

Searching for Molecular Solutions – Cited Notes

CHAPTER 7

These Files contain details on all references to this ftp site within **Chapter 7** of *Searching for Molecular Solutions*. The page numbers of the book where the reference is made are shown in the Table below, the corresponding page number for this file, and the title of each relevant section.

Contents:

Book Reference Page Number	Page Number in this File	Section	
		No.	Title
235	2	<u>18</u>	T Cell Receptor Technology and Applications
245	12	<u>19</u>	Minimized Antibodies

Section 18: ***T Cell Receptor Technology and Applications***

Cited on p. 235 of *Searching for Molecular Solutions*

T cell receptors have taught us some intriguing aspects of molecular recognition, introduced in Chapter 3 of *Searching for Molecular Solutions*. In particular, the T cell receptor's 'obsession' with recognizing peptides only in the context of major histocompatibility complex (MHC) proteins is of interest. This demonstrates that a protein-mediated binding interaction can combine recognition of highly diverse elements within a specific constant framework. For this to be possible, an extended recognition surface is required, such as that provided by T cell receptor (and antibody) complementarity-determining regions (CDRs). A generalized artificial analog of T cell recognition would be the engineering of a library of binding molecules against targets with both constant framework regions (equivalent to MHC) and many natural variants (equivalent to MHC-bound peptides). In practice, it is usually easier to use antibody libraries or a variant thereof to find binders for such targets, but it is also possible to display libraries of T cell receptors in an equivalent manner (as we will see shortly).

The general design similarities between immunoglobulin and T cell receptor antigen recognition structures might lead one to propose that it should be possible to obtain antibodies whose CDRs could fulfill the same recognition feats towards peptide in the context of MHC as routinely performed by T cells. In other words, why should it not be possible to come up with an antibody which acts like a T cell receptor in terms of what it 'sees'? Although this proved difficult with conventional hybridoma technology, the limitations were not with antibody structures themselves, but the inherent biological imperatives involved. T cell receptors have an innate bias towards the recognition of MHC (mediated through the limited germline variability of their CDR1 and CDR2 regions, as noted in

Chapter 3 of *Searching for Molecular Solutions*), which is not the case for antibodies. Also, B cells whose surface immunoglobulins have affinity towards self-MHC will tend to be ablated, and significant *in vivo* amounts of antibodies against peptide : MHC complexes could seriously interfere with normal immune signaling. But *in vitro* antibody display libraries have no such limitations, and through this play antibodies with selective binding for peptide : MHC complexes have indeed been isolated ^{1,2}. Nonimmunized Fab fragment libraries have successfully yielded clones binding specific complexes of peptide and human MHC (HLA), by selecting *in vitro* with peptide : MHC bearing an affinity tag ³. Expression within T cells of these antibody variable regions (fused with T cell receptor domains which mediate intracellular signaling) enabled a cytotoxic T cell response to be mounted against cells expressing the target peptide : MHC complex ⁴. Studies have also shown that 'T cell receptor-like' antibodies can be used as direct immunotoxin conjugates against target cells expressing the peptide : MHC complex of interest ⁵.

Yet the biology of T cells restricts normal *in vivo* responses to foreign peptides in the context of self-MHC. Another pathway for inserting antibodies into what are normally T cell functions has been the fusion of antibody recognition domains with signaling domains for T cell-related activities. These 'T-bodies' (Fig. 7.Na) then can combine the generalized antigen recognition of antibodies with the cellular effector functions of T cells ♡, especially cytotoxicity ^{8,9}. In particular, T-bodies are no longer constrained by T cell MHC restriction. For example, when expressed within T cells, T-bodies have potential for adapting the host T cell into a cytotoxic effector towards a non-MHC-associated surface viral or tumor antigen in any human individual ⁸, whereas a comparable conventional cytotoxic T cell could only be active in individuals whose MHC background matched its recognition specificity.

♡Since T cell receptors lack large cytoplasmic domains, T cell receptor signaling acts through accessory proteins, particularly CD3 ^{6,7}. Other accessory signaling domains have been useful for T-body construction ⁸.

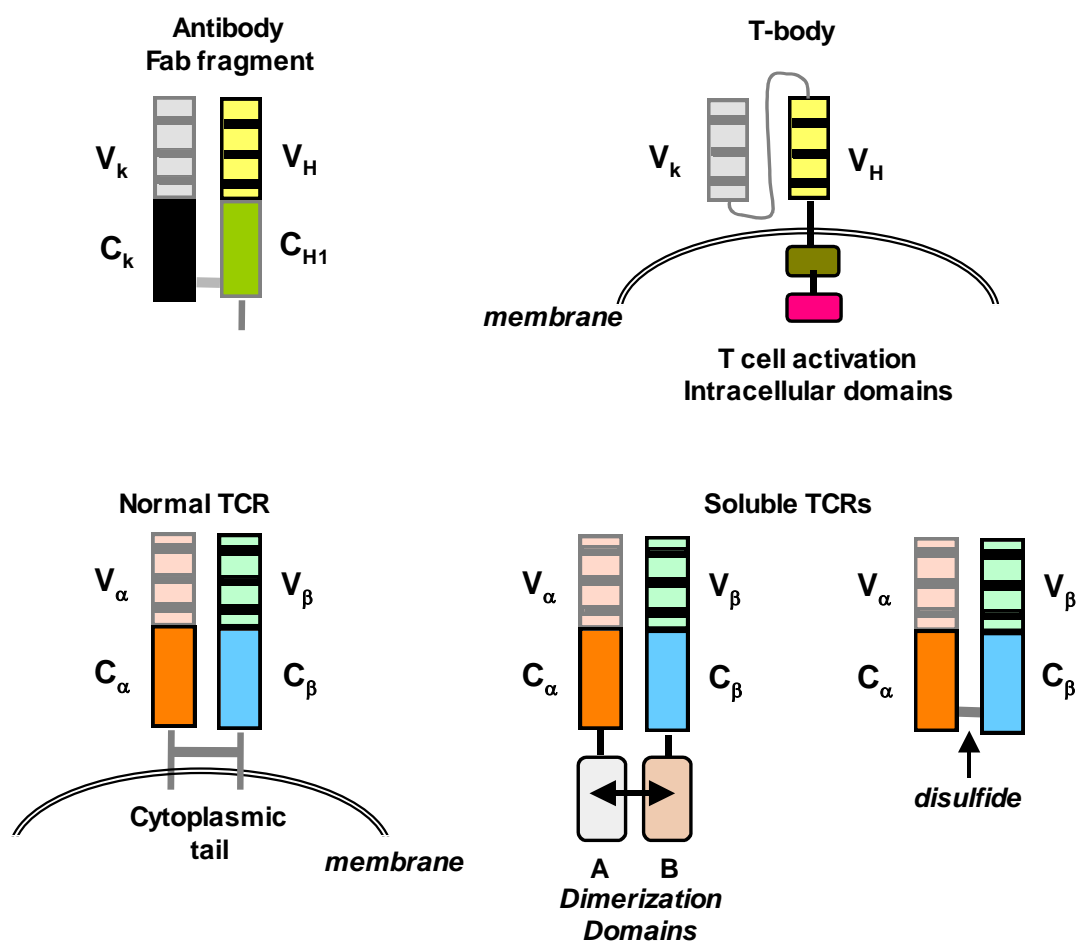


Fig. 7.Na

Antibodies anchored like T cells ('T-bodies'), and soluble T cell receptors (TCRs). The representative T-body schematic (top) is a single-chain Fv fragment appended to a transmembrane segment and separate intracellular signaling domains for T cell activation. Soluble T cell receptors have the transmembrane segment replaced by domains promoting the formation of heterodimers (A and B) or via engineered disulfide linkages.

The opposite of rendering antibodies more like T cell receptors is (naturally enough) to generate T cell receptors with antibody-like properties. A very pronounced difference between antibodies and T cell receptors is the exclusive expression of the latter as membrane-bound proteins, while antibodies naturally have both membrane and soluble forms. Although normally membrane-anchored, T cells can be artificially engineered into a soluble form by the procedure of replacing the C-terminal regions (including the transmembrane domain) with a soluble alternative conducive to α / β chain dimer formation. Single-chain α / β T cell surface receptors joined by a flexible linker (in an analogous manner as for antibodies) can be expressed, but appear to require the presence of one of the constant domains (C β □ unlike antibody scFv fragments (Figs. 7.2, 7.3 of *Searching for Molecular Solutions*; and also the color version of latter from the same ftp site).¹⁰ The best generalizable approaches for making soluble T cell receptors have fused the α and β chains with self-interactive segments capable of driving the desired heterodimerization^{11,12}, or introduced new disulfide linkages within the constant regions ♥ (Fig. 7.Na)¹³. A soluble T cell receptor retains its original peptide : MHC specificity, and thereby becomes (at the level of recognition) functionally analogous to antibodies selected for recognition of peptide : MHC complexes, which we considered above. The recognition modes of the various antibody and T cell receptor constructs we have been considering are shown schematically in Fig. 7.Nb.

♥T cell receptor α and β chains are naturally stabilized by a disulfide proximal to the cell membrane (as depicted in Fig. 7.Na), but this has been found unsuitable for generation of soluble α / β heterodimers¹³.

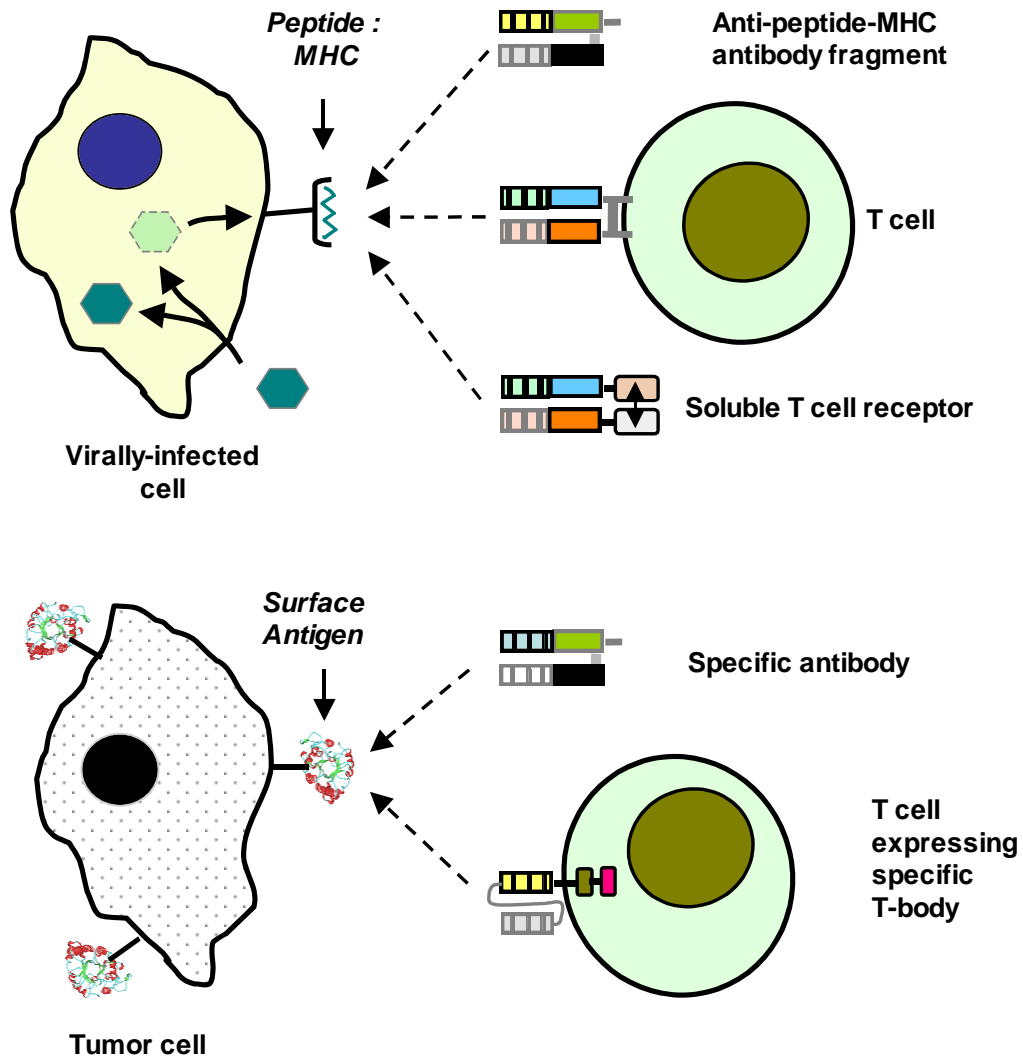


Fig. 7.Nb

Top, Depiction of recognition of viral peptide : MHC on surface of an infected cell by conventional T cell, soluble T cell receptor, or antibody selected *in vitro* for same specificity against peptide : MHC as for corresponding T cell receptor. *Bottom*, tumor cell expressing a surface antigen (not presented by MHC), recognized by conventional antibody or T cell expressing transfected T-body construct for corresponding antigen recognition and T cell signaling for activation of cytotoxicity.

Another feature of T cell biology is the nature of selected binding affinities towards specific peptide : MHC by cognate T cell receptors. We noted in Chapter 3 of *Searching for Molecular Solutions* the great importance of controlling T cell self-reactivity, and that T cell clones with high affinity towards self-MHC were actively selected against. The affinity and kinetics of T cell receptor interaction with specific peptide : MHC also has a major bearing on immune activation and regulation ¹⁴. These biological constraints mold the normal spread of T cell receptor affinities into the low to moderate range ¹⁵. We have seen how the natural 'affinity ceiling' of antibodies has been easily surpassed through *in vitro* evolutionary display (Chapters 3 and 7), and phage display of T cell receptors has also yielded variants whose binding affinities are much greater than natural precedent ♥ ¹⁶⁻¹⁸. The antibody mimics of T cell receptors noted above can also achieve high affinities ⁴. Yet binding affinities in complex biosystems may converge to an optimum which is far below the maximum possible, for compelling evolutionary reasons. (The blind hand of selection as usual will favor the best arrangement which favors persistence and reproduction of the biosystem as organism). So if only *in vitro* uses for a biomolecular interaction are planned, then artificial tweaking of the binder-ligand affinity may be very profitable.

On the other hand, if *in vivo* application is the goal, caution may be called for. The relatively low affinity of T cell receptors for MHC acts as a strategy to minimize cross-reactivities between different peptides presented by the same MHC molecule. Excessive affinity for MHC (mediated by the CDR2 regions) might then be predicted to come at the cost of specificity (Fig. 7.Nc). Some investigations using isolated peptide : MHC complexes have suggested that T cell receptor CDR2 mutations which favor increased affinity retain a surprising degree of specificity ¹⁹, and certain mutations may act to stabilize specific conformations of CDR1 or CDR2 without specificity loss ²⁰. Nevertheless, other studies with human T cells transfected with mutant T cell receptors have

♥ This has the case for peptides presented by either Class I or Class II MHC.

indicated that high receptor affinity comes at an unacceptable cost in loss of effective specificity²¹.

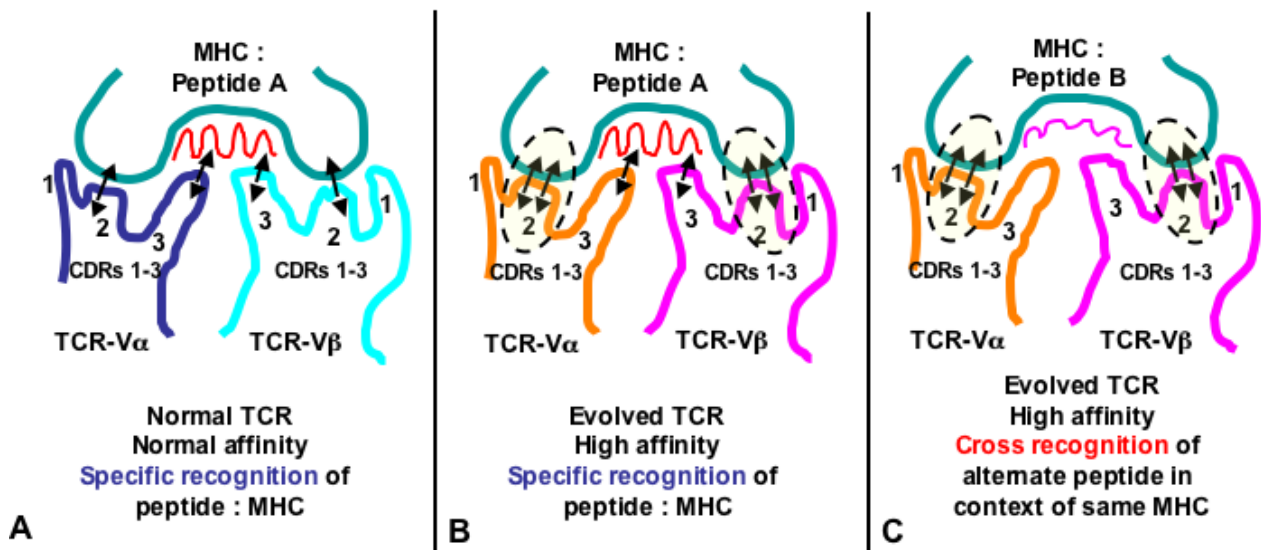


Fig. 7.Nc

Schematic depiction of interactions between peptide : MHC complexes between (A) a normal T cell receptor (TCR; numbers represent complementarity-determining regions (CDRs)), (B) a T cell receptor with enhanced affinity *in vitro* mediated via CDR2 regions contacting MHC (dotted ovals), and (C) cross-reaction engendered by high-affinity receptor variant by CDR2 interactions even when peptide is altered (C).

At the same time as one is thinking about T cell receptors, it is hard to avoid including MHC, as we have seen. As a component of a receptor –ligand complex interaction, MHC technology often parallels (and mirrors) corresponding T cell advances. Consider that identifying cells expressing a specific peptide-MHC complex on their surfaces can be performed using fluorescent-labeled soluble T cell receptor or corresponding anti-peptide : MHC antibody (as in Fig. 7.Nb). But

how is the converse task performed, where one wishes to identify T cells which recognize a specific peptide : MHC complex? The obvious proposal of using soluble MHC complexed with specific peptide runs aground through the low affinity of normal T cell receptor ligand binding, as the binding with monomeric MHC : peptide is too weak for practical purposes. But if multimeric MHC : peptide complexes are formed *in vitro*, multiple surface T cell receptors can be engaged, taking the effective affinity above the threshold of utility for routine fluorescent staining and other applications. The initial work in this regard used biotin-labeling of Class I MHC to generate avidin-linked tetramers²², but Class II MHC multimers have also been used in an analogous manner²³.

It is sometimes the case that the recognition ligand for an immune receptor is not immediately defined in an experimental system. For example, if a single-cell component of the adaptive immune system is randomly isolated and cloned, the sequence of its surface receptors can be readily obtained, but the target binding specificities of the same receptor molecules is not so easily accessible. This circumstance is also encountered with tumor cells whose normal progenitors derive from immune cells such as T and B lymphocytes at various stages of maturation. Even if the original specificity of an immune receptor is known, it may be very useful to identify a simple peptide which binds with high affinity to the same receptor, and thus acts as a '*mimotope*' or epitope mimic. Display-based technologies are an efficient approach towards solving these problems. Phage display with peptide libraries has thus been used to identify ligands for surface immunoglobulins on B cell lymphomas^{♥ 24,27}. But how is this applicable to T cell receptors and MHC? As we may recall from Chapter 4, any specific MHC

♥ Any T or B cell which has undergone somatic receptor diversification could in principle also undergo other pathological genetic changes leading to cellular immortalization, and thus the specificity of the immunoglobulin or T cell receptor of a transformed immune cell is essentially arbitrary. Binding of lymphoma surface receptors by recognized ligand can lead to cell death and a potential treatment²⁴, but each tumor in each patient thus requires a tailor-made peptide for this purpose. An alternative exploitation of the unique aspects of surface immune receptors in lymphomas is the generation of anti-idiotypic vaccines^{25,26}.

molecule can bind a large set of peptides, although far less than the total possible even for peptides of 8-9 residues. Display technologies can be used to define the range of peptide-binding specificities of MHC proteins, and to thereby identify conserved anchor residues within the peptide ligands²⁸. Peptide libraries are not useful, though, for the identification of T cell receptor specificities, since recognition in these circumstances only occurs in the context of MHC. Display libraries of peptide : MHC complexes themselves are then required for such an undertaking^{29,30}. Peptide : MHC complexes are stabilized by the invariant protein β 2-microglobulin[▼]. Rather than covalently linking MHC to a phage, a viable alternative is to use soluble MHC in conjunction with phage-linked peptide libraries and β 2-microglobulin itself³¹. In this way, MHC molecules can assemble with peptide and stabilizing β 2-microglobulin on a phage surface, allowing selection for specific complexes which bind to an individual T cell receptor. Selected phage from such libraries (displaying appropriate peptide : MHC / β 2-microglobulin complexes) then allow identification of peptides recognized by the T cell receptor of interest in the context of a specific MHC molecule. Still, a single display system is technically advantageous, and it might be expected that a eukaryotic alternative could potentially offer improvements in MHC protein folding and complex assembly. Eukaryotic peptide : MHC display systems using insect baculovirus have therefore become a competitive option for screening of soluble T cell receptors for peptides which mimic the original MHC-presented ligands to which they were directed³².

Since antibody catalysis was considered in *Searching for Molecular Solutions*, and this section has noted cases of 'cross-over' function between engineered antibodies and T cell receptors, we might wonder whether enzymatic catalysis is applicable in the T cell receptor domain as it is for antibodies. One could conceive of circumstances where a catalytic T cell receptor might be useful, as in

▼ See Chapter 3 (Fig. 3.4) of *Searching for Molecular Solutions*, and the corresponding color version from the same ftp site.

regulating autoimmunity by cleaving specific self-peptide epitopes presented on cell surfaces in the context of MHC (and thereby preventing activation of other self-reactive T cells). Yet T cell receptor catalysis would seem much more limited than that seen with abzymes, by the very nature of T cell receptor recognition, and the constraints imposed by the recognition process itself. A considerable portion of the total recognition interface of the T cell receptor is necessarily devoted to interaction with MHC, and thus reduces the available sequence space variation in which a catalytic residue combination might be found. Also, it would be a challenge to generate a suitable peptide transition state analog - MHC complex and raise a complementary T cell receptor with resulting desired catalysis towards a native peptide presented in the context of the same MHC. While perhaps impossible *in vivo*, this might be attainable in principle at least using *in vitro* display systems. Before leaving this speculative area, we could be reminded that at least one instance of catalysis associated with the α/β T cell receptor has been documented, in the form of the oxidative release of reactive oxygen products, in common with the corresponding antibody process ♥³³.

And this topic may also serve to remind us that while antibodies and T cell receptors are amazing results of the blind creativity of natural evolution, they are not necessarily the optimal molecular design solutions for all problems which humans can meet with. Accordingly, many workers have thought about alternative designs for molecular recognition, and acted on such ideas, which is taken further in Chapter 7 of *Searching for Molecular Solutions*.

♥ This area is addressed within the file SMS–Extras-Ch7/Section A11; from the same ftp site.

Section 19: **Minimized Antibodies**

Cited on p. 245 of *Searching for Molecular Solutions*

In the quest for obtaining a minimal antigen-recognition unit, it is important that one does not introduce other problems, or unnecessarily sacrifice certain useful functional features. For example, while scFv antibody fragments (Fig. 7.2 of *Searching for Molecular Solutions*) are (at ~25 kDa) much smaller than full-size immunoglobulins, they are more unstable *in vivo*³⁴. Size reduction at the expense of stability is an unacceptable trade-off for most envisaged therapeutic applications. It has been observed that scFv fragments with a long flexible linker have a propensity to dimerize, a structurally permitted topological consequence of the linker region itself, as depicted in Fig. 7.Nd. Since the linker length is a critical factor in such arrangements, it was realized that a linker too short to be compatible with monomer formation could still allow dimerization, and in effect drive the process in this direction (Fig. 7.Nd). Heterodimers between two linked 'cross-over' pairs of heavy and light chains enabled the formation of two antigen binding sites in a single molecule, and thus conferred bispecificity on the resulting 'diabodies'³⁵. Single-chain diabodies can be constructed with an additional connecting linker (Fig. 7.Nd)³⁶.

The promise of enhanced stabilities of diabodies and their *in vivo* targeting of tumor antigens prompted further tinkering with dimerization approaches, including fusions of scFv fragments with the CH3 constant region domain from IgG1 (designated as 'minibodies')³⁴. Juggling of antibody domains in general has allowed the generation of a variety of forms, including those with trivalency and above³⁷. Bispecific antibodies have many applications where a bridging function of some kind is desirable, which is often called for in therapies aimed at focusing an immune effector cell upon a target. In the latter circumstances, an appropriate

bispecific antibody recognizes structures on both the immune cell surface and the target, and thereby acts as a bridging agent ³⁸.

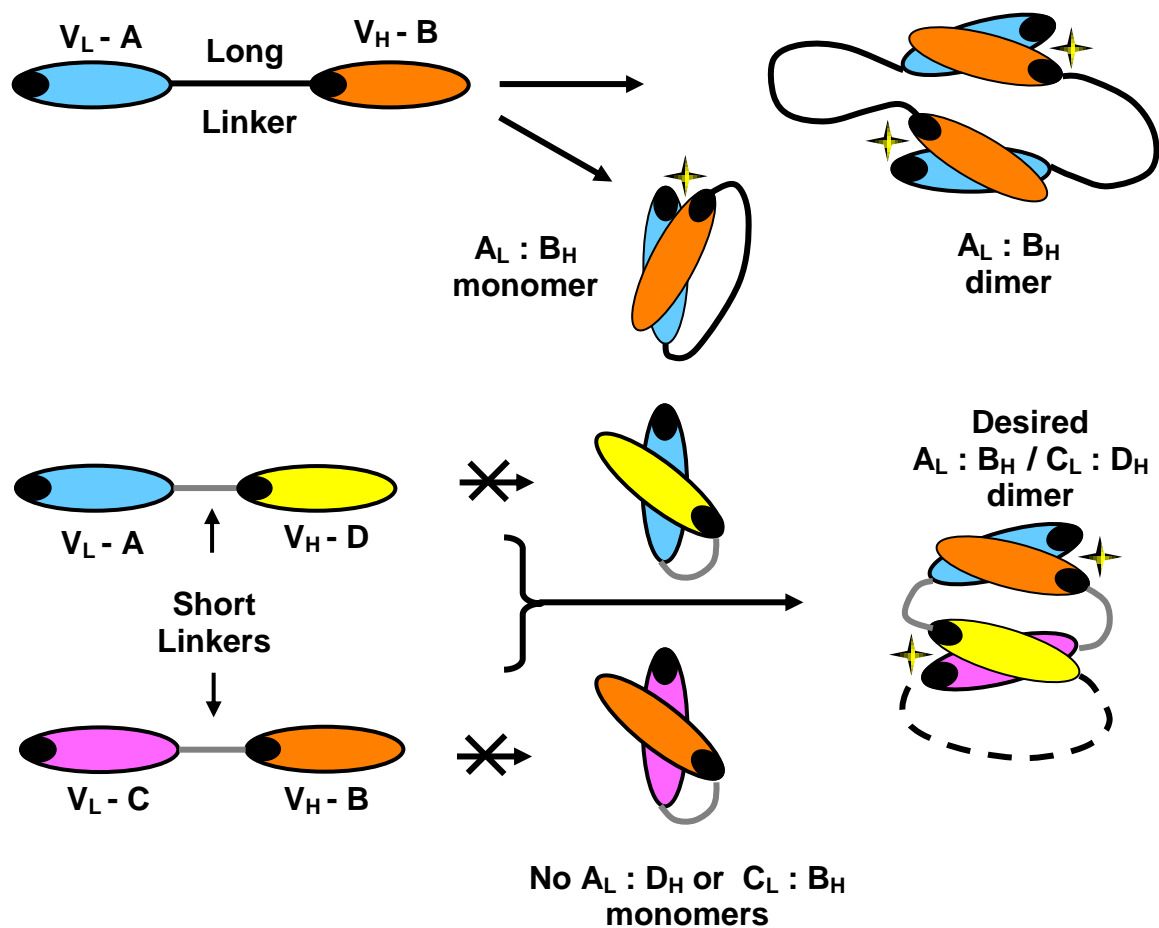


Fig. 7.Nd

Schematic depicting the formation of dimers for scFv fragments, and the 'forced' formation of dimers with scFv with shorter linkers (diabodies). Black ovals denote N-terminal regions for each antibody heavy and light chain domain; yellow stars denote the correct assembly of an antigen binding site. For the diabody fragments, only the bispecific heterodimeric forms can bind the original antigen targets. The dotted line denotes the position of an optional longer third linker for producing a single-chain diabody.

While these kinds of manipulations are biotechnologically important, they are not improvements on the size issue raised earlier. Considerably smaller single-domain antibodies have been identified as a result of cases of 'natural protein engineering' from some seemingly unlikely sources. First we should consider the notion of applying isolated immunoglobulin heavy chain segments for antigen-binding³⁹. Although this was initially considered a likely pathway towards a low-molecular weight general recognition element, this strategy proved disappointing in several important ways. Theoretically, isolated heavy chains might show significant limitations in their range of antigen recognition and affinities, in comparison to complete immunoglobulin antigen-binding regions. But a more immediate practical issue comes from the observation that both human and mouse immunoglobulin heavy chains have an exposed hydrophobic patch in the absence of a normal partner light chain⁴⁰, and accordingly suffer from problems of aggregation and poor solubility. Human ingenuity might have solved these problems, but as it eventuated nature has obviated such a project by providing us with some 'engineered' isolated heavy chains which come ready-made as functional, soluble antigen-binding molecules. This topic directs us the camelid and shark natural single domain antibodies considered in Chapter 7 of *Searching for Molecular Solutions*.

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