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Review

Liquid chromatography of recombinant proteins and protein drugs☆

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Abstract

Many recombinant proteins (rPRTs) have a high bioactivity and some of them may eventually be classified as drugs beneficial to human health, recombinant human protein drugs (rPDs). rPDs are a high-technology product with all the associated economic benefits, therefore the liquid chromatography (LC) of rPRT is different from that of proteins isolated in laboratory scale for purely research purposes. The design of a purification scheme for an rPRT depends on the intended function of the purified rPRT, as a pure sample for research in small scale, or as a product for industrial production. This review paper mainly deals with the latter instance, producing rPD at a large scale. Pharmaceutical economics is considered not only for each step of purification, but also the whole production process. This strategy restricts the content of this review paper to the factors affecting the optimization source, the character of rPRT in up-stream technology and the purification of the rPRT in down-stream production. In the latter instance, the purification step is required to be as efficient as possible and LC is the core of the refined purification method, which is either a single LC method or combination of LC methods, sometimes, it may be a combination of LC and other non-LC separation methods comprising an optimized purification technology. Here some typical examples of rPRT purification at the large scale, recent developments, such as protein folding liquid chromatography, short column chromatography, and new packing material and column techniques are introduced.

Keywords: Liquid chromatography; Biotechnology; Recombinant protein; Protein drug; Purification; Protein folding; Large scale; Packings; Column techniques; Pharmaceutical economics

Contents

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1. Introduction

Proteins are the basis of life; proteins are one of the most important biological components and protein science is a fundamental part of life science. A protein of high purity is vital for investigating the molecular structure, character, and bioactivity of an unknown protein, whether it occurs naturally or is produced by recombinant DNA technology, known as recombinant protein (rPRT). If the protein obtained in this manner is the same as that human protein, it is so called as a recombinant human protein, many of them are drugs beneficial to human health, and are known as recombinant protein drugs (rPDs).

The type of purification employed depends on what the pure sample is required for, is it for research, or is it required at a larger scale for industrial production. For the former, two factors, the purity and the bioactivity are most important, because the amount required is low, therefore, mass recovery, expenses and complex purification technologies are not a major consideration. For the latter production at an industrial scale, in addition to the quality of the rPD (purity $\geq$95%) and various impurities at a low level, only one other factor stands out, gaining the largest benefit for the effort, without spending more money to obtain the rPD at the highest purity possible. Therefore, the cost of producing an rPD must be reduced by optimization from a pharmaceutical economics standpoint. As discussed above the two requirements of purity and profit have led to two quite different modes of thought. This is probably the reason why it is difficult for scientists and/or engineers to scale-up their purification technology from the laboratory to the industry.

This review paper covers all aspects involved in the development of rPRTs and rPDs, including initial characterization, purification and production economics.

1.1. Pharmaceutical economics

Several investigations have discussed the process economics of industrial monoclonal antibody (Ab) manufacture and the importance of liquid chromatography (LC) and pharmaceutical economics [1–5]. The production of rPRT involves an up-stream system containing a DNA recombinant method, the selection of an expression system, production of the protein through fermentation, and the down-stream technology, which consists of a series of purification and other associated steps. Pharmaceutical economics is a specific branch of science which studies areas such as manufacturing cost, the establishment of a reasonable evaluation system for a specific drug (optimization theory) and market analysis.

rPDs can be divided into three types, antibodies, vaccines, and therapeutic proteins. The importance of pharmaceutical economics in the purification of rPDs can be demonstrated taking antibody drug production as an example. It was previously reported [5] that an antibody is a very efficient drug for cancer patients, and a cancer patient need about $35,000/y to buy such drugs. This price is too high for cancer patients in most countries. The question is how to lower the manufacturing cost or what are the factors affecting the cost of rPD production? The cost for manufacturing rPD is dominated by the fixed cost of sourcing from its source, production scale, and the ratio of the expenses of up-stream express to down-stream production. That they are separated, is demonstrated by the following examples. (1) The source of the antibody drug, or the up-stream expression system. Young et al. [6] and Mison and Curling [7] both separately employed the expression system of the Chinese hamster ovary (CHO), a transgenic goat and transgenic corn to express an antibody. For the same output of 100 kg/y, the CHO expression system is the most expensive, while the transgenic corn is the cheapest, costing a sixth to a sixtieth of that of the CHO

system; (2) the production scales for the same transgenic corn, the cost to output of 1000 kg/y is only a third to a quarter of that of 100 kg/y; (3) the ratio of the cost of up-stream to down-stream for producing monoclonal antibody, is 46/54 for an output of 6.2 kg/y, but 20/80 for an output of 100 kg/y [8]. The main expense in the down-stream production of an rPD is LC [9]. Still taking the antibody as an example. Protein A is a very effective media used for the affinity chromatography (AFC) purification of antibodies, its cost is $7500/L corresponding to a 200 L bioreactor and $4–5 million for 10,000 L/y, that is a really significant contribution to the cost of antibody production. Schubert and Freitag [10] recently reported a cheaper ceramic hydroxyl fluoapatite material in place of the expensive protein A column for purifying recombinant human antibody from a CHO cell culture supernatant this will be discussed further in the "refined purification-AFC" section later.

1.2. Characteristics of recombinant proteins

Down-stream technology is tightly related to the source of the target protein, the state of the target protein (native or unfolded), the concentration of the target protein as well as that of other components in the sample. The expression system of an rPRT can be prokaryotic (*E. coli*) or eukaryotic (based on yeast, CHO, insect cell line, animal cell line, or plant cell line). The *E. coli* expression system has many advantages such as, the high expression of the target protein, a short growing period, and the low cost and simple operation, but it also has a main disadvantage, the target protein is often produced in an unfolded state, forming inclusion bodies, which are insoluble in an aqueous salt solution. A target protein in an inclusion body has the correct primary molecular structure, but the incorrect three-dimensional molecular structure. Before performing any protein purification steps, the unfolded protein must be refolded to its native state, protein renaturation. The renaturation process is very difficult and has a very low efficiency, thus increasing the complexity and cost of producing the target protein. Because the purity of the target protein in the inclusion body is usually in the range of 30–70%, its purification is relatively easy [11–14].

Eukaryotic expression systems have various host-dependent advantages and disadvantages. With the yeast expression system, growth is rapid and high cell densities can be achieved, the target protein is produced in its native state, but some target proteins have problems with low expression levels, incomplete signal peptide processing and product stability. With the CHO expression system, the target protein is obtained in its native state and does not require post-translational modification, but cell growth is slower, and CHO cells are expensive to culture. However, if the titers can be increased up to 10 g/L and beyond, the price of CHO and transgenic production may decrease. Comparing plants and animals as bioreactors, plants appear to be safer, easier to produce and less expensive than using animals. In transgenic animals the proteins may be generated as products in, milk, egg white, blood, urine, and insects [15,16].

Irrespective of the expression system used to produce the target proteins they will all be in a solid or liquid state after production and will need further treatment before proceeding to final purification.

1.3. Strategic consideration

The establishment of a purification scheme for a recombinant protein depends on five factors. (1) The important characteristics of an rPRT include solubility, hydrophobicity, iso-electric point. (2) The purpose for purifying the target protein for research, or industrial production. (3) LC acts as a core purification method combining with other cheaper separation methods. Based on the theoretical evaluation of separation science by Giddings [17], chromatography and electrophoresis are the most efficient separation methods, but only the former can be employed at the preparative and industrial scale. Because of the cost of LC, other traditional separation methods, such as precipitation, centrifugation and membrane filtration, have been employed to supplement LC in the purification of rPRT in the modern pharmaceutical manufacturing setting. (4) Pharmaceutical economics dominates the whole production process. (5) Establishing a universal technique platform suitable not only for one type of rPRT, but also for a group of rPRT, thereby shortening the time to market. This is especially important for accelerating a new kind rPRT to market in future.

From the foregoing discussion (Sections 1.1–1.3), the following conclusions may be obtained. (1) The purity and bioactivity of a protein at the small scale are the most important factors for research but little consideration is given to expense, while for the production of a protein at the large scale, aside from satisfying drug quality standards; pharmaceutical economics dictates the whole production process. (2) As long the expression system up-stream is fixed, the cost of rPRT production at the large scale is lower than that of the small scale, further, for larger the production scale, more expense is incurred by the down-stream technology, mainly from LC; (3) LC for the purification of rPRT not only involves LC itself, but also includes the use of other forms of separation techniques.

2. Pre-treatment

2.1. Sample treatment

If an rPRT is derived from a eukaryotic expression system and it exists in a solid state, all of the soluble components should initially be converted from the solid state to the liquid state. With an ultrasonic processor, the solid sample can be converted to a homogenized followed by a liquid–solid separation performed by centrifugation. Both the fats on top of the liquid phase and the particles in the pellet below the liquid phase are removed. The retained liquid layer contains the rPRT, which requires further coarse separation. Also if the original sample is in a liquid state, it also needs a centrifugation separation step, in case the sample contains very small particles. The supernatant containing the rPRT, as shown in Fig. 1, then goes for subsequent coarse separation.

Recombinant protein derived from *E. coli* is often harvested in inclusion bodies, and it is referred to as inclusion body pro-

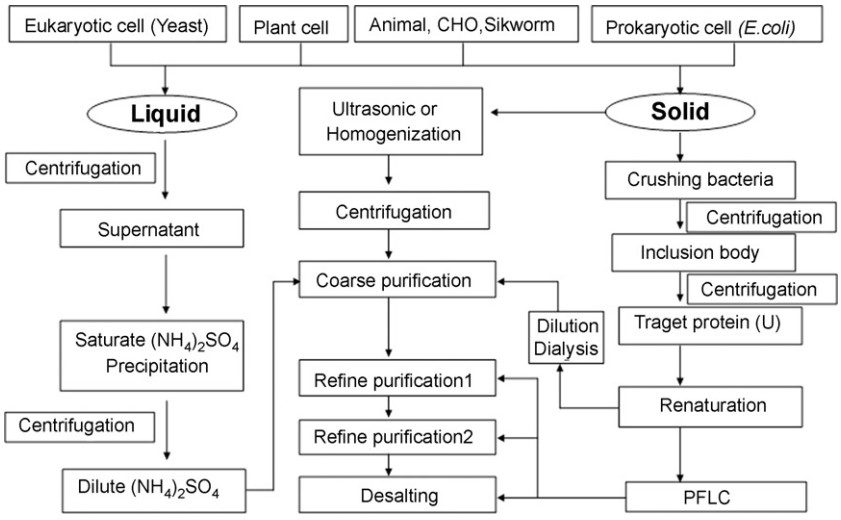

Fig. 1. Scheme of sample treatment of recombinant proteins.

tein. The inclusion body protein inside *E. coli* actually has the correct amino acid sequence, but has an incorrect three-dimensional structure. Inclusion bodies occur because *E. coli* as a prokaryote lacks the correct folding machinery to fold a protein derived from a eukaryote. Therefore the unfolded protein experiences hydrophobic interactions among the exposed hydrophobic amine residues then the recombinant protein molecules form aggregates, which usually do not dissolve in any kind of aqueous salt solution, except 7.0 M guanidine hydrochloride (GuHCl), or 8.0 M urea solutions which return the target recombinant protein exist to its monomeric state. Inclusion body proteins may be, either bacterial in origin (from the host) or the target recombinant protein itself, all existing in a solid state [18,19], and requiring special sample treatment. The bacteria are placed in a buffer and crushed using an ultrasonic processor, or other mechanical means. The inclusion bodies are pelleted at a low centrifugal speed. After several rounds of cleaning with a suitable buffer, the clean inclusion body is usually dissolved using a solution containing a chaotropic agent. The chaotropic agent can disrupt hydrogen and hydrophobic bonding thus dissolving the inclusion body proteins allowing them to exist in an unfolded monomeric state. Sometimes, a reductant is included with the chaotropic agent in the solution to disrupt any disulfide bonds and ensure that the inclusion body dissolves rapidly and completely. Even still with a mixed-solution, some particles may still exist, and an additional centrifugation separation must be performed. The harvested solution, sometimes, has a very high viscosity due to the presence of DNA and RNA, these very large molecular mass molecules are broken into small segments using ultrasonic disruption in an un-continuous manner in an ice-water bath and this reduces the viscosity of the sample [20–22]. The rPRT obtained in the chaotropic agent solution must be renatured before any additional separation processes can be performed.

2.2. Renaturation of inclusion body proteins

According to Anfinsen's Theory, as long as a protein has the correct primary structure, or correct amino acid sequence, the protein can spontaneously fold to its correct three-dimensional structure, or native state, as this has the lowest energy state. In other words, a protein is thermodynamically stable in the native state but unstable in the unfolded state. Based on Anfinsen's Theory, by removing the denaturant (chaotropic agents such as urea and GuHCL) from the environment the denatured target should spontaneously renature, or refold to its native state with the correct three-dimensional structure. Many methods may be employed to accomplish this removal or reduction in the concentration of the chaotrobic agent [23,24]. However Anfinsen's Theory does not hold true for many proteins, especially those with a strong hydrophobicity and/or many sulfide bonds, which are hard to refold correctly, and sometimes cannot refold at all.

2.2.1. Dilution

The principle of the dilution method is based on the fact that protein molecules in the unfolded state can spontaneously refold to their native state when the chaotropic agent is removed from the environment. Usually, diluting the sample solution by 10–100-folds accomplishes this process. Although the chaotropic agent cannot be completely removed, its concentration is so reduced that its effect does not retard protein refolding. On the contrary, some proteins have strong hydrophobicity and their hydrophobic amino residues can interact with each other to make partial polymers and/or precipitates of the protein, even in the presence of chaotropic agents at low concentrations. The presence of the chaotropic agent with a suitable concentration may partially prevent the formation of these associations, thereby favouring protein refolding.

During the dilution process, the rPRT in the unfolded state starts to refold. For some proteins the refolding process occurs very fast, taking less than a second, to obtain a high recovery of protein folding, while others fold slowly. In the latter instance, the concentration of the chaotropic agent may be too low locally; some polymers, and/or precipitates form, decreasing the efficiency of refolding. Therefore, in many instances, protein refolding requires the presence of urea at a concentration ranging from 2 to 4 M [25]. To increase the efficiency of pro-

tein folding, a continuous elution method was developed [26]. Even though many improvements have been developed, some precipitates still form, making a centrifugation step necessary 8–12 h after dilution. Dilution is a simple method not requiring any special equipment, but the sample solution volume increases increasing the difficulty of the subsequent purification process.

2.2.2. Dialysis

To overcome the intrinsic disadvantage of the dilution method due to the increase in the final volume of the sample, dialysis is adopted. The sample solution is placed in a membrane packet and is immersed in a refolding buffer, usually for 24 h, and undergoes buffer exchanging several times. Salts and low molecular mass substances (<10,000 Da) diffuse though the membrane, whereas the high molecular mass rPRT remains within the dialysis packet. This operation is simple but has a long cycle time, also some precipitates of target protein may still form in the membrane packet, thus filtration, or centrifugation is necessary before going through the subsequent separation [27,28].

Although scientists have been working on it for many years and many methods have been employed for the refolding of rPRT, the efficiency of protein refolding usually only ranges from 5% to 20%. A new method really needs to be developed to increase the efficiency of protein refolding.

2.2.3. Protein folding liquid chromatography (PFLC)

Protein folding liquid chromatography is a new division in science is "a kind of liquid chromatography, incorporating various biochemical and/or physicochemical processes originally accomplished in solution, which can result in either increasing the efficiency, or reducing the time of protein folding" [29,30]. The definition includes both dynamic and thermodynamic considerations. From its nomenclature, it may be thought to imply two functions, protein folding and protein purification. This true, but aside from its two main functions, it also results in the removal of chaotropic agents and the easy recovery of chaotropic agent [29–31]. A monograph and review paper of PFLC was recently published to introduce this new kind of LC as a tool for protein folding in molecular biology [29–31]. It was reported that ion exchange chromatography (IEC), size exclusion chromatography (SEC), hydrophobic interaction chromatography (HIC), and affinity chromatography can be employed to have either all, or some of these four functions simultaneously [32–36]. Also many types of chromatography, such as expanded bed chromatography (EBC) [37,38], continuous annular chromatography (CAC) [39–41], simulated moving bed chromatography (SMBC) [42,43], and chromatographic cake [44–49] have been successfully used in PFLC at both the small and large scales, this will be discussed further in Section 4.2.2.

The operation of both PFLC and normal liquid chromatography are essentially the same. A sample solution containing the rPRT is directly injected into a suitable chromatographic column, and then the fractions containing the renatured target protein are collected. However, the principle of PFLC is significantly different from LC. From a molecular mechanism standpoint, each type of PFLC has its own special format, but from a thermodynamics standpoint, all of them function in the same way.

All types of PFLC share the following steps, (1) to hold up protein molecules in an unfolded state within the stationary phase of LC thereby diminishing the formation of polymers and/or precipitates and allowing proteins to spontaneously refold as they are eluted, in other words only the stationary phase contributes to protein folding; (2) the stationary phase, mobile phase, their association, the flow rate of the mobile phase and the gradient elution mode, all contribute to protein folding, in other words all chemical equlibria dominate protein folding as shown in Fig. 2. From a chemical equilibrium viewpoint, the separation principle of normal liquid chromatography solely depends on the partition coefficient of a protein in two phases (shown as the dash line rectangle in the bottom in Fig. 2). While protein refolding, based on Anfinsen's Theory, the folding efficiency is dominated by the competition for a thermodynamic equilibrium between

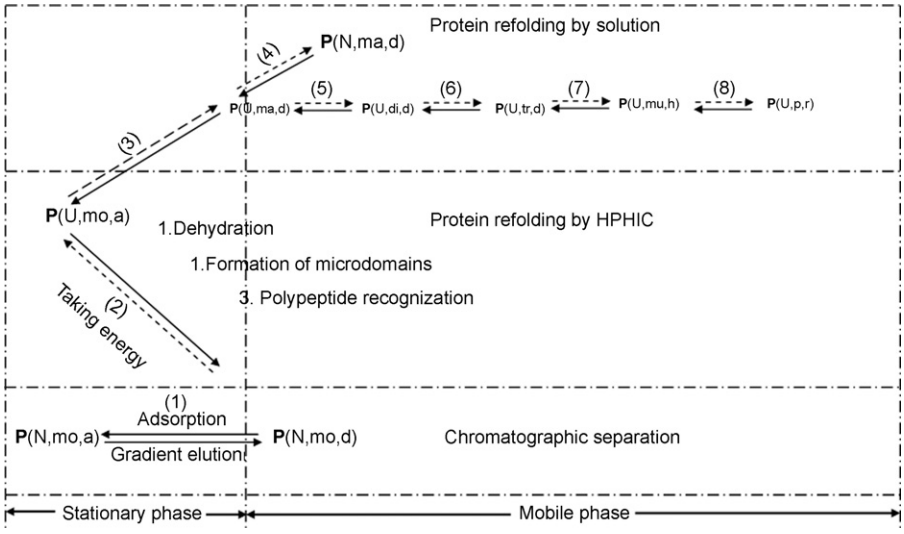

Fig. 2. Scheme of a general principle by PFLC. U, unfolded state; N, native state; mo, monomer; di, dimmer; mu, multimer; pr, precipitate.

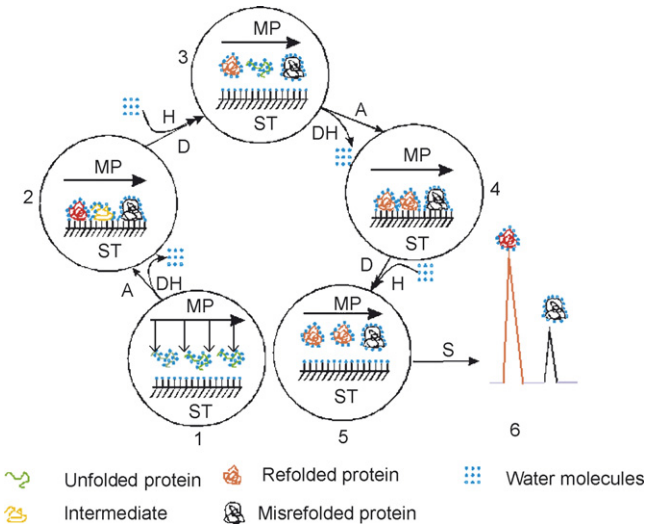

Fig. 3. Scheme for the refolding of denatured protein with HPHIC. A, adsorption; D, desorption; DH, dehydration; H, hydration; MP, mobile phase; ST, stationary phase.

monomers in the unfolded state and polymers and/or precipitates, and the association and disassociation of the unfolded protein molecules (denoted by dash and solid arrows rectangle on the top in Fig. 2). Both adsorption and desorption equilibria are connected together through the association of the mobile and stationary phase of LC during elution (shown as the dash line rectangle on the middle just for HIC in Fig. 2). Depending on the kind and magnitude of molecular interactions between the stationary phase of liquid chromatography (STLC) and the target protein, the molecular mechanism is quite different from one form of LC to another. Taking HIC as an example [31], the molecular mechanism here includes three processes acting simultaneously (also shown in the middle in Fig. 2). With the chromatographic running, the association of the stationary and mobile phases combine to propel the serious chemical equilibria along the solid arrow direction to move the target protein forward from its polymeric to monomeric form in the unfolded state until it refolds to its native state, and in this manner accomplish complete protein folding.

Fig. 3 [46] shows how PFLC works. (1) The unfolded protein molecules in the mobile phase (MP) are pushed forward into tight contact with the stationary phase of HIC (STHIC) to form a stable complex; (2) the unfolded protein molecules take a high enough energy at the molecular level and the stationary phase simultaneously has three functions, retarding the unfolded protein molecules preventing aggregation (Fig. 2), instantaneous dehydration and the formation of the correct micro domains [31,46]; (3) the association of both the stationary and mobile phases creates the chemical equilibrium necessary to transition from mis-folding to correct folding; (4) the unfolded protein molecules are re-retarded by the STHIC to accomplish the second cycle of adsorption–desorption to ensure the protein molecules with correct folding become more and more folded; (5) after many of these cycles, the target protein is completely folded; (6) the completely folded target protein having the same retention time as that of its native state is finally eluted.

Theoretically, urea solution is a neutral charge chaotropic agent and thus can be used in many kinds of LC, while GuHCl solution cannot be loaded onto an ion-exchange column, because of its very high ionic strength. In practice, a sample solution containing either chaotropic agent, especially at high concentrations is never directly injected onto a column for any kind of LC, as protein precipitates form as the sample makes contact with the mobile phase (an aqueous salt solution). This problem is solved and discussed in Section 4.2.

Although many inclusion body proteins have been successfully refolded by PFLC, it is a technique that is still developing and a lot of problems require resolution. Some inclusion body proteins still cannot be completely refolded, or cannot be refolded at all, especially those containing more than four-pairs of disulfide bonds. Although inclusion body proteins have a problem with refolding, the purity of inclusion body proteins is usually relative high, indicating that the subsequent purification of the target protein should be relatively easy.

2.2.4. Other methods

Molecular chaperones and artificial chaperones can act as additive agents in the buffer solution, and can also assist with protein folding [51–53]. Compared to PFLC, all of those other refolding methods cannot be employed to separate, or incompletely separate impure proteins, chaotropic agents and other small solutes.

2.3. Coarse separation

As outlined above, LC is only one efficient method for the refined separation of rPRT at the large scale, a lot of samples containing rPRTs require an initial coarse separation, as they contain many kinds of contaminants, such as RNA and DNA with a large molecular mass, or fats and nutrient components from the cell culture broth may tightly stick to the top of the column, thereby blocking the column. In most circumstances, an initial coarse, or non-chromatographic separation is required. Precipitation, centrifugation, membrane filtration, and crystallization are the most commonly employed techniques.

2.3.1. Precipitation separation

Precipitation is a routinely employed method for the coarse separation of rPRTs at both the small and large scales. The precipitation separation of proteins is based on the solubility characteristics of proteins and is the oldest protein purification technique, but it is still employed in modern pharmaceutical manufacturing. It can be accomplished by changing the result in protein becoming less soluble in aqueous media, including the addition of salt and other precipitation agents. Based on the production scale and the importance of the rPRT, several different methods can be selected. For research purposes, any organic solvents, such as acetone, trifluroacetic acid (TFA), ethanol, 2-propanol, organic polymer, inorganic salt, ammonium sulphate, or the adjustment of the pH can be used as a precipitating agent. For the large-scale purification of proteins, precipitations combined with centrifugation have been employed in most circumstances. The advantage of this method is that it is cheap and

easy to operate at the large scale. Based on the "salt-out effect", the saturated ammonium sulphate solution (60%, w/w) results in the precipitation of most proteins. After centrifugation, the resulting pellet can be re-dissolved in a dilute ammonium sulphate solution. If a small volume of dilute ammonium sulphate solution is used to re-dissolve the precipitate, an enrichment process of the target protein should also be performed. As long as the rPRT molecules do not aggregate during this process and the pellet contains the rPRT, it can be re-dissolved completely. The volume of the dilute ammonium sulphate solution should be kept as small as possible, resulting in a highly concentrated rPRT sample. However, a disadvantage of this process is that the resulting protein solution often needs to be dialyzed to obtain an ionic strength sufficient low to allow ion exchange chromatography. Also, with this process, the mass recovery and bioactivity of the rPRT may be low, usually; the recovery is in the range of 50–80% [54]. This problem sourcing from ammonium sulphate as precipitant can be avoided by using an organic precipitant or novel polymer.

The most widely used polymer is polyethylene glycol (PEG), with an average molecular mass of 6000–20,000 Da. The main advantage of PEG over organic solvents is that it is easier to handle. It is inflammable, uncharged, non-toxic, and inexpensive. Low concentrations are sufficient (often less than 25%) to precipitate most proteins. Organic polymers function in a way analogous to that of organic solvents. One disadvantage is that concentrated solutions of PEG are highly viscous. Polyethylene glycol can also be difficult to remove from protein solutions. However, after dilution with buffer the viscosity decreases, and since the substance is uncharged, the solution may be applied directly to an ion-exchange column to further eliminate residual PEG [55]. However, this type of precipitation, like ammonium sulphate precipitation, is nonspecific and often leads to an impure or "mixed" precipitate. Affinity precipitation can also be employed to improve the selectivity. Phospholipid solobilized in an aqueous solution by non-ionic surfactant was used for the precipitation of avidin and was reported to have a high yield and purity [56]. Soybean trypsin inhibitor (STI) was immobilized on polymerized liposome (PLC) as an affinity agent for trypsin precipitation from (1) an artificial solution containing BSA and trypsin and (2) a crude pancreatic extract. In this instance, 91% of trypsin was coprecipitated and 87% of the precipitated trypsin was recovered by elution with 0.01 M NaOH [57].

Centrifugation and filtration must be employed together. If the composition of the supernatant contains salts; it may not be suitable for the subsequent refined separation by LC. Some proteins may associate to form dimers, trimers or polymers during the dissolving process, or they may not dissolve at all in the dilute ammonium sulphate solution. Even though there is no precipitate formation very soon, when storing the supernatant, some precipitations can appear and in this case re-centrifugal separation must be performed. Prior to loading a sample solution onto a chromatographic column, it must be ensured that there is not any particle in the sample solution.

Crystallization is also an important method for protein separation and purification. Crystallization is the nucleation and growth of a solid phase in a saturate solution. It can be used as a separation technique, as the process results in the separation of the mother liquor and the solid phase. If the mother liquor and the solid phase have different compositions, it can also be used as a purification technique. In such a case, the mother liquor must contain impurities. Protein and antibodies are often purified by crystallization. Hedrich et al. developed a large-scale procedure for the purification of lipases from the fungus *G. candidium* after two LC steps of IEC and HIC [58]. For a more extensive discussion on crystallization refer to Sandana [59].

2.3.2. Membrane filtration

Membrane filtration is a very simple method for removing substances according to their molecular size. Force, such as gas pressure, is used to push the liquid, together with smaller molecules through the membrane, impurities with either a greater or smaller molecular mass, than the target protein, can be separated with simultaneous enrichment. It has a problem with a low loading capacity for the rPRT, as the largest protein molecules are retained and increase in concentration behind the membrane. Hollow-filter membrane and membrane filter equipment may be employed to enlarge the scale for purifying the protein, but it is only employed as a relatively coarse separation of large proteins from the smaller ones. This method is simple, if it can be used in conjunction with an accompanying centrifugation, or ultrafiltration (UF) step, the filtration process can be accelerated.

To improve the resolution and reduce the number of purification steps and elution times for proteins, conventional UF was improved and developed into a new purification technique, known as high-performance tangential flow filtration (HPTFF). HPTFF is a two-dimensional purification method that exploits differences in both the size and charge characteristics of proteins and nucleotides [60]. Conventional UF is limited to the separation of solutes that have at least a 10-fold difference in size. With HPTFF it is possible to separate biomolecules with the same molecular mass and even possible to retain one biomolecule while passing a larger molecular mass species through the membrane. It can be combined with IEC, UF and SEC in three separate steps for purification; concentration and buffer exchange and all of these steps can be performed in a single-unit operation, thereby reducing production costs. Since HPTFF builds on existing UF technology, there is already a well-established industrial infrastructure in place for the implementation of HPTFF processes. It can provide a high-resolution purification while maintaining the inherent high throughput and high yield characteristics of conventional UF and can therefore be used in the initial, intermediate, and final purification of rPRTs. It is also useful for the purification of monoclonal antibodies and for the separation of product variants that differ in as little as one of more than 150 amino acids [61]. It can be also used for the purification of IgG-BSA and BSA monomer–oligomer mixtures and the ultrafiltration of human pharmaceuticals produced by recombinant DNA methods [62].

3. Refined purification—a series of LC

Refined purification of rPRTs is employed to obtain a highly pure target protein. It should be carried out after course separation. LC with its high resolution and its flexibility lends itself well to this purpose. It can be employed as either a single-step process, or as in most instances, in combination with a number of other kinds of LC. Both the single-step and combination methods always need an initial coarse separation step as well as other associated steps, such as buffer exchange, thereby providing a total purification technology. In a few instances, a single LC step accompanied by other non-LC separation techniques may be enough to accomplish protein purifications with a purity ≥95%, but in most circumstances, a combination of several LC steps must be used. Some special rPDs, such as rhinsulin, require a purity ≥99%, and it is impossible to accomplish this demanding purification with just a single-step LC. For some protein drugs, viral clearance must be established also. This requires employing multiple, orthogonal purified steps, even if other purity criteria are satisfactory. It involves the selection of many kinds of LC, and arranging a specific order. A general understanding of the characteristics of each type of LC is not enough; the LC must be also assessed for its ability to be included in an optimized purification technology, where it is a step in a series of LC steps, in the downstream production of an rPRT. It is also important to review the optimized purification from a pharmaceutical–economic standpoint.

3.1. Separation of recombinant proteins and general proteins

rPRTs and naturally occurring proteins can both simply be called proteins, the difference between them is only their source, the former is derived from a DNA recombinant, while the latter exists naturally in prokaryotic and eukaryotic systems. If proteins from two sources are in their native state, the principle and method of their separation and purification does not differ. The only difference between them is the sample preparative method. An important point for naturally occurring proteins is to ensure that they remain their native state as they transfer from a solid to a solution and accompanying concentration. Some native proteins exist at a very low concentration in the sample and have a low bioactivity due to the existence of other non-functional proteins (known as impure proteins) at a high concentration; it is very easy to lose the bioactivity of the target protein as the impure proteins are removed. This is a very difficult scenario to overcome in proteome investigations. For rPRTs, as pointed out in the previous section (Section 2.2), the sample preparative step is based on the source of the expression system. It is hard to refold protein from inclusion bodies, especially protein molecules, with over four pairs of disulfide bonding. Compared to the very low abundance of some functional proteins of interest to proteomics, the expressed amount of an rPRT is generally quite high. Except in these cases, the subsequent methods for the separation and purification of the two alternatively sourced proteins are the same.

3.2. Retention mechanism of proteins in LC

LC is central to protein purification technology; therefore the principles of protein purification should be briefly introduced. The principle of protein purification by any kind of LC should be based on the retention mechanism of the protein from the standpoint of the thermodynamic equilibrium. This can be further explained by either a quantitative relationship between solute retention and other chromatographic parameters in LC or the calculated magnitude of molecular interaction forces relating to the structural characteristics of the protein molecules. The former is simple and easy to perform in practice, while the latter is very complicated and hard to adapt to applications. Even though for the former, the retention mechanism of solutes, even for small simple solutes, is not yet fully understood. For example, reverse phase liquid chromatography (RPLC) is the most popular method employed for small solute separation in high-performance liquid chromatography (HPLC), in it, at least, ten retention mechanisms have been identified, and the four most important of these are reviewed [63]. Protein molecules are much more complicated than small solutes and therefore cannot be explained in terms of solute interactions. Fortunately, the stoichiometric displacement theory for retention (SDT-R) was presented by one of the authors of this paper and Regnier and co-workers [64] in 1984. Snyder et al. [65] introduced it as the retention mechanism of biopolymers not only for RPLC, but also for HPLC. Since then, SDT-R as the protein retention mechanism has been demonstrated in IEC [66], AFC [67], HIC [50], and RPLC. This fact indicates that there is only one mechanism of protein retention, SDT-R, the principle of protein separation can be simply elucidated.

The core of SDT-R is that when a mole of protein is adsorbed by a stationary phase of a HPLC, a stoichiometric mole of the displacer Z, at the contact region between the protein and the stationary phase is necessarily released. Its expression can be shown as:

$$\log k' = \log I - Z \log[\mathrm{D}] \quad (1)$$

Here, k' is the capacity factor of protein; $\log I$ is a set of constants (five molecular interactions in HPLC) and relates to the affinity of a protein for the stationary phase; [D] is the molar concentration of the displacer in the mobile phase. The displacer is an organic solvent in RPLC, salt, or $[H^+]$ in IEC and AFC, water in HIC. Eq. (1) indicates the common character of protein retention in HPLC, while the different kinds of displacers depend on the kind of HPLC employed. From Eq. (1), with an increasing [D], the retention of the protein decreases. This point will be explained in greater detail for each type of HPLC, from the standpoint of molecular interactions later.

3.3. Membrane chromatography (MC)

In order to increase the sample loading and the selectivity of a specific rPRT, or a group of proteins, the membrane surface can be chemically modified with various groups including ligands of RPLC, IEC, AFC, and HIC to increase the selectivity.

The modified membrane is known as membrane chromatography (MC). So far, the resolution of MC has been demonstrated in some special circumstances, performing at a high resolution comparable with normal LC at analytical scale [68]. If some of the chemically modified membrane possesses immobilized metal ion, ion exchange, mixed-mode interaction, and isoelectric interaction groups, and so on, the MC can raise the selectivity and resolution of proteins. In this circumstance, MC is actually one of the refined purification methods at both the small and preparative scale. With the MC, the result is a more efficient adsorption–desorption cycle of target proteins, allowing a considerably higher flow rate and thus considerably shorter separation times. This area was reviewed by Thömmes and Kula [69]. Knudsen et al. [70] used an anion-exchange membrane in a flow-through mode to replace the traditional ion-exchange columns for large-scale production of recombinant monoclonal antibodies. They provided a reasonable alternative to columns for the removal of low levels of impurities, such as DNA, host cell protein, and virus. Freitag et al. [71] employed a control mixed-model interaction chromatography on a membrane absorbent (MAs) binding to anion-exchange and Cibacron Blue affinity groups to purify the recombinant human antithrombin III (rh-AT III). The protein can be separated in a single run from the major protein impurities present in the fermenter supernatant, namely transferrin and BSA. Hu et al. [72] purified VP3 protein of infectious bursal disease virus using nickel ion-immobilized regenerated cellulose-based membranes. The purification efficiencies of rPD using Ni^{2+}-NTA commercial agarose gels and Ni^{2+}-IDA regenerated cellulose-based membranes at 4 °C were compared. Breton et al. [73] reported that a mutant of interleukin-6, known as δ22-Il-6 Cys 3, 4, could be fractionated in a multi-compartment electrolyser with isoelectric membranes, which allowed the collection of the more alkaline species for characterization. Nachman et al. [74] used a membrane-based receptor affinity chromatography (MRAC), which utilizes the molecular recognition between an immobilized receptor and its soluble protein ligand, to develop it for the purification of human interleukin-2 and related biomolecules. The multi-purpose affinity membrane used in this study consists of a soluble form of interleukin-2 receptor (IL-2R) chemically bonded to hollow-fiber membranes in an oriented fashion. The MRAC was found to be a viable, scalable and an extremely productive affinity purification method.

3.4. Size exclusion chromatography or gel permeation chromatography (GPC)

The principle of protein separation by SEC or gel permeation chromatography is like membrane filtration, based on the molecular size of the proteins. The size of protein molecules vary over a large range, but most have a molecular mass in the region of 20,000–200,000 Da. Suppose a protein is in its native state and has a perfect sphere, their diameter is roughly 3–8 nm packing in SEC, or GLC columns are porous with pores of a size similar to the required proteins. The largest proteins cannot penetrate the beads because the pores are too small, so they flow quickly around the outside of the beads and elute out first. The smallest proteins are able to penetrate the pores in the beads and thus get isolated from the flow of the mobile phase temporarily after a while, they elute out. The gel beads have a range of pore size, so that intermediate sized proteins can spend some time inside in the beads, but not as much as the smallest proteins. Consequently, proteins can be separated by their molecular size. Both SEC and GPC, are employed to do "group separation" of a sample of unknown composition. In this case, SEC alone is really a kind of coarse separation for proteins. On the other hand, SEC can be used together with other sizing techniques, such as ultracentrifugation, to check for aggregates.

SEC is also a refined purification method for the final desalting step in the down-stream process. The main advantage is that it may be employed for the purification of any kind of protein and the mobile phase employed in SEC is a dilute buffer, the separation proceeds under isocratic elution conditions without molecular interactions between the protein and the SEC stationary phase, ensuring that the purified protein retains its bioactivity.

The resolution of SEC is the poorest among all LC; it cannot distinguish a pair of proteins with a molecular mass difference of 500 Da. However, It can be employed to identify the presence of aggregates, and is a very powerful tool for investigating protein folding.

For the ideal SEC, there is not any interaction between the protein and the stationary phase, but, in practice, some of the selective interactions always exist to various extents between proteins and their stationary phases, resulting in not only the partial retention of proteins, but also a reduction in mass recovery or bioactivity of the rPRT. Fortunately, Ejima et al. [75] recently reported a method to partially solve this problem. They added an aqueous arginine solution (0.2–0.75 M) to the mobile phase and used both silica-based and polymer-based GPC columns for the purification of a mouse monoclonal antibody and the recombinant proteins, human activin, interleukin-6, basic fibroblast growth factor, and interferon-γ. They found that the existence of arginine in the mobile phase can decrease this selective interaction with proteins, resulting in increased mass recovery. There are two new kinds of protein separation by size, but they have a quite different separation principle from the traditional SEC, or GPC. They are hydrodynamic chromatography (HDC) [76] and slalom chromatography (SC) [77] which will discussed later in Section 4.1.

SEC is known as a tool for protein renaturation with simultaneous purification for over 10 y [78]. Rolland et al. [79] recently reported that the purification of different forms of recombinant Hepatitis B (HBc) virus core protein by either ultracentrifugation or SEC as the last purification step. The result shows that the resolution of SEC is even poorer than that of ultracentrifugation, but the recovery is greater. Amari and Mazsaroff [80] used a two-dimensional SEC–RPLC to analyze the recombinant human interleukin-11 fusion protein (rhIL-11 FP) expressed from *E. coli*. The other application of SEC is to separate and identify monomers from dimers, and/or polymers. Chang et al. [81] investigated a high-performance SEC method for the determination of the potency of recombinant bovine somatotropin (rbST) monomer and the estimation of dimer and soluble aggre-

gates in bulk drug substances. Strömqvist et al. [82] identified the glycosylation of the recombinant extracellular superoxide dismutase.

3.5. Ion exchange chromatography

The separation principle of the rPRT by IEC is that all rPRT have charges on them as a result of amino acid side chains, which contain aspartate, glutamate, histidine, lysine and argine. The retention of rPRT on an IEC column depends on the strength of electrostatic interactions between the stationary phase and the rPRT. This is the reason why IEC can be used as a universal-type of LC for protein separation. The net charge on a given rPRT depends on its exact composition, and on pH. Consequently, at a given pH different rPRT will have various net charges, and a shift in pH will change this value for each rPRT, although for all of them it will become more negative at a higher pH, and more positive at a lower pH. However, Kopaciewicz et al. [66] reported that the "net charge" model is inadequate. Deviations may result from charge asymmetry, since it appears that only a fraction of the protein surface interacts with the stationary phase. They presented and tested the SDT-R for protein separation by IEC.

This fact provides a very broad and flexible method to optimise the IEC conditions for the resolution of rPRT with a very similar chemical and physiochemical characteristics. A column of anion exchange chromatography (AEX) has a positive charge that attracts negatively charged rPRT, and under neutral pH conditions, most native rPRT is negatively charged. For the minority of positively charged (high isoelectronic point) rPRT, cation exchange chromatography (CAX) is used. The IEC column can be chemically modified with various ligands to have four kinds of LC, strong cation exchange chromatography (SCX), weak cation chromatography (WCX), strong anion chromatography (SAX) and weak anion chromatography (WAX), which provide many options for the optimization of the rPRT purification. The advantage of IEC in purifying proteins is that most of the proteins remain bioactive, indicating its usefulness for rPRT protein purification. However, in some cases, if the bioactive sites of an rPRT are on, or very close to its salt-bridges and the electrostatic interaction is very strong, the salt-bridge may break due to the changes in pH and/or salt concentration, the rPRT may lose partial, or even its whole bioactivity. IEC requires the salt concentration in the sample solution to be very low and the pH must be adjusted so the target protein strongly retards on the LC column, otherwise, a buffer exchange is required.

IEC is a very popular and effective method for rPRT purification and also as a tool for protein refolding with simultaneous purification as outlined by Lu et al. [83]. Christodouloua et al. [84] reported on the thermostable chitinase (Chi40) from various expression vector systems in *E. coli* as secreted forms. The Chi40 was initially purified by IMAFC and followed by IEC at the large scale. The protein from *E. coli* was highly active but not homogeneous, since a considerable proportion of the cytosolic form of Chi40 protein was incorrectly folded. But the Chi40 protein secreted into the culture medium was purified by HIC and IEC and high amounts of correctly folded and active Chi40 protein could be recovered in a short time. Baumgartner et al. [85] presented a scaleable process for the production and purification of gram quantities of recombinant lectin Phaseolus vulgaris phytohemagglutin in E-form (PHA-E), derived from yeast. The PHA-E was secreted at approximately 100 mg/L at the 2 and 200 L scales and was purified to 95% homogeneity in a single step using CAX. McDonald et al. [86] purified the fibroblast growth factor-saporin (rFGF-2-SAP) mitotoxin in a large-scale expression from *E. coli*, the first step was EBC (streamline SP) with subsequent SP-sepharose. The loading amount and recovery were 0.376 g and 65% for the first step, 0.115 g and 76.5% for the next step, respectively. After that, heparin-affinity, and SEC were used for the last two purifications. The final product had a purity of 96.6% and a recovery of 23.1%. Sticha et al. [87] investigated the purification of recombinant hamster polymorphic arylamine *N*-acetyltransferase as a dihydrofolate reductase fusion protein. DEAE column was employed to partially purify the fusion protein and then the cleaved thrombin was purified with DEAE again, resulting in not only the separation of rNAT2-70D from FLAG-L54F DHFR, but also the purification of rNAT2-70D to near homogeneity. The IEC method of purifying rNAT2-70D is inexpensive and simple and yields more than 8 mg of pure enzyme from 1 L of cell culture. Yun et al. [88] presented an effective separation of PEGylated recombinant human granulocyte colony-stimulating factor (rhG-CSF) with novel "PEG-pellet" PEGylation mode and IEC. CAX was initially employed to separate PEGylated rhG-CSF from intact rhG-CSF, followed by ACX. It should be pointed out that of the foregoing three purification technologies, the first two [86,87] employed a combination with the same kind of IEC, i.e., CAX–CAX and DEAE–DEAE combination, and the last one [88] was a CAX–ACX combination. In most circumstances, IEC should be combined with other kinds of LC and/or non-LC methods, IEC may be the first, middle, or last step. This is really an unusual choice, continuously employing two CAX steps, but they obtained good results. Feng et al. [89] purified the recombinant human Apolipoprotein AI (rhApoAI) derived from a *Pichia pastoris* expression system at a concentration of 160 mg/L in a 14 L fermenter by using a coarse separation of cold acetone precipitation followed by Q-Sepharose Fast Flow IEC with 60% recovery. The purified rhApoAI had a specific binding activity with liver cells SMC7721 and native human ApoAI could inhibit binding. Dassa et al. [90] reported that by combining IEC and IMAFC in the presence of an aminoxide detergent, the ADP/ATP carrier-iso 1 cytochrome C fusion protein (Anc2-Cyc1(His6)p) derived from yeast was purified at a large scale. Maurice et al. [91] presented a scheme for the purification of protein A from Achromogenic Atypical Aeromonas salmonicida: the induced protein was isolated from an inclusion body by a simple solubilization–renaturation procedure and purified by IEC on Q-Sepharose to a purity of over 95% pure monomeric protein. It was reported at the time, that this represents the first large-scale production of biologically active recombinant A-protein. Tan et al. [92] purified the recombinant ethionine-α-deamino-γ-mercaptomethane-lyase as a novel anticancer therapy including a heat step, two steps of DEAE Sepharose FF ion exchange, and ActiClean Etox endotoxin-AFC at a large scale. The multi-gram

level per batch has a high yield (>60%), high purity (>98%), high stability, and low endotoxin. Wang et al. [93] developed a unique approach for high-level expression and production of a recombinant cobra neurotoxin containing four pairs of disulfide bonds in *E. coli*. The fusion protein was released into the solution at a low ionic strength under an osmotic shock treatment, and purified by IEC and then GPC. More importantly, this protocol can be easily used for the production of the toxin at a larger scale and at a low cost. The approach outlined in this report is suitable for the production of other recombinant proteins also.

3.6. Hydrophobic interaction chromatography

Two kinds of molecular interaction forces, electrostatic interactions corresponding to IEC and hydrophobic interactions corresponding to both RPLC and HIC can be employed for the purification of various proteins, being common to several types of LC. Electrostatic interaction forces are common to four types of LC, SCX, SAX, WCX and WAX, while hydrophobic interaction forces are common to only two types of LC, RPLC and HIC. RPLC is usually not suitable for the purification of rPDs as it involves denaturing most of the native protein, therefore HIC is the most suitable method for the purification of rPDs and rPRTs with various hydrophobicities. Andrews [94] demonstrated the point that modern optimized purification procedures for recombinant proteins typically consist of two LC separation stages, IEC followed by HIC. The advantage of HIC is that the chromatographic conditions are very close to the physiological conditions of the human body, such as a neutral pH, an aqueous salt solution, and room temperature, all of which are favourable to the maintenance of the proteins bioactivity. However, the application of HIC has not been as broad as that of IEC yet, because of the packing material problem. The criticism by Oscarsson et al. [95] is that "classical commercial hydrophobic absorbents are inadequate for down-stream processing because of their high hydrophobicity". Jennissen [96] essentially agree with this criticism in that "the major problem encountered on such hydrophobic gels is that proteins can be very effectively adsorbed but elution in the native state is often impossible". The reported the octyl ligands are not very useful [95] but phenyl and butyl ligands have many applications. The gels in this case are polysaccharide-based HIC. This instance has been continuing until to up date, to appear an improvement. Tsumoto et al. [97] reported on a method, which partially overcomes this shortfall. They added arginine to the mobile phase of HIC to improve protein elution. This modification was examined through the interaction between recombinant human interleukin-6 (IL-6), activin-A from phenyl-sepharose. These results show that arginine facilitated the elution of IL and activin-A, resulting in a greatly improved recovery of the native protein by HIC. Arginine acts by weakening hydrophobic interactions between IL-6 and activn-A to the phenyl-sepharose. It should be mentioned here that silica-based HIC packing does not experience a problem with protein elution at all (see the Section 4 latter).

Several retention mechanisms for proteins in HIC have been reported and recently reviewed by Lienqueo et al. [98]. The SDT-R for HIC considers eight molecular interactions including three conformational changes in proteins [50]. From the SDT-R here, water is the displacer and salt also contributes to protein retention but only as a diluting agent. From theoretical calculations, ammonium sulphate is the best salt for protein retention and it is employed accompanying potassium dihydrogen phosphate to compose the mobile phase of HIC. The protein retention process can be explained thus, as a hydrated protein molecule is pushed forward by hydrophobic interaction forces from the aqueous salt solution of the mobile phase and when it arrives at the hydrated stationary phase it interacts with the stationary phase via the hydrophobic amino acid residues of protein molecules, while the hydrophilic amino acid residues in the protein molecule face the mobile phase and simultaneously release water at the contact surface region. Meanwhile, the molecular conformation of the protein changes after adsorption and dehydration. The decrease in water (displacer) concentration during gradient elution, results in decreases in the hydrophobic interactions with the mobile phase, the protein molecule disassociates from the stationary phase with simultaneous re-hydration of both the stationary phase and the protein thereby completing the cycle of protein adsorption–desorption. Proteins elute based on their different hydrophobicities. The more the hydrophobic the protein, the longer the retention is.

Geng et al. [29] reported that HIC could be a tool for protein purification with simultaneous renaturation. As long as the rPRT in the denaturing solution is directly injected into the HIC silica-based column, the purity obtained for the refolded and purified target protein is usually greater than 85%, and can be even up to 95%. In the latter instance, the collected fraction can be directly desalted. Geng et al. [46] presented a new technology for the purification with simultaneous renaturation of rhIFN-γ at the industrial scale using only one step of HIC. All of those rPDs sourced from *E. coli.* If an rPRT is derived from a secretion-type expression system, HIC can still be employed as a one step LC for its purification, but in most cases, it is combined with other LC and/or none-LC methods. Goyal et al. [99] reported that hydrophobic interaction expanded bed adsorption chromatography (HI-EBAC) was an easy method for the purification of recombinant streptokinase (rSK) from an inclusion body. After diluting the extract in a 8.0 M urea solution, the sample solution was directly loaded on to an EBAC column containing streamline phenyl. By passing the solution four times through the column, total protein loading, 405 mg, 82.7% rSK activity could be recovered with a 3-fold increase in the specific activity of rSK. Kepka et al. [100] presented a method to purify the recombinant cutinase produced from *E coli* using a combination of an aqueous two-phase extraction and HIC. The interfacing of a poly(ethylene glycol) (PEG)–phosphate aqueous two-phase system was employed followed by HIC. The yield and purity were investigated with respect to ligand hydrophobicity, dilution of loaded top phase and elution conditions. The tagged ZZ-cutinase-$(WP)_4$ was obtained in a PEG-free phase and purified to >95% purity with a total yield of 83% during the two-step recovery process. Mendonça et al. [101] purified the recombinant human thyrotropin (rhTSH) derived from a CHO secreting cell. The rhTSH is a glycoprotein hormone produced by a secreting CHO cell line in a condi-

tioned medium, which was diafiltered and then adjusted to a sodium chloride concentration of 1 M. This was finally loaded onto a HIC column packed with phenyl-sepharose CL 4B. The product obtained had a purity of 90–99% with a 37% recovery. The purified factor increased its concentration 28-fold by adopting this two-step strategy. Ehlermann et al. [102] developed a new strategy for isolating active S100A1 protein using the precipitation–HIC combination. After EDTA extraction of Ca^{2+}-binding protein, produced by either myocardium or recombinant bacteria. S100A1 remains in supernatant fractionated by ammonium sulfate but was strongly adsorbed by the stationary phase of HIC, octyl-sepharose. The usual Ca^{2+} washing buffer can eliminate any unspecific hydrophobic binding of proteins mediated by ammonium sulfate, while the target protein (Ca^{2+} bonding protein) was eluted by elution buffer. This yielded 1.4–2.0 mg/100 g of wet tissue and 0.7–1.0 mg/100 mL of bacterial culture. Lienqueo et al. [103] recently reviewed the current insights into protein behaviour in HIC including the retention mechanism for molecular interactions in HIC.

3.7. Affinity chromatography

The separation principle of protein by AFC is based on the interactions between a protein and the AFC stationary phase, which has a high selectivity or specificity and it is only one of the non-universal types of LC. The selective interaction may occur between a protein and low molecular mass substance and between two or several biopolymers. Although AFC is a non-universal type of LC, it is a very powerful method for protein purification, because of its high selectivity. This is particularly true when a target protein is a minor component of a complex mixture and the isolation involves an rPRT in an extract. Many kinds of AFC are employed to perform the purification of rPRTs, but we are only concerned with those used at the preparative scale in this paper. Based on the various ligands, AFC can be divided into many types, such as, inhibitor, lectin, nucleic acid, hormone, vitamin, sugar, immobilized metal ion affinity chromatography (IMAC) and immunoaffinity chromatography [104–106], the last two are the most popular ones employed. Azarkan et al. [107] recently reviewed the development and application of AFC as a tool for proteomics investigations, and also listed a lot of selective and specific interactions between proteins and the stationary phase of AFC. IMAC is often employed for the purification of rPRTs containing sulfide groups, in particular histidine (His). Metal ions act as a chelater(metal-) when bound to a chromatographic media to fix the metal ion to a solid support, enabling the separation to take place. Because histidine is relatively rare, representing only 2.2% of the amino acids, across all proteins with many containing none, or none accessible on their surface. This provides a built-in selectivity for certain native proteins. The use of rPRT to introduce a His tag further exploits the selectivity of His. AFC can be also employed to do the purification with simultaneous renaturation of both rPDs and rPRTs [108]. Compared to SEC, IEC, and HIC, even though the IMAC has a very high selectivity, it still needs to be combined with other LC and/or non-LC separation methods. Liu et al. [109] reported the large-scale preparation and purification of recombinant human parathyroid hormone (rhPTH) 1–84 from *E. coli*. The soluble fusion protein His_6-thioredoxin-hPTH (1–84) was purified in combination with IMAC–IEC–SEC. Finally, over 300 mg/L of intact hPTH (1–84) with a high purity of up to 99% was obtained. The purified rhPTH (1–84) was shown to have full bioactivity. Schmidt et al. [110] purified the baculovirus-mediated large-scale expression and purification of a polyhistidine-tagged rubella virus capsid protein using IMAC. The final yield was 5 mg of purified protein per liter of cell culture. Compared to IMAC, immunoaffinity chromatography has even a higher selectivity; also it can use a protein, which will capture/bind the target specifically. Antibodies and their corresponding antigens are one example. The traditional immunoadsorbent based on polyclonal antibody preparations have largely been replaced by adsorbent based on monoclonal antibodies. There are several advantages of using monoclonal antibodies as adsorbents. For minor protein components, single-step purification factors of several thousand folds are possible. The disadvantages are high cost and the high risk of fouling and irreversible chemical denaturation and notable proteolytic degradation. To prevent this, a coarse separation, or preliminary purification step is necessary. Protein A-based AFC is one of the most commonly employed forms of AFC. A matrix bonding to protein A is a very powerful tool for the purification of antibody, or in a contrary manner, it binds to antibody for purifying protein A, both have the same interaction forces. The most important application of protein A-AFC is to capture antibody in a highly specific manner, even from dilute stream feed. However, protein A is antibody subtype specific and not all antibodies can be isolated using this ligand. Since protein A binds to the fc-region of the antibodies, molecules lacking this portion, e.g., single chain antibodies or fab-fragments, are also excluded. Elution of the target molecule from the protein A column generally requires drastic conditions, a step gradient is required at a low pH, rapid neutralisation of the eluting fractions, indicating that it is suitable for small (mL-range) columns/fractions, but becomes increasingly difficult as the column/fraction volume increases. As a ligand it has a problem that sometimes, it may leak from the stationary phase into the target rPD and also, Protein A is expensive, it is nevertheless widely used and it is featured in almost all commercial scale processess for clinically approved monoclonal antibodies. To reduce cost and overcome the disadvantages of protein A, Schubert and Freitag [10] recently developed a cheaper column, which could be used instead of the expensive protein A column. He did a comparison of ceramic hydroxy- and fluoroapatite versus protein A/G-based resins for the isolation of a recombinant human antibody from a CHO cell culture supernatant. Apatite stationary phases including a novel ceramic fluoroapatite material were employed to purify the recombinant protein A. The result indicates that the yields can approach the maximum (of ca. 90%). No traces of contaminants were observed in the analysis gel. It was reported that it is the first time that yields of 90% with such high purities have been obtained as the result of a single chromatographic step. Thömmes et al. [111] investigated the isolation of monoclonal antibodies from cells containing hybridoma broth using a protein A coated adsorbent (Streamline rProteinA) in expanded

beds. A clarified and highly concentrated (up to 50-fold) eluate of high purity was obtained. A scale up of the MAb purification is demonstrated from lab scale (250 mg MAb per purification cycle) to a small pilot scale (2 g MAb per cycle). Low product concentrations in the broth in combination with the high capacity of the adsorbent resulted in long sample application cycles (10–11 h). Yasuda et al. [112] employed an efficient and rapid purification procedure for recombinant human α-Gal A which is responsible for the metabolism of neutral glycosphingolipids by AFC with Concanavalin A (Con A)–sepharose and an immobilized thio-α-galactoside (thio-Gal) agarose column. This procedure is especially useful for the purification of mutant forms of α-Gal A, which are not stable under conventional purification techniques. The recovery and purity were 62% and 69%, respectively.

3.8. Reversed-phase liquid chromatography

Due to the strong hydrophobility of the stationary phase of RPLC (STRPLC), native proteins often denature after purification by RPLC. The disadvantages of RPLC include; the odour and poisonous nature of the organic solvents and the necessity for special buildings due to their explosive nature, also they are flammable, and finally recycling the waste solvent is problematic. These considerations, limit the more widespread application of RPLC for the purification of rPRT. However, some rPRTs are able to spontaneously refold to their native state as organic solvent is removed from the collected fraction. The principle of protein separation by RPLC is the same as that for HIC and a correlation between RPLC and HIC was reported by Chang et al. [113]. Differences with HIC are that an ion-pairing agent, such as TFA, or potassium dihydrogen phosphate must be added into the mobile phase of RPLC to allow proteins elute more easily. The action of TFA was found to have three functions, adjusting the pH of the mobile phase to 1.5–2, partially denaturing the protein, and finally as a secondary displacer [114]. RPLC was mainly employed at the analytical scale for proteins and peptides. In terms of resolution, acetonitrile is the best organic solvent for protein separation. Compared to other kinds of LC, the resolution of RPLC is the best; some proteins are still purified by RPLC, even at a large scale.

Kroeff et al. [115] reported a process for the purification of the biosynthetic human insulin (BHI) at the kilogram scale by a silica-based RPLC. The RPLC procedure was successfully integrated into the multimodal chromatographic production process used to purify large amounts of BHI. The insulin product obtained has a high chemical purity and retains full biological activity. To overcome the intrinsic disadvantages of RPLC, Fahrner et al. [116] employed a non-flammable solvent for preparing the rhinsulin-like growth factor-I. They used a gradient elution with hexylene glycol, a non-flammable solvent to replace acetonitrile. The separation produced an equivalent yield, purity and throughput to RPLC with acetonitrile as a solvent. Mills et al. [117] presented a method of one-step purification of a recombinant protein from a whole cell extract by RPLC. The recombinant protein, TM 1–99 (113 amino acid residues, 12,837 Da) derived from an *E. coli* cell lysate in the cell contents was extracted with a 0.1% aqueous TFA solution and directly loaded onto an RPLC column and eluted using a shallow gradient elution. They loaded 23 and 48 mg of lyophilized crude cell extract on to a RPLC column and produced 2.4 and 4.2 mg of purified product respectively with a >94% purity. Compared to AFC, the results obtained show the excellent potential for one-step RPLC in the purification of rPRTs from cell lysates, where high yields of purified product and a greater purity are achieved. Olson et al. [118] reported a preparative isolation of the recombinant human insulin-like growth factor 1 by RPLC. A process scale LC column packed with a larger-size particle to reduce back-pressure and cost was reported. Since TFA counter-ion binds tightly to proteins and is difficult to subsequently dissociate, a combination of acetic acid and NaCl was substituted and a shallow gradient elution using premixed mobile phase buffers at the same linear velocity was found to give an equivalent separation at low load levels and minimized solvent degassing. By optimizing the pH, ionic strength and temperature, a high-capacity preparative separation of rhIGF-1 from its related fermentation variants, was scaled up by 1305-fold and this resulted in superimposable chromatograms, with a 96% recovery and with a >99% purity. Jin et al. [119] employed RPLC to purify hybrid antibacterial peptide CA–MA [cecropinA(1–8)–magainin2(1–12)] derived from P. pastoris SMD1168. The recombinant CA–MA was purified by RPLC and 22 mg of pure active CA–MA was obtained from a 1L fermentation culture. Wilkinson et al. [120] purified the recombinant salmon insulin-like growth factor-II derived from *E. coli* using a combination of immobilized-Ni-AFC and RPLC. It should be pointed out here that from the foregoing examples, although some of them reported one step of RPLC for the rPRT purification, the purity of ≥95% was only obtained once by Olson et al. [118]. Therefore, although RPLC has the highest resolution of all LC, it still needs to be combined with other kinds of LC.

3.9. Optimization of purification technology

The optimization of a purification and/or production technology for an rPRT is based on both scientific and economic considerations. A single step with a low cost is the best option, but it rarely exists. The best combination of several LC steps depends on many factors: (1) the selectivity and cost of the packing material of the chosen LC. A strong retention of the target protein has strong enough retention, but the existing contaminations cannot, or very weak retention on the selected LC column. A monoclonal antibody-AFC (mAc-AFC) column should be the first choice, but is only valid for some very special rPRTs and has the problem of high cost. Even though other kinds of AFC column, IMAC, polyclonal-AFC column, which can adsorb more kinds of proteins can also be selected, they still have the same problem with the mAc-AFC column. (2) The combination of a series of LC methods, compared to AFC, has a low cost and one column can be used many times for the separation of various kinds of target protein. The problem is its hard to optimize as mentioned above; (3) column size. For protein separation, apart from SEC, or GPC, the resolution of protein by other kinds of

LC is almost independent of column length, indicating that the protein purification does not require a long column. A longer column is favourable only when loading larger sample sizes, but it has a long cycle running time for protein purification, and results in more loses in mass yield and bioactivity, and in a higher cost; (4) optimization of a combination of cheap non-LC separation methods and other steps including the content of the sample solution, the manner of the buffer exchange, and so on.

3.9.1. Buffer exchange and desalting

When the composition of the sample solution from the previous purification step is unsuitable for the subsequent purification, or when it has a compatibility problem, such as a high salt sample solution; a sample solution with a high content of desorption agent, or the pH of the sample solution is not suitable for the subsequent LC step, then the composition of the sample solution must be changed, this is known as buffer exchange. The first way to change the buffer composition of a sample solution is by dialysis, it usually takes 24 h per cycle, the second way is to perform separation by SEC or GPC. The latter is faster often taking less than one hour at the small scale, but this is dependent on the equipment and the volume. Ultrafiltration is the third way of changing the buffer composition of a sample solution. With this technique the sample solution is diluted with the desired buffer, concentrated to the original volume, diluted again and so on. Within a number of cycles the original buffer has in practice changed to the diluted buffer. The new buffer required depends on the circumstances; many different buffers have been used in other publications [121–123]. Many purification technologies for rPRTs contain a buffer exchange step, sometimes; two or three steps are required. It usually does not cost very much, but it takes a long time, especially for the large-scale operation by dialysis (24 h a cycle), which results in a decrease ~~in~~ the throughput/year of the rPRT.

Desalting is actually a special form of buffer exchange but is only used for the final product in which the salt concentration may be too high; the pH of a protein solution may not be suitable for long-term storage or distribution. This is usually the last separation step of the target protein from small solutes before packaging the rPRT into a bottle, for storage, or lyophilizing. If desalting is required for an rPD, the original solution components must be completely displaced by those of a more suitable solution.

3.9.2. Order of a series of liquid chromatography

LC as the core of a refined purification method accompanied with other non-LC separations forms an optimized purification technology, which dominates the whole down-stream production technology of rPRT, but it has not found a general role to follow. If the selection and arrangement in the order of a series of LC is carried out correctly, it should establish an optimized production technology for the rPRT concerned. Based on the characteristics of the target protein and the composition of the sample solution, it is possible to select the best initial step, using LC for a subsequent step for further purification. Theoretically, except for AFC, every type of protein can be separated by any kind of universal-type LC. The composition of a sample solution basically does not effect the separation by SEC, AFC, or even RPLC, but does affect IEC, only adsorbing protein from a dilute salt solution and HIC, requiring protein adsoption from a high concentration salt solution. The composition of the culture broth of a secreted rPRT is that of a dilute salt solution, indicating that except for HIC, all other kinds of LC will be totally suitable for the first LC step to adsorb the target protein. However, HIC is suitable as the first LC step when the target protein is re-dissolved in an ammonium sulphate solution.

Besides the examples described for each kind of LC in Sections 3.3–3.7, taking the statistical data from the most recently published (2006 to up right now) 20 examples for rPRT purification schemes shown in Table 1 indicate methods for arranging the order of a series of LC. It is apparent from Table 1 [124–143], that all 20 rPRTs are derived from four sources, yeast (11), insects (4) and CHO (4), and egg (1). Each purification scheme shown here, like those described in Sections 3.3–3.7 before, contains one, two, three, or even more LC steps in combination with other non-LC separation methods. The average separation step is as follows: coarse separation, 3.1; buffer exchange, 0.6; LC, 1.9; purity obtained from the data is $\geq$95%, indicating most of them have a complicated purification technology. The amount purified ranges from 0.15 to 542 mg, and an average of 86.4 mg for the available data shown here, which summarises the purification of 13 rPRTs. The top three throughputs are greater than 100 mg denoted by an underline they could not be taken into account, because they have a different calculation method. The first two [125,127] are expressed as the throughout per litter, the third one [133] is from a total throughout of a 15 L fermenter (about 9 L of culture broth). If the top three data points are excluded, the average throughout is only 19.4 mg. All of these data indicate that these purification technologies are still at the small scale. It would be expected that some of the purification technology may be further scaled up by up to 10-fold, resulting in throughputs of 200 mg, and 864 mg of rPRT, respectively. However, they are probably hard to scale up to an industrial level, such as $\geq$1 g.

Excluding non-LC separation steps, six of the rPRTs are purified using only a single-step LC, indicating the significant progress for rPRT purification using the LC method. This is the ideal instance irrespective of the subsequent purification step. However, most of them need a combination of several LC's. As long as the first LC step is fixed, subsequent LC selection is easier, because, usually an LC with a different interaction force is employed. From Table 1 IEC is employed in 11 instances while AFC is employed in 9 instances both are comparable. In the latter, five of them are IMAC which is cheaper than the other four, which are AFC columns bonded to proteins. In other words, the cheaper LC employed accounts for 80% of the total number of LC columns employed.

This can be explained by the fact that the salt concentration in the culture broth from the four expression systems used is relatively low, and therefore suitable for the adsorption of the rPRT by IEC and AFC. It is reasonable that IEC as one of the universal-types of LC and also a cheap form of LC is the most commonly employed. HIC is usually more suitable for protein adsorption from a solution with high salt concentration,

Table 1
Example of the Eukaryon Expression systems in 2006–2007

Recombinant protein[a]	Expression systems	Coarse separations (steps)[b]	BE (steps)	LC (steps)	Con (steps)	Total protein	Purify (%)	Ref.
hPSP94	Yeast	C-UM-BE	**1**	WAX	U	23.4 mg	96	[124]
hIFN-α2b	Yeast	R-C-D	–	SAX, SEC	U	298 mg	95	[125]
rhBAFF	Yeast	C-P-C-BE	**1**	WAX, SEC	PEG		95	[126]
rhcatalase	Yeast	C-P-C-R-D-C-D	**3**	SAX, AFC	–	102 mg	95	[127]
YlLip2	Yeast	C-UM-D	**1**	SAX	U	45 mg	–	[128]
Nictaba	Yeast	C-F	–	SAX, AFC	–			[129]
rhIFN-λ	Yeast	C-D-P-Di-F	**1**-	SCX, SEC	–	57%	98	[130]
rhACP	Yeast	C-P-D-C	**1**	Ni^{2+}-AFC	F	6.15 mg	–	[131]
rhIGF	Yeast	C-F-D	–	SCX, HIC	–	70.44 mg	95	[132]
rHSA/IFNα2b	Yeast	C-D	∓	Dye-AFC, HIC, SAX, SEC	–	542 mg	98	[133]
GlcAT-I	Yeast	C-H-C-C-D	–	WAX, WCX, HiTrap-AFC	–	1.6 mg	–	[134]
rhPTX3	CHO	Con-UM	∓	SAX, HIC, SEC	U	68%	95	[135]
rhEPO	CHO	C-Con	–	Ni^{2+}-AFC, SEC, WAX	U	–	98	[136]
rhEpo-Fc	CHO	C	–	ProteinA-AFC	–	96%	98	[137]
VPAC2	CHO	R-S-C	–	Chitin-AFC	–	6.5 mg	95	[138]
rLBD	Insect	R-S-C-D-C	**2**	IMAC, SAX, SEC	U	6.8 mg	97	[139]
rhCBG	Insect	S-C-H-C	**1**	Ni^{2+}-AFC, SAX	–	13 mg	95	[140]
L1/ECD	Insect	C-S-C	–	IMAC	–	–	–	[141]
hG	Insect	R-C	–	GIP–AFC		1.6 mg	–	[142]
hHA	Egg	C-R-C	**1**	SAX–SCX		6.4 mg	99	[143]

[a] hPSP94: human prostate secretory protein of 94 amino acids; rhIFN-2b: recombinant human interferon alpha2b; rhBAFF: B recombinant human lymphocyte stimulator; rhcatalase: recombinant human catalase; YlLip2: the extracellular lipase gene from Yarrowia lipolytica; Nictaba: Nicotiana tabacum lectin; rhIFN-λ: recombinant human interferon-lambda; rhACP: recombinant human acid phosphatase; rhIGF: recombinant human insulin-like growth factors; rHSA/IFNα-2b: recombinant human serum albumin-interferon-α2b; GlcAT-I: Galactose-1,3-glucuronosyltransferase I; rhPTX3: recombinant human long pentraxin; rhEpo: recombinant human erythropoietins; rhEpo-Fc: recombinant human erythropoietin-Fc; VPAC2: VPAC2 agonist; rLBD, recombinant ligand-binding domains; rhCBG: recombinant human cytosolic-glucosidase; rL1/ECD: recombinant L1 cell adhesion glycoprotein; hG: human glutaminase; hHA:human hemagglutinin.

[b] BE, buffer exchanged; C, centrifugation; Con, condensation; D, dialysis; F, filter; H, homogenized; MF, membrane filter; P, precipitated; R, resuspended; S,sonication; UM, ultrafiltration membrane.

and also for the purification with simultaneous renaturation of rPRT derived from *E. coli*, in some cases, a purity ≥95% with only one step is required [47,99]. It is actually the case that some proteins, such as human growth hormone bind nicely to HIC resins at low salt concentrations, but here it was employed the least one.

Although the statistical data from Table 1 represents a new development for rPRT purification technology from secreted-type proteins, it is still valid for the refined purification of a sample from *E. coli*, after renaturation and for coarse separation and proteins in general also.

4. Packings and column techniques

4.1. Packings

A lot of new kinds of packing material with numerous and specific characteristics are reported each year, but most of them are limited to the separation of small solutes. For biopolymers, many of them use AFC. Scientists and engineers are most interested in universal and cheap packing for purifying many kinds of proteins, i.e., one kind of LC column can be employed for the various requirements of protein purification. The resolution and selection of proteins mainly depends on the characteristics of the employed packing material. In most cases protein purification at the large scale is carried out using a soft, or a semi-rigid based-matrix, because they have a very good compatibility with proteins. Porous silica-based packing material, which is chemically modified with various groups, can also be used for protein purification, but, in most cases, it has been employed for small solute separation. Its high cost dictates that it is only employed in special circumstances for protein purification at the large scale. Garcia et al. [144] reported that very fast protein purification can be accomplished with a perfusion chromatographic column, which was developed, 15 y ago and it is still employed now. This packing material has an average pore size ranging from 30 to 100 nm and experiences less non-reversed adsorption. The highest linear velocity covers the range of 1000–5000 cm/h without a significant resolution loss. Kaufmann [145] investigated a new kind of packing material, tentacle support, which is chemically modified and has a linear and long polymer chain consisting of up to 50 monomers corresponding to a length of about 10 nm. This support has a pore size ranging from 100 to 500 nm and can be chemically bonded with various types of ligands, for example, IEC groups. The tentacle IEC packing material obtained can be used at a wide pH range. Compared to the usual packing material in which its ligands interact with only part of the surface protein molecules, the long tentacle ligands can almost enclose the whole surface of protein molecules and interact with more amino acid residue of the protein molecule, increasing the capacity for protein binding by 3–4-fold. Kanda et al. [146] reported that a mixed-functional stationary phase having small holes bonded to a hydrophobic group can adsorb hydrophobic solutes deep in the hole, but large protein molecules with

hydrophility cannot enter the small hydrophobic hole and interact with the hydrophilic surface, resulting in the separation of proteins from small hydrophobic solutes. Charoenrat et al. [147] reported that a multi-model ligand adsorbent has several kinds of groups, such as a thioether group, a carboxylic group, and an aromatic group, which can interact with more sites on protein molecules, increasing the adsorbed amount of recombinant β-glycosidase EBC.

Hahn et al. [148] experimentally compared the performance of 15 commercially available protein A media. Equilibrium and dynamic binding capacity for human IgG were determined and the capture of IgG from a crude feed-stock was investigated. They found that agarose-based media exhibited a higher binding capacity and a higher maximum equilibrium binding capacity and the dissociation constants derived from adsorption isotherms were smaller. The other media exhibited higher apparent rate constants, indicating a faster mass transfer. This study can be useful as a guide for the optimization of large-scale purification processes. Blank et al. [149] reported self-immobilizing recombinant antibody fragments as ligands for general immunoaffinity chromatography. It is based on fusion proteins of scFv fragments with several chitin-binding domains, which can be immobilized directly from a crude bacterial lysate onto inexpensive chitin beads for the purification of proteins without any gradient or detector. The result from the determination of parallel processing of 24 different samples on a milligram scale indicates that the method can be used for an anti-His tag antibody either alone or directly coupled to IMAC to obtain a very pure protein.

A kind of silica-based HIC packing material was reported to provide two functions of the purification and renaturation of proteins [49,150,151]. A chromatographic cake (1 cm in length, 5 cm in diameter) packed with silica-based HIC packing material with a small particle size (0.7 nm and pore diameter of 30 nm) was reported to successfully separate seven standard proteins [29,49]. Based on the SDT [152], the retention of solute only depends on the contact surface area between the stationary phase and the solute, as long as the contact surface area of the small particle packings in a short column, a so called S–S combination, is comparable to that of large particle packings packed into a longer column, a so called L–L combination, the resolution of both should be theoretically identical. Because inter and intra particle diffusion at different flow rates differ for both combinations, the resolution exhibits little difference. It was reported that the Kelin Fast Protein Purification Column (Shaanxi Xida Kelin Gene-Pharmacy Co., Xian, China) [29,30,47] could be used to separate five standard proteins. It can also be employed for a fast coarse separation [150,151]. The fact that both S–S and L–L combinations have a good resolution and renaturation of proteins (see next section) indicates that silica-based HIC packing material has no problem with irreversible adsorption. The L–L combination can also be used for coarse separation, such as a group separation of protein, fast screening a suitable column, and also for the manufacture of rPD by using disposable equipment and material [5].

Mixed-mode chromatography has been widely employed. Kennedy et al. [153] reported on a multimodal LC column for protein separation with a mix of AEX-HIC. Wei et al. [154] reported that HIC has the same mixed-mode and established a theoretical model for the evaluation of retention for the mixed-mode of protein. That means the two mechanisms contribute to protein retention in a competitive manner. Later, many research groups specially synthesized some packing materials with two or more groups to mimic the protein adsorption and/or separation of two types of LC or even more. Columns such as HIC–IEC, AFC–IEC and AFC–RPLC can be used at both analytical and preparative scales, but not for protein separation by two kinds of LC alone, respectively. The principle of this separation by mixed-mode AFC–IEC chromatographic separation can be explained using the example of a resin called "MEP HyperCel" made by Pall Life Sciences. The resin ligand containing 4-mercaptoethylpyridine (MEP) in which it contains a heterocyclic ring and a thioether linkage is known to have an affinity for antibodies. MEP HyperCel allows antibody binding at a neutral pH directly out of the feedstock and, unlike protein A, binds all species and isotypes, including IgM. Elution can be carried out at pH of 4 or higher. As the pH decreases, the pyridine ring picks up a positive charge, so the adsorbent nature changes from hydrophobic to one that has a positive charge (similar to an anion exchanger). Burton et al. [155] invented a hydrophobic interaction chromatographic resin with ionizable groups (HIC–IEC mixed-mode). Gao et al. [156] discussed the mechanism of protein salt-tolerant adsorption onto a commercial mixed-mode adsorbent Streamline Direct HST. Reif [157] used an IMAC-membrane mixed-mode column for the isolation of a recombinant fusion protein (EcoRV), which carried a polyhistidine sequence (HIS_6-tag) at the N-terminus. The advantages of the mixed-mode resin are that it can adsorb more rPRT with hydrophobic groups and ion group from an aqueous medium together, such as a fermentation broth, as well with an affinity group for selective adsorption from sample solutions. In other words, proteins can adsorb with the mixed-mode but elution conditions only by a required mechanism, resulting in selectively eluting protein. Freitag et al. [158] employed a controlled mixed-mode interaction chromatography for anion exchange and Cibacron Blue affinity on membrane adsorbers, recombinant human antithrombin III (rh-AT III) can be separated in a single run from the major protein impurities present in the fermenter supernatant, namely transferrin and BSA. Battersby et al. [159] employed an affinity-reversed-phase liquid chromatography assay to quantitate recombinant antibodies and antibody fragments in fermentation broth.

4.2. Column techniques–industrial scale

Guiochon [2,160] recently published two review papers named, "preparative liquid chromatography" and "Csaba Horvath and preparative chromatography", involving the theory and principal methods for the implementation of preparative liquid chromatography, and displacement chromatography for the extraction of rPRT. On the applied front, the availability of instruments for simulated moving bed separations at the scale needed for both preparative and expanded bed for the extraction of recombinant proteins from fermentation broths were reviewed. A survey of the literature dealing with prac-

tical applications and recent meetings shows that preparative chromatography is becoming a well-established separation and purification method in the pharmaceutical industry. Kroeff et al. [115] employed a silica-based RPLC packing material packed in an axial compression column to purify BHI at the kilogram scale and obtained an insulin product with a high chemical purity and full biological activity.

4.2.1. Short column and chromatographic cake

Purification of a protein by a short column, or chromatographic cake has some advantages, such as a resolution comparable to that of normal LC, but it can work at a high flow rate. This cake can be employed in some extreme conditions which are never used, such as for samples with a very high viscosity, or when precipitates form during sample injection, or as PFLC for the target proteins derived from *E. coli*. Therefore, the short column technique is developing very fast.

It is known that the resolution of small solute separation in LC depends on the column length and it is usually separated with the isocratic elution mode, but the resolution for biopolymer is almost independent of column length and it depends on the gradient elution mode.

Yamamoto et al. [161] and Kato et al. [162] recently reported that the resolution of a protein may be affected by many factors, column length, flow rate, gradient mode and temperature. These reports indicate that the comparison of the resolution of proteins purified from two column lengths should be performed under limited conditions. Anyhow, short column chromatography and/or chromatographic cake, have both been reported to have many advantages both at the analytical and preparative scales.

Vovk et al. [163] employed a caky monolith methacrylate disc column with CM groups (3 mm in length, 12 mm in diameters) to isolate the tomato pectin methylesterrase (PME) isoform and polygalacturonase (PG1). The result indicates that the following could be obtained from 6 kg of fresh tomato flesh, 28 mg of purified de-salted PME, 12.5 mg of purified and active PGI and finally 4 mg of PG2 fraction contaminated with salt-dependent PME isoform. Although the separated product is not a recombinant protein, it does provide some indication for the efficiency of purification of recombinant proteins sourced from a plant bioreactor. This size of the chromatographic cake can be employed at the preparative scale [31]. A chromatographic cake with length, 1 cm and diameter enlarged to 20 cm, five standard proteins were also successfully separated under a flow rate of mobile phase of 100 mL/min.

Although short column phenomena were examined using the SDT for proteins [64] by both Tennikov et al. [164] and Belenkii et al. [165], but only qualitatively. To allow further development of the short column technique it needs the support of a new theory for short column protein separation.

4.2.2. Column for protein folding

As was pointed out in Section 2.2 once a sample in 7 M GuHCl, or 8 M urea solution makes contact with an aqueous solution, some rPRT with strong hydrophobicities will precipitate and block the column employed. To solve this problem, several kinds of chromatographic columns and techniques were reported. Choi et al. [38] employed packed and expanded bed adsorption chromatography (EBA) as the solid-phase for the refolding of an inclusion body protein, Lipoprotein kringle "LK68" sourced from *E. coli*.

Compared to conventional solution refolding, the EBA process resulted in 4.3- and 1.7-fold higher yields. A novel application of SMB to protein folding was reported previously by Park et al. [43]. They used a four-zone simulated moving bed process based on SEC to overcome the disadvantages associated with inclusion body refolding in a batch dilution and chromatography. The refolded protein was obtained continuously with a high productivity, low consumption of the refolding buffer, and a high efficiency of the SEC medium. Computer simulations and several SMB experiments showed that the standing wave design and the proposed process could achieve a high level of protein recovery (96%), a high specific protein-folding yield (96%), and a low degree of protein aggregation. Continuous matrix-assisted refolding of proteins was also reported by Schlegl et al. [41]. They described a refolding reactor for continuous matrix-assisted refolding of proteins. The reactor was composed of an annular chromatography system and an ultrafiltration system to recycle aggregated proteins produced during the refolding reaction. The system was tested with bovine α-lactalbumin as model protein. Superdex 75 PrepGrade was used as a size-exclusion medium. The yield of 30% active monomer in the batch process was improved to 41% at a recycling rate of 65%. Assuming that the aggregates can be redissolved and recycled into the feed stream in a quantitative manner, a refolding yield close to 100% is possible.

Compared to normal chromatographic columns, a chromatographic cake has a much bigger diameter. If little precipitates form on the surface of the filter above the top of the cake, it only blocks a very small fraction of the total filter surface, not affecting the chromatographic run. Wu et al. [32] reported that periodically washing the cake with a strong solvent, acted not only in re-dissolving the precipitates, which formed, but also increased both the mass and bioactive recovery of the target protein. Geng et al. [46] reported on a new technology for the purification with simultaneous renaturation of rhIFN-γ from *E. coli* using a large size (1 cm in length and 30 cm in diameter packed with HIC packing material) chromatographic cake, in this instance, this cake was referred to as "the unit of simultaneous renaturation and purification of protein (USRPP)" An extract containing 2 g of rhIFN-γ in a 7.0 M GuHCl solution of 700 mL was directly pumped into the large cake for renaturation with simultaneous purification. A flow rate of 120 mL/min and a four hour one step gradient elution, produced a purity and specific bioactivity of rhIFN-γ approaching 95% and 8.7×10^7 IU^{-1} mg, respectively. A comparison to the data from the literature [166] is shown in Fig. 4. In terms of purity, mass and bioactive recovery, cost in time and materials, the chromatographic cake is much better than the traditional column.

4.2.3. Monolith column

Monolith column is also called continuous bed column and it can be made from synthetic organic or inorganic material and

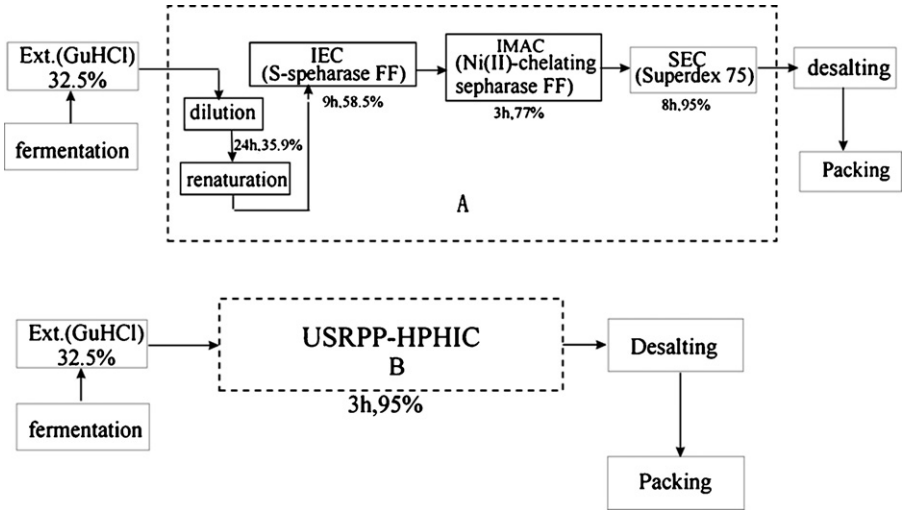

Fig. 4. Scheme for the comparison of the new and usual production technologies of the rhIFN-γ produced by *E. coli* with the unit of simultaneous renaturation and purification of proteins (USRPP). (A) Usual production technology; four steps, 44 h, purity, >95%; increases in bioactivity recovery, 1.6-folds; (B) new production technology; one step, 3 h; purity, >95%; increases in bioactivity recovery, 61-folds. Ext., extract; GuHCl, guanidine hydrochloride.

can work with a fast flowing mobile phase. The basis for fast separations with such media is a reduced mass transport resistance owing to the fact that pore diffusion is practically non-existent and film diffusion from the core of the mobile phase to the surface of the matrix is the only transport resistance Therefore, the period of time required for the separation is reduced by at least one order of magnitude when compared to conventional columns packed with bulk supports. Brne et al. [167] recently separated immunoglobin G (IgG) from immunoglobin M (IgM) using different ion-exchange methacrylate monoliths. The strong anion-exchange column had the highest dynamic binding capacity reaching more than 20 mg of IgM/mL. Branovic et al. [168] reported a semi-industrial monolithic column (500 mL CIM DEAE) for down-stream processing of clotting factor IX. This column can be employed for purification after solid-phase extraction with DEAE-sephadex. With a loading volume of 1830 mL containing a total of 27.6 g of protein and with a flow rate of 50 mL/min, it only takes 120 min, and the recovery for bioactivity and mass are 53.7% and 92.7%, respectively. The result indicates that it is better than the usual DEAE column and takes a short time to accomplish. Jungbauer [169] reported that polymethacrylate monoliths were scaled to 8000 mL.

4.2.4. Hydrodynamic chromatography (HDC) and slalom chromatography (SC)

Hydrodynamic chromatography (HDC) and slalom chromatography were reported as two new kinds of LC for the separation of proteins by molecular size [76,77]. HDC was developed in the 1970s by Marzio et al. [170] and employed for polymer separation. The separation principle for HDC is to create a parabolic flow profile occurring in the interstitial space between the particles packed into column. The separation occurs due to the exclusion of solutes from the low velocity regions near the surface of the particles. A large polymer molecule will be more excluded from the low velocity regions near the wall than a smaller molecule. As a result, the large polymer will experience a higher mean solvent velocity and will be transported through the system faster than the mobile phase. Thus the elution order is the same as in SEC. Venema et al. [171] established a theoretical model to describe the migration behaviour of polymers in HDC. Stegeman et al. [172] investigated the migration rate of macropolymers on porous particles and established a simple theoretical model to describe a molecular mass calibration graph, which includes both HDC and SEC. Stegeman et al. [173] found that with HDC of linear random coil polymers in columns packed with 1.5-μm non-porous particles, polymers with high molecular masses (10^4–10^7), the resolution appears to be almost independent of the elution velocity.

Two independent groups discovered SC in 1988 as a novel size-fractionation method for relatively large DNA molecules (>5 kbp) [174,175]. The mechanism of SC can be explained as being derived from hydrodynamic principles. The character of SC is that the separation occurs via a hydrodynamic phenomenon rather than the equilibrium based one. That is in SC larger DNA molecules are eluted much later than smaller ones, and the mechanism of SC can be explained as the result of the hydrodynamic principle. Hirabayashi et al. [176] used seven kinds of RPLC for improving the resolution of DNA separation using a mixed-mode SC-HIC. Smith et al. [177] employed AEX to separate linear, open circular, and super coiled plasmid topoisomers. Rittich et al. [178] reported the separation of lambda DNA and its fragment and plasmid pBR322 DNA by a polymer-based on the copolymer 2-hydroxyethyl methacrylate and ethylene dimethacrylate and proved it was based on SEC mechanism. Hirabayash et al. [179] investigated the effects of DNA topology, temperature and solvent viscosity on DNA retardation in SC Perrin et al. [180] employed a C_1 column to investigate the mobile phase viscosity dependence on DNA separation in SC. A connection between the SC and HDC process was predicted to link the two processes in a global separation mechanism based on a non-equilibrium principle.

Although two kinds of LC have the same mechanism, there are some differences; first, the elution order in HDC is the same as that in SEC, but that in SC is the opposite of SEC and HDC, second, in HDC there is a critical effect, but SC does not have a critical effect; third, the separation by HDC depends on the particle size of the column packing and the flow rate, and not on the pore size of the packing or the chemical character in SC, but in SC, the degree of DNA retardation is significantly affected by various hydrodynamic factors, such as the particle size of the packings, the flow-rate and the solvent temperature, whereas chemical factors, such as the chemical nature and pore size of packings, and solvent hydrophobicity, do not have a major effect.

5. Future

With the increase in the number of expression systems for rPRTs and the enlargement of the production scale, the complexity of the sample, which is required for purification also increases, introducing more difficulties for purification technology at both the small and large scales, especially for industrial production. LC as a core of purification technology could play an increasingly important role in not only guaranteeing product quality, but also in lowering the cost of the whole production process for rPRTs. The development of rPRT purification and production requires chromatographers to understand the general production process of rPRTs, including the down-stream and up-stream technologies, as well as how these are governed by pharmaceutical economics. This point has dominated rPRT purification and production in the past, does so now, and will do so into future.

The cost of LC is mainly derived from the consumption of packing media. AFC packing material is excellent for the purification of a specific protein, or a group of proteins, but its very high price limits its more widespread application. However, developing a more effective, cheaper, and universal-type of LC packing material has a great potential to lower the commercial price resulting in more economic benefits, because of the huge market.

Some recently developed new methods and techniques in the LC region, such as PFLC, monolith and short column chromatography have demonstrated many advantages for the purification of rPRTs both at the small and large scales, but they are just at the development stage, they should be paid a lot more attention to thoroughly investigate and explore their uses. It would be expected that short columns including monolith column may change protein purification in the future, because of this, a theory of short column chromatography for protein separation needs to be established.

LC of rPRT is actually an integrated systematic technology, including a combination of various kinds of purification methods from separation science, enlarging the production scale in the engineering field, using molecular biology for the up-stream expression system, and performing economic optimization through pharmaceutical economics, and thus, it should be established as an efficient, broad cooperation of many experts working in various scientific and technological fields.

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