19.5 cm, total length 27 cm; 25 mM potassium phosphate buffer, pH 6.8, with 10% v/v 2-propanol; UV detection at 313 nm; temperature, 25°C; constant-applied electrical field, 7 kV, 36 μA; electrokinetic injection, 3 s, 1 kV. From ref. 41. Used with permission.
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sensorgrams. Reproducible results were obtained only with immobilization levels of antibody between 10,000–20,000 Resonance Units (RU) as dictated by the weak affinities and the small size of the antigens (< 1000 Daltons). The design of control experiments is very important when studying interactions in the $K_a = 10^3 \text{ M}^{-1}$ range (Note 4.6). This applies to WAC as described earlier, but is even more pronounced in the biosensor experiments. The analyte response in the 39.5 system was less than 50 RU, which is in the range of the noise contributed from differences in properties of the immobilized ligand and variations in the analyte concentration of the samples (bulk refractive index), as well as pH and temperature fluctuations. This is illustrated in Fig. 5 where the discrepancies create a "hook effect." The reference cell should therefore mimic the active flow cell both in terms of ligand characteristics and immobilization level, and the bulk refractive index should not exceed the analyte response. We believe that this technique will become useful for reliable screening for weak affinity ligands, as discussed shortly.

4. Traditional screening methods in monoclonal antibody production, such as ELISA, are generally designed for selection of high-affinity antibodies. Consequently, there is always a risk that valuable low-affinity antibodies can be lost in the early stages of finding a suitable antibody. Usually, there is an abundant supply of interesting low-affinity clones present after making e.g. hybridomas that are ignored due to a lack of analytical procedures. If new screening techniques can be introduced to detect the weak antibodies or antibody fragments, we should be able to find the ligand among a larger spectrum of antibodies including the very weak at $K_a < 10^3 \text{ M}^{-1}$. Typically, ELISA and similar immunoassay procedures can be modified to include weak binders by allowing the antibodies to bind simultaneously to several epitopes in a well of a microtiter plate, for example. By doing so we can pick the weak affinity antibodies due to their avidity effects on binding several weak sites at the same time. Other screening techniques are available, most notably biosensors and affinity chromatography. Weak affinity chromatography based on immobilized epitopes (45) is of special interest as it can be used for antibodies or fragments that cannot be selected in avidity based immunoassay or for very weak antibody-based binding sites. Another advantage with chromatography is that quantitative information on affinity in terms of binding constants can be elucidated. This is of special importance when fine selecting from a large pool of plausible candidates.

5. It is worth noting that IgM antibodies have proven rather difficult to purify, and whereas the methods we have employed for their purification (a combination of strong ion-exchange and size-exclusion chromatography) have worked satisfactorily, they have required considerable optimization. In the case of IgG purification the situation is often much brighter, as a range of bacterially derived antibody binding proteins are commercially available. The most widely used is protein A, but for some antibodies protein G (46) is better suited. All these matrices possess high capacity, which allows rapid purification of large
amounts of antibodies under standardized protocols. As an alternative, it may be worth considering the use of immobilized antigen for an affinity based purification of the ligand, which is common practice for high-affinity systems. This procedure has previously been successfully applied for the purification of low-affinity antibodies (47).

6. Many different approaches are available for coupling of the antibody ligand onto the solid support (33). As the number of active binding sites in the column is a key factor for the performance of the weak affinity system, the choice of immobilization method is crucial. It is noteworthy that some methods for directed immobilization are described (48), which can prove helpful to improve system homogeneity. As a rule, however, we prepare the support by traditional immobilization procedures such as coupling with aldehyde activation, a reliable method that generally gives high coupling yields. This method is particularly useful as the level of coupling sites can be regulated easily by the periodate oxidation used in the immobilization method. If the nature of the antigen does not make it amenable for a reaction with the coupling agent, it can be included to protect the antigen-binding region of the ligand. It is also convenient to perform the coupling reaction directly in situ in the column (49). However, we have found that the coupling yields, using this approach, are generally lower than in batch operation.

Porous silica supports are available with pore sizes ranging from 50–4000 Å. The choice of support involves a decision for each unique case with regard to the

Fig. 5. Binding of maltose to immobilized 39.5 in Biacore X. (A) Equilibrium responses at different analyte concentrations were corrected by the reference signal from a flow cell with no immobilized protein. A "hook-effect" describing an apparent loss in activity at higher analyte concentrations is shown. (B) The signal was corrected using a flow cell with immobilized irrelevant antibody, thus mimicking the active flow cell. The corrected signal forms a typical saturation curve. From ref. 44. Used with permission.
balance between pore size and surface area. Smaller pore sizes inherently give rise to higher surface areas. However, as both the ligand and ligate must be easily accommodated within the pores for the corresponding surface to become available, silica with pore sizes smaller than the size of the ligand–ligate complexes are not recommended. The surface coverage of one IgG molecule is approximately $150 \times 150$ Å$^2$, and we have selected 300 Å pore size for IgG and 300–500 Å for the larger IgM when separating low molecular weight antigens (<1000 Daltons).

7. The use of frontal affinity chromatography (50) for the estimation of $K_a$ and the binding capacity ($Q_{\text{max}}$) for various compounds is convenient and reliable provided that the binding-site population is not heterogeneous in nature. This procedure involves saturation of the column by the analyte at various concentrations ([A]), which renders chromatograms describing elution profiles each composed of an elution front and a plateau. The elution volume ($V$) depends on [A] and the affinity ($K_a$) between the analyte and the immobilized ligand and is determined by the inflection point of the front. $V_0$ describes the front volume when no adsorption exists. By plotting $1/([A](V - V_0))$ vs $1/[A]$, in analogy with the Lineweaver–Burk plot of enzyme kinetics, $-K_a$ can be calculated from the intercept on the abscissa. The intercept on the ordinate reflects $1/Q_{\text{max}}$.

8. A reference system is important to provide information as to whether the weak interactions observed really occur between the antigen-binding site of the antibody and the epitope of the antigen, or if they are mainly of separate (nonspecific) origin. The choice of a relevant system is vital and presents a delicate task. Ideally, it should be identical to the “real” antibody column in all aspects except for the antigen-binding site of the immobilized antibody, which should not bind at all. This is not easily accomplished, but our ways to surmount this problems have been (1) to immobilize polyclonal antibodies of the same species, (2) to use monoclonal antibodies of the same subclass but immunized with a different antigen, and (3) to prepare columns absent of antibody.

9. Column packing is an obstacle for many people working with HPLC method development. This is probably due to certain safety issues arising from the relatively high pressures involved (>300 bar), which pose a limit to the number of people volunteering for this task. However, the procedure is not difficult and neither is it expensive. It is generally agreed that a balanced density solvent mixture should be employed when applying the slurry to the packing bomb (51), to inhibit particle size segregation and particle aggregation. To counteract aggregation further, surfactants in the packing slurry have been found useful (52). However, as many of the commercially available stationary phases today are of very narrow size distribution, we have found that the use of balanced density solvents is mostly not necessary for commercially available materials. Any buffer, which preserves the activity of the ligand, is likely to work.

The operational packing pressure should be maximized to the limit set by the stability of the solid support. Typically this is 300–340 bar for porous silica. A vertical orientation of the column in the packing system is important, whereas the direction of packing (upward or downward) is not essential.
To ascertain that the column is fully packed, we usually employ at least a 50% excess of the solid phase in the packing slurry. Moreover, to minimize the void in the upper end of the column, we use a simple “topping-up” technique: 20–30 mg of solid phase is suspended in 1 mL of acetone in an Eppendorf tube. This suspension is added dropwise to the column with a pasteur pipet, allowing the acetone to partly evaporate between each addition until the column is filled with material and the surface acquires a smooth appearance. The method is also useful when trying to bring new life to older columns.

Finally, as an alternative for those not keen on packing columns with a high-pressure packing apparatus, it is also possible to pack under lower pressure using the HPLC pump together with the POROS® Self Pack™ system (Perseptive Biosystems, Framingham, MA). We have found that this method actually yields columns of comparable quality to those prepared by high-pressure packing, provided that the specified commercial supports are used. Miniaturized systems also allow the use of the HPLC pump for column packing, and a mL-range injection loop is often adequate as the slurry reservoir.

10. A standardized description of the retention of a chromatographic peak is the capacity factor, $k'$, which is calculated from the elution volume of the retained analyte, $V_r$, and the system void volume, $V_0$. If the chromatographic peak follows a symmetrical gaussian distribution, $V_r$ equals the elution volume of the peak maximum. It is noteworthy that the true measure of $V_r$ is found at the point of the peak where 50% of the analyte has been eluted, meaning that if the peak is asymmetrical, peak maximum does not in general equal the volume of the peak maximum (3). This is a fact that most chromatography data-handling software packages do not account for, but it is possible in several spreadsheet programs to create and apply a macro string, which may help surmount the problem. In addition, a manual integration command is generally available within the application by which the true $V_r$ can be estimated via a digitized “cutting and weighing” approach.

References

Affinity Chromatography
Methods and Protocols
Bailon, P. (Ed.)
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