**Chromatography**

Lecture-2

Chromatography

- 1 Ion-exchange Chromatography (IEX)
- 2 Affinity Chromatography
- 3 Hydrophobic Interaction Chromatography (HIC)
- 4 Gel Filtration Chromatography

## CHROMATOGRAPHY

The major step(s) in most downstream processes is purification of the target molecule by chromatography.

## PRINCIPLES OF LIQUID CHROMATOGRAPHY

### 1- The Partition Coefficient

Chromatography is a core technique of biochemical investigations and is used extensively to purify a protein of interest from a complex mixture. Chromatography can be analytical or preparative, but the basis of all forms of chromatography is the **distribution** or **partition coefficient** (*P*), which describes the way in which a compound (the analyte) distributes between two immiscible phases. For two such phases, A and B, the value for this coefficient is a constant at a given temperature and is given by the expression:

:

$$P = \frac{c(\text{solute})_{\text{phase A}}}{c(\text{solute})_{\text{phase B}}}$$

The term **effective distribution coefficient** is defined as the total amount, as distinct from the concentration, of analyte present in one phase divided by the total amount present in the other phase. It is in fact the distribution coefficient multiplied by the ratio of the volumes of the two phases present.

**2- Column Chromatography:** Chromatographic systems used for protein purification consist of a **stationary phase**, which is typically an **immobilised** solid, and a liquid **mobile phase**, which is passed through the stationary phase after the mixture of **analytes** to be separated has been applied to the column. The mobile phase, commonly referred to as the **eluent**, is passed through the column either by use of a pumping system or gravity (atmospheric pressure).

The stationary phase is either coated onto discrete small particles (the matrixالحشوة ) and packed into the column or applied as a thin film to the inside wall of the column. During the chromatographic separation, the

**analytes** continuously pass back and forth between the two phases, exploiting differences in their distribution coefficients, and emerge individually in the **eluate** as it leaves the column.

## 3 Chromatography Components for Protein Purification

A typical chromatographic system suitable for protein purification consists of the following components, ideally be situated in a cold room or refrigerator to maintain protein stability:

- **A stationary phase**: Chosen to be appropriate for the analytes to be separated; typically, an aqueous buffer with sufficient ionic strength to maintain a soluble protein. Certain additives sometimes required.
- **A column**: Research-lab-sized liquid chromatography columns range in length from 5–100 cm, with internal diameters from 4 mm to 6 cm and made of stainless steel, plastic or glass, depending on the application and hence the system pressure involved. The column has to be carefully packed to generate reliable separations. Often, columns are obtained pre-packed from commercial suppliers.
- **A mobile phase and delivery system**: Chosen to complement the stationary phase and hence to discriminate between the sample analytes and to deliver a constant rate of flow into the column.
- **An injector system**: To deliver test samples to the top of the column in a reproducible manner.
- **A detector with data acquisition**: To give a continuous record of the presence of the analytes in the eluate as it emerges from the column. Detection is usually based on the measurement of a physical parameter such as visible or ultraviolet absorption or fluorescence. A typical chromatography system has a computer to control the system and acquire readouts of the monitored physical parameter. The plot of the monitored parameter versus elution time is called a **chromatogram.**
- **A fraction collector**: For collecting the separated analytes for further biochemical studies.

Depending on the particle size of the stationary phase (and the desired resolution), the chromatography may be carried out as **gravity-driven**, **low-pressure** or **high-pressure liquid chromatography**. Gravity-driven chromatography is often used with cartridges for a quick single-step purification of small-scale samples and without inline detection systems. Larger samples are processed in pumped systems. In **low-pressure liquid**

**chromatography**, the flow of the eluent through the column is achieved by a low-pressure pump, frequently a **peristaltic pump.**

**chromatography matrix**

Another important consideration is the matrix and ideally this should be:

- **reusable**;
- **chemically stable** (not leach during processing);
- **physically stable** (i.e. withstand the required flow conditions);
- **have appropriate internal structure**/porosity to give suitable mass transfer and capacity;
- **be commercially available** from a reliable source to meet regulatory requirements;
- **be cost effective**.

Typical matrix types often encountered include **agarose** and **crosslinked agaroses**, **cross-linked dextran**, **composites of dextran and polyacrylamide or dextran and agarose**, **cellulose and organic polymer-based beads**.

**There are four main chromatographic techniques used for the purification of proteins: ion-exchange, affinity, gel filtration** and **hydrophobic interaction**. The principle of each, examples and the usual stage at which they are used during downstream processing are detailed in Table1. Of the four, ion-exchange, affinity and hydrophobic interaction chromatography are adsorptive processes whereby binding and elution are undertaken.

**Table 17.1** Details of the four major chromatographic methods used for downstream processing of biologics.

| *Chromatographic method* | *Basis of separation* | *Example ligands/ systems* | *Principle* | *Stage of purification procedure applied* |
|---|---|---|---|---|
| Ion exchange | Charge | Cation exchange, *e.g.* carboxy-methyl (CM) and sulfopro-pyl (SP) | Positively charged mole-cules bind | Usually early in puri-fication process, giv-ing high recovery and purification factor |
| | | Anion exchange, *e.g.* diethylami-noethyl (DEAE) and quaternary amine (Q) | Negatively charged mole-cules bind | |
| Affinity | Biospecific affinity | Protein A (for IgGs) | Specific interac-tion between protein and ligand | Usually at early stage, high resolution and purification factor |
| Hydrophobic interaction | Polarity | Phenyl-Sepharose | Polarity, interac-tion increases with ionic strength | Often after salt precipitation |
| Gel filtration | Size | Sephadex | Large molecules elute first | Polishing step late in purification |

**A survey of approximately 100 papers** confirms that **affinity chromatography achieves the highest purification factors** of all these approaches (Table 2).

**Table 17.2** The average and maximum purification factors of the four major chromatographic methods utilised during downstream processing as determined from a survey of the primary literature.

| *Chromatography method* | *Purification factor* | |
|---|---|---|
| | *Average* | *Maximum* |
| Ion exchange | 7 | 143 |
| Affinity | 57 | 499 |
| Hydrophobic interaction | 10 | 79 |
| Gel filtration | 5 | 26 |
| Precipitation[a] | 4 | 33 |

[a]Precipitation is not a chromatographic method but is included for comparison.

However, **ion-exchange chromatography is the most commonly utilised chromatographic approach**, comprising approximately 41% of all purification steps used, and gel filtration chromatography (17%) is the second most widely used approach. **Precipitation is used almost exclusively as a first step**, whereas **ion exchange is used as a bulk purification step and as a polishing step late** in the workflow (Figure.2).

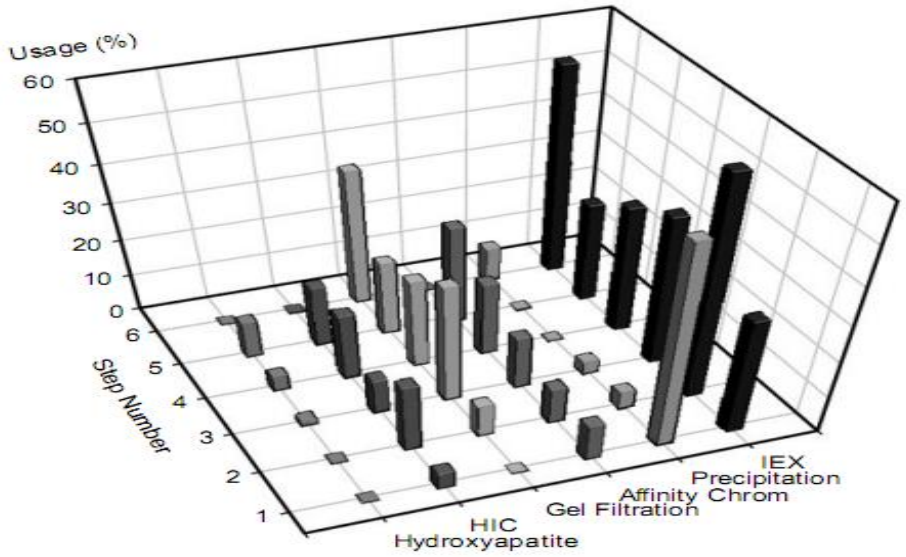



**Figure 17.2** The use of chromatography and precipitation methods as a percentage of the total number of unit operations at a given step throughout downstream processing as determined from a survey of the primary literature. IEX, ion exchange; HIC, hydrophobic interaction chromatography.

## 1– Ion-exchange Chromatography (IEX)

Ion-exchange chromatography is the most commonly used chromatographic step in downstream bioprocessing and is an adsorptive binding and elute method. التبادل الأيوني هي الخطوة الأكثر استخداما في المعالجة الحيوية النهائية وهي طريقة ربط امتزازي وثم إزالة

Ion exchange can be **subdivided into cation exchange**, whereby positively charge molecules bind, and **anion exchange**, whereby negatively charged molecules bind the resin. In both cases, the bound material is usually **eluted by increasing the salt concentration**. The target protein of interest must therefore be **in a buffer of the appropriate pH and salt concentration** before being applied to the column. As outlined in Table.1, **commonly used ligands include carboxymethyl and diethylaminoethyl (DEAE)** for cation and anion exchange, respectively.

Ion exchange dominates as the method of choice in most steps (Figure2) due to the fact it is used as a **bulk purification method and a polishing step** and the fact that **a weak binding high-capacity ion-exchange step** is followed by a **lower capacity strong affinity binding step**.

**2– Affinity Chromatography**

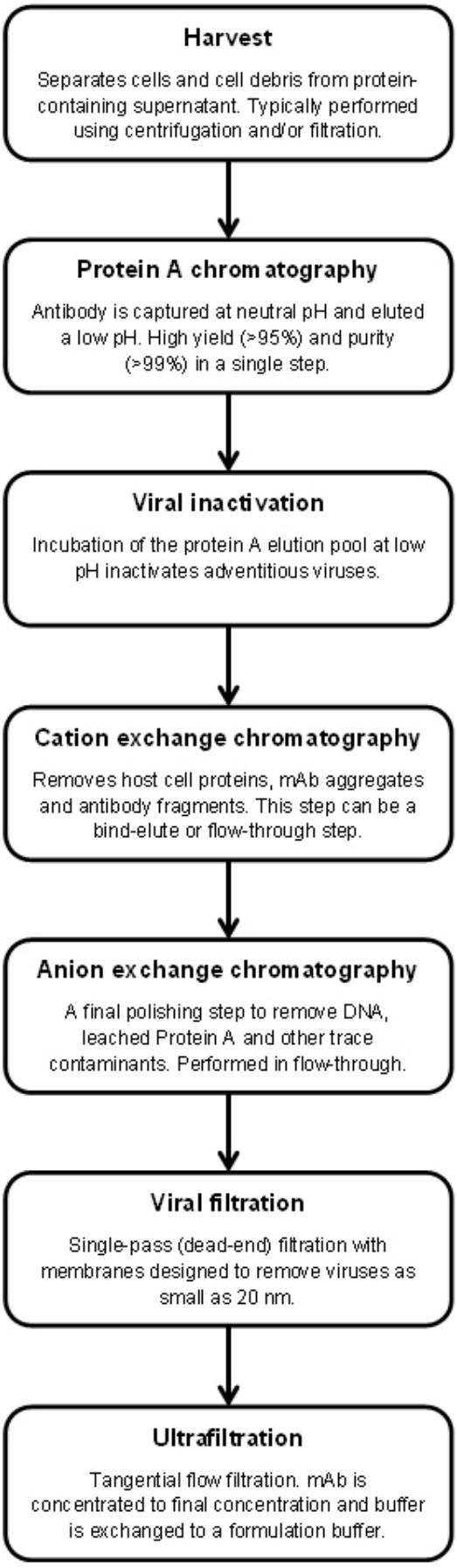
Applied correctly and with the appropriate ligand, affinity chromatography is **the most powerful chromatographic purification method available to yield high purification factors in a single step**. Due to the high biospecificity of this approach, it is used as a fundamental capture step in most processes where an appropriate ligand is available and this is one of the limitations in using the approach. The approach is thus **based upon the affinity of the target molecule for a ligand immobilised on an appropriate resin**.

Protein A capture of monoclonal antibodies expressed from *in vitro* cultured mammalian cells is one of the most widely used affinity chromatography steps. Indeed, industrially **the gold standard purification of monoclonal antibodies is to use protein A chromatography as the first step in the purification process after primary recovery steps.** For example, in 2002 the demand for protein A resins exceeded 10 000 L and an already $50 million a year market was growing at a rate of 50% per year. However, increased yields of recombinantly produced monoclonal antibodies coupled with the high cost of protein A resins mean that investigators are currently focused upon **improving the capacity and throughput of this approach** in order to continue making this approach economical. A further area of concern with the use of protein A is **the stability of these resins upon cleaning,** and therefore there is much interest in finding alternatives to protein A chromatography for antibody purification.

**Affinity ligands are also used at the industrial and laboratory scale.**

**Dye-based ligands** are perhaps the largest industrially utilised approach, largely for the purification of **industrially produced enzymes**.

**Immobilised metal affinity chromatography** also widely used at the laboratory scale, particularly **the use of poly His-tags**, whereby **the target protein of interest is engineered to**



**contain a His-tag** usually of six consecutive His residues at either the C- or N-terminal end of the protein. This approach **is popular due** to the **high binding capacity**, **stability**, **robustness** and **recovery** offered by poly His-tags. However, **a drawback of such an approach** is that it is **often necessary to remove the His-tag during bioprocessing** to maintain the authenticity of the biomolecule, **adding further steps and costs to the process**. **It is therefore most widely applied to high-throughput research approaches where there is a need to purify a large number of different proteins for further study.**

Although the development of affinity separations is one of enormous potential, **the growth of regular packed bed affinity chromatography** seems to have plateaued over the past few years. **The limitations** do not lie in affinity based separations but **in packed bed chromatography columns** and the **lack of cheap ligands available** for use at an industrial scale.