Chapter 7
Miniantibodies

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Abbreviations

IPTG	Isopropylthiogalactoside
PBS	Phosphate buffered saline
scFv	Single-chain Fv fragment
tet	Tetracycline
LB	Luria-Bertani media
SB	Super broth media

7.1 Introduction

7.1.1 Motivation

The usual motivation to create a multivalent molecule is to increase its functional affinity (avidity) to a corresponding multimeric antigen structure, which can be a cell surface, a virus surface or a fibrous polymer. Obviously, no increase in affinity to a soluble monomeric antigen can be expected. However, an increased functional affinity to a formally monovalent antigen will usually be observed when it is immobilized on a densely packed surface (e.g. on an ELISA plate or a BIAcore chip). The increased size of the antibody fragment upon multimerization, in conjunction with higher functional affinity, can also lead to improved tumor localization (Hu et al. 1996; Todorovska et al. 2001; Deyev et al. 2003; Kubetzko et al. 2006). Besides causing an avidity gain, bivalent binding might also result in agonistic activity, which may or may not be desired in a particular application.

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It is essential to understand that this avidity gain, which can be quantified (see, e.g. Crothers and Metzger (1972); Plückthun and Pack (1997); Müller et al. (1998b)), is a phenomenon closely linked to geometry. The bivalent molecule must be able to reach two epitopes at the same time. Thus, both the distance and the relative orientation of the binding sites will be crucial. It is, therefore, not the same if the two binding sites are linked head to tail, head to head, or tail to tail, even when connected with flexible linkers.

In the IgG molecule, the two binding sites are far enough apart to often reach epitopes from two adjacent protein molecules. The inherent (approximate) C2 symmetry of the antibody is ideal if the two antigens are also related by an approximate twofold axis. In a membrane, where proteins have some mobility, such an arrangement is very often possible. From such considerations we designed the "miniantibodies" to mimic the geometry of IgG molecules, using components which can be made conveniently in bacteria.

A further advantage of this format is that two different specificities can be combined within one miniantibody, offering numerous applications in biotechnology, diagnostics, and potentially therapy. Such applications can include the cross-linking of two cells or, alternatively, binding to two epitopes on the same cell can increase avidity and possibly selectivity. Finally a bispecific molecule can bring a payload to a cell by one arm binding to the cell, the other to the payload.

Dimeric miniantibodies can also be of interest as capture molecules: when immobilized on plastic support, at least one of the molecule's binding sites usually remains functional, while the other eventually may denature upon binding to the surface. In contrast, monovalent scFv fragments normally lose their antigen binding capability upon immobilization to plastic.

7.1.2 Overview of Multimerization

Three principal concepts exist to multimerize antibody scFv fragments (Plückthun and Pack 1997). The first – being the subject of this chapter – is to connect them, usually via a flexible hinge region, to a module or domain that will itself multimerize. We have termed the resulting constructs "miniantibodies" (Pack and Plückthun 1992), as they recreate the basic flexible disposition of the two binding sites of IgG molecules in a smaller assembly. The ability to multimerize antibody formats other than scFv fragments by using the same strategy is readily apparent.

The second possibility is to shorten the linker between the antibody V_L and V_H domains so that a monovalent fragment cannot form (Holliger et al. 1993; Todorovska et al. 2001), creating so-called dia-, tri- or tetrabodies. The third alternative is to connect two or more scFv fragments linearly with flexible linkers (Kellner et al. 2008). These two latter approaches have also been combined successfully in the past (Kipriyanov et al. 1999). While easy to draw as a cartoon, it must be remembered that domains of natural antibody domains are quite aggregation-prone, especially during folding (i.e. expression) and will in such assemblies also

generate partially folded domains that can lead to inactive and heterogeneous products. It is not secured, therefore, that the desired molecular assembly will actually form in reality in good yield in every case, nor that it will have the prescribed binding properties.

The "miniantibody" concept has been the attempt to create a structure with the same "wing span" as an IgG, but composed of modules which can be readily expressed in *E. coli*. This is achieved by the oligomerization domains which are not directly located downstream of the C-terminus of the scFv fragment but rather separated via a spacer, such as the upper hinge region from murine or human IgG3. This confers rotational freedom and flexibility very similar to that of the Fab-arms of full-length antibodies. As the oligomerization domains in the vectors presented here are encoded in different modular gene cassettes, it is possible to switch formats readily between these multimerized antibody fragments by simple cloning, independent of their structural properties (Willuda et al. 2001). Also, many fusions to other oligomeric modules, to enzymes and other proteins have been previously analyzed regarding their dimerization or multimerization potential (Plückthun and Pack 1997; Zhang et al. 2004).

7.1.3 Miniantibodies

The basic concept for all miniantibody constructs is the fusion of the scFv fragment to an oligomerizing element. In the simplest case, this self-associating peptide domain is an amphipathic, α-helix-forming stretch of amino acids (usually between 16 and 40 residues, see Table 7.2 for details) attached to the scFv via a flexible hinge region, giving both partners enough steric freedom to fold individually. As schematically outlined in Fig. 7.1, this leads to dimeric or tetrameric miniantibodies, depending on the oligomerization motif chosen. Most conveniently, the miniantibodies are expressed in the bacterial periplasm to allow the formation of disulfide bonds in their scFv part. All amphipathic oligomerization helices presented in Table 7.1 were therefore chosen to be compatible with periplasmic folding and the transport through the bacterial membrane, causing no significant problems in the folding and expression of most scFv fragments tested.

7.1.4 Dimeric Miniantibody Constructs

While most methods for the formation of bivalent or bispecific antibody fragments require a significant reconstruction of the format compared to the scFv, the generation of dimeric miniantibodies is simply achieved by adding an oligomerizing sequence to the C-terminus of the scFv fragment. Examples for such self-associating modules are the naturally occurring dimerization helix from the yeast transcription factor GCN4 (Fig. 7.1a) (scFv-ZIP; O'Shea et al. 1991; Dürr et al. 1999), the C_H3/

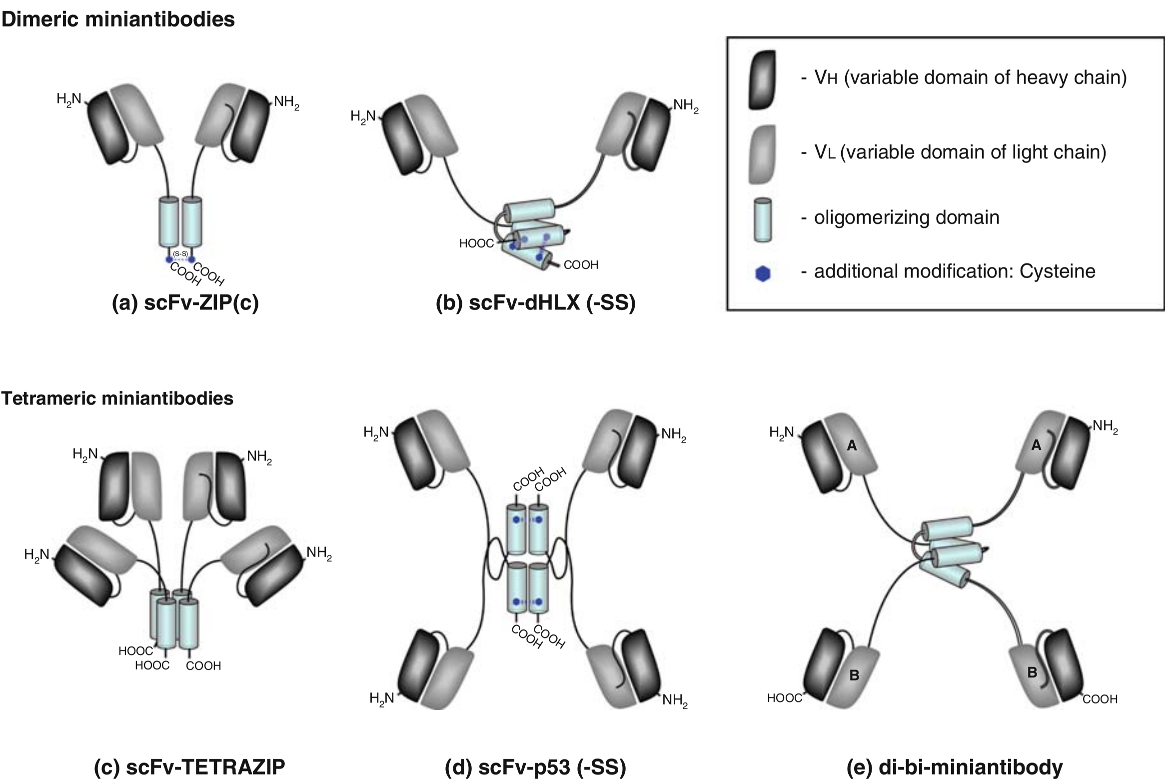

Fig. 7.1 *Schematic representation of oligomeric miniantibody formats*. (**a**) Dimeric GCN4 leucine zipper, scFv-ZIP, the optional disulfide bond is shown in parentheses and with a *dotted line*; (**b**) dimeric helix-turn-helix module, scFv-dHLX, the optional disulfide bonds are shown as *dotted lines*; (**c**) tetrameric modified GCN4 leucine zipper, scFv-TETRAZIP; (**d**) tetramerizing domain of human p53, scFv-p53, the optional disulfide bonds are shown as *dotted lines*; and (**e**) $(scFv)_A$-hinge-dHLX-hinge-$(scFv)_B$ arrangement, di-bi-miniantibody. In each case, V_H and V_L domains of the scFvs are represented in *darker* and

Table 7.1 Cross-references between oligomerizing elements and corresponding plasmids/literature

Construct	Upper hinge	Self-associating peptide	Modifications	Plasmid	Reference
Bivalent					
scFv-ZIP	Murine IgG3	GCN4 leucine zipper	–	pACKZIP	A, B, C, D
scFv-ZIPc	Murine IgG3	GCN4 leucine zipper	C-terminal Cys	pACKZIPc	C, D
scFv-dHLX	Murine IgG3	Helix1-turn-Helix2	–	pAK500	H
scFv-dHLX-SS	Murine IgG3	Helix1-turn-Helix2	Internal Cys	pAK500-SS	J
Bispecific					
scFv-JUN	Murine IgG3	JUN leucine zipper	–	pACKIHJUN	I
scFv-FOS	Murine IgG3	FOS leucine zipper	–	pACKFOS	I
CH1-CL	Murine IgG3	CH1 and CL from IgG	–	pKM30425 M1CHCL	F
Tetravalent					
scFv-TETRAZIP	Murine IgG3	GCN4 leucine zipper, modified	–	pACKtZIP	A, D
scFv-p53	Human IgG3	Oligomerization domain of human p53	–	pMStetp53His	E
scFv-p53-SS	Human IgG3	Oligomerization domain of human p53	Internal Cys	–	J
Tetravalent/bispecific					
di-bi	Murine IgG3	Helix1-turn-Helix2	–	pKM310M1dhlx 425h	G

Important elements of various miniantibody formats are listed as overview. For exact amino acid sequences of the elements, see Table 7.2. Vectors carrying miniantibody genes in these formats and references are given. Letters in the reference column denote: (A) Pack et al. 1995; (B) Pack et al. 1993; (C) Pack and Plückthun 1992; (D) Ge et al. 1995; (E) Rheinnecker et al. 1996; (F) Müller et al. 1998c; (G) Müller et al. 1998b; (H) Krebber et al. 1997; (I) Plückthun and Pack 1997; (J) Kubetzko et al. 2006

Fig. 7.1 (continued) *lighter color*, respectively. Linker and hinge regions are shown as *black lines* (either filled or not, indicating different polypeptide chains within homo- or heterodimeric constructs). The respective helical elements responsible for oligomerization are depicted as grey cylinders. Their orientation is derived from the published crystal structures of the coiled coil (PDB 2zta) (O'Shea et al. 1991), tetrazipper (PDB 1gcl) (Harbury et al. 1993), the NMR structure of the designed dHLX motif (PDB 1qp6) (Hill and Degrado 1998) and both NMR and X-ray structures of the p53 tetramerization domain (PDB 1aie) (Jeffrey et al. 1995; Mittl et al. 1998). Several of the constructs have also been modified with additional cysteines to allow disulfide formation (Kubetzko et al. 2006). This is schematically indicated by *dots* for the cysteines and *dotted lines* for the disulfide bonds. These cysteine modifications are optional, and the expression yield is generally higher when not using the cysteine modified modules

F_c-domains of antibodies (see Chap. 30) or synthetically designed two- or four-helical bundle elements (Eisenberg et al. 1986). The latter motif consists of a helix-turn-helix motif fused to the scFv with two of them "clasping" each other (see scFv-dHLX in Fig. 7.1b).

Bispecific miniantibodies can be created if two different scFvs are chosen and fused to modules forming specific heterodimers. However, as not all heterodimerizing modules also work well in vivo, problems of homodimerization and proteolytic susceptibility have to be taken into consideration. The question of specific heterodimerization was tackled using an in vivo selection approach with different libraries for both helices (Arndt et al. 2000) resulting in coiled coil helices which showed very good behavior with regard to stability, heterospecificity, and resistance to proteases (Arndt et al. 2001).

7.1.5 Tetrameric Miniantibody Constructs

Specific amino acid exchanges in all hydrophobic contact positions a and d of the GCN4 zipper (reviewed by Woolfson 2005) result in the self-assembly of a stable tetrameric bundle (Harbury et al. 1993), and fusing this modified zipper version to a scFv leads to tetrameric miniantibodies (scFv-TETRAZIP in Fig. 7.1c; Pack et al. 1995). A low immunogenicity can be expected for the fusion of a humanized scFv with the tetramerization domain of human p53 (Jeffrey et al. 1995; Rheinnecker et al. 1996), as it also uses a human IgG3 hinge (Table 7.1).

7.1.6 Extensions of the Miniantibody Concept

To further stabilize the multimeric formats, intermolecular disulfide bonds were designed and introduced in variants of the scFv-dHLX and scFv-p53 formats, resulting in the respective SS mutants (see Table 7.2). The presence of the newly introduced cysteine residues (two in the dimerization motif dHLX and one in p53) was shown to cause the formation of covalent cross-links of the self-associated peptides, thus increasing their stability (Kubetzko et al. 2006). On the basis of similar considerations, single cysteine residues can also be added to the C-terminus of the leucine zipper (c variant in Fig. 7.1a), resulting in covalent linkage by disulfide bond formation (Pack and Plückthun 1992). However, it has to be kept in mind that incorrect disulfides may also be formed in these constructs with additional cysteines, leading to slightly lower yields of correctly folded multimeric antibody fragments.

A combination of directed bivalency with bispecificity can be obtained by using so-called "di-bi-miniantibodies" (Müller et al. 1998a). In this construct, a second scFv is fused downstream the dimerization motif, resulting in a $(scFv)_A$-hinge-dHLX-hinge-$(scFv)_B$ arrangement (Fig. 7.1e).

Table 7.2 Amino acid sequences of hinges and oligomerizing elements

Construct	Element	Amino acid sequence
	Murine IgG3 upper hinge	PKPSTPPGSS
	Human IgG3 upper hinge	TPLGDTTHTSG (present in scFv-p53 constructs)
scFv-ZIP	GCN4 leucine zipper	RMKQLEDKVEELLSKNYHLENEVARLKKLVGER
scFv-ZIPc	GCN4 leucine zipper – Cys	RMKQLEDKVEELLSKNYHLENEVARLKKLVGER–GG**C**GG
scFv-dHLX/di-bi	Helix1-turn-helix2 – spacer – $(His)_5$	GELEELLKHLKELLKG-PRK-GELEELLKHLKELLKG–*GSGGAP*–HHHHH
scFv-dHLX-SS	Helix1-turn-helix2 with two internal disulfide bonds	GELEELLKHLKELLKG-PRK-GEL**C**ELLKHLKEL**C**KG–*GSGGAP*–HHHHH
scFv-JUN	JUN leucine zipper	RIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVMNY
scFv-FOS	FOS leucine zipper	LTDTLQAETDQLEDKKSALQTEIANLLKEKEKLEFILAAH
scFv-TETRAZIP	GCN4 leucine zipper, modified	R**L**KQ**I**EDK**L**EE**I**LSK**L**YH**I**ENE**L**AR**I**KKL**L**GER
scFv-p53	Oligomerization domain human p53 – spacer – $(His)_5$	KPLDGEYFTLQIRGRERFEMFRELNEALELKDAQAGKEP–*GGSGGAP*–HHHHH
scFv-p53-SS	Oligomerization domain human p53 with one internal disulfide bond	KPLDGEYFTLQIRGRERFEMFRELNE**C**LELKDAQAGKEP–*GGSGGAP*–HHHHH

The amino acid sequences (one-letter code) of various oligomerizing modules and hinges are given. In the modified GCN4 leucine zipper, which leads to tetramerization, the exchanged amino acids are in *bold-face* as are the cysteine residues introduced for stabilization purposes in other variants. The histidine-tags for detection and purification purposes are *underlined*. The amino acids EF and the end of some constructs were introduced for an *Eco*RI-restriction site. Cross-references to the corresponding vectors and literature are listed in Table 7.1

7.2 Materials

- Standard molecular biology equipment and reagents for the following objectives:
  - Performing PCR reactions
  - Digesting and gel purifying DNA
  - Ligating and transforming DNA
  - Performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent immunoblotting
- An appropriate vector system for expression of scFvs fused to an oligomerization domain (see Table 7.1 and Fig. 7.1, see also Chaps. 3 and 27)
- Cell disrupting instrument like a French Press (Aminco Rochester, NY, USA) or a TS 1.1 benchtop (Constant Systems Ltd. UK)

7.3 Procedure

The cloning of the miniantibodies (Figs. 7.1 and 7.2) follows standard procedures. We describe here the expression of miniantibodies in the periplasm of *E. coli* in shake flasks. A purification scheme using rapid coupled two-column purification is given in Chap. 27.

The procedure essentially follows the protocol as described earlier in Lindner and Plückthun (2001), which was based on Pack and Plückthun (1992). Detailed information on high-cell-density fermentation of miniantibodies on gram scales is given in Pack et al. (1993), Horn et al. (1996), Plückthun et al. (1996), and Schroeckh et al. (1996).

1. Select one of the presented formats for the chosen scFv (Table 7.1) and pick the appropriate vector (Table 7.2 and Fig. 7.2).

   Note: The vectors shown here and those in Chaps. 3 and 27 are modular and largely compatible because of matching restriction sites. In the pAK vectors, the scFv fragment is cloned between the (asymmetric) *Sfi*I sites, as discussed in detail in Chap. 3. The different dimerization or multimerization elements (Table 7.2) can be exchanged between the *Eco*RI and *Hin*dIII sites in most vectors (except, currently, the ones for the scFv-ZIPc and di-bi constructs). The rest of the backbone (e.g. between *Hin*dIII und *Xba*I) can be exchanged to coexpress a molecular chaperone (Chap. 27). In the pAK vectors, the region upstream of the scFv fragment (e.g. between *Xba*I and *Sfi*I, Fig. 7.2) can be exchanged to replace the Shine–Dalgarno sequence with a stronger version (Chap. 3).
2. If recloning from another expression vector without compatible *Sfi*I sites, design suitable primers, using, if desired, the information in Chap. 3 as a guide.

   Note: A protocol on how to PCR amplify an antibody with an unknown sequence from hybridoma or spleen cells and how to convert it into a scFv format compatible with this vector is given in Chap. 3.

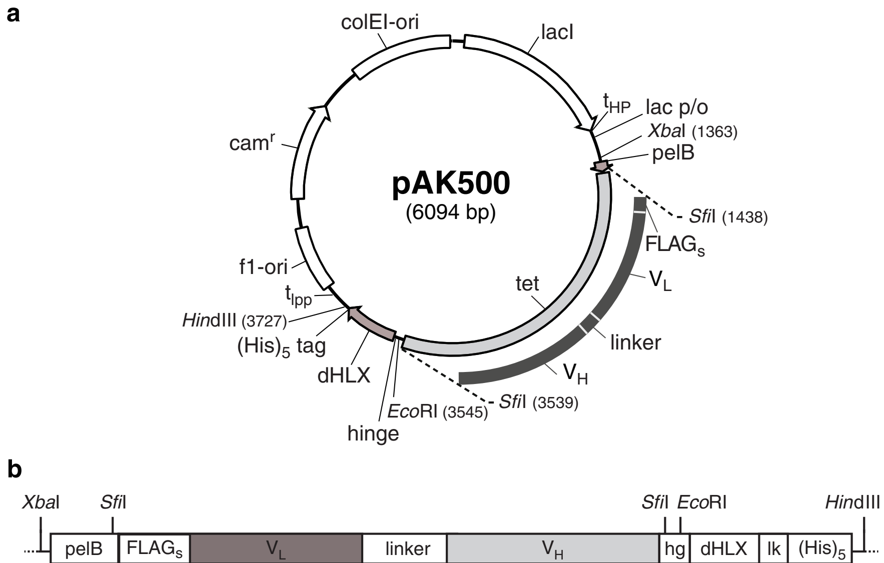

Fig. 7.2 *Expression vector pAK500 and schematic organization of the scFv-dHLX*. (**a**) Vector pAK500 (Krebber et al. 1997) encoding the dHLX cassette for the creation of a scFv construct in the scFv-dHLX format is shown as an example of a dimerization module. Other dimerization modules can be placed similarly between *Eco*RI and *Hin*dIII. The vector shown still contains the tetracycline-resistance cassette as stuffer to be replaced by the antibody scFv gene using the *Sfi*I cleavage sites (see Chap. 3). The resulting scFv insert is shown as the outer segment. Because of the compatibility between pAK vectors and the pJB series (Chaps. 3 and 27), elements (e.g. a stronger Shine Dalgarno sequence, absence of f1-ori, or a chaperone coexpression element) can be exchanged between different vectors. *lacI*: *lac* repressor; t_{HP}: strong upstream terminator to prevent read-through from LacI expression; *lac* p/o: *lac* promoter/operator; *pelB*: signal sequence (pectate lyase gene of *Erwinia carotovora*), modified to contain an *Sfi*I site; *tetR*: tetracycline resistance "stuffer" cassette (contains *tetA* and *tetR*-genes; 2,101 bp); *hinge*: murine IgG3 hinge region (see Table 7.2); *dHLX*: double helix element (see Table 7.2); *(His)*$_5$ *tag*: stretch of 5 histidine residues for IMAC purification (Lindner et al. 1992) and detection with an anti-his tag antibody (e.g. 3D5-phosphatase fusion (Lindner et al. 1997; Kaufmann et al. 2002)); t_{lpp}: downstream terminator; *f1 ori*: intergenic region of phage f1 (for production of single-stranded DNA); *cam*^{r}: chloramphenicol-acetyl-transferase gene; *colE1-ori*: plasmid replication origin (derived from pUC-plasmid series). (**b**) Schematic overview of the miniantibody construct (V_L-linker-V_H-dHLX fusion). *FLAG*$_s$: shortened (DYKD) version of FLAG tag for western blot detection (Knappik and Plückthun 1994); *hg*: murine IgG3 hinge region (see Table 7.2); *lk*: linker. Note: The size of the genetic elements is not drawn to scale. For more detail of the upstream and downstream region of the constructs, see Chap. 3

3. PCR amplify and clone the scFv in the selected vector. Confirm the correct arrangement of scFv and oligomerization domain in the final vector by DNA sequencing.

   Note: Some scFv fragments, especially when multimerized by any method, can become aggregation prone and potentially lead to growth defects of the

strain. In order to properly characterize the final construct before expression, it is recommended to add 1% glucose to all growth media in order to reduce expression before induction. The *lac* promoter/operator systems used here are under the control of the catabolite activator protein (CAP) and thus require the absence of glucose for full induction, or, conversely, are repressed by high glucose.

4. For expression, transform an *E. coli* host suitable for periplasmic expression (e.g. JM83 (Yanisch-Perron et al. 1985), RV308 (Maurer et al. 1980) or SB536 (Bass et al. 1996)).

   Note: JM83 is a generally robust strain that appears to lead to less lysis of the outer membrane upon periplasmic expression of some antibody fragments than some other strains (see also Chap. 27), RV308 is a strain that produces very little (inhibitory) acetate during growth to high cell densities and thus supports fermentation very well, and SB536 is deficient in two periplasmic proteases, HhoA (or DegQ) and HhoB (or DegS).
5. Inoculate a 20 ml pre-culture in LB medium (containing the appropriate antibiotic and 1% glucose) with a single bacterial colony harboring the plasmid encoding the respective scFv fragment. For this volume, use at least a 250 ml shaking flask. Incubate at 24°C overnight.
6. From this overnight culture, inoculate the main culture in SB medium containing 0.1% glucose at a starting OD_{600} of 0.1. Use a baffled shake flask for higher final cell densities to secure aeration. Shake at 24°C and add 1 mM IPTG (final concentration) at an OD_{600} of 0.5.

   Note: For most scFv fragments or miniantibody constructs, usage of only 0.1% glucose in the expression culture upon starting is recommended. In the majority of cases, this amount of glucose is enough to efficiently repress protein expression for 3–4 h until the culture has reached the OD required for induction. If higher concentrations of glucose are used, IPTG-induced protein expression might fail or be delayed in these CAP-regulated systems. However, there are some aggregation-prone scFv fragments which require the presence of 1% glucose at the time of inoculation. Whether this applies to the scFv of interest has to be tested individually.

   Note: The growth at room temperature is generally very beneficial for increasing the yield. At higher temperatures, not only does a more significant portion of many antibody fragments end up in the insoluble periplasmic fraction, but also incorrectly folded antibody fragments (or aggregates) interfere with membrane assembly, leading to an induced leakiness of the outer membrane and product loss.

   Note: If the expression vector carries the *skp* or *fkpA* gene (Chap. 27), much higher cell densities can be obtained, as the cells usually do neither lyse nor stop growing after induction (for details, see Chap. 27). Alternatively, a set of chaperones can be coexpressed on a second plasmid (Chap. 27).
7. Harvest the cells 4 h after induction by centrifugation (5,000 *g* for 10 min at 4°C)

   Note: This expression time is an average value, which depends on the aggregation properties of the construct and any proteolytic degradation, e.g.

in linker regions of fusion proteins. Robust constructs or constructs expressed in combination with overexpression of chaperones (Chap. 27) can be expressed for longer times.

8. Resuspend the cell pellet carefully in 1/100 column volume of loading buffer and add Benzonase (Merck) to a final concentration of 10 U/ml for removal of nucleic acids. Which loading buffer to use depends on the subsequent purification method chosen for the miniantibody. If the construct carries a His tag for IMAC purification (as for some of the constructs in Table 7.1 or pAK500, Fig. 7.2), it is recommended to use cold 50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0.

   Note: This and all subsequent steps should be carried out at 4°C in order to minimize protease activity and to stabilize the protein of interest.

   Note: To reduce protein degradation, protease inhibitors can be added to the solubilized cells. Proteolysis can be an issue for some fusion proteins, especially with positively charged residues in or near the linker region. However, the commercial protease inhibitor cocktails are mostly targeting eukaryotic proteases and are thus not very effective against *E. coli* proteases. Also, proteolysis, if it occurs by periplasmic enzymes, frequently begins during the induction phase, and can therefore only partially be combated with inhibitors.

   Note: In the product literature, Tris buffers are generally not recommended for IMAC, as their amines might interact with immobilized metal ions. However, we and others found that such buffer conditions do not influence the absorption of proteins containing hexa-histidine-tags, but rather keep some *E. coli* proteins from nonspecifically interacting with the chelating column matrix.

   Note: For troubleshooting, aliquots of the original culture and the supernatant after centrifugation should be kept and analyzed for scFv expression by SDS-PAGE and immunoblotting. These samples could pinpoint problems of the expression itself, compared to difficulties with the isolation and purification steps afterwards.

9. Disrupt the cells using a French Press (20,000 psi, 4°C in a cold room), the TS 1.1 benchtop, or sonification. For the French Press, perform at least three passages for optimal lysis of the cells. For all methods, take care that the cell suspension is not heated by the treatment.

10. Centrifuge the crude extract in order to separate insoluble cell debris from soluble protein (20,000 *g*, 30 min at 4°C). Carefully separate supernatant from pellet.

    Note: The soluble/insoluble distribution of the miniantibody expression can be analyzed by performing a western blot (see also Chap. 27). Since antibody fragments can form soluble aggregates, however, a mere inspection of western blots may be misleading. Therefore, a serious characterization of an antibody construct must include gel chromatography, ideally coupled with multi-angle light scattering. This will give a very clear description of the amount of soluble aggregates in a preparation, or their development over time.

Note: Successful transport to the periplasm can be inferred by the correct processing of the signal sequence. This can be detected by the anti-FLAG M1 antibody Sigma-Aldrich recognizing the processed FLAG tag at the very N-terminus ($^{+}H_3N$-DYKD. . .) (Knappik and Plückthun 1994), as the antibody does not recognize the tag when it is not at the N-terminus. This N-terminal short FLAG is present in the vector systems used here (this chapter, Chap. 3, Chap. 27).

11. Filter the supernatant through a 0.22 μm filter (use filters with low protein binding properties, e.g., Durapore filters from Millipore).
12. Apply the filtered supernatant of step 10 to the appropriate chromatography column.

    Note: Purification of antibody fragments using a rapid, directly coupled two-column procedure (IMAC and ion exchange chromatography) is presented in detail in Chap. 27.

7.4 Troubleshooting

While in general, the miniantibody strategy has been found to be quite robust, an intrinsic aggregation tendency of the scFv fragment is amplified by having several copies in one molecular assembly. If the protein of interest is mainly insoluble, the following procedures might be beneficial:

(a) Co-express one or several molecular chaperones which may increase the level of soluble expression (see Chap. 27, Bothmann and Plückthun 1998, 2000). Note, however, that the coexpression may merely shift insoluble aggregates to soluble aggregates. It is mandatory, therefore, to properly analyze the purified protein for oligomeric state by gel filtration, ideally coupled with multi-angle light scattering.

(b) Refold the protein from inclusion bodies. For this purpose, first reclone the scFv without any signal sequence into a plasmid with the strong T7-expression system (Ge et al. 1995). Refolding has to be optimized for each protein individually, but Huston et al. (1991), Ge et al. (1995) and Rudolph and Lilie (1996) give some initial guidelines. Commercial refolding kits are available, facilitating the screening for optimal conditions (Hampton Research, Laguna Niguel, CA, USA).

(c) Either introduce mutations in the scFv gene which may support proper folding or transplant the CDRs to a well-folding framework, thus leading to reduced aggregation. For an initial guidance, see Ewert et al. (2004). For additional discussions on this topic, see Knappik and Plückthun (1995), Jung and Plückthun (1997), Nieba et al. (1997), Willuda et al. (1999), Kaufmann et al. (2002), Honegger et al. (2009) or Kügler et al. (2009).

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