

*Review*

## Antibody Fragments and Their Purification by Protein L Affinity Chromatography

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**Abstract:** Antibodies and related proteins comprise one of the largest and fastest-growing classes of protein pharmaceuticals. A majority of such molecules are monoclonal antibodies; however, many new entities are antibody fragments. Due to their structural, physiological, and pharmacological properties, antibody fragments offer new biopharmaceutical opportunities. In the case of recombinant full-length antibodies with suitable Fc regions, two or three column purification processes centered around Protein A affinity chromatography have proven to be fast, efficient, robust, cost-effective, and scalable. Most antibody fragments lack Fc and suitable affinity for Protein A. Adapting proven antibody purification processes to antibody fragments demands different affinity chromatography. Such technology must offer the unit operation advantages noted above, and be suitable for most of the many different types of antibody fragments. Protein L affinity chromatography appears to fulfill these criteria—suggesting its consideration as a key unit operation in antibody fragment processing.

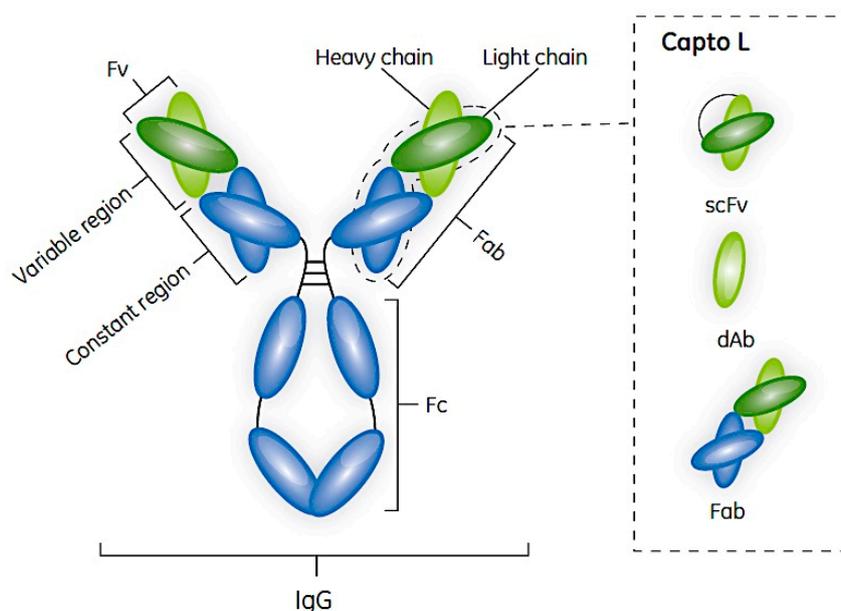
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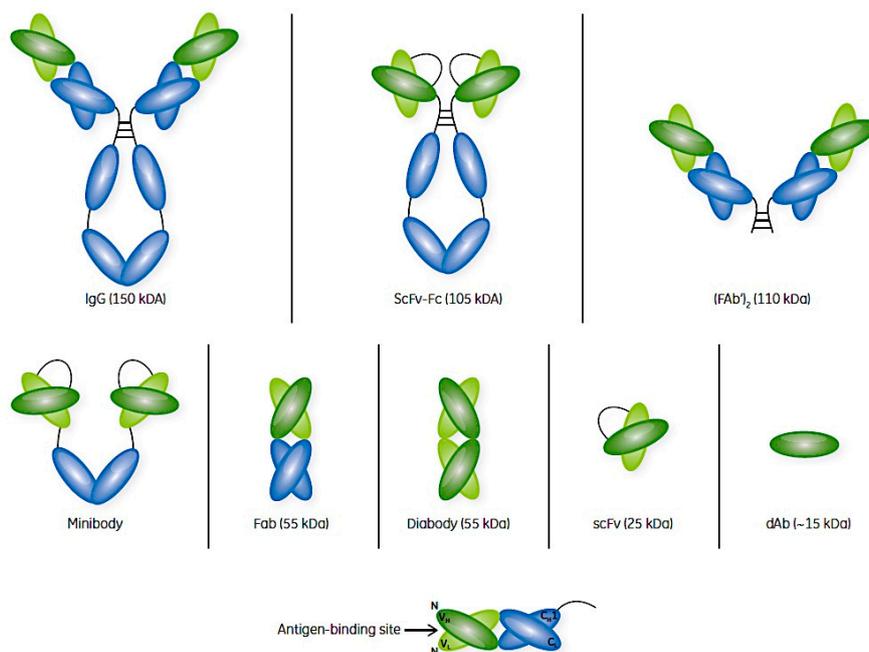
## 1. Introduction

There are in excess of 35 therapeutic monoclonal antibodies (mAbs) approved for clinical use [1–4] with hundreds of new mAbs in various stages of clinical development or evaluation. There may be over one thousand mAbs currently under study as biopharmaceutical agents, with approximately 300 new candidates being added each year [5]. Several of these may be biosimilars of already successful therapeutic mAbs [6]. The literature base for therapeutic antibodies is equally vast, with more than 200,000 published references on the subject [7]. Much recent interest is also directed towards antibody fragments (Ab-fragments). Some mAb therapeutics may be developed into Ab-fragments displaying alternate pharmacokinetics and other desired properties. Several Ab-fragment-based biotherapeutics have been approved, including ReoPro® (abciximab, Janssen/Eli Lilly), Lucentis® (ranibizumab, Roche-Genentech), and Cimzia® (certolizumab pegol, UCB). In 2010 those three products had total sales of over \$3.5 billion (USD) [7]. Many more Ab-fragments are under development [3,8–20].

Antibody fragments (Figures 1 and 2) are of varied structure, but typically smaller than native antibodies, and lack an Fc domain. Their smaller size offers more binding events per mass of protein purified and administered, less viscous formulations (and, thus, ease of injection) and possibly improved tissue delivery. The lack of an Fc domain is one reason Ab-fragments exhibit reduced circulation half-lives and immune system activation [11]. The latter is advantageous in some applications (see below). Ab-fragments, including fragment antigen-binding fragments (Fabs), single-chain variable fragments (scFvs), such as pexelizumab (Alexion), and single-domain antibody fragments (dAbs) (Figure 2) are examples of how antibody-based biotherapeutic agents are expanding in class and scope. Other examples of Ab-based therapeutics include bi-specific antibodies, with two different antigen binding regions, such as Removab® (catumaxomab, Fresenius/Trion), or antibody drug conjugates, such as Adcetris® (brentuximab vedotin, Seattle Genetics).



**Figure 1.** Structure of a typical full-size IgG antibody molecule showing its various domains. Protein L binds to the variable region of the kappa light chain without interfering with the antigen binding site.



**Figure 2.** IgG and various fragments thereof together with detail of the antigen binding Fab region.

Although lack of an Fc region should reduce the circulation half-life of Ab fragments various recombinant chemical modifications such as “PEGylation” can enhance Ab-fragment circulation life [3,12–15]. Cimzia® is one example of a PEGylated Ab fragment.

The ease with which Ab-fragments can be genetically or otherwise altered to improve target affinity, extend *in vivo* half-life, or facilitate tissue targeting, has helped support rapid growth in studies involving antibody fragments. Yet, while robust methods to purify mAbs at relatively large (many Kg) scale are well established [16], there are still challenges associated with the production and purification of antibody fragments [8,17–20]. Downstream processing presently constitutes a roadblock to delivering clinically efficacious Ab-fragments at effective unit costs.

The three approved Ab-fragment pharmaceuticals noted above verify such fragments can be produced, purified, and formulated at large scale. However they are purified using somewhat varied processes which can include different chromatographic or non-chromatographic operations. This contrasts with mAbs which are generally purified by rapidly adapting common, proven, robust, “toolbox” approaches. In almost all cases such processes include an initial Protein A affinity capture chromatography operation. Due to the fact that Ab-fragments lack Fc most Ab-fragments do not bind to Protein A resins with the high affinity or capacity required in modern bioprocessing. More modern affinity based operations may take advantage of Protein L (see below), Protein G [17], or other affinity ligands capable of appreciably binding Ab fragments.

The following sections consider various types of antibody fragments that can be generated, how they present unique purification challenges, and a possible role for Protein L chromatography as a “generic” antibody fragment purification method. The review ends with summaries of two Protein L-based Ab-fragment purification processes.

## 2. Antibodies and Their Fragments

Full-size “intact” antibodies (Figure 1) are Y-shaped multi-domain protein molecules comprised of two light (L) chains (either kappa or lambda) and two heavy (H) chains (either alpha, gamma, delta, epsilon, or mu) linked by a series of disulfide bonds. Each L chain displays one variable (V) and one constant (C) domain, while each H chain contains one V and three C domains. The antigen-binding specificity of an antibody resides in the V domains located at the N-terminal regions of the L and H chains. Thus antibodies are typically bivalent molecules, capable of binding two similar antigens. Three “hypervariable”, or complementarity-determining regions (CDRs), are found within each  $V_H$  and  $V_L$  domain. Sequence variability is concentrated in these regions, and loop formation occurs here. The remaining areas of the  $V_H$  and  $V_L$  domains show less variation and comprise the framework (Fr) regions which structurally support the loops [21].

Carboxy terminal-associated sections of the two H chains comprise the fragment crystallizable (Fc) region involved in recruiting effector functions, such as complement fixation or immune cell activation, as well as in stabilizing the Ab (to reduce aggregation). Fc promotes enhanced serum half-life, and also binds to Protein A [22–24]. The multi-domain structure of antibodies supports the creation of smaller molecular weight Ab-fragments which offer antigen recognition [25] and the possibility to combine specific binding affinities [26,27]. The methodologies with which antibody fragments have been generated range from proteolytic cleavage [28,29] to modern genetic engineering approaches. Further diversification comes from their being covalently or recombinantly enhanced with other functional properties.

New forms of Ab-fragments with novel physical and pharmacological properties continue to emerge. As such they can present varied challenges in their production and purification. Ab-fragments are especially amenable to genetic manipulations (see below) or covalent modifications (e.g., [13,30]) aimed at improving their affinity, stability, and function. Antibody fragments have been linked with enzymes [31], toxins [32], and radionuclides for cancer treatment [33–35], as well as viruses for gene therapy, liposomes or nanoparticles for improved drug delivery, and dye or other sensing substances [11,36–39].

Figure 2 depicts some Ab-fragments. Fabs are the oldest class [8] and were first generated by cleavage of an intact antibody with an enzyme, such as papain [28]. Cleavage yields two monovalent Fab fragments, each composed of one  $V_H$  and one  $V_L$  chain linked by disulfide bonds and displaying a single antigen-binding site. Fabs exhibit a number of properties that make them attractive as biotherapeutic agents [40].

Single-chain Fv fragments (scFvs) are monovalent structures, with affinity for a single antigen. Approximately 25 kDa in size, an scFv contains the V regions of an antibody’s heavy and light chains fused into a single polypeptide chain via a short flexible linker. An scFv comprises the complete antigen-binding site of its parental antibody molecule [41] but, lacking the Fc region, is unable to initiate effector functions [42] or bind as appreciably as intact Abs to Protein A. Phage display and related technologies [43–45] are readily adapted to scFv development. As such scFv’s are currently viewed as promising therapeutic fragments, with several having been in clinical trials [3,7,9,46]. Recently scFv fragments which can be internalized by cells were suggested as potentially novel agents for tumor modulation [47].

Single domain antibody fragments (dAbs), consisting of  $V_H$  [48] or  $V_L$  [49] domains of 12–15 kDa, are some of the smallest functional antibody fragments that retain full antigen-binding specificity. They

are approximately one-tenth the molecular weight (MW) of a normal antibody (150–160 kDa), one fourth the MW of an average Fab (50 kDa) and half the MW of a typical scFv (25 kDa) [8,50–52]. Although dAbs contain only three of the six CDRs from the parent antibody, they exhibit antigen binding specificity and affinity [52]. They can be remarkably stable under harsh conditions of temperature, pressure, and denaturing chemicals [53].

It is known that  $V_H$  and  $V_L$  domains can exist *in vivo* in certain human diseases, such as heavy chain disease [54,55] and multiple myeloma [56]. Single-domain antibodies also occur naturally in animals. Camelids produce a class of heavy chain antibodies ( $V_{HH}$ ) that are devoid of light chains [57,58]. Single-domain antibodies (called V-NAR) have been discovered in sharks [59,60]. Studies of such single domain antibodies provide insight to the design of recombinant Ab-fragments.

### 3. mAbs and Their Fragments as Therapeutic Agents

mAbs are firmly entrenched as the most successful and widely used antibody-related reagents for clinical and diagnostic applications, with sales in the US and Europe in the tens of billions of dollars [5,7,61]. While mAbs are effective therapeutic agents when the antigenic target is well-defined and accessible, such as in some cancers and autoimmune diseases, many diseases offer less accessible (e.g., intracellular), cryptic, or hypoallergenic targets [40,62]. In such circumstances, antibody fragments may provide advantages due to their smaller size and structure, and the ease with which they can be produced and genetically modified [11,21,27,47,63–66]. Ab-fragment-related biomedical applications include tumor treatment and imaging [67], viral replication suppression [68–70], toxin and venom neutralization [41,71], and receptor blockage [36,72].

Due to their Fc region mAbs can persist in circulation for days to weeks. While this is advantageous in some conditions, in others it is not [73]. Ab-fragments tend to be more readily cleared from the body. While this may reduce their usefulness for some clinical applications [74], antibody fragments may be better suited than Abs for other applications [8,75,76]. These include those requiring shorter-term tumor penetration and imaging, where diagnosis depends on greater uptake of the labeled antibody by tumor than normal tissue [26,48,62,77–79].

While Ab-fragments are typically monovalent, they can be combined (pre- or post-initial processing) into multivalent entities, such as diabodies, triabodies, and tetrabodies [15,27,73,80,81]. Modification in valency may lead to recruitment or modification of effector functions [11] which may be important in different treatment regimens.

When prolonged serum half-life, but not the other physiological functions associated with Fc is desired, Ab-fragments can be modified by various chemicals or polymers [3] or recombinantly fused to serum albumin, or antibody constant regions [51]. “PEGylation” is currently the most established route, due to the maturity of the technology and the success of several PEGylated biopharmaceuticals. Methods to PEGylate antibodies and antibody fragments have been in existence for close to forty years [12,14], as have methods to purify PEGylated proteins [82]. PEGylation typically reduces target affinity by a few Logs but does not eliminate affinity or other interactions [13,15,18].

#### 4. Antibody Fragment Production and Purification

Antibody fragments often differ in physical properties from the antibodies they are derived from. For example, lack of Fc region may result in their exhibiting greater tendency to aggregate [83,84] during purification protocols.

Microbial, fungal, plant, and mammalian expression systems have been successfully used with Ab-fragments [75,85]. Initially the most commonly used system was periplasmic expression in *E. coli* [51]. Production of Lucentis and Cimizia involve such expression [17]. Scanlan *et al.* [20] recently noted the trend for Ab-fragments to be expressed in high cell density prokaryotic or yeast cell fermentations [86]. This places significant demands on clarification and primary capture steps. They also noted that for Ab-fragments which do not contain Fc domains, primary capture can be achieved via cation exchange (CEX) chromatography. Some well-established CEX resins can offer dynamic binding levels  $\gg 100$  g/L for Ab-fragments [87]. Several vendors now sell CEX resins that function over broad ranges of conductivity and pH. When an even wider range of potential operating conditions are required mixed mode resins may be used in bind/elute mode [88]. Other approaches are possible depending on the separation challenge' for examples see [10,89–91]. Follow-on orthogonal unit operations may then involve ion exchange, hydrophobic interaction or size exclusion. While several processing options exist for Ab fragment purification, no methods of choice have yet emerged. An ideal purification platform would be generically applicable for a wide range of antibody fragments, and allow rapid processing with high product yield and purity [2].

The major drawback to non-affinity-based primary capture is that significant process development effort must be applied to every fragment under consideration. In the case of mAbs, industry met a similar challenge by using platforms where, to a large extent, highly-selective primary capture is afforded by Protein A-based affinity chromatography. This allows more effort to focus on optimizing follow-on chromatography, filtration or other operations to remove impurities and/or contaminants. It is, therefore, of interest to consider if Protein L-based affinity resins can play a similar role in Ab-fragment processing. Protein A is somewhat limited in regard to the antibody classes it interacts with (Table 1). A Protein L-based production platform may not accommodate all Ab-fragments, but a suitable affinity toolbox method could help make purification of many Ab-fragments cost-effective (for antibody processing costs see [92]).

#### 5. Protein L and Its Use in Antibody Fragment Bioprocessing

Several virulence-associated antibody-binding proteins isolated from the cell walls of bacteria have proven to be useful for purification of antibodies, with Protein A (from *Staphylococcus aureus*) [93] and Protein G (from groups C and G *streptococci*) [94,95] perhaps being the most well-known. Protein L (from *Peptostreptococcus magnus*) [94,96] offers options for purification attributed to its rather unique binding specificities (Table 1).

**Table 1.** Protein A, G and L binding \*.

Species and Antibody Class	Protein A *	Protein G *	Protein L
<b>1. General</b>			
Kappa LC 1, 3, 4	None	None	Strong
Lambda LC	None	None	None
Heavy chain **	Strong	Strong	None
Fab, ScFv, Dab	Weak ***	Strong	Strong
<b>2. Human</b>			
IgG <sub>1</sub> , IgG <sub>2</sub>	Strong	Strong	Strong
IgG <sub>3</sub>	Weak	Strong	Strong
IgG <sub>4</sub>	Strong	Strong	Strong
IgA, IgM	Weak	None	Strong
IgE	Weak	None	Strong
IgD	None	None	Strong
<b>3. Mouse IgG<sub>1</sub></b>			
IgG <sub>2a</sub> , IgG <sub>2b</sub>	Strong	Strong	Strong
IgG <sub>3</sub> , IgM	Strong	Strong	Strong
<b>4. Rat IgG<sub>1</sub></b>			
IgG <sub>2a</sub> , IgG <sub>2b</sub>	None	Strong	Strong
IgG <sub>2c</sub>	Strong	Weak	Strong
<b>5. Pig total IgG</b>			
<b>6. Dog total IgG</b>			
<b>7. Chicken IgG</b>			None
<b>8. Cow IgG<sub>1</sub></b>			
IgG <sub>2</sub>	Strong	Strong	None
<b>9. Goat IgG<sub>1</sub></b>			
IgG <sub>2</sub>	Strong	Strong	None
<b>10. Sheep IgG<sub>1</sub></b>			
IgG <sub>2</sub>	Strong	Strong	None

Notes: \* References [97,98], typical “Strong” affinities will reflect dissociation constants (Kd’s) < 10<sup>-7</sup> M. \*\* Protein A and G both bind to antibody Fc regions, however they exhibit some antibody subclass differences. For example, Protein G binds more strongly than Protein A to polyclonal IgG from cow, sheep and horse. Unlike Protein A, Protein G binds polyclonal rat IgG, human IgG3 and mouse IgG1 [99]. Protein A binds to some VH-3 subclass heavy chains [100]. Protein G exhibits CH1 interaction [101]. \*\*\* Protein A only interacts with the VH3 region of antibody fragments lacking Fc, hence binding to Fab, scFv or Dab will be dependent on the heavy chain chosen.

Proteins A, G, and L all bind to different target sites in conserved framework regions of antibodies, without compromising an antibody’s ability to interact with its antigen [100]. Protein A-based affinity media are most frequently used to purify full-size antibodies because they recognize targets possessing the Fc domain, however antibody fragments typically lack the Fc region which is why Protein A resins are of limited use in Ab-fragment purification (see Table 1).

Protein L is present at the surface of about 10% of *Peptostreptococcus magnus* strains [102]. It is a 76 to 106 kDa protein containing four or five highly homologous, consecutive extracellular immunoglobulin (Ig) binding, or B, domains (depending on the bacterial strain from which it is isolated) [103,104]. These B domains are responsible for the protein’s interaction with Ig kappa light chains [94]. Given that its

target is a light chain, Protein L will bind to representatives of most antibody classes, including IgG, IgM, IgA, IgE, and IgD; however, in many cases where high affinity and high capacity binding are required Protein A may be favored for practical reasons.

Protein L-based affinity chromatography resins for research applications have been offered commercially by several vendors for many years. Such affinity resins have been used in the noncommercial purification of scFv's [31,41,80,89,105,106], Fab's [91,106–108], and single-domain antibodies [52,91,98,103,108,109].

Affinities of Protein L for various antibodies and fragments are indicated in Table 1. It should be noted that the native form of Protein L does not recognize antibodies (or related fragments) from certain animal species [99]. Genetic engineering could possibly allow Protein L to expand its versatility and specificity (see below). As the binding site for Protein L resides in framework region 1 of the variable domain of kappa light chain subtypes (kappa I, III, and IV from humans and kappa I and V from mice) [94,96,110], fragments derived from antibodies that have kappa light chain subtypes can be purified using Protein L [100]. Since Protein L interacts with the light chain, it has no immunoglobulin class restrictions and offers the potential of being a “broadly if not generally” useful affinity ligand [109]. Approximately 60% of mammalian IgG light chains are kappa chains, with the remaining 40% being lambda chains that lack binding sites for Protein L [73,98]. For Ab-fragments containing the lambda light chain, Camelid-protein-based [79] affinity resins may be employed [19].

Genetic engineering may also allow Ab-fragments to acquire enhanced ability to bind to Protein L [111–113]. Boes *et al.* [113] and Muzard *et al.* [114] demonstrated that Protein L binding activity could be transferred from high-affinity Protein L binding antibodies to antibodies or scFv fragments that normally did not react with the ligand. These modifications had little effect on either antigen binding activities or antibody yields. The presence of the binding site provided a convenient mechanism for detection of the antibody through use of labeled Protein L.

While Protein L is restricted to recognition of specific kappa light chains, there are examples in the scientific literature where Protein L has been fused with other bacterial cell wall proteins, such as Protein G and Protein A, to generate highly versatile affinity ligands with broad binding specificity [115–118].

## 6. Alternatives to Protein L: Peptide Tags and Mimetics

As with other recombinant proteins, antibody fragments can be genetically engineered to display peptide tags, such as those possessing several histidines to facilitate purification by immobilized metal affinity chromatography (IMAC) or other affinity approaches such as GST-C [119]. Das *et al.* [89] compared immobilized metal affinity chromatography to Protein L affinity for the purification of histidine-tagged scFv fragments, and found Protein L to be a more versatile and robust method.

Peptide tags may exhibit other challenges in regard to incorrect folding of the fragment during expression, or with association of the V domains causing problems with affinity and specificity [120]. Proteases released during cell disruption may degrade tags [42]. Peptide tags may enhance aggregation of antibody fragments, leading to product loss during purification [121]. However the most significant drawback to the use of tags is the perceived need to remove potentially immunogenic tags by including tag cleavage and removal steps in production. Use of Protein L circumvents tag-specific challenges (Table 2).

**Table 2.** Ab fragment affinity purification using protein L *versus* rTags such as GST-C or 6HIS IMAC \*.

Criteria	rTag	Protein L
Effective	Yes	Yes
Alters Ab Fragment	Possibly	No
Requires Tag Cleavage	Maybe	No
Bioprocess Resins Available	Yes	Yes
Typical Dynamic Capacity (g/L) <sup>+</sup>	NK	25 g Fab
Working pH stability	3–12	2–10
Effective cleaning in place	Yes <sup>++</sup>	Yes <sup>+++</sup>
Available Ligand or Tag Assays	Perhaps	Yes
Regulatory Support	Yes	Yes

\* For definitions see text. <sup>+</sup> 10% breakthrough, 2.4 min residence time in 20 cm bed. NK = not known as can vary, see text. <sup>++</sup> Metal ion stripping may be required. <sup>+++</sup> e.g., 100 mM 1-thio-glycerol reducing agent, followed by 0.05 M NaOH.

In the case of Protein L robust synthetic affinity ligands that mimic the action of the natural biological ligand may provide an alternative for both mAb and Ab-fragment processing [122–124]. In time it will be interesting to see if the improvements offered by synthetic ligands can match those offered by genetic engineering of already highly evolved affinity proteins, e.g., [125–127]. Twenty years of Protein A resin development tends to favor the latter approach, but synthetic ligand development, screening methods, and performance are continually improving.

## 7. Protein L Bioprocess Resins

Several vendors supply protein L-coupled chromatography resins for laboratory screening and use (e.g., [128,129]). GE Healthcare offers Capto™ Protein L suitable for biopharmaceutical processing. One might expect that “second generation” (engineered protein) Protein A and Protein L resins would exhibit similar processing costs for mAb and Ab fragments respectively, with resin dynamic capacities and cleaning-in-place (CIP) stabilities as important process variants [92]. Table 3 compares performance of a MabSelect™ Protein A and Capto L resins, which are based on similar base matrices and ligand linkage chemistries.

**Table 3.** Commercial protein A and L resins for large scale antibody and fragment processing \*.

Criteria	MabSelect	Capto L
Rigid 85 µm Matrix	Yes	Yes
≤500 cm/h in 20 cm high bed	Yes	Yes
rProtein Ligand (non-animal)	Yes	Yes
Typical Dynamic Capacity (g/L) <sup>+</sup>	30 g IgG	25 g Fab
Working pH	3–10	2–10
Standard cleaning in place <sup>++</sup>	Yes	Yes
Leached Ligand Assays	Yes <sup>**</sup>	Yes <sup>***</sup>
Regulatory Support	Yes	Yes

\* www.gelifesciences.com. <sup>+</sup> 10% breakthrough, 2.4 min residence time in 20 cm bed. <sup>++</sup> e.g., 100 mM 1-thioglycerol reducing agent followed by 0.15 M NaOH for Protein A, or 0.02 to 0.05 M NaOH for Protein G. <sup>\*\*</sup> e.g., rProtein A ELISA kit from Repligen Corp., USA. <sup>\*\*\*</sup> e.g., Protein L ELISA kit, Medicago AB, Sweden. Example ligand leakage values are given in Section 8.

Cleaning-in-Place (CIP) studies using the approach noted in Table 3 indicate that MabSelect can survive more than 110 CIP cycles with no significant reduction in performance. More modern ligands derived from Protein A antibody binding domains [125,126] offer enhanced basic pH stability and several hundred CIP cycles using relatively inexpensive 0.1 to 0.5 M NaOH solutions. Present Protein G resins can be CIP'd with NaOH but are not as chemically stable as Protein A, so an interval of 0.02–0.05 M NaOH is suggested. It is expected that next-generation Protein L resins may benefit from similar recombinant technology [125–127].

## 8. Examples of Protein L-based Purifications

Various publications and patents describe non-affinity based Ab-fragment purification processes using ion exchange [10,90] or hydroxyapatite [130] chromatography. Spitali *et al.* [10] describes a process to purify an Ab-fragment from a periplasmic cell extract based on cation exchange target-capture chromatography, followed by anion exchange target-flow-through chromatography. Development and verification of two different Protein L affinity based separation processes were recently described for Fab [131] and Dab [88] targets. Both examples involved three step processes with Protein L affinity chromatography followed by two other chromatographic steps. In this sense they mimic the three column processes common to production of most mAb biopharmaceuticals.

In the first example, purification of a kappa subclass Fab, of theoretical pI 8.5, in *E. coli* supernatant at 1 g/L, involved Protein L affinity chromatography followed by target-capture cation exchange chromatography to reduce Fab aggregates. This was followed by target-flow-through anion exchange chromatography. Unit operation yields were >90%. High total process yield (>87%) was achieved with Fab aggregate content reduced from 3.5% to 0.8% over the process. *E. coli* host protein was reduced over four Logs to 13 ppm following Protein L chromatography, and to 6 ppm at the end of the process. Feed endotoxin content of 1.7 million EU/mg of target protein was reduced to 11 EU/mg following Protein L chromatography, and to <0.1 EU/mg at the end of the process. Protein L ligand leakage was <6 ng/mL (analyzed using an ELISA kit from Medicago AB).

In the second example, [88] Capto™ L affinity and two mixed mode chromatography operations (Capto MMC ImpRes and Capto adhere ImpRes) were used for domain antibody (Dab) purification. The third column was again operated in target flow-through mode. The Dab target, of theoretical pI 9.2 and MW 12.9 kDa, was expressed (0.3 g/L feed) in the periplasm of *E. coli*. As in the previous example, the three-step purification process was developed using high throughput screening tools and then verified at larger scale. Total process yield was >80%, with a Protein L step yield of 99%. *E. coli* protein in the starting sample was more than 200,000 ppm whereas the final sample contained approximately 6 ppm. Endotoxin content was reduced from approximately 2 million EU/mg in the feed to below the limit of quantification (<0.1 EU/mg) in the purified sample. Protein L leakage was also at undetectable levels.

## 9. Conclusions

While the therapeutic potential for Ab-fragments is significant, their various structural formats present challenges to production and purification. These challenges are more varied, but not unlike, those associated with mAb production. For therapeutic use, large quantities of active ingredients are needed, so large-scale production and purification must be efficient from the standpoint of product yield, purity,

and cost. While there are different methodologies available for purification of mAbs, Protein A-based affinity chromatography has emerged as the dominant primary capture method. With the advent of suitable resins and ligands it is assumed that Protein L may possibly come to play a similar role in Ab-fragment processing. Several Protein L-based resins are sold by different vendors and at least one vendor offers Protein L resin suitable for GMP antibody fragment production, which offers performance similar to Protein A resins already used in production of biopharmaceutical mAbs. In addition the techniques used to improve Protein A resin capacity, lifetime, and ease of cleaning-in-place should be readily applicable to improving Protein L resins.

### Supplementary Materials

Supplementary materials can be accessed at: <http://www.mdpi.com/2073-4468/4/3/259/s1>.

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### Conflicts of Interest

The authors are, or were previously, employed by GE Healthcare Life Sciences, Uppsala, Sweden.

### Abbreviations

Ab, antibody; Ab-fragment, antigen-binding antibody fragment; mAb, monoclonal antibody; CHO, Chinese hamster ovary; CIP, cleaning-in-place; ELISA, enzyme linked immunosorbent assay; Fc, crystallizable Ab fragment which may bind to protein A; GMP, Good Manufacturing Process; GST-C, glutathione transferase (affinity tag) based chromatography; HCP, host cell protein; His, histidine; Ig, immunoglobulin; IMAC, immobilized metal affinity chromatography; LC, light chain; PEGylation, covalent modification with poly(ethylene glycol) polymers; Protein A, Fc-binding protein of bacterial surface origin; Protein G, Ab binding protein of bacterial surface origin; Protein L, Ab light chain-binding protein of bacterial surface origin; rTag, recombinant affinity tag such as 6His based tags for IMAC; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

### References

1. Lain, B. Protein A: The life of a disruptive technology. *BioProcess Intl.* **2013**, *11*, 29–38.
2. Malpiedi, L.P.; Diaz, C.A.; Nerli, B.B.; Pessoa, A., Jr. Single-chain antibody fragments: Purification methodologies. *Process Biochem.* **2013**, *48*, 1242–1251.
3. Herrington-Symes, A.P.; Farys, M.; Khalili, H.; Brocchini, S. Antibody fragments: Prolonging circulation half-life. *Adv. Biosci. Biotech.* **2013**, *4*, 689–698.
4. Walsh, G. Biopharmaceutical benchmarks 2014. *Nat. Biotechnol.* **2014**, *32*, 992–1000.
5. Therapeutic Antibody Data Base. Available online: [tabs.craic.com](http://tabs.craic.com) (accessed on 25 May 2015).

6. Socinski, M.A.; Curigliano, G.; Jacobs, I.; Gumbiner, B.; MacDonald, J.; Thomas, D. Clinical considerations for the development of biosimilars in oncology. *mAbs* **2015**, *7*, 286–293.
7. Elvin, J.G.; Couston, R.G.; van der Walle, C.F. Therapeutic antibodies: Market considerations, disease targets and bioprocessing. *Int. J. Pharm.* **2013**, *440*, 83–98.
8. Nelson, A.L. Antibody fragments: Hope and hype. *mAbs* **2010**, *2*, 77–83.
9. Lin, G.M.; Li, Y.H.; Jaiteh, L.E.; Han, C.L. Pexelizumab fails to inhibit assembly of the terminal complement complex in patients with ST-elevation myocardial infarction undergoing primary percutaneous coronary intervention. Insight from a substudy of the Assessment of Pexelizumab in Acute Myocardial Infarction (APEX-AMI) trial. *Am. Heart J.* **2012**, *164*, 43–51.
10. Spitali, M.; Symmons, J.; Whitcombe, R.; Pearce-Higgins, M.R. Protein Purification. Patent WO21201368A2, 2 February 2012.
11. Holliger, P.; Hudson, P.J. Engineered antibody fragments and the rise of single domains. *Nat. Biotech.* **2005**, *23*, 1126–1136.
12. Abuchowski, A.; McCoy, J.R.; Palczuk, N.C.; van Es, T.; Davis, F.F. Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase. *J. Biol. Chem.* **1977**, *252*, 3582–3586.
13. Karr, L.J.; Shafer, S.G.; Harris, J.M.; Van Alstine, J.M.; Snyder, R.S. Immuno-affinity partition of cells in aqueous polymer two-phase systems. *J. Chromatogr. A* **1986**, *354*, 269–282.
14. Jain, A.; Jain, S.K. PEGylation: An approach for drug delivery. A review. *Crit. Rev. Ther. Drug Carrier Sys.* **2008**, *25*, 403–447.
15. Chapman, A.P. PEGylated antibodies and antibody fragments for improved therapy: A review. *Adv. Drug Deliv. Rev.* **2002**, *54*, 531–545.
16. Kelley, B. Industrialization of mAb production technology. The bioprocessing industry at a crossroads. *mAbs* **2009**, *1*, 443–452.
17. Jalalirad, R. Efficient chromatographic processes for elevated purification of antibody fragment (Fab D1.3) from crude *Escherichia coli* culture. *Biotechnology* **2013**, *12*, 74–80.
18. Koussoroplis, S.-J.; Heywood, S.; Uyttenhove, C.; Barilly, C.; Van Snick, J.; Vanbever, R. Production, purification and biological characterization of mono-PEGylated anti IL-17 antibody fragments. *Int. J. Pharm.* **2013**, *454*, 107–115.
19. Eifler, N.; Medaglia, G.; Anderka, O.; Laurin, L.; Hermans, P. Development of a novel affinity chromatography resin for platform purification of lambda Fabs. *Biotechnol. Prog.* **2014**, *30*, 1311–1318.
20. Scanlan, C.; Shumway, J.; Wagner, M.; Waghmare, R. Downstream purification and formulation of Fab antibody fragments. *BioPharm. Intl.* **2014**, *27*, 42–44.
21. Weisser, N.E.; Hall, J.C. Applications of single-chain variable fragment antibodies in therapeutics and diagnostics. *Biotechnol. Adv.* **2009**, *27*, 502–520.
22. Camber, A.J.; Ward, E.S.; Winter, G.; Parnell, G.D.; Wawrzynczak, E.J. Comparative stabilities *in vitro* and *in vivo* of a recombinant mouse antibody fvcys fragment and a bisFvCys conjugate. *J. Immunol.* **1992**, *149*, 120–126.
23. Sanz, L.; Cuesta, A.M.; Compte, M.; Alvarez-Vallina, L. Antibody engineering: Facing new challenges in cancer therapy. *Acta Pharmacol. Sin.* **2005**, *26*, 641–648.

24. Nelson, A.L.; Reichert, J.M. Development trends for therapeutic antibody fragments. *Nat. Biotechnol.* **2009**, *27*, 331–337.
25. Hu, S.; Shively, L.; Raubitschek, A.; Sherman, M.; Williams, L.E.; Wong, J.Y.; Shively, J.E.; Wu, A.M. Minibody: A novel engineered anti-carcinoembryonic antigen antibody fragment (single-chain Fv-CH3) which exhibits rapid, high-level targeting of xenografts. *Cancer Res.* **1996**, *56*, 3055–3061.
26. Roque, A.C.; Lowe, C.R.; Taipa, M.A. Antibodies and genetically engineered related molecules: Production and purification. *Biotechnol. Prog.* **2004**, *20*, 639–654.
27. Monnier, P.P.; Vigouroux, R.J.; Tassew, N.G. *In vivo* applications of single chain Fv (variable domain) (scFv) fragments. *Antibodies* **2013**, *2*, 193–208.
28. Porter, R.R. The hydrolysis of rabbit Y-globulin and antibodies with crystalline papain. *Biochem. J.* **1959**, *73*, 119–126.
29. Inbar, D.; Hochman, J.; Givol, D. Localization of antibody-combining sites within the variable portions of heavy and light chains. *Proc. Natl. Acad. Sci. USA* **1972**, *69*, 2659–2662.
30. Chapman, A.P.; Antoniow, P.; Spitali, M.; West, S.; Stephens, S.; King, D.J. Therapeutic antibody fragments with prolonged *in vivo* half-lives. *Nat. Biotechnol.* **1999**, *17*, 780–783.
31. Coelho, V.; Dervede, J.; Petrausch, U.; Panjideh, H.; Fuchs, H.; Menzel, C.; Dübel, S.; Keilholz, U.; Thiel, E.; Deckert, P.M. Design, construction and *in vitro* analysis of A33scFv:CDy, a recombinant fusion protein for antibody-directed enzyme prodrug therapy in colon cancer. *Int. J. Oncol.* **2007**, *31*, 951–957.
32. Kim, G.; Wang, Z.; Liu, Y.Y.; Stavrou, S.; Mathias, A.; Goodwin, K.J.; Thomas, J.M.; Neville, D.M. A fold-back single chain diabody format enhances the bioactivity of an anti-monkey CD3 recombinant diphtheria toxin-based immunotoxin. *Protein Eng. Des. Sel.* **2007**, *20*, 425–432.
33. Carter, P. Improving the efficacy of antibody-based cancer therapies. *Nat. Rev. Cancer* **2001**, *1*, 118–129.
34. Kobayashi, N.; Odaka, K.; Uehara, T.; Imanaka-Yoshida, K.; Kato, Y.; Oyama, H.; Tadokoro, H.; Akizawa, H.; Tanada, S.; Hiroe, M.; *et al.* Toward *in vivo* imaging of heart disease using a radiolabeled single-chain Fv fragment targeting tenascin-C. *Anal. Chem.* **2011**, *83*, 9123–9130.
35. Matsuda, T.; Furumoto, S.; Higuchi, K.; Yokoyama, J.; Zhang, M.R.; Yanai, K.; Iwata, R.; Kigawa, T. Rapid biochemical synthesis of <sup>11</sup>C-labelled single chain variable fragment antibody for immuno-PET by cell-free protein synthesis. *Bioorg. Med. Chem.* **2012**, *20*, 6579–6582.
36. Zarschler, K.; Prapainop, K.; Mahon, E.; Rocks, L.; Bramini, M.; Kelly, P.M.; Stephan, H.; Dawson, K.A. Diagnostic nanoparticle targeting of the EGF receptor in complex biological conditions using single-domain antibodies. *Nanoscale* **2014**, *6*, 6046–6056.
37. Saerens, D.; Frederix, F.; Reekmans, G.; Conrath, K.; Jans, K.; Brys, L.; Huang, L.; Bosmans, E.; Maes, G.; Borghs, G.; *et al.* Engineering camel single-domain antibodies and immobilization chemistry for human prostate-specific antigen sensing. *Anal. Chem.* **2005**, *77*, 7547–7555.
38. Hariri, G.; Zhang, Y.; Fu, A.; Han, Z.; Brechbiel, M.; Tantawy, M.N.; Peterson, T.E.; Mernaugh, R.; Hallahan, D. Radiation-guided P-selectin antibody targeted to lung cancer. *Ann. Biomed. Eng.* **2008**, *36*, 821–830.
39. Zeng, X.; Shen, Z.; Mernaugh, R. Recombinant antibodies and their use in biosensors. *Anal. Bioanal. Chem.* **2012**, *402*, 3027–3038.

40. Enever, C.; Batuwangala, T.; Plummer, C.; Sepp, A. Next generation immunotherapeutics—honing the magic bullet. *Curr. Opin. Biotechnol.* **2009**, *20*, 1–7.
41. Devaux, C.; Moreau, E.; Goyffon, M.; Rochat, H.; Billiad, P. Construction and functional evaluation of a single-chain antibody fragment that neutralizes toxin AaH1 from the venom of the scorpion *Androctonus australis* hector. *Eur. J. Biochem.* **2001**, *268*, 694–702.
42. Isaksen, M.L.; FitzGerald, K. Purification and analysis of antibody fragments using proteins L, A and LA. In *Antibody Engineering*; Kontermann, R., Dübel, S., Eds.; Springer Verlag: Berlin, Germany, 2001; Chapter 21, pp. 282–291.
43. McCafferty, J.; Griffiths, A.D.; Winter, G.; Chiswell, D.J. Phage antibodies: Filamentous phage displaying antibody variable domains. *Nature* **1990**, *348*, 552–554.
44. Marks, J.D.; Hoogenboom, H.R.; Bonnert, T.P.; McCafferty, J.; Griffiths, A.D.; Winter, G. By-passing immunization: Human antibodies from V-gene libraries displayed on phage. *J. Mol. Biol.* **1991**, *222*, 581–597.
45. Clackson, T.; Hoogenboom, H.R.; Griffiths, A.D.; Winter, G. Making antibody fragments using phage display libraries. *Nature* **1991**, *352*, 624–628.
46. Pucca, M.B.; Bertolini, T.B.; Barbosa, J.E.; Galina, S.V.; Porto, G.S. Therapeutic monoclonal antibodies: scFv patents as a marker of a new class of potential biopharmaceuticals. *Braz. J. Pharm. Sci.* **2011**, *47*, 31–39.
47. Fitting, J.; Blume, T.; Ten Haaf, A.; Blau, W.; Gottenlöhner, S.; Tur, M.K.; Barth, S. Phage display-based generation of novel internalizing antibody fragments for immunotoxin-based treatment of acute myeloid leukemia. *mAbs* **2015**, *7*, 390–402.
48. Ward, E.S.; Güssow, D.; Griffiths, A.D.; Jones, P.J.; Winter, G. Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*. *Nature* **1989**, *341*, 544–546.
49. Pereira, B.; Benedict, C.R.; Le, A.; Shapiro, S.S.; Thiagarajan, P. Cardiolipin binding a light chain from Lupus-prone mice. *Biochemistry* **1998**, *37*, 1430–1437.
50. Harmsen, M.M.; De Haard, H.J. Properties, production, and applications of camelid single-domain antibody fragments. *Appl. Microbiol. Biotechnol.* **2007**, *77*, 13–22.
51. Holt, L.J.; Herring, C.; Jespers, L.S.; Woolven, B.P.; Tomlinson, I.M. Domain antibodies: Proteins for therapy. *Trends Biotechnol.* **2003**, *21*, 484–490.
52. Cossins, A.J.; Harrison, S.; Popplewell, A.G.; Gore, M.G. Recombinant production of a VL single domain antibody in *Escherichia coli* and analysis of its interaction with peptostreptococcal protein L. *Protein Expr. Purif.* **2007**, *51*, 253–259.
53. Dumoulin, M.; Conrath, K.; Van Meirhaeghe, A.; Meersman, F.; Heremans, K.; Frenken, L.G.J.; Muyldermans, S.; Wyns, L.; Matagne, A. Single-domain antibody fragments with high conformational stability. *Protein Sci.* **2002**, *11*, 500–515.
54. Hendershot, K.; Bole, D.; Köhler, G.; Kearney, J.F. Assembly and secretion of heavy chains that do not associate post-translationally with immunoglobulin heavy-chain binding protein. *J. Cell Biol.* **1987**, *104*, 761–767.
55. Prelli, F.; Frangione, B. Franklin's disease: Ig gamma2H chain mutant BUR. *J. Immunol.* **1992**, *248*, 949–952.

56. Hilschmann, N.; Craig, L.C. Amino acid sequence studies with Bence-Jones proteins. *Proc. Natl. Acad. Sci. USA* **1965**, *531*, 1403–1409.
57. Ungar-Waron, H.; Elias, E.; Gluckman, A.; Trainin, Z. Dromedary IgG purification, characterization and quantitation in sera of dams and newborns. *Israel J. Vet. Med.* **1987**, *43*, 198–203.
58. Arbabi Ghahroudi, M.; Desmyter, A.; Wyns, L.; Hamers, R.; Muyldermans, S. Selection and identification of single domain antibody fragments from camel heavy-chain antibodies. *FEBS Lett.* **1997**, *414*, 521–526.
59. Roux, K.H.; Greenberg, A.S.; Greene, L.; Strelets, L.; Avila, D.; McKinney, E.C.; Flajnik, M.F. Structural analysis of the nurse shark (new) antigen receptor (NAR), molecular convergence of NAR and unusual mammalian immunoglobulins. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 11804–11809.
60. Nuttall, S.D.; Krishnan, U.V.; Hattarki, M.; De Gori, R.; Irving, R.A.; Hudson, P.J. Isolation of the new antigen receptor from wobbegong sharks, and use as a scaffold for the display of protein loop libraries. *Mol. Immunol.* **2001**, *38*, 312–326.
61. Dimitrov, D.S. Therapeutic proteins. In *Therapeutic Proteins, Methods in Molecular Biology*; Voynov, V., Caravella, J.A., Eds.; Humana Press Clifton: New Jersey, NJ, USA, 2012; pp. 1–26.
62. Jain, R.K. Physiological barriers to delivery of monoclonal antibodies and other macromolecules in tumors. *Cancer Res.* **1990**, *50*, 814S–819S.
63. Reichert, J.M. Monoclonal antibodies as innovative therapeutics. *Curr. Pharm. Biotechnol.* **2008**, *9*, 423–430.
64. Revets, H.; De Baetselier, P.; Muyldermans, S. Nanobodies as novel agents for cancer therapy. *Exp. Opin. Biol. Ther.* **2005**, *5*, 111–124.
65. de Marco, A. Biotechnological applications of recombinant single-domain antibody fragments. *Microb. Cell Fact.* **2011**, *10*, 44.
66. Rahbarizadeh, F.; Ahmadvand, D.; Sharifzadeh, Z. Nanobody: An old concept and new vehicle for immunotargeting. *Immunol. Invest.* **2011**, *4*, 299–338.
67. Tanaka, T.; Williams, R.L.; Rabbitts, T.H. Tumour prevention by a single antibody domain targeting the interaction of signal transduction proteins with RAS. *EMBO J.* **2007**, *26*, 3250–3259.
68. Ibanez, L.I.; De Filette, M.; Hultberg, A.; Verrips, T.; Temperton, N.; Weiss, R.A.; Vandeveld, W.; Schepens, B.; Vanlandschoot, P.; Saelens, X. Nanobodies with *in vitro* neutralizing activity protect mice against H5N1 influenza virus infection. *J. Infect. Dis.* **2011**, *203*, 1063–1072.
69. Pant, N.; Hultberg, A.; Zhao, Y.; Svensson, L.; Pan-Hammarstrom, Q.; Johansen, K.; Pouwels, P.H.; Ruggeri, F.M.; Hermans, P.; Frenken, L.; *et al.* Lactobacilli expressing variable domain of llama heavy-chain antibody fragments (lactobodies) confer protection against rotavirus-induced diarrhea. *J. Infect. Dis.* **2006**, *194*, 1580–1588.
70. Dekker, S.; Toussaint, W.; Panayotou, G.; de Wit, T.; Visser, P.; Grosveld, F.; Drabek, D. Intracellularly expressed single-domain antibody against p15 matrix protein prevents the production of porcine retroviruses. *J. Virol.* **2003**, *77*, 12132–12139.
71. Hussack, G.; Arbabi-Ghahroudi, M.; van Faassen, H.; Songer, J.G.; Ng, K.K.; MacKenzie, R.; Tanha, J. Neutralization of *Clostridium difficile* toxin A with single-domain antibodies targeting the cell receptor binding domain. *J. Biol. Chem.* **2011**, *286*, 8961–8976.

72. Tang, Z.; Feng, M.; Gao, W.; Phung, Y.; Chen, W.; Chaudhary, A.; St Croix, B.; Qian, M.; Dimitrov, D.S.; Ho, M. A human single-domain antibody elicits potent antitumor activity by targeting an epitope in mesothelin close to the cancer cell surface. *Mol. Cancer Ther.* **2013**, *12*, 416–426.
73. Willems, A.; Leoen, J.; Schoonoghe, S.; Grooten, J.; Mertens, N. Optimizing expression and purification from cell culture medium of trispecific recombinant antibody derivatives. *J. Chromatogr. B* **2003**, *786*, 161–176.
74. King, D.J.; Turner, A.; Farnsworth, A.P.; Adair, J.R.; Owens, R.J.; Pedley, R.B.; Baldock, D.; Proudfoot, K.A.; Lawson, A.D.; Beeley, N.R. Improved tumor targeting with chemically cross-linked recombinant antibody fragments. *Cancer Res.* **1994**, *54*, 6176–6185.
75. Anderson, D.C.; Reilly, D.E. Production technologies for monoclonal antibodies and their fragments. *Curr. Opin. Biotechnol.* **2004**, *15*, 456–462.
76. Chan, A.C.; Carter, P.J. Therapeutic antibodies for autoimmunity and inflammation. *Nat. Rev. Immunol.* **2010**, *10*, 301–316.
77. Yokota, T.; Milenic, D.E.; Whitlow, M.; Schlom, J. Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. *Cancer Res.* **1992**, *52*, 3402–3408.
78. Johdi, N.A.; Harman, R.; Sanjuan, I.; Kousparou, C.; Courtenay-Luck, N.; Deonarain, M.P. Production and binding analyses of a humanised scFv against a cryptic epitope on tumour-associated fibronectin. *Protein Expr. Purif.* **2013**, *88*, 157–163.
79. De Vos, J.; Devoogdt, N.; Lahoutte, T.; Muylldermans, S. Camelid single-domain antibody-fragment engineering for (pre)clinical *in vivo* molecular imaging applications: Adjusting the bullet to its target. *Expert Opin. Biol. Ther.* **2013**, *13*, 1149–1160.
80. Aubrey, N.; Devaux, C.; Sizaret, P.Y.; Rochat, H.; Goyffon, M.; Billiad, P. Design and evaluation of a diabody to improve protection against a potent scorpion neurotoxin. *Cell. Mol. Life Sci.* **2003**, *60*, 617–628.
81. Todorovska, A.; Roovers, R.C.; Dolezal, O.; Kortt, A.A.; Hoogenboom, H.R.; Hudson, P.J. Design and application of diabodies, triabodies and tetrabodies for cancer targeting. *J. Immunol. Methods* **2001**, *248*, 47–66.
82. Fee, C.J.; Van Alstine, J.M. PEG-proteins: Reaction engineering and separation issues. *Chem. Eng. Sci.* **2006**, *61*, 924–939.
83. Bird, R.E.; Hardman, K.D.; Jacobson, J.W.; Johnson, S.; Kaufman, B.M.; Lee, S.M.; Lee, T.; Pope, S.H.; Riordan, G.S.; Whitlow, M. Single-chain antigen-binding proteins. *Science* **1988**, *242*, 423–426.
84. Ewert, S.; Huber, T.; Honegger, A.; Plückthun, A. Biophysical properties of human antibody variable domains. *J. Mol. Biol.* **2003**, *325*, 531–553.
85. Frenzel, A.; Hust, M.; Schirrmann, T. Expression of recombinant antibodies. *Front. Immunol.* **2013**, *4*, 217.
86. Zarschler, K.; Witecy, S.; Kapplusch, F.; Foerster, C.; Stephan, H. High yield production of functional soluble single-domain antibodies in the cytoplasm of *Escherichia coli*. *Microbiol. Cell Fact.* **2013**, *12*, 97–108.
87. Ljunglöf, A.; Lacki, K.M.; Mueller, J.; Harinarayan, C.; van Reis, R.; Fahrner, R.; Van Alstine, J.M. Ion exchange chromatography of antibody fragments. *Biotech. Bioeng.* **2009**, *96*, 515–524.

88. Three step purification process for dAb expressed in periplasm of *E. coli*. GE Application Note 29-0655-41AA. Available online: [www.gelifesciences.com](http://www.gelifesciences.com) (accessed on 14 August 2015).
89. Das, D.; Allen, T.M.; Suresh, M.R. Comparative evaluation of two purification methods of anti-CD19-c-myc-His<sub>6</sub>-Cys ScFv. *Protein Expr. Purif.* **2005**, *39*, 199–208.
90. Spitali, M. Downstream processing of antibody fragments. In *Process Scale Purification of Antibodies*; Gottschalk, U., Ed.; John Wiley and Sons, Inc.: New York, NY, USA, 2009; pp. 349–372.
91. Danielsson, Å.; Widehammar, J.; Rodrigo, G. Preparation and high resolution analysis of a Fab fragment. *FASEB J.* **2013**, *27*, Ib157.
92. Franzreb, M.; Müller, E.; Vajda, J. Cost estimation for protein A chromatography: An in silico approach to Mab purification strategy. *BioProcess Intl.* **2014**, *12*, 44–52.
93. Forsgren, A.; Sjöquist, J. “Protein A” from *S. aureus*. I. Pseudo-immune reaction with human gamma globulin. *J. Immunol.* **1966**, *97*, 822–827.
94. Björck, L. Protein L. A novel bacterial cell wall protein with affinity for Ig L chains. *J. Immunol.* **1988**, *140*, 1194–1197.
95. Reis, K.J.; Ayoub, E.M.; Boyle, M.D. Streptococcal Fc receptors. I. Isolation and partial characterization of the receptor from a group C streptococcus. *J. Immunol.* **1984**, *132*, 3091–3097.
96. Akerstrom, B.; Nilson, B.H.; Hoogenboom, H.R.; Björck, L. On the interaction between single chain Fv antibodies and bacterial immunoglobulin-binding proteins. *J. Immunol. Methods* **1994**, *177*, 151–163.
97. Protein G Sepharose Fast Flow 4 Instruction Document 71-7083-00 AI. Available online: [www.gelifesciences.com](http://www.gelifesciences.com) (accessed on 14 August 2015).
98. De Chateau, M.; Nilson, B.H.; Emtell, M.; Myhre, E.; Magnusson, C.G.; Åkerström, B.; Björck, L. On the interaction between protein L and immunoglobulins of various mammalian species. *Scand. J. Immunol.* **1993**, *37*, 399–405.
99. Hage, D.S.; Bian, M.; Burks, R.; Karle, E.; Ohnmacht, C.; Wa, C. Bioaffinity Chromatography. In *Handbook of Affinity Chromatography*, 2nd ed.; Hage, D.S., Cazes, J., Eds.; CRC Press: Boca Raton, FL, USA, 2005; p. 103.
100. Enever, C.; Tomlinson, I.M.; Lund, J.; Levens, M.; Holliger, P. Engineering high affinity superantigens by phage display. *J. Mol. Biol.* **2005**, *347*, 107–120.
101. Derrick, J.P.; Wigley, D.B. The third IgG-binding domain from Streptococcal Protein G: An analysis by x-ray crystallography of the structure alone and in a complex with Fab. *J. Mol. Biol.* **1994**, *243*, 906–918.
102. Kastern, W.; Holst, E.; Nielsen, E.; Sjöbring, U.; Björck, L.; Protein, L. A bacterial immunoglobulin-binding protein and possible virulence determinant. *Infect. Immun.* **1990**, *58*, 1217–1222.
103. Murphy, J.P.; Duggleby, C.J.; Atkinson, M.A. The functional units of a peptostreptococcal protein L. *Mol. Microbiol.* **1994**, *12*, 911–920.
104. Kastern, W.; Sjöbring, U.; Björck, L. Structure of peptostreptococcal protein L and identification of a repeated immunoglobulin light chain-binding domain. *J. Biol. Chem.* **1992**, *267*, 12820–12825.
105. Kriangkum, J.; Xu, B.; Gervais, C.; Paquette, D.; Jacobs, F.A.; Martin, L.; Suresh, M.R. Development and characterization of a bi-specific single-chain antibody directed against T cells and ovarian carcinoma. *Hybridoma* **2000**, *19*, 33–41.

106. Rouet, R.; Lowe, D.; Dudgeon, K.; Roome, B.; Schofield, P.; Langley, D.; Andrews, J.; Whitfield, P.; Jeremius, L.; Christ, D. Expression of high-affinity human antibody fragments in bacteria. *Nat. Protoc.* **2012**, *7*, 364–373.
107. Hu, W.-G.; Jager, S.; Chau, D.; Mah, D.; Nagata, L.P. Generation of a recombinant full-length human antibody binding to botulinum neurotoxin A. *Appl. Biochem. Biotechnol.* **2010**, *160*, 1206–1216.
108. Kouki, T.; Inui, T.; Okabe, H.; Ochi, Y.; Kajita, Y. Separation method of IgG fragments using protein L. *Immunol. Invest.* **1997**, *26*, 399–408.
109. Housden, N.G.; Harrison, S.; Roberts, S.E.; Beckingham, J.A.; Graille, M.; Stura, E.; Gore, M.G. Immunoglobulin-binding domains: Protein L from *Peptostreptococcus magnus*. *Biochem. Sci. Trans.* **2003**, *31*, 716–718.
110. Nilson, B.H.K.; Solomon, A.; Björck, L.; Åkerstöm, B. Protein L from *Peptostreptococcus magnus* binds to the kappa light chain variable domain. *J. Biol. Chem.* **1992**, *267*, 2234–2239.
111. Nilson, B.H.; Lögberg, L.; Kastern, W.; Björck, L.; Åkerström, B. Purification of antibodies using protein L-binding framework structures in the light chain variable domain. *J. Immunol. Methods* **1993**, *164*, 33–40.
112. Zahid, M.; Loyau, S.; Bouabdelli, M.; Aubrey, N.; Jandrot-Perrus, M.; Billiald, P. Design and reshaping of an scFv directed against human platelet glycoprotein VI with diagnostic potential. *Anal. Biochem.* **2011**, *417*, 274–282.
113. Boes, A.; Spiegel, H.; Delbrück, H.; Fisher, R.; Schillberg, S.; Sack, M. Affinity purification of a framework 1 engineered mouse/human chimeric IgA2 antibody from tobacco. *Biotechnol. Bioeng.* **2011**, *108*, 2804–2814.
114. Muzard, J.; Adi-Bessalem, S.; Juste, M.; Laraba-Djebari, F.; Aubrey, N.; Billiad, P. Grafting protein L-binding activity onto recombinant antibody fragments. *Anal. Biochem.* **2009**, *388*, 331–338.
115. Kihlberg, B.-M.; Sjöbring, U.; Kastern, W.; Björck, L. Protein LG: A hybrid molecule with unique immunoglobulin binding properties. *J. Biol. Chem.* **1992**, *267*, 25583–25588.
116. Vola, R.; Lombardi, A.; Tarditi, L.; Björck, L.; Mariani, M. Recombinant proteins L and LG: Efficient tools for purification of murine immunoglobulin G fragments. *J. Chromatogr. B* **1995**, *668*, 209–218.
117. Svensson, H.G.; Hoogenboom, H.R.; Sjöbring, U.; Protein, L.A. A novel hybrid protein with unique single-chain Fv antibody- and Fab-binding properties. *Eur. J. Biochem.* **1998**, *258*, 890–896.
118. Svensson, H.G.; Hoogenboom, H.R.; Sjöbring, U. The ultimate Ig-binding protein. *BIA J.* **1999**, *2*, 21–23.
119. Lichty, L.L.; Malecki, J.L.; Agnew, H.D.; Michelson-Horowitz, D.J.; Tan, S. Comparison of affinity tags for protein purification. *Protein Expr. Purif.* **2005**, *4*, 98–105.
120. Goel, D.; Colcher, D.; Koo, J.S.; Booth, B.J.; Pavlinkova, G.; Batra, S.K. Relative position of the hexahistidine tag affects binding properties of a tumor-associated single-chain Fv construct. *Biochim. Biophys. Acta* **2000**, *1523*, 13–20.
121. Schmeisser, H.; Kontsek, P.; Esposito, D.; Gillette, W.; Schreiber, G.; Zoon, K.C. Binding characteristics of IFN-alpha subvariants to IFNAR2-EC and influence of the 6-histidine tag. *J. Interf. Cytok. Res.* **2006**, *26*, 866–876.

122. Lowe, C.R. Combinatorial approaches to affinity chromatography. *Curr. Opin. Chem. Biol.* **2001**, *5*, 248–256.
123. Roque, A.C.; Taipa, M.A.; Lowe, C.R. An artificial protein L for the purification of immunoglobulins and fab fragments by affinity chromatography. *J. Chromatogr. A* **2005**, *1064*, 157–167.
124. Roque, A.C.; Lowe, C.R. Rationally designed ligands for use in affinity chromatography: An artificial protein L. *Methods Mol. Biol.* **2008**, *421*, 93–109.
125. Hober, S.; Nord, K.; Linhult, M. Protein A chromatography for antibody purification. *J. Chromatogr. B* **2007**, *848*, 40–47.
126. Pabst, T.M.; Palmgren, R.; Forss, A.; Hunter, A.K. Engineering of novel Staphylococcal protein A ligands to enable milder elution pH and high dynamic binding capacity. *J. Chromatogr. A* **2014**, *1362*, 180–185.
127. Watanabe, H.; Matsumaru, H.; Ooishi, A.; Feng, Y.; Odahara, T.; Suto, K.; Honda, S. Optimizing pH response of affinity between protein G and IgG Fc. *J. Biol. Chem.* **2009**, *284*, 12373–12383.
128. Protein L–Agarose from *Peptostreptococcus magnus*. Available online: [www.sigmaaldrich.com](http://www.sigmaaldrich.com) (accessed on 14 August 2015).
129. Thermo Scientific™ Pierce™ Protein Biology. Available online: [www.piercenet.com](http://www.piercenet.com) (<https://www.thermofisher.com/se/en/home/brands/thermo-scientific/pierce-protein-biology.html>) (accessed on 14 August 2015).
130. Gagnon, P.S. Enhanced purification of antibodies and antibody fragments by apatite chromatography. US8093364 B2, 2012.
131. A platform approach for the purification of antibody fragments (Fabs). Application Note 20-0320-66AA, GE Healthcare Life Sciences, 2013. Available online: [www.gelifesciences.com](http://www.gelifesciences.com) (accessed on 14 August 2015).

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