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Review

Single-chain antibody fragments: Purification methodologies

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ABSTRACT

At present, single-chain variable fragments (scFv) of antibodies are considered one of the most important tools in human therapies. Wide applications of antibodies are being exploited in different medical, pharmaceutical and research areas. These molecules maintain the same binding functionality that full length antibodies but possess several advantageous features as quickness to penetrate the tissues, easy manipulation, fast elimination of their immunocomplex and the possibility of being produced in simple expression systems like bacteria and yeast. The increasing demand in antibody based methodologies is driving advances in the production and purification of genetically engineered antibodies and antibody fragments. While advances in expression systems allow the production of high titers of antibodies, there exist some limits imposed by the downstream methodologies which are not efficient enough to ensure their industrialization.

The main aim of this review is to highlight the principal characteristics of single-chain variable fragments of antibodies addressing advances and perspectives on scFv purification.

Contents

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1. Introduction

Antibodies, also known as immunoglobulins, are one of the most important components of the animal immune system. Some of them can act as receptors of specific antigens while others act as effectors of the humoral system. All immunoglobulins, irrespective of their specificity, have a common structure with four polypeptide chains (Fig. 1a), two identical heavy (H) chains and two identical light (L) chains, joined together by disulphide bonds [1]. All four polypeptide chains contain constant (C) and variable (V) regions, found at the carboxyl and amino terminal portions respectively. The V regions of both heavy and light chains together with their first constant regions combine to form two identical antigen binding sites (Fab). Effector functions of antibodies, such as placental transport or antigen-dependent cellular toxicity, are mediated by structural determinants within the crystallizable region (Fc) [1,2].

Since1890, when Kitasato, von Behring and Calmette were the first at describing antibody activity against diphtheria and tetanus toxins [3], numerous scientific lines of investigations were directed to the discovery of antibody structure and functionality. The subsequent important advance was the hybridoma technology, developed by Köhler and Milstein [4], which resulted in the ability to produce monoclonal antibodies (mAbs) and to increase the specificity of antibody-based tools.

According to a recent report from The Antibody Society (2013/2/28), nowadays there are 36 therapeutic monoclonal antibodies approved or in review in either The European Union or United State. The most common mAbs formats are chimeric, humanized, fully human and of murine origin. Other novel therapeutic formats, as for example single-chain variable fragments (scFv) and subtypes, are in development or are undergoing clinical testing [5,6].

In contrast to the well-established mAbs industrial bioprocessing [7], scFv production and purification still present some limits imposed by the technology, equipment and/or facilities that are available. In this context, numerous research lines are exploring multiple ways of production and purification processes. The addressed strategies include reducing raw materials costs and time consumption, as well as searching for platform methodologies which could ensure the purification of a wide range of scFv molecules and derivatives with a few purification steps.

2. Structure of scFv antibodies

Several scientific advances as recombinant DNA technology and phage-display techniques allowed the construction of engineered antibodies fragments [8], being single chain variable fragment (scFv) one of the smallest immunoglobulin with antigen-binding activity [9]. As it can be seen in Fig. 1b, this molecule consists of the variable regions of heavy and light chains joined together by a flexible peptide linker [10]. In alternative formats, both regions can also be joined non-covalently (Fv fragments) or by disulfide bonds (dsFv) [11]. The peptide linker length generally vary from 10 to 25 amino acids and typically include hydrophilic amino acids, being the peptadecapentide $(Gly_4Ser)_3$ the most common sequence [8]. The variable regions can be connected in either the VH-linker-VL or VL-linker-VH orientation (Fig. 1b and c), being the former the most common arrangement. It has been reported by several authors that these orientations may affect expression efficiency as well as binding capability [12].

Some of the drawbacks of scFv structure are those related to its low stability and its short pharmacokinetics. Some strategies to overcome these problems may include fusion with albumin, PEG [13,14] or Fc region (IgG CH2 and CH3 domains) [8]. Conjugations with nanocarriers [15,16] or multimer formation [17] comprise other alternatives to improve scFv biodistribution. Shorter linkers (0–10 amino acids) are known to favor multimer formation and are frequently used when dimmers or trimmers are the preferable format [18,19]. Another methodology to produce scFv dimmers comprises connecting two scFv molecules covalently by means of a third linker, thus generating the single-chain diabody $(scFv)_2$ which is represented by the format VH-linker-VL-linker-VH-linker-VL (see Fig. 1d) [20].

3. Applications of scFv antibodies

Single-chain variable fragments of antibodies comprise a wide range of molecules that present different functionalities. At present, there are thousands of examples of scFv and scFv-derivatives being in development or in advanced phases of clinical trials [5,6].

ScFv presents several advantageous properties, related mainly to its low molecular weight, that make it highly promising. For example, scFv is considered a powerful tool in immunotherapy since it penetrates more rapidly and evenly to tumors and other tissues in comparison with whole antibodies [21]. They can also be fused with other molecules like drugs or radionuclides in order to deliver these agents specifically toward target cells [22,23]. Other therapeutic applications are the treatments of inflammatory and tumor diseases [24,25], as well as drug addiction [26]. They are also used in scientific research projects, for example, Hust et al. [27] have expressed more than 400 scFv molecules directed to different human proteins in order to improve the proteome research as well as to develop biomedical diagnostics or treatments. ScFv are also currently applied in standard laboratory assays like immunoblot analysis, flow cytometry and immunohistochemistry [28]

4. ScFv preparation

The selection of the desired antibody fragment can be carried out using display libraries or affinity binding. Phage display, ribosome display and cell surface display in bacteria or yeast systems are the methodologies most commonly applied to search for the scFv of interest [29]. Upon completion of the selection process, it is important to decide what expression system will be the most convenient. Nowadays, there are several options such as bacterial and yeast systems; animal, plant and insect cells as well as cell-free machineries. Generally, scFv sequence and final product application have marked influence in the choice of expression host. Once the scFv production is standardized, it is crucial to select a downstream process which leads to a final product with high levels of purity and elevated yield recovery. Despite the high initial expression yield for some scFv proteins, purified yields, however, are still generally low [29]. For this reason, many efforts are still needed to develop high-throughput scFv bioprocess platforms for a recovery of high yields of scFv. Next sections will review some downstream processing strategies commonly used to purify antibody fragments

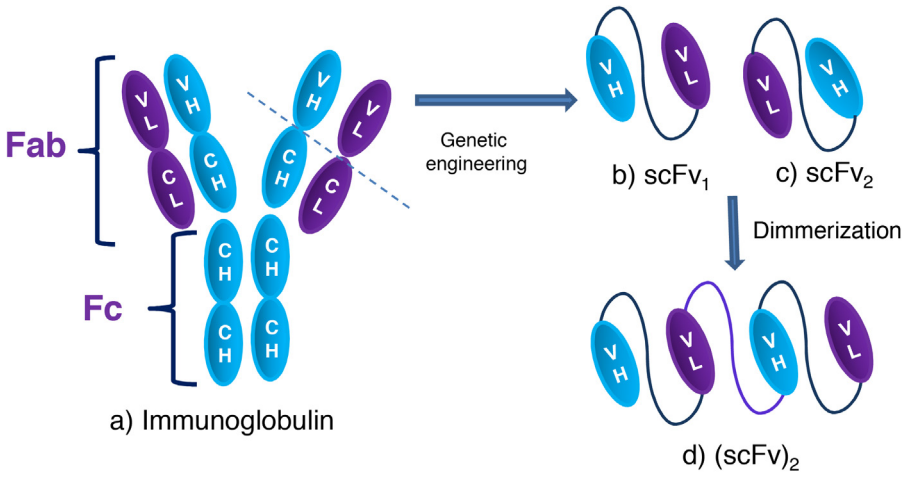

Fig. 1. (a) Schematic representation of the immunoglobulin structure. Each molecule contains different fragments (Fab and Fc), which include heavy (H) and light chains (L). Each heavy chain consists of one variable domain (VH) and three constant domains (CH) while each light chain presents a single variable domain (VH) and a constant region (CH). (b and c) ScFv molecules are composed by the linking of the variable domains of heavy and light chains by means a peptide-linker.(d) scFv dimmers can be generated by means of a third linker.

and will mention some advances in scFv purification methodologies.

5. Downstream processing of scFv

Downstream processing is a key point for the development of commercial antibodies [7]. Purification protocols should be designed to target the best possible performance combined with the minimum cost. Selection of an extraction technique depends on the equipment available, the scale of operation and the type of sample. As it is shown in Fig. 2, a traditional complete purification process involves an initial stage of primary recovery (clarification and treatment of samples to downstream purification), an intermediary purification step (in which purity of 40–80% is reached) and polishing (achievement of the final high purity and the optimal conditions for the protein stability). At present, the downstream processing of mAbs is standardized and mature, with good estimation of production and purification costs and yields [7]. However, scFv-related bioprocesses are being developed and generic protocols to estimate antibody fragments purification performances as well as the final product cost are still lacking [30]. Nevertheless, different unit operations, which will be mentioned in next sections, are being tested in each downstream processing stage in order to find the best cost/benefit relationship.

6. ScFv primary recovery

The clarification stage may be a significant bottleneck in both clinical and commercial manufacturing due to the high cell densities achievable in cell culture processes. Many process impurities are negatively charged at the extraction pH and are able to form colloids during the cell culture and harvest processes. Alternatively, residual impurities may also precipitate and increase turbidity during processing and even interfere with the performance of the capturing chromatographic step [31].

Generally, the first step used to harvest cell culture is centrifugation. Most large-scale applications use disk stack centrifuges (DSCs) to remove cells and cell debris [32]. These machines are preferable since they are scalable, perform continuous operation, and exhibit capacity to handle a wide variety of feedstock [33]. The combination of centrifugation-depth filtration systems is another methodology widely applied to harvest large-scale cell culture or fermentation processes [34]. According to depth filtration methodology, one or more depth filters can be used sequentially to optimize the filtration. In each successive filtration step, smaller particles are progressively removed by means of mechanical sieving and adsorption. The use of several steps during the primary recovery, however, could lead to subsequent lost of material of interest. In this context, alternative strategies to reduce or eliminate depth filtration have been considered, for example, the inclusion of flocculating agents in the cell culture fluid [34].

Microfiltration is another preferable technique to manage high-density fermentations, such us with yeast cell. There are several filter formats, e.g. pleated-sheet microfilter, tubular microfilter, and hollow fiber ultrafiltration (UF). Large-scale studies demonstrated that tangential flow filtration (TFF) is a robust unit operation to harvest of mammalian cell culture while cross-flow filtration (CFF) is commonly used to harvest of yeast cell product [33].

7. ScFv capture/purification

The first capture or primary purification of scFv seems to be the most controversial stage when defining a generic scFv downstream process. In contrast with the purification protocols of mAbs, which consist of an almost unchanged Protein A-based chromatography [35], the first capture of scFv does not comprises an unique unit operation applicable to a wide range of scFv proteins [30]. Affinity-based chromatography is the most common methodology used as a first extraction step of antibodies fragments. However, alternative strategies such as non-affinity chromatographies, precipitation, liquid–liquid extraction and cryogels, have also been satisfactorily used by several authors.

Next sections will address examples of different scFv capturing methodologies and the advantages and disadvantages of each one will be discussed.

7.1. Affinity-based chromatography

Affinity chromatography relies on the specific interaction between the antibody molecule and a complementary ligand. The antibody of interest selectively binds to the carrier after passing a solution containing it through the chromatographic column under favorable conditions [36]. This methodology reduces non-specific interactions, increases operational yields and facilitates the elimination of undesirable contaminants, even from diluted extracts. Affinity-based chromatographic procedures represent the

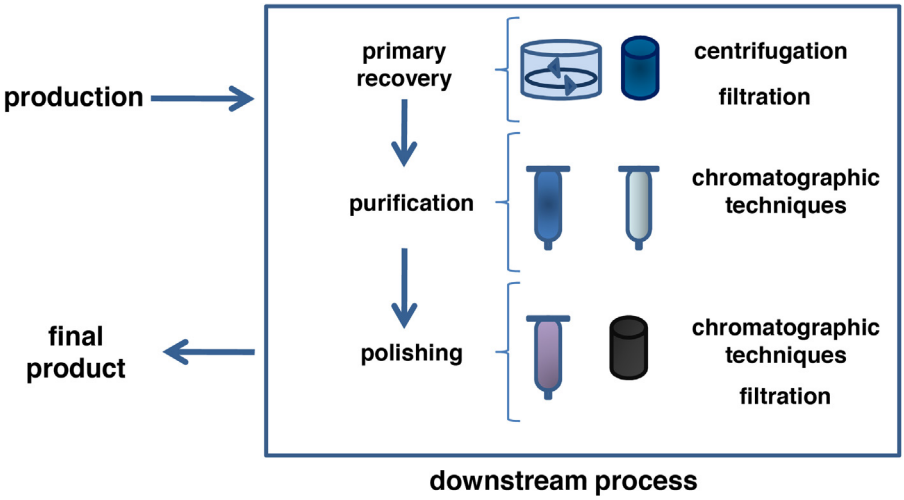

Fig. 2. Flow chart depicting the basic steps of a conventional downstream process.

methodology most frequently used for a first capture of the targeted antibody, leading to high product purity. Several molecules, with different structure and function, are commercially available as potential ligands.

7.1.1. Natural binding ligands

Naturally IgG-binding proteins comprise a group of molecules expressed on the cell surface of several species of bacteria which possess high binding affinity for mammalian immunoglobulins [37]. Peptostreptococcal Protein L (PpL) is widely applied to purify scFv fragments due to its high affinity for the Fab portion of antibodies, especially κ-light chains [38]. PpL can also be used as probe reagent to detect antigen–scFv complexes in immunoassays since it interacts with κ-light chains practically without affect the antigen binding property of scFv [37,38].

A drawback respect to Protein L-based resin is that not all mammalian immunoglobulins present a high PpL affinity [39]. To overcome these limits, leading groups have modified genetically antibody fragments sequences in order to improve its interaction with PpL, thus providing a potential method universally applicable to purify and to detect scFv antibodies [30].

7.1.2. Tag ligands

Tag ligands represent a group of molecules that share the property of binding with high affinity both natural and synthetic protein stretch. The affinity stretch or tags can be short polypeptide sequences or whole proteins co-expressed as fusion partners with the target proteins [40].

At present, immobilized metal affinity chromatography (IMAC), which employs chelating metal ions as ligand, is one of the most extended methodology used to purify recombinant antibodies [41,42]. Several vector systems, which express proteins containing a stretch of 5–6 histidines in the final product, are commercially available [40]. Binding takes place around neutral pH and elution can be carried out by either reducing pH or using competitors (e.g. imidazole) [43]. A distinctive advantage of IMAC over other affinity techniques is its applicability under denaturing conditions. This is often necessary when recombinant proteins are over-expressed in *Escherichia coli* in the form of inclusion bodies [44]. Additionally, IMAC offers a series of important features for large-scale purification such as ligand stability, low cost, high protein loading, mild elution conditions and easy regeneration [44]. Nevertheless, in certain cases, separation performances of IMAC have resulted to be lower than those obtained from PpL-based chromatography. For example, in a work developed by Das and colleagues [45], which compares IMAC with PpL-based purification, IMAC was found to need of a complementary chromatographic step to reach a final purity comparable to that of PpL-based separation. Other disadvantages associated with the use of IMAC may include: need of extensive work to process; controlled oxidative reduction conditions inside the column; metal-induced cleavage that may damage the protein backbone; toxicity of metal ions leaching from the solid support.

There are other tags that have been incorporated into the scFv structure to perform affinity purification, but their use is not so extended. The RPAS® Purification Module from Amersham Biosciences for example, allows the production of soluble single chain variable fragments (ScFv) of antibodies from *E. coli* using the pCANTAB 5E expression vector. This scFv carries a C-terminal 13 amino acid peptide tag (E-Tag) which is recognized by an Anti-E Tag monoclonal antibody [46]. Another peptide tag is the amino acid epitope sequence – RPAP, which allows the purification of scFv derivatives containing the peptide tag APDTRPAPG by means of affinity interaction [47]. A cheaper version of this tag was more recently tested by Lan et al. in expanded bed adsorption [48], with recovery yields of more than 85%. Another example of scFv purification by affinity stretches is the expression of biotinylated scFv and its ulterior purification with the natural biotin ligand avidine/streptavidin [49].

Irrespective of the tag nature, its effective application for downstream purification purposes needs of a careful consideration since the additional steps required for removing tags could lead to complicate the procedure.

7.1.3. Biomimetic ligands

Biomimetic ligands are synthetic molecules that have emerged from novel technologies as combinatorial techniques, sophisticated molecular modeling approaches, designing and screening programs [40,50]. These molecules represent low cost and robust alternatives to the use of conventional biological affinity ligands. Triazine based ligand are the most efficient for scFv purification. Particularly, ligand 8/7 (artificial protein L) exhibits the capability of binding both fragments and full sized immunoglobulins of different classes and from sources [51]. This property resulted in the isolation of immunoglobulins from crude samples, under non-optimized conditions, thus achieving a high degree of purity (up to 95%) [52]. An important advantage of ligand 8/7 respect to natural Protein L, is its capability of binding a wide range of human κ-light chains [51].

The non-peptidyl triazine ligand from Prometic Biosciences [53], is a more recent biomimetic ligand, alternative to Protein L, that captures and purifies antibody fragments directly from biological feedstocks. The ligand can purify scFv and Fab fragments from a wide range of mammalian sources including human, bovine, ovine and murine [54,55].

Perhaps the main disadvantage when working with biomimetic ligands, is the little knowledge upon their mechanism of interaction with antibodies, which is necessary to optimize the binding process. For this reason, the interaction between synthetic ligands and immunoglobulins are being characterized by leading research groups [50].

7.2. Non affinity-based chromatography

Although affinity-based chromatography is the most widely applied technique, other chromatographic methodologies such as ion exchange, size exclusion, and hydrophobic interaction can be used to purify antibody fragments. Table 1 shows a comparison between the purification parameters of various scFv proteins obtained by different packed bed chromatographic techniques. As it can be appreciated, affinity separations show low recovery percentage but the highest final purity (considering that it is the first capture step in most of the examples showed). In certain cases, however, purification performances are highly dependent on the scFv whole structure. Moosman and co-workers [59], for example, have successfully purified PEGylated scFv by combining ion exchange and hydrophobic interaction chromatographies, reaching final purities of more than 90% and recoveries higher than 50%. The authors concluded that PEG not only improved scFv pharmacokinetics but also facilitated the purification of the complex after its synthesis. Similar results were obtained by Evans et al., who demonstrated that scFv-albumin fusion presented better recovery yields when purified with ion exchange chromatography (IEX) instead of the single scFv protein [56]. Another chromatographic methodology which shows high final purity is hydrophobic interaction chromatography (HIC). This technique presents the advantage of removing effectively DNA and host protein contaminants, thus achieving scFv purity near 100% [59]. However, the high salt concentrations, required to elute antibodies and its low yield recovery make its use restricted [62]. Size exclusion chromatography is another example of a methodology widely used for scFv purification. However, it is not used for capturing recombinant antibodies due to its low selectivity [63], remaining as a complementary methodology to remove impurities and inactive antibody fragments such as aggregates and degradation products [41].

7.3. Non chromatographic methodologies

Although chromatographic separations are far away the most developed ones, alternative methodologies such precipitation and liquid–liquid extraction are worth of consideration due to its simplicity and low cost:

7.3.1. Precipitation

Precipitation remains as an attractive technique because it allows concentrating the sample to a solid, thus offering the maximum degree of volume reduction and benefiting the subsequent downstream purification [62]. Protein precipitation achieves separation by the conversion of soluble proteins to an insoluble state, which subsequently can be removed by several means. The methods to reduce the solubility of proteins comprise the manipulation of medium conditions such as pH, metal ions, nonionic polymers, organic solvents, specific ligands and polyelectrolytes [64]. Up to date, some applications to purify scFv antibodies include metal chelate affinity precipitation and purification with smart polymers [65]. Kumar et al. [66] have developed a simple methodology to purify His-tagged scFv molecules by employing metal affinity macroligands (MAL) loaded with Cu(II) or Ni(II) cations, reaching high yield of recombinant antibodies (see Table 2).

7.3.2. Liquid–liquid extraction

Aqueous two-phase extraction has proved to be a powerful unit operation for the downstream processing of biomolecules, allowing simultaneously its clarification, concentration and partial purification [67]. Aqueous two phase systems provide a suitable environment to maintain biological activity and protein solubility due to its high biocompatibility, high water content and low interfacial tension, minimizing product degradation [68,69]. However, there are some limitations related to the complex interactions of the multiple components involved as well as in scaling up.

Several systems have been explored to purify immunoglobulins from plants [70] and mammalian cells with purity of 70–95% and recoveries greater than 90% [71,72]. Nevertheless, purification of scFv by partitioning in aqueous two-phase systems has not been widely studied. At present, there are just a few reports about the

Table 1
Reported scFv purification parameters through different chromatographic methodologies.

Chromatographic method	scFv clinical relevance	scFv target	Yield percentage	Final purity percentage[a]	Purification factor	Reference
Affinity	[b]	scFv-albumin	75	–	–	[56]
	Anticoagulant	scFv-His_6	15	83	2.02	[41]
	Radioimage	scFv-His_6	–	93	–	[57]
	Diseases treatment	scFv-His_6	53	>90	–	[58]
	Tumor therapy	scFv	–	>90	–	[59]
Ion exchange	[b]	scFv	20	–	–	[56]
	[b]	scFv-albumin	40	–	–	[56]
	Tumor therapy	scFv-PEG(5)	94	90	–	[59]
	Tumor therapy	scFv-PEG(30)	94	–	–	[59]
	Cancer therapy	scFv	84	82	2.30	[60]
	Cancer theraphy	scFv	73	–	–	[58]
Hydrophobic interaction	Tumor therapy	scFv-PEG(5)	72	>90	–	[59]
	Tumor therapy	scFv-PEG(30)	67	98	–	[59]
	Cancer theraphy	scFv	33	82	1.12	[60]
	[c]	tribody	94	98	2.00	[61]
Size exclusion	Anticoagulant	scFv-His_6	89	96	1.15	[41]

[a] Considering previous extractive steps.
[b] The main aim of the work is to improve scFv pharmacokinetics.
[c] Authors focused in purification techniques rather than triabody characteristics.

Table 2
Reported antibody fragments purification parameters employing methodologies alternative to the classical packed chromatographic columns.

Methodology	scFv source	scFv concentration (mg/L)	Yield percentage	Final purity percentage[d]	Purification factor	References
Non-chromatographic techniques						
MAL–Cu(II) precipitation	*E. coli*	22	91	–	16	[66]
MAL–Ni(II) precipitation	*E. coli*	19	80	–	21	[66]
Protanal LF precipitation	*E. coli*	–	30	≥90	–	[92]
Protanal LF precipitation	*E. coli*	–	26	≥90	–	[92]
AMTPS	Human IgG lysate	–	33	100	–	[73]
AMTPS	*P. pastoris*	60	88	–	3	[e]
Alternative chromatographic formats						
Macroporous cryogels	*E. coli*	–	81–87	–	13–15	[81]
EBA	*P. pastoris*	15	–	≥90		[79]
EBA[a]	*E. coli*	14	92	–	–	[48]
EBA[b]	*E. coli*	15	93	–	–	[48]
EBA[c]	*E. coli*	30	88	–	–	[48]
Monolithic column	*E. coli*	–	64	≥90	–	[91]
SBM I	*B. Megaterium*	50	–	–	–	[84]
SBM II	*B. Megaterium*	70	–	–	–	[84]

[a] Clarified.
[b] Unclarifid.
[c] Concentrated unclarified feedstocks.
[d] Considering previous extractive steps.
[e] In preparation.

application of reversed micellar aqueous two phase systems to fractionate antibody fragments from human IgG lysates (Table 2) [73].

Particularly, our research group is developing a methodology to purify His_6-scFv, directly from yeast broth by using affinity-based aqueous micellar two-phase systems (AMTPS). Preliminary results, presented in Table 2, shows recovery yields of 88% and purification factor near of 3 (data not published yet).

8. Trends in scFv capture

Antibody production has gone under a significant increase over the past years, mainly, as a consequence of the optimization and the scale up of antibody fragments expression. The advances in scFv production is shifting the bottleneck of antibodies fragments industrialization from upstream production processes toward downstream processes [29,63,74].

As it was mentioned previously, a conventional multi-step downstream process could result in low recovery yields (Table 3), thus affecting the cost/benefit relationship. Generally, a high lost of material takes place along clarification and first capture step, representing in certain cases more than 50% of the initial protein content [41]. For this reason, the integration of these unit operations could lead to a better operational economy [72,76]. Precipitation as well as extraction with aqueous two-phase phase systems (ATPS) are pioneer techniques that had allowed the integration of clarification and first capture unit operations [66,71,72]. However, the high purity degree needed for therapeutic molecules could make both methodologies insufficient to be used as unique purification steps. For this reason, different strategies are being developed to overcome some drawbacks concerning to both the low selectivity of classical integrative techniques (precipitation, ATPS) and the needing of clarification step previous to packed bed-chromatographic techniques.

8.1. Expanded bed adsorption

The initial purification of the target molecule was traditionally carried out by adsorption chromatography with conventional packed bed of adsorbent and a clarification step was always required before loading the crude feed into the chromatographic column. In contrast, adsorption in expanded beds (EBA) enables proteins to be recovered directly from particulate-containing feedstocks, such as fermentation broths and preparations of disrupted cells [77]. The adoption of this technique greatly reduces the complexity of downstream processing by eliminating certain filtration, centrifugation and concentration steps [78,79]. Initially, EBA performance was highly dependent on the media viscosity, presenting, in certain cases, problems with fouling [62]. Fortunately, technological advances have solved most of its inherent troubles [62], thus at present it is possible to purify recombinant antibodies with high recovery yields and purity.

Particularly, Lan et al. [48] applied EBA to purify dbFv molecules and compared the recovery performance when using different feedstocks (clarified, unclarified and concentrated unclarified). The Authors found that the values of operational productivity for clarified and unclarified feedstock as well as its yield recovery were similar (see Table 2). This result has demonstrated that a previous clarification step can be omitted without affect the purification performance. Other example of scFv purification by means of EBA is that from Vigor and co-workers who successfully purified His-tagged anti-CEA scFv using expanded bed immobilized metal affinity chromatography.

8.2. Super macroporous cryogels

In the last years, polymeric (macro- to mega-) porous materials have been introduced for protein capture. Generally, this methodology allows the purification of biomolecules directly from fermentation broths [80].

At present, there are a wide range of cryogels matrices, which could be composed with different polymeric blends, offering different superficial properties. Cryogels can also be loaded with affinity ligands in order to purify specific molecules [80]. The main disadvantages related to this methodology are its low binding capacity and limited surface area, both of them, as a consequence of the high internal volumes [67,80]. Nevertheless, in 2004, Dainiak et al. developed a robust and scaleable chromatographic technique to isolate antibody fragments from cell culture fluids by using continuous supermacroporous dimethylacrylamide (DMAA) cryogel column [81]. The cited procedure approached scFv purification factors of 13–15 with recovery yields of more than 80% (see Table 2). Most recently, Jain and Kumar [82] presented a protocol to recover Hist-tagged proteins directly from bioreactor. The methodology includes the use of metal affinity columns based on cryogel matrix to extract urokinase and mAbs [82]; however, the procedure can be adapted to different his-tagged proteins.

Table 3
Reported scFv purification parameters employing conventional downstream processes.

No. of purification steps	Yield percentage	Final purity percentage	Total purification factor	References
2[a]	66	>90	–	[59]
2[a]	63	98	–	[59]
3	8	96	2.34	[41]
3	53	93	–	[58]
4	24	92	–	[57]
4	25	≥90	2.56	[60]
5	3	≥90	–	[75]

[a] From pre-purified and PEGylated scFv samples.

8.3. Continuous multicolumn chromatographic separation

There exists an increasing interest to replace the classical single column discontinue chromatography for cost-effectiveness procedures. Interesting alternative chromatographic configurations are those based on continuous multicolumn methodologies. Isocratic simulated moving bed (SMB) process, for example, is one of the most exploited continuous chromatographic separation [83]. These techniques offer several advantages over classical batch separations such as better productivity, reduced buffer consumption and high throughput [74]. A recent work developed by Martinez Cristancho et al. [84] compared purification parameters of histidine-tagged D1.3 scFv with those from the theoretical purification through SBM. The productivity resulted to be two times higher and the final product was fivefold concentrated in the continuous methodology [84]. However, experimental data would be necessary to confirm the real purification performance.

8.4. Magnetic separations

The use of magnetic particles to capture antibody fragments represents another methodology alternative to conventional packed bed chromatography [62]. This technique involves the use of magnetic microspheres, covered with specific ligands, which can be added into complex biological feedstocks in order to purify different biomolecules. The main benefit of this methodology is its ability to reduce the numbers of purification steps by allowing product purification without a previous clarification step [36]. Nowadays, different magnetic separation formats, e.g. magnetically stabilized fluidized beds (MSFB) and high gradient magnetic fishing (HGMF), are well developed [36,85]. Particularly, HGMF presents better separation of small particles in comparison to MSFB [86], thus offering a higher throughput purification.

At present, several magnetic microspheres are commercially available to capture scFv and scFv-derivatives from biological media. For example, Gray et al. [87] used MagnaHis™ Protein Purification System, from Promega, to purify His-tagged scFv directly from *E. coli* lysates. Other companies also offer Protein L-covered particles to specifically capture both antibodies and antibody fragments. On the other hand, streptavidine magnetic beds is routinely used to capture scFv-displaying phages during antibody fragments selection [88,89].

Even though there is an extended use of magnetic separation at micro-scale procedures, some strategies such as HGMF, still has not been industrialized because presents limited capacity and cannot work continuously [86].

8.5. Efficient processing of inclusion body

Even though the inclusion body formation is not desired, the proteins expressed into them present some advantages such us low contamination with native proteins and high protection from

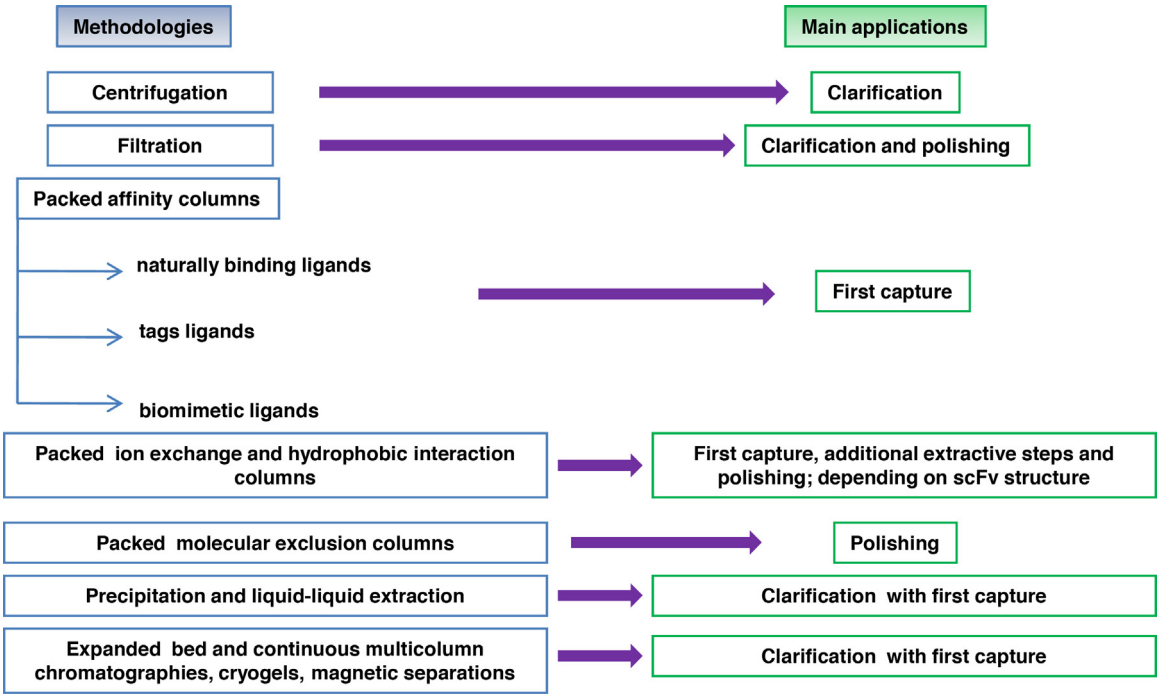

Fig. 3. Different unit operation applied to purify scFv with their main application along the whole downstream process.

endogenous proteolytic degradation [90]. Some research lines has taken advantages of inclusion bodies by coupling the purification and refolding of scFv particles to reach a final product of high purity degree. Sushma et al. [91], for example, integrated the solubilization of an anti TNF-α scFv with its purification in a Ni(II)-monolithic column, thus obtaining an homogeneous final product with a yield of 65%. Gautam et al. [92] coupled refolding and precipitation of anti-HIV-1 antibody fragments from *E. coli* inclusion bodies. The researchers used alginate to purify and fold antibodies fragments (see Table 2). The cited smart polymer worked as pseudochaperonins and pushed the protein down the correct refolding pathway. The methodology, however, presented low recovery yields (26–30%) [92].

9. Polishing

It is a frequent fact that certain particles remain as sample contaminants irrespective of the capturing methodology applied. These impurities can be salts, organic components, DNA and molecules with chemical surface properties similar to those of the target molecule [93]. Usually, unfolded, degradated or multimers antibody fragments are also found after capturing steps. The main aim of polishing steps is to remove these minor contaminants as well as to transfer the purified molecule into a suitable buffer and if necessary, to concentrate it.

Filtration was one of the first techniques used to polish and several advances in filtration media maintain it as a methodology frequently applied. This technique allows the separation of remaining contaminants with molecular weight different to the scFv proteins, the concentration of the sample as well as the changing of buffer solution.

Other methodologies widely used to polish are non-affinity chromatographies [35,55]. Anion exchange is frequently utilized as the last chromatography step because of its ability to scavenge endotoxins that may have entered to the process via contaminated manufacturing materials or inappropriate sample handling [94]. Its proven ability to reduce other key contaminants such as nucleotides and virus make it an even stronger candidate [94]. Gel-filtration based chromatography is also widely applied as a final purification methodology. Several works reported the successful polishing ability of gel-filtration chromatography that allowed high final purities [41,95].

10. Conclusion

As more scFv molecules are produced, the greater is the interest in finding downstream processing methodologies to be applied over a wide range of antibody fragments purification. Fig. 3 summarizes the methods addressed in this review with their main application in the different stages of scFv downstream processing. The first capture methodology seems to be the most controversial stage when searching for platform techniques. Several unit operations, such as chromatographic and non-chromatographic steps, have been assayed for different authors. Up to date, packed bed affinity-based chromatographies represent the methodology most widely used in the first capture step, being the immobilized metal affinity chromatography (IMAC) and Protein L-resins the most frequently utilized to capture scFv. Non-affinity chromatographies comprise another group of alternative methodologies that could be successfully used to capture scFv molecules. However, as it can be seen from Fig. 3, their use is highly dependent on antibody fragment structure. Alternative capturing strategies, such as precipitation and liquid–liquid extraction, present the advantage of being simple methodologies that integrate clarification and purification steps. However, its inherent low selectivity makes its use restricted to early extractive steps, needing of a second purification technique.

Some techniques more recently developed, such as expanded bed absorption, simulated moving bed, macroporous cryogles, magnetic separation, as well as the integration of inclusion body solubilization with scFv purification, seems to solve some of the drawbacks of the actual scFv downstream processing protocols, however further assays still should be accomplished to demonstrate its actual performance.

Based on all the issues addressed, it could be concluded that many efforts are still needed to standardize the purification of scFv and scFv-derivatives molecules. Nevertheless, the founding of a generic protocol to purify antibody fragments seems to be limited due to the wide range of scFv structures available. For this reason, scientists will probably have to rest satisfied with finding generic basic extractive steps able to be adaptable for each particular scFv.

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