

## **New Strategies in Polypeptide and Antibody Synthesis**

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### **ABSTRACT**

The synthesis of radioligands can benefit considerable from optimized recombinant protein production, both on the aspect of economy of production as on the level of improving the targeting and pharmacokinetics of the ligand. This paper first describes a general production optimization strategy, but also elaborates on a protein design strategy tailored to targeting applications. Production in *Escherichia coli* will benefit from economy of goods and time as compared to other organisms. In order to increase the chance of finding a successful production system in this host, we have assembled a large number of expression strategies in a single, uniform expression system (FastScreen). The system allows rapid optimization of direct production of native proteins or via a fusion protein strategy with subsequent recovery of the desired protein. As an example of recombinant radioligand synthesis for improved targeting and clearing, a manifold of intermediate molecular size was synthesized by fusing one Fab and two single chain variable fragments (scFv) antibody binding fragments into a trifunctional molecule (Tribody). Due to the use of the specific heterodimerization of the Fab chains, trispecific, bispecific or trivalent antibody derived targeting reagents can easily be obtained. Recombinant production techniques also allow for specific incorporation of amino acids favoring a site specific labeling (labeling tags).

### **INTRODUCTION**

Radioimmunosciintigraphy (RIS) or radioimmunotherapy (RIT) often uses proteinaceous ligands for targeting tumor cells or other cellular disorders. Peptides have been shown to be useful in some cases, while the natural protein ligands of many tumor associated receptors are obvious candidates for developing diagnostic or even therapeutic reagents. In order to qualify as successful candidates the ligands need to

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be obtainable in a highly purified form, either from a natural source, or by using recombinant DNA production methods. Being able to produce an active form of the ligand or targeting protein is a key factor in the successful development of a RIS/RIT product. An active form should be defined by two key parameters: first, the larger part of the protein preparation should be able to bind the ligand with high affinity. Second, the folding of the protein should be stable enough not to influence the clearing and biodistribution behavior. For some ligands, it is even worthwhile developing stabilized mutants of the wild type protein, in order to improve their pharmacokinetic (PK) parameters. Indeed, proteins can fold into an unstable conformation that retains all or part of the native interaction with the receptor. However, these conformations will be more prone to protease degradation and subsequent clearing, or even to specific removal from the serum by the liver. These factors should be considered both during design of a production process, and when considering a labeling strategy to obtain the radioligand.

There is a vast body of knowledge and technology to produce proteins in recombinant organisms. Several hosts have been developed for recombinant production of polypeptides, with *Escherichia coli*, yeast (*Pichia pastoris*), Baculoviruses and mammalian expression systems amongst the most popular. The successful expression system must often be determined empirically, and varies from protein to protein. The gram negative bacterium *Escherichia coli* is still one of the favored systems, due to its short development time, the ease of use and favorable economy of use. For these reasons it is often worthwhile to try to optimize the expression of a particular protein in *E. coli* even upon initial failure, since a fast and efficient expression system might open more opportunities and influence the success rate of the project further down the line. The first part of this paper will discuss the strategies we currently use in order to develop a high-efficiency expression system for *E. coli*.

Recombinant proteins can be modified in order to tailor the proteins for optimal use in any particular application. As an example, antibodies can be trimmed down to their respective binding domains which can be used as a fast-clearing targeting reagent. We will discuss the possibilities to apply manifold engineering in order to tailor targeting, uptake as well as clearing of the recombinant antibody fragments to specific applications.

Successful RIS/RIT will not only depend on the synthesis of the ligand and the PK of the reagent, but also on the method of labeling of the polypeptide. Especially when using protein ligands, care must be taken not to influence the interaction of the radiolabeled ligand with the receptor, but also not to destabilize the protein by the labeling chemistry. Destabilized proteins are expected to have a different clearing route and PK as compared to a stable derivative. We will discuss the possibilities of recombinant protein production to modify the protein with target sequences for labeling reactions. These specific “labeling-tags” then can be incorporated into the protein sequence and serve as a preferred site for specific incorporation of the radioisotope.

## **NEW STRATEGIES IN POLYPEPTIDE SYNTHESIS: FAST OPTIMIZATION OF RECOMBINANT PRODUCTION IN *E. COLI***

Despite the amount of technology developed for recombinant protein production, optimization of the expression strategy is often needed for a large number of proteins or peptides. Factors that need to be considered are production and recovery in sufficient yield of the active polypeptide. Also, the production system of choice should yield stable folded polypeptide. In many cases, intermediate folding forms of proteins are known to be able to still bind their receptor, but are thought to have a faster clearing in the liver, and are particularly prone to protease degradation. Also, stable folding intermediates or non-native isoforms can have a higher tendency of sticking to surfaces, or forming a heterodispersed protein solution (micro-aggregates).

Expression system optimization usually considers a number of host cells to be used for protein production. Due to its short development time, low cost, high density growth on cheap media, and availability of technology for genetic engineering, *Escherichia coli* is still a host of choice for heterologous protein production. Even if only relative low yields of stable and biologically active polypeptide can be recovered from an *E. coli* culture, the process might still be favorable considering economy and the time frame necessary to produce the requested amount of protein. These considerations make it worthwhile to spend more effort on optimizing an *E. coli* production system.

Several aspects in an *E. coli* expression system can be optimized. A good promoter is a first prerequisite. Not only does the promoter need to be strong, it is also highly advisable to choose a well regulated promoter. Also the method of induction needs some consideration. Promoters that can be induced in a chemical way (e.g. by the addition of lactose or IPTG), can usually be induced at any temperature. This is important, since protein production at lower temperatures can increase the amount of correctly folded, active protein. The level of repression is a highly underestimated promoter feature. A leaky promoter will continuously synthesize a low level of heterologous protein of interest. This can lead to loss of the expression plasmid if the strain is grown in non-selective medium (note that this is always the case in high cell density cultures due to consumption of the antibiotic). However, when maintained under antibiotic selection, the selection superimposed by the activity of the leaky promoters tend to favor the growth of mutants that have a genetic alteration in one of the elements of the expression system (promoter, translation initiation region or gene)<sup>1</sup>. To increase the chance of obtaining an expression system for a given protein, or to make the expression system compatible with scale-up procedures, a well controlled promoter can be a serious advantage.

Bacteriophage lambda has some very stringently controlled promoters, which are thoroughly silenced when the phage is growing parasitically when integrated into the *E. coli* genome, but strong enough to outcompete any cellular promoter when the phage induces its bacteriolytic program. The most efficient way to induce the lambda promoters made use of a thermosensitive repressor *cI857* that allowed induction after shifting the culture from 28°C to 42°C. This temperature induction however also induces heat-shock proteases and increases the tendency of most proteins to aggregate in inclusion bodies (IB). In order to avoid this, we have created a regulatory system for this highly controlled promoter that is based on the chemical induction of an

antirepressor of *cI* (the *ant* protein of phage P22). In this way expression can be based on a strong, but extremely well repressed promoter ( $\lambda P_L$ ), thus avoiding counterselection of efficient expression clones which can happen as soon as after plating the cells for the first time.

The next aspect that needs optimization is the efficiency of translation initiation of the gene. Especially mRNA secondary structures, but also suboptimal codon usage, can have a negative effect on the final expression level of a particular heterologous gene. Since the translation initiation region extends also in the 5'-translated region of the gene, often a pragmatic approach is used to optimize translation initiation. Either different ribosomal binding sites can be assessed, or mutations can be introduced in the 5'-coding region of the gene of interest. Rapid assessment of a possible enhancement of the mutations on the level of expression can then be tested using an *in vitro* protein transcription/translation system<sup>2</sup>. *In vitro* systems can even be used to synthesize small amounts of ligands<sup>3,4</sup>, while it is possible to incorporate radioactive labeled amino acids directly in the protein. An alternative and more general method to ascertain efficient translation initiation for a non-optimized heterologous gene also translational coupling can be used. Here, an upstream cistron that is well-translated is placed in front of the gene of interest, with the stop-codon of the first cistron close to the start codon of the second cistron (gene of interest). The first cistron's translating ribosomes then iron out possible mRNA secondary structures that can inhibit translation initiation<sup>5</sup>.

One major problem when overproducing proteins is the formation of protein aggregates, known as inclusion bodies (IB). IB can be denatured chemically, and renatured in an optimized buffer system. However, the buffer system and refolding method needs to be investigated for each particular protein and even then, the overall yield can be very low for some proteins. Also, different isoforms are known to be formed after chemical refolding, which might reduce the specific activity and/or stability of the protein as compared to the natural protein. Changing culture conditions (e.g. lowering the induction temperature) is not always sufficient to produce the protein as a soluble ligand. One way to overcome this is to make use of fusion protein technology. Here, the protein of interest is produced coupled to a highly expressed and/or highly soluble protein, by genetic fusion of the gene of the fusion partner to the gene of interest. Fusion proteins are often highly expressed as a soluble protein while the native protein is not. When the fusion partner is placed at the N-terminus of the gene of interest, this strategy also solves the problem of translation initiation optimization since the fusion partner is selected to already have a good translation initiation region. Several fusion partners are proposed in the literature, and most research groups have their favorite fusion partner, albeit that this fusion partner differs depending on the authors. In our experience, there is no one tool that fits all. Certain fusion partners can have a solubilizing effect on one particular protein, but not on another, while the situation can differ for a second protein of interest. So it makes sense to try out at least a few fusion partners. When fusion protein technology is used, also an affinity tag can be incorporated that allows for rapid and selective isolation of the protein of interest, thus speeding up the production process, making the production more economical and allowing to start production from smaller culture amounts since the overall recuperation yield is higher.

In most cases it is desirable or necessary to remove the fusion part after production. This can be done by using proteases that are specific for a certain amino acid sequence. Several proteases are known to do this. Some of them also have a specificity at position P<sub>1</sub>' or even P<sub>2</sub>' (the first and second amino acid after the cleavage site). This leaves the protein of interest with a small modification. However, a few proteases have been described that allow for the recuperation of a truly native protein from the fusion protein (Table 1). The use of the proteases to efficiently cut a fusion protein however is also non-predictable. The site-preference of these proteases is generally relaxed, so proteins even not containing an obvious target sequence can also be degraded. Also, a certain fusion protein might be processed only very inefficiently by a certain protease, while another protease would perform better. So it is advisable to also optimize the protease site during expression strategy optimization.

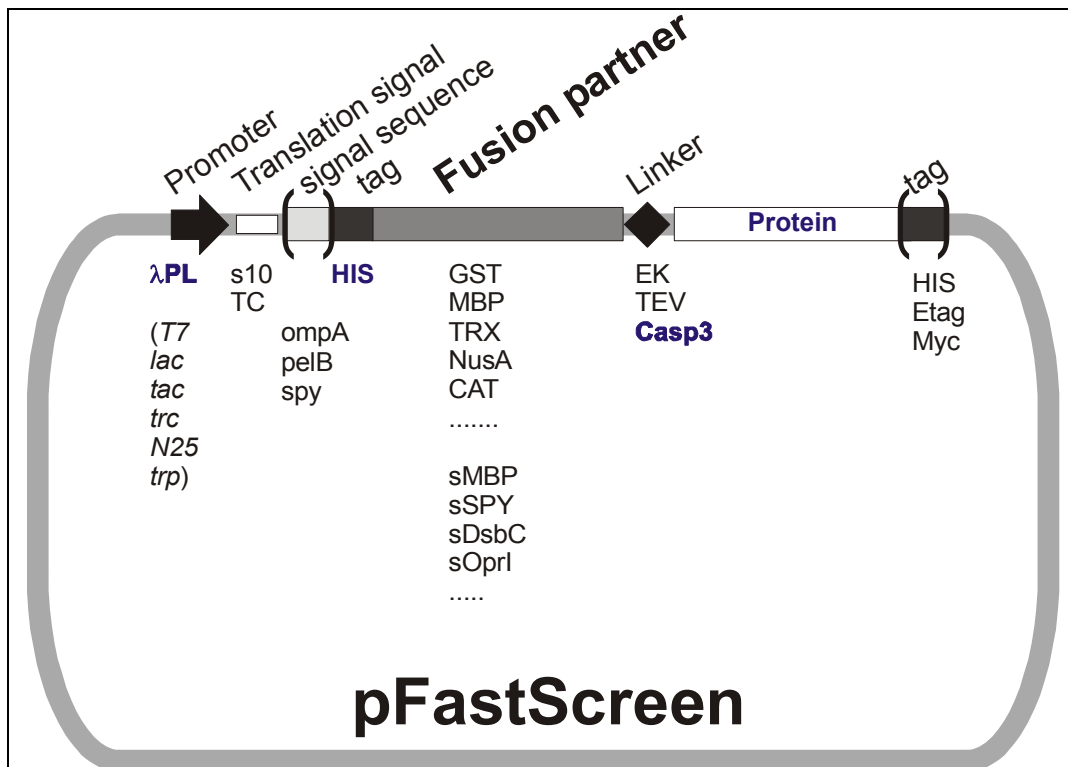
Enzymes with specificity also in P <sub>1</sub> '	SEQUENCE
IgA protease	Thr-Pro-Ala-Pro-Arg-Pro-Pro  <sup>^</sup> <i>Thr-Pro</i>
Collagenase	Pro-Xxx  <sup>^</sup> <i>Gly-Pro</i>
HRV3C (PreScission)	Leu-Glu-Val-Leu-Phe-Gln  <sup>^</sup> <i>Gly-Pro</i>
TEV	Glu-Xxx-Xxx-Tyr-Gln  <sup>^</sup> <i>(Gly/Ser)</i>
Enzymes to obtain authentic protein:	
Trypsin	Arg  <sup>^</sup> , Lys  <sup>^</sup>
Thrombin	Xxx-Xxx-Pro-Arg  <sup>^</sup>
Factor Xa	Ile-Glu-Xxx-Arg  <sup>^</sup>
Enterokinase	Asp-Asp-Asp-Lys  <sup>^</sup>
Caspase-3/7	Asp-Glu-Val-Asp  <sup>^</sup>

**Table 1:** Recognition sites for proteases with a more restricted action. Some proteases need certain defined amino acids after the cleavage site, and thus result in a non-native N-terminal sequence of the protein of interest. Tobacco Etch Virus protease 3C (TEV) has been described to also accept other amino acids at the P<sub>1</sub>' position, although with a lowered efficiency of processing<sup>6</sup>. The Caspase-3 and Caspase-7 enzymes were evaluated to be highly processive, while almost no difference was seen upon changing the P<sub>1</sub>' amino acid (NM, EVD, AK. unpublished results).

Some polypeptides need to be able to form disulphide bonds in order to fold into a stable protein. This can be achieved by fusing the protein to an *E. coli* signal sequence, which will guide the protein to the more oxidizing environment of the periplasmic space. Also here, the choice of the signal sequence might need some optimization when combined with a certain protein of interest. Interestingly, fusion protein strategies can also be used to produce proteins in the periplasm, so this strategy can also be used with secreted proteins.

For the purpose of shortening the duration of analyzing all these different strategies, we have integrated all the possibilities discussed into a single, modular vector system. The resulting vector set (pFastScreen) allows for assessment of two translation initiation sites and three different signal sequences for native expression optimization in the cytoplasm or the periplasm respectively, fusion to N-terminal or C-terminal detection or purification tags (removable or non-removable), and most of the popular fusion partners described in the literature (either for cytoplasmic or periplasmic production). Fusion partners that have been described to enhance the expression and solubility of a target protein that are incorporated in the FastScreen expression system are e.g. glutathion-S-transferase GST<sup>7</sup>, maltose binding protein (MBP, with or

without signal sequence for secretion)<sup>8</sup>, thioredoxin (TRX)<sup>9</sup>, chloramphenicol acetyl transferase (CAT), the transcription factor NusA<sup>10</sup>, secreted DsbA or DsbC<sup>11</sup>, or the outer membrane factor OprI<sup>12</sup>; but within FastScreen these fusion partners are always combined with a polyhistidine tag to allow for efficient capture and purification of the fusion protein after expression<sup>13</sup>. Also, the possibility to evaluate three different protease sites is incorporated in the vector system (Fig 1). A first screening however is usually performed with a chosen promoter (the  $\lambda P_L$ /ant system discussed above), and one protease recognition site. We have efficiently used Caspase enzymes for processing the fusion protein. The caspase enzymes in this case are also polyhistidine tagged so the enzyme can be extracted together with the unprocessed fusion protein and the processed fusion partner in a single step, using immobilized metal affinity chromatography (IMAC) that has a specific affinity to the adjacent imidazol groups.



**Figure 1.** Overview of the FastScreen method for rapid strategy optimization of production of a protein of interest in *E. coli*. The vector framework is optimized for stability and high-level expression<sup>14-16</sup> and by default uses the well-controlled  $\lambda P_L$  promoter. Factors that need to be empirically optimized are assessed in a parallel approach. Several vectors are assayed for optimal production of soluble (active) protein. A large variety of fusion protein strategies is incorporated in the vector series, but these can be removed by site specific proteolysis of the linker sequence. Also the protease maturation is an empirical factor that can be assessed in the vector system.

Changing promoters or protease recognition sites however is easily performed once one or some successful expression strategies are defined, and further optimization might be needed. The FastScreen method thus selects a set of about 10-15 expression vectors for a parallel evaluation of different expression strategies, possibly followed

by a second optimization round by changing some of the elements (promoter, protease recognition site, tags), or optimizing the host strain and culture conditions.

The procedure is applicable to small peptides (or peptide multimers), as well as to larger proteins. Since all is done in a standardized expression system, all variable factors can be truly compared for efficiency. A typical FastScreen analysis only takes 4-8 weeks and evaluates most of the current expression strategies for *E. coli* to answer the question whether or not a given protein can be produced in *E. coli*, and this with a higher certainty than when the decision is based on a single experiment.

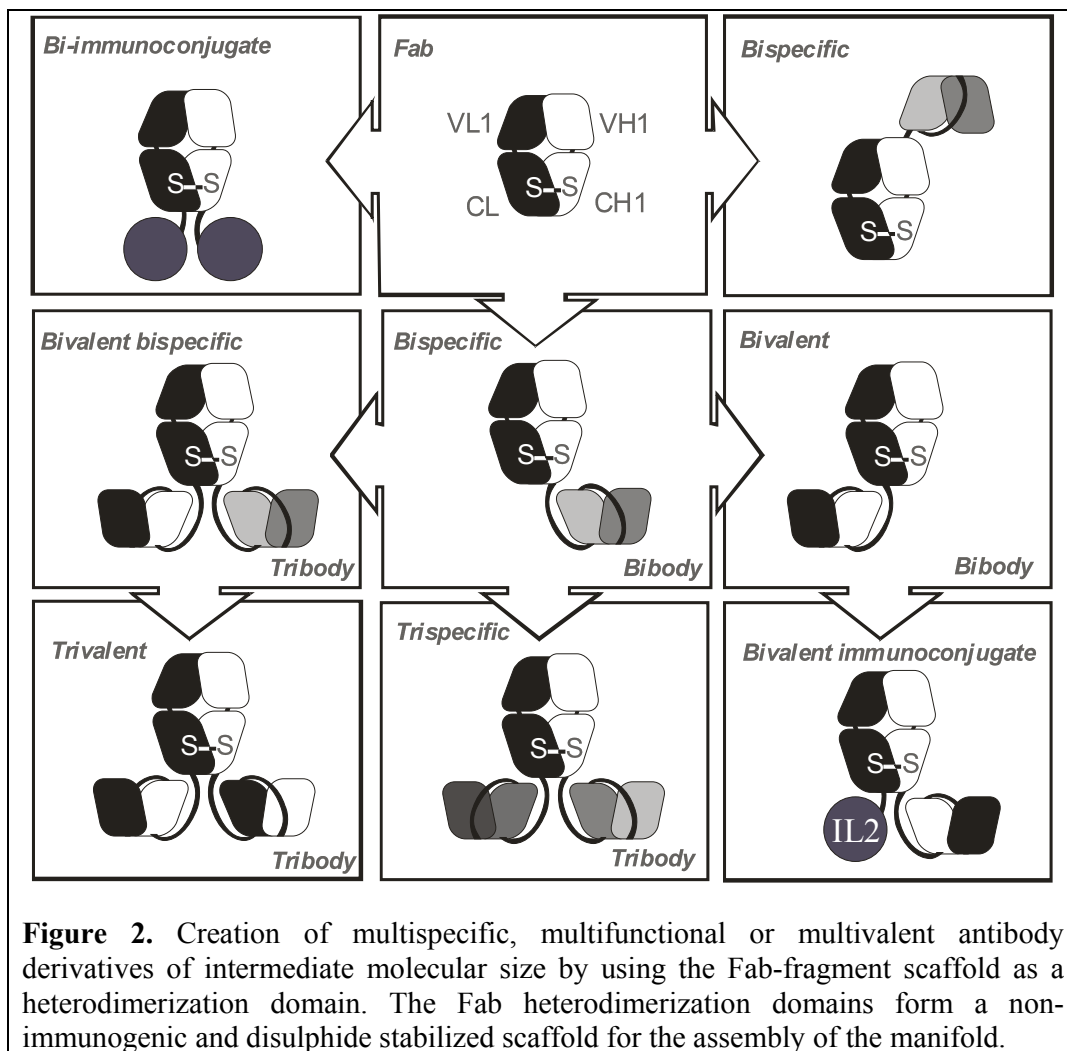
## **NEW STRATEGIES IN ANTIBODY SYNTHESIS: TAILORING MANIFOLDS FOR OPTIMAL CLEARING AND TARGETING**

Monoclonal antibodies (moAbs) have been extensively used for RIS applications, and recently the first radiolabeled moAbs have been approved for therapy. However, the PK characteristics of moAbs for targeting radiolabel can be improved. Due to its large molecular size (150 kDa) moAbs are hindered in passing the venal lining (>100 kDa) and in diffusing into the extracellular matrix (ECM) (>60 kDa). Also, moAb can be expected to interact with granulocytes, macrophages and lymphocytes due to the interaction with Fc-receptors present on these cells, leading to non-specific targeting. Last but not least, moAb can have a serum half life of several days (IgG molecules have a half life up to 20 days), due to the protective interaction of the Fc part with the FcRn receptors in the liver<sup>17</sup>.

Antibodies consist of a constant Fc tail, coupled to two antigen binding fragments (Fab). These Fab fragments can be further divided in a constant region and a variable fragment region. Both of these regions consist of two domains (VH and CH1 derived from the heavy chain H and VL and CL of the light chain L). Since all the information to bind an antigen is contained uniquely in the variable fragment, this fragment can be isolated as the smallest antibody derived targeting reagent. The F(ab')<sub>2</sub> fragment (100 kDa) or the monovalent Fab fragment (50 kDa) can be derived from the native antibody by enzymatic digestion. The variable domains Fv (25 kDa) however, need to be expressed in a recombinant way by isolating the gene sequences encoding the Fv. The Fv fragment consists of the VH and a VL fragment that need to interact with each other to form a fully functional antibody fragment or a stable protein. However, the interaction between these domains is usually low (10 mM K<sub>D</sub> range), so that upon dilution the fragments dissociate and either the Fv is unable to bind and/or the protein is rapidly degraded. Several solutions have been proposed for stabilizing the Fv molecules. Fv molecules can be synthesized as a single chain polypeptide (scFv), as a bivalent protein that will fold as a diabody ((scFv)<sub>2</sub>), or stabilized by engineering an extra disulfide bond between the domains. These small antibody fragments have shown promise due to a faster tumor penetration, and a faster clearing than the moAb. This results in a more optimal contrast of tumor/blood radiolabel early after the injection, as well as a reduction of collateral radiodamage. However, scFv, diabodies and Fab fragments are easily cleared by the kidney glomerula, who have a pore size around 8-10 nm. An average Fab fragment measures 8 x 5 nm, and thus passes relatively freely through the pores. Since a F(ab')<sub>2</sub> fragment (100 kDa) contains the very flexible antibody hinge region, these molecules easily adapt to an extended configuration that will also result in kidney clearance. Also charge is an important factor: the fractional clearance of cationic proteins is more enhanced as compared to

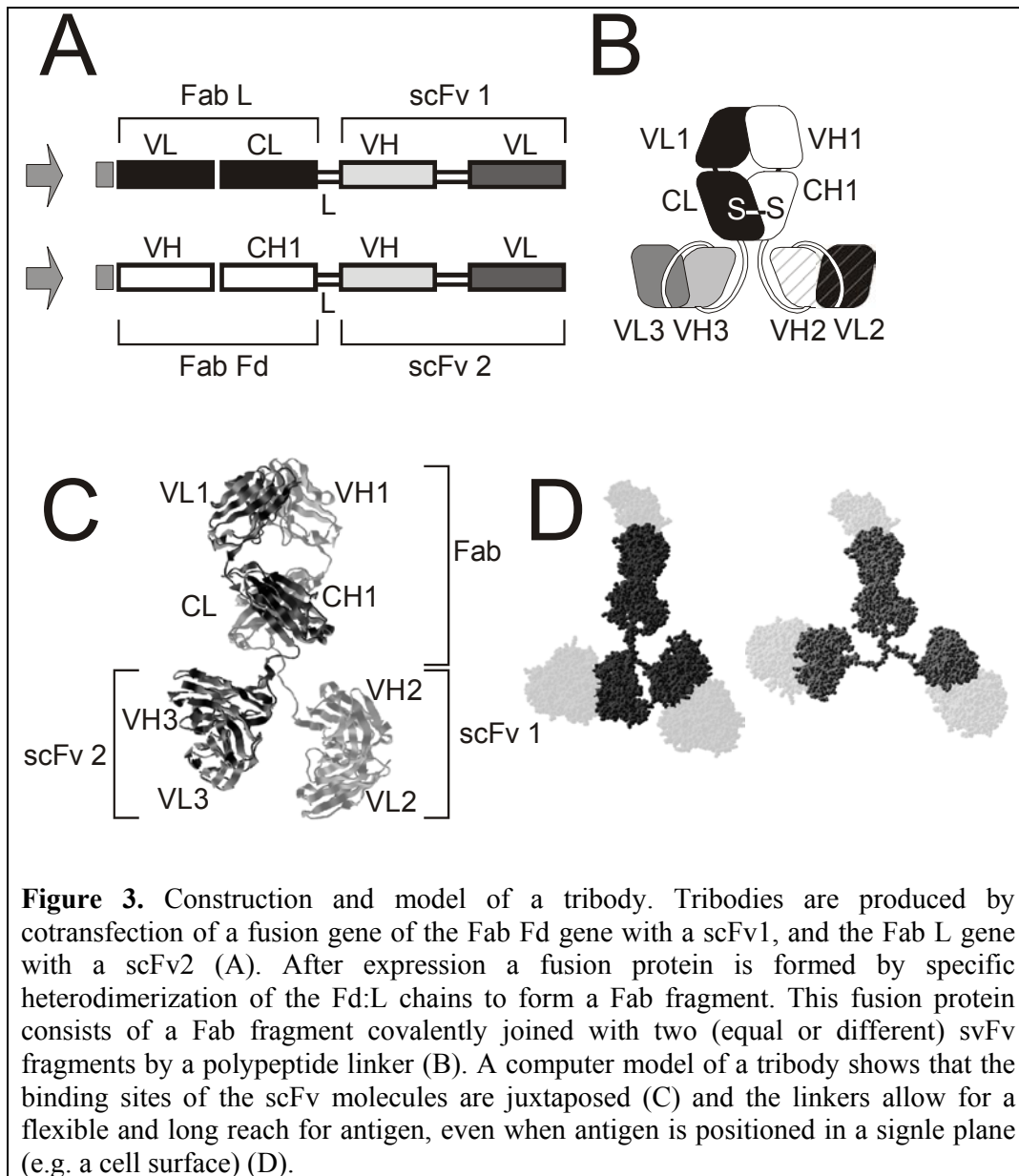
neutral or anionic proteins. Proteins in the filtrate are then at least in part reabsorbed by the proximal kidney tubule cells by binding to endocytic transport receptors, degradation of the protein and possible anchorage of the radiolabel in the kidney, with potential nephrotoxicity (especially in RIT)<sup>18</sup>.

Since the glomerular filtration rate is estimated to be about 7,5 liters per hour (125 ml/min), serum half life of small proteins (4-6 nm diameter, <60 kDa) can not be expected to be more than 0,5 – 1 hour, what is confirmed in numerous PK studies<sup>19-21</sup>. However, these small antigen binding proteins can be used as building blocks in a larger manifold, thus increasing the molecular size above the kidney removal threshold and allowing the incorporation of more than one function. Anna Wu and coworkers have demonstrated that by increasing the molecular size of the antibody manifold, the uptake in the kidney was reduced, while the somewhat longer serum half life of the “minibodies” (an 80 kDa (scFv-CH3)<sub>2</sub> fusion protein) resulted in a better tumor targeting and a better tumor/blood contrast, although at a somewhat later time point as compared to a 50 kDa diabody<sup>22, 23</sup>.



We have applied molecular manifold modeling to create multispecific antibodies of intermediate molecular size (Fig 2). Multispecific antibodies can be used to target

more than one receptor, or more than one epitope on target receptors. Also, multispecificity can be used in a pretargeting strategy where in a first step the antibody reagent is bound to the tumor cells, after which a small and fast clearing radiolabeled reagent is administered<sup>24</sup>. Bispecific antibodies can also be used to redirect immun effector cells towards tumor cells, in such a way that the immun system is now reprogrammed to kill the tumor cells. In order to synthesize bispecific antibodies of intermediate size, a specific heterodimerization domain will enhance the production of the correct product.



**Figure 3.** Construction and model of a tribody. Tribodies are produced by cotransfection of a fusion gene of the Fab Fd gene with a scFv1, and the Fab L gene with a scFv2 (A). After expression a fusion protein is formed by specific heterodimerization of the Fd:L chains to form a Fab fragment. This fusion protein consists of a Fab fragment covalently joined with two (equal or different) svFv fragments by a polypeptide linker (B). A computer model of a tribody shows that the binding sites of the scFv molecules are juxtaposed (C) and the linkers allow for a flexible and long reach for antigen, even when antigen is positioned in a single plane (e.g. a cell surface) (D).

One very efficient heterodimer formed in eukaryotic cells is the Fab fragment. Indeed, B-cells contain a quality control system on the Fab heterodimerisation based on the interaction of the major chaperone protein of the endoplasmic reticulum lumen BiP with the CH1 domain in the heavy chain fragment (Fd), that is only displaced by interaction of the Fd chain with the L chain<sup>25</sup>. We have shown that it is possible to

extend both of the Fab chains with extra protein domains such as scFv molecules<sup>26</sup>. Our preferred site for extension is at the C-terminus of the Fab chain, thus avoiding to introduce conformational interference for binding the antigen recognized by the Fab, but Fab chains can generally also be extended at their N-terminus (Fig. 3). Bivalent or bispecific antibody derivatives can thus be created by fusing a Fab fragment to a scFv (75 kDa “*bibody*”). When two scFv genes are genetically coupled to the genes encoding the Fab-chains, a Fab-(scFv)<sub>2</sub> molecule will be produced which we refer to as “*tribody*”. Tribodies can be made trivalent, bispecific with a bivalent binding for one target, or trispecific. Also, tribodies and bibodies can be extended with other peptides or small proteins e.g. IL2, which can bring an immun adjuvating activity to the tumor<sup>27</sup> but also increases the vascular permeability and thus might enhance radioligand uptake.

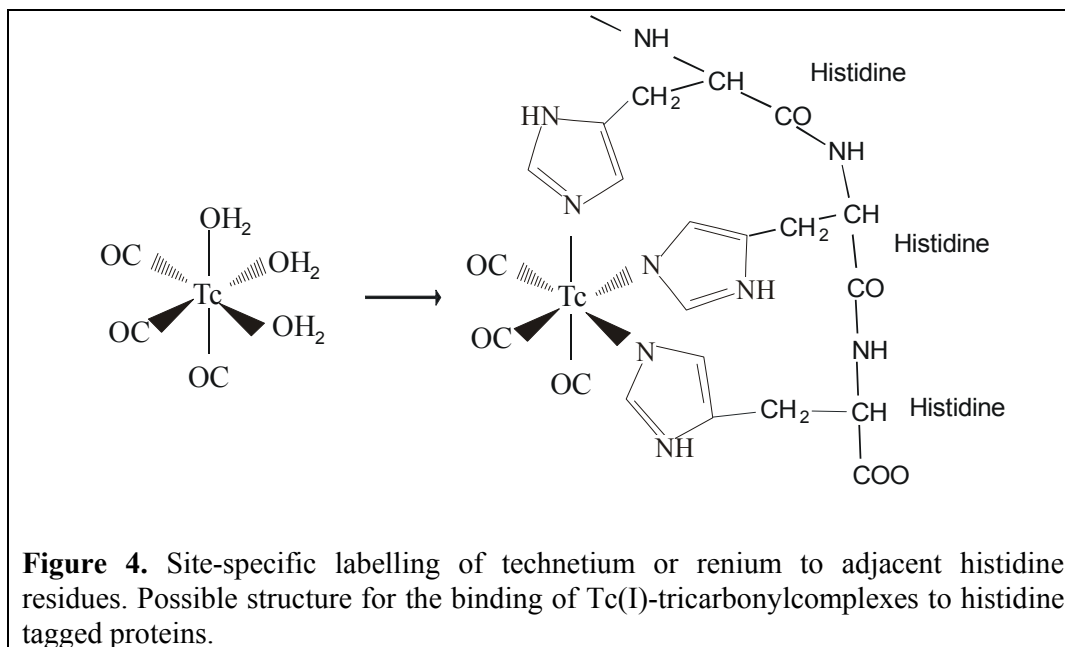
As mentioned, mammalian cells are a preferred host for producing these molecules since exclusively heterodimers will be formed, apart from L:L light chain homodimer mispairing (Bence-Jones molecules) that are easily removed during purification and usually do not interact with the targeted antigen efficiently. Production levels depend largely on the antibody used and vary between 1 – 20 mg/l in transiently transfected HEK293T cells grown in T-flask, and 5 – 200 mg/l in stably transfected SP2/0 myeloma cells grown in roller bottles. Tribody molecules can be efficiently recovered from the cell culture supernatants<sup>28</sup>, and at least bibody molecules (Fab-scFv) have been successfully produced in *E. coli*<sup>29</sup>. In our experience, the tribody molecules (Fab-(scFv)<sub>2</sub>) have a lower tendency to aggregate as compared to non-fused (scFv) or dimeric scFv (sc(Fv)<sub>2</sub> and (scFv)<sub>2</sub> diabody) manifolds. After production a small percentage of the molecules was found to be a dimer, possibly by extended scFv-scFv interactions between two tribody molecules as was observed also with (scFv-CH3)<sub>2</sub> minibodies<sup>23</sup>, but all tribodies we produced so far could be isolated as a monomer after purification. Using a trispecific molecule, three different antigens could be bound with different linker sequences connecting the Fab chains with the scFv, varying in size between 5 and 15 amino acids<sup>28</sup>. We could show cell-cell crosslinking along two different axes of a model trispecific antibody, demonstrating the flexibility in binding of a tribody manifold<sup>26</sup>. Molecular modeling could confirm the very flexible nature of antigen binding in this manifold (Fig 3). The tribody molecule (100 kDa) is stable upon incubation at 37°C in either PBS or mouse serum, and indeed shows a slower serum clearance as compared to a sc(Fv)<sub>2</sub> dimer (50 kDa), with a half life of 5 hours as compared to 0,5 h measured for the sc(Fv)<sub>2</sub> dimer. A clear binding improvement could be obtained when an increased avidity was engineered into the molecule, where for at least some antigens, trivalent molecules even outperform the bivalent binders. Depending on the receptor targeted, a multivalent binding of the reagent will likely be more favorable to induce “antigen modulation”, or internalization of the antibody – receptor complex. Certainly for RIS/RIT applications this is considered an advantage.

In conclusion, bibodies and tribodies are a novel antibody manifold that can be used to efficiently create multivalent and/or multispecific reagents of intermediate molecular size, thus optimizing a trade-off between efficient targeting and clearing by increasing the binding and uptake by engineering avidity, and avoiding massive passage through and accumulation in the kidney. Due to its assembly based on heterodimerisation, the manifold allows a precise control on the valence and place of any specific building block incorporated in the fusion molecule.

## ADAPTING PROTEIN TO SEQUENCE SPECIFIC LABELING

The standard method for the radioiodination of polypeptides and proteins involves electrophilic substitution of the radioiodine directly on one of the amino acids of the protein, mainly on tyrosine residues (predominately in ortho position of the hydroxyl group), but iodination on tryptophan and histidine residues has also been described<sup>30, 31</sup>. The iodination involves the use of oxidants such as iodogen or chloramines T or the application of enzymes such as lactoperoxidases. The method is very simple but a significant disadvantage is that they are deiodinated *in vivo* extensively<sup>32</sup>. Indirect methods include conjugation of radioiodinating reagent such as Bolton-Hunter<sup>33</sup> and SIB (N-succinimidyl 3-iodobenzoate)<sup>34</sup>. SIB has the advantage that competitive hydrolysis is minimized and conjugation efficiency to the proteins or polypeptide is therefore increased compared with the original Bolton-Hunter reagent.

Different methods are described for labelling of Mabs with <sup>99m</sup>Tc. Beside the many bifunctional agents (forming coordination bonds with technetium) that have been described coupling to the amino- or carboxy terminal of proteins other labelling methods have been published. One of these methods involve reducing intramolecular disulfide bonds to generate thiol groups which have high affinity for Tc(V). Several reviews have been given for this direct protein labelling method<sup>35, 36</sup>. One of the disadvantages of the method is that great care must be taken into account during the reduction of the disulfide bonds of the protein in order to maintain the immunoreactivity of the molecule. The recombinant introduction of several (two an even more) cysteine tags, whether or not interrupted by other amino-acids, at either the amino- or carboxy terminus could offer new challenges for this method<sup>37, 38</sup>.



The organometallic Tc(I) labelling technique using  $fac-[^{99m}\text{Tc}(\text{OH}_2)_3(\text{CO})_3]^+$  has nowadays been established as potential alternative to the currently applied Tc(V) methodology<sup>39, 40</sup>. The superior *in vivo* stability of the  $fac-[^{99m}\text{Tc}(\text{OH}_2)_3(\text{CO})_3]^+$  and the many possibilities of coupling the biomolecules, is one of the most convincing advantage of this approach. The amino acid histidine offers three potentially

coordinating functionalities. Histidine is able to coordinate tridentately and facially to a metal center with only small changes in the geometry of the molecule<sup>41</sup>. Also polyhistidine sequences can form a chelating complex with a tricarbonyl technetium or rhenium<sup>40</sup> (Fig 4). Polyhistidine tags are also used as purification tags since a genetic fusion of a protein with a polyhistidine tag allows a one step purification using immobilized metal affinity chromatography (IMAC), with chelating resins loaded with nickel, zinc, cobalt or copper<sup>13</sup>.

These examples illustrate a combination of recombinant protein design and novel labelling chemistry to obtain a more site-specific method for protein labelling. This approach is likely to deliver overall synthesis methods with a higher conservation of biological activity, and thus producing better radioligands.

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## **REFERENCES**

1. Mertens N, Remaut E, and Fiers W, Tight transcriptional control mechanism ensures stable high-level expression from T7 promoter-based expression plasmids. *Biotechnology (N Y)*, 13(2):175-9 (1995).
2. Betton JM, Rapid Translation System (RTS): A Promising Alternative for Recombinant Protein Production. *Curr Protein Pept Sci*, 4(1):73-80 (2003).
3. Kim DM and Swartz JR, Prolonging cell-free protein synthesis with a novel ATP regeneration system. *Biotechnol Bioeng*, 66(3):180-8 (1999).
4. Spirin AS, *Cell-Free Translation Systems*. 2002: Springer, ISBN3540420509
5. Spanjaard RA and van Duin J, Translational reinitiation in the presence and absence of a Shine and Dalgarno sequence. *Nucleic Acids Res*, 17(14):5501-7 (1989).
6. Kapust RB, Tozser J, Copeland TD, and Waugh DS, The P1(′) specificity of tobacco etch virus protease. *Biochem Biophys Res Commun*, 294(5):949-55. (2002).
7. Smith DB and Johnson KS, Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene*, 67(1):31-40 (1988).
8. di Guan C, Li P, Riggs PD, and Inouye H, Vectors that facilitate the expression and purification of foreign peptides in *Escherichia coli* by fusion to maltose-binding protein. *Gene*, 67(1):21-30 (1988).
9. LaVallie ER, DiBlasio EA, Kovacic S, Grant KL, Schendel PF, and McCoy JM, A thioredoxin gene fusion expression system that circumvents inclusion

- body formation in the E. coli cytoplasm. *Biotechnology (N Y)*, 11(2):187-93 (1993).
10. Wilkinson DL and Harrison RG, Predicting the solubility of recombinant proteins in Escherichia coli. *Biotechnology (N Y)*, 9(5):443-8 (1991).
  11. Collins-Racie LA, McColgan JM, Grant KL, DiBlasio-Smith EA, McCoy JM, and LaVallie ER, Production of recombinant bovine enterokinase catalytic subunit in Escherichia coli using the novel secretory fusion partner DsbA. *Biotechnology (N Y)*, 13(9):982-7 (1995).
  12. Cote-Sierra J, Jongert E, Bredan A, Gautam DC, Parkhouse M, Cornelis P, De Baetselier P, and Revets H, A new membrane-bound OprI lipoprotein expression vector. High production of heterologous fusion proteins in gram (-) bacteria and the implications for oral vaccination. *Gene*, 221(1):25-34 (1998).
  13. Crowe J, Dobeli H, Gentz R, Hochuli E, Stuber D, and Henco K, 6xHis-Ni-NTA chromatography as a superior technique in recombinant protein expression/purification. *Methods Mol Biol*, 31:371-87 (1994).
  14. Mertens N, Remaut E, and Fiers W, Versatile, multi-featured plasmids for high-level expression of heterologous genes in Escherichia coli: overproduction of human and murine cytokines. *Gene*, 164(1):9-15 (1995).
  15. Mertens N, Fiers W, and Remaut E, *An advanced vector system for high-level recombinant gene expression in E. coli*, in *Biotechnology International II: Latest developments in the Biotechnology industrie and research*, T.H. Connor, H.U. Weier, and F. Fox, Editors. 1999, Universal Medical Press: San Francisco. p. 165-172.
  16. Mertens N, Remaut E, and Fiers W, Increased stability of phage T7g10 mRNA is mediated by either a 5'- or a 3'-terminal stem-loop structure. *Biol Chem*, 377(12):811-7 (1996).
  17. Ward ES, Zhou J, Ghetie V, and Ober RJ, Evidence to support the cellular mechanism involved in serum IgG homeostasis in humans. *Int Immunol*, 15(2):187-95 (2003).
  18. Flynn AA, Pedley RB, Green AJ, Dearling JL, El-Emir E, Boxer GM, Boden R, and Begent RH, The nonuniformity of antibody distribution in the kidney and its influence on dosimetry. *Radiat Res*, 159(2):182-9 (2003).
  19. Baxter LT, Zhu H, Mackensen DG, and Jain RK, Physiologically based pharmacokinetic model for specific and nonspecific monoclonal antibodies and fragments in normal tissues and human tumor xenografts in nude mice. *Cancer Res*, 54(6):1517-28 (1994).
  20. Beresford GW, Pavlinkova G, Booth BJ, Batra SK, and Colcher D, Binding characteristics and tumor targeting of a covalently linked divalent CC49 single-chain antibody. *Int J Cancer*, 81(6):911-7 (1999).

21. Willuda J, Kubetzko S, Waibel R, Schubiger PA, Zangemeister-Wittke U, and Pluckthun A, Tumor targeting of mono-, di-, and tetravalent anti-p185(HER-2) miniantibodies multimerized by self-associating peptides. *J Biol Chem*, 276(17):14385-92 (2001).
22. Wu AM, Chen W, Raubitschek A, Williams LE, Neumaier M, Fischer R, Hu SZ, Odom-Maryon T, Wong JY, and Shively JE, Tumor localization of anti-CEA single-chain Fvs: improved targeting by non-covalent dimers. *Immunotechnology*, 2(1):21-36 (1996).
23. Wu AM, Tan GJ, Sherman MA, Clarke P, Olafsen T, Forman SJ, and Raubitschek AA, Multimerization of a chimeric anti-CD20 single-chain Fv-Fc fusion protein is mediated through variable domain exchange. *Protein Eng*, 14(12):1025-33 (2001).
24. Devys A, Thedrez P, Gautherot E, Faivre-Chauvet A, Sai-Maurel C, Rouvier E, Auget JL, Barbet J, and Chatal JF, Comparative targeting of human colon-carcinoma multicell spheroids using one- and two-step (bispecific antibody) techniques. *Int J Cancer*, 67(6):883-91 (1996).
25. Lee YK, Brewer JW, Hellman R, and Hendershot LM, BiP and immunoglobulin light chain cooperate to control the folding of heavy chain and ensure the fidelity of immunoglobulin assembly. *Mol Biol Cell*, 10(7):2209-19 (1999).
26. Schoonjans R, Willems A, Schoonooghe S, Fiers W, Grooten J, and Mertens N, Fab chains as an efficient heterodimerization scaffold for the production of recombinant bispecific and trispecific antibody derivatives. *J Immunol*, 165(12):7050-7. (2000).
27. Reisfeld RA and Gillies SD, Recombinant antibody fusion proteins for cancer immunotherapy. *Curr Top Microbiol Immunol*, 213(Pt 3):27-53 (1996).
28. Willems A, Leoen J, Schoonooghe S, Grooten J, and Mertens N, Optimizing expression and purification from cell culture medium of trispecific recombinant antibody derivatives. *J Chromatography*, 786(1-2):161-176 (2003).
29. Lu D, Jimenez X, Zhang H, Bohlen P, Witte L, and Zhu Z, Fab-scFv fusion protein: an efficient approach to production of bispecific antibody fragments. *J Immunol Methods*, 267(2):213. (2002).
30. Fraker PJ and Speck JC, Jr., Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a,6a-diphrenylglycoluril. *Biochem Biophys Res Commun*, 80(4):849-57 (1978).
31. Hunter W and Greenwood F, Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature*, 194:495-496 (1962).
32. Smallridge RC, Burman KD, Ward KE, Wartofsky L, Dimond RC, Wright FD, and Latham KR, 3',5'-diiodothyronine to 3'-monoiodothyronine

conversion in the fed and fasted rat: enzyme characteristics and evidence for two distinct 5'-deiodinases. *Endocrinology*, 108(6):2336-45 (1981).

33. Bolton AE and Hunter WM, A new method for labelling protein hormones with radioiodine for use in the radioimmunoassay. *J Endocrinol*, 55(2):xxx-xxxi (1972).
34. Vaidyanathan G and Zalutsky MR, Protein radiohalogenation: observations on the design of N-succinimidyl ester acylation agents. *Bioconjug Chem*, 1(4):269-73 (1990).
35. Eckelman WC and Steigman J, Direct labeling with <sup>99m</sup>Tc. *Int J Rad Appl Instrum B*, 18(1):3-7 (1991).
36. Hnatowich DJ, Is technetium-99m the radioisotope of choice for radioimmunoscinigraphy? *J Nucl Biol Med*, 38(4 Suppl 1):22-32 (1994).
37. Bogdanov A, Jr., Simonova M, and Weissleder R, Design of metal-binding green fluorescent protein variants. *Biochim Biophys Acta*, 1397(1):56-64 (1998).
38. George AJ, Jamar F, Tai MS, Heelan BT, Adams GP, McCartney JE, Houston LL, Weiner LM, Oppermann H, and Peters AM, Radiometal labeling of recombinant proteins by a genetically engineered minimal chelation site: technetium-99m coordination by single-chain Fv antibody fusion proteins through a C-terminal cysteinyl peptide. *Proc Natl Acad Sci U S A*, 92(18):8358-62 (1995).
39. Alberto R, Schibli R, Waibel R, Abram U, and Schubiger AP, Basic aqueous chemistry of  $M(OH)_2(CO)_3 + (M = Re, Tc)$  directed toward radiopharmaceutical preparations. *Coord. Chem. Rev.*, 190:901-919 (1999).
40. Waibel R, Alberto R, Willuda J, Finnern R, Schibli R, Stichelberger A, Egli A, Abram U, Mach JP, Pluckthun A, and Schubiger PA, Stable one-step technetium-99m labeling of His-tagged recombinant proteins with a novel Tc(I)-carbonyl complex. *Nat Biotechnol*, 17(9):897-901 (1999).
41. Schibli R, La Bella R, Alberto R, Garcia-Garayoa E, Ortner K, Abram U, and Schubiger PA, Influence of the denticity of ligand systems on the in vitro and in vivo behavior of (<sup>99m</sup>Tc(I)-tricarbonyl complexes: a hint for the future functionalization of biomolecules. *Bioconjug Chem*, 11(3):345-51 (2000).