Stable one-step technetium-99m labeling of His-tagged recombinant proteins with a novel Tc(I)–carbonyl complex

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We have developed a technetium labeling technology based on a new organometallic chemistry, which involves simple mixing of the novel reagent, a ^{99m}Tc(I)–carbonyl compound, with a His-tagged recombinant protein. This method obviates the labeling of unpaired engineered cysteines, which frequently create problems in large-scale expression and storage of disulfide-containing proteins. In this study, we labeled antibody single-chain Fv fragments to high specific activities (90 mCi/mg), and the label was very stable to serum and all other challenges tested. The pharmacokinetic characteristics were indistinguishable from iodinated scFv fragments, and thus scFV fragments labeled by the new method will be suitable for biodistribution studies. This novel labeling method should be applicable not only to diagnostic imaging with ^{99m}Tc, but also to radioimmunotherapy approaches with $^{186/188}$Re, and its use can be easily extended to almost any recombinant protein or synthetic peptide.

Keywords: organometallic technetium, recombinant proteins, radiolabeling, targeting, imaging

In vivo tumor imaging with radiolabels is an important diagnostic tool in cancer treatments. Nonspecific compounds such as technetium-99m sulfur colloids[1] or technetium-99m Sestamibi[2,3] are routinely used today in scintimammography and myocardial perfusions. (Sestamibi is ^{99m}Tc[MIBI]$_6^+$, where MIBI is 2-methoxy isobutyl isonitrile[4]). More advanced applications involving targeted tumor therapy[5] require specific compounds for initial diagnostics, such as those provided by tumor-specific antibodies. Single-chain antibody fragments (scFvs) have the potential for good tumor targeting, since they penetrate rapidly and yield high ratios of tumor to background at early time points[6]. As scFv fragments[7] are becoming available from combinatorial libraries on a routine basis, a general and facile radiolabeling procedure, robust enough to be carried out in a hospital setting, will be required to exploit this technology. To match the in vivo half-life of scFv fragments, ^{99m}Tc with a half-life of 6 h is most attractive, since it is readily and economically available through a ^{99}Mo/^{99m}Tc generator system and has an ideal photon energy.

Unfortunately, there had been no convenient method for ^{99m}Tc labeling of scFv fragments. All strategies employed to date were dependent on free sulfhydryl groups, introduced by a thiol-containing chelator, which either requires chemical modification or a genetically introduced free cysteine. This has been done by adding a single free cysteine[9,10] to the C-terminus of the scFv fragment[8], or a small C-terminal peptide like CGCG or G_4C to the C terminus (refs 11–13), or even by fusing metallothionein[14]. However, the unpaired cysteine presents a problem for routine production and storage, because it can interfere with the disulfides in the scFv fragment and favor misfolding, leading to poor production yields and covalent aggregates. Furthermore, trans-chelation to other sulfur-containing ligands has been shown to be an important route of radiopharmaceutical instability[15].

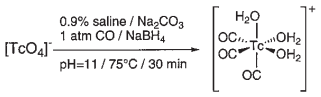
All the Tc complexes mentioned that have been used for Tc labeling of proteins are "classical Werner type" coordination compounds without direct metal–carbon bonds. Technetium is usually in the oxidation state +V as $M{=}O^{3+}$ core, wrapped by tetradentate N- or S-ligands, which provide high thermodynamic stability because of their chelate effect.

Organometallic precursors in lower oxidation states have not received much attention because their synthesis was perceived to be difficult, and they had been presumed to be unstable against hydrolysis and oxidation. Probably the only important radiopharmaceutical with technetium in a low oxidation state is ^{99m}Tc[MIBI]$_6^+$ (Sestamibi)[4], which contains only monodentate M-C bonds, and is perfectly stable in vivo, but completely inert against substitution and thus not suitable for labeling purposes.

In this paper we present a new approach to solve these problems. By using a Tc(I)–carbonyl compound, we can obtain extremely stable complexes to the His-tag, which may be one of the most convenient attachment sites, as it is a widely used and general purification tag for immobilized metal ion affinity chromatography (IMAC)[16]. We found a very convenient method of obtaining routine synthesis of the organometallic aqua-ion $[^{99m}Tc(H_2O)_3(CO)_3]^+$, abbreviated TcCO, directly from $[^{99m}TcO_4]^-$ in 0.9% sodium chloride solution under 1 atm of CO. Using this compound, we obtained high specific activities and very stable complexes. To delineate these fundamental advantages, in particular for the purpose of protein labeling and for understanding the coordination chemistry, we also investigated some model complexes of TcCO. We demonstrated the specificity of the reaction for histidine, and finally the proof of principle, by labeling a recombinant scFv and demonstrating its in vivo utility.

Results and discussion

Synthesis. To date, typical syntheses of carbonyl complexes of technetium or rhenium have usually employed high pressure and/or



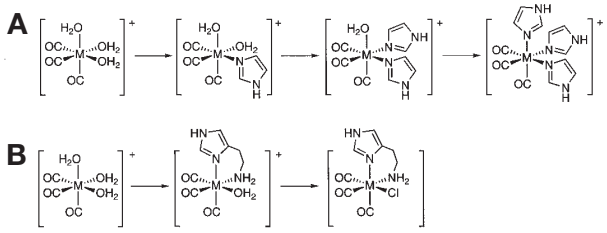
Scheme 1. New synthesis of ^{99m}Tc/^{188}Re carbonyl aqua-ion.

high-temperature autoclave reactions[17,18]. Obviously, such a procedure is not convenient for radioactive material. Therefore, we developed a novel approach and recently presented a synthesis of $[^{99}TcCl_3(CO)_3]^{2-}$ directly from $[^{99}TcO_4]^-$ (ref. 19). Only 1 atm of CO and ambient conditions were required for a quantitative yield. This represents one of the rare examples in which, under such conditions, carbonyl complexes are directly accessible from oxo-precursors. We have now further simplified the synthesis by eluting $[^{99m}TcO_4]^-$ or $[^{188}ReO_4]^-$ directly from the generator in 0.15 M NaCl solution into a vial containing $NaBH_4$ and filled with CO (Scheme 1). Synergistic interactions between the reductant ($NaBH_4$) and the stabilizing agent (CO) make the synthesis of TcCO or $[^{188}Re(H_2O)_3(CO)_3]^+$ (denoted ReCO) possible. No intermediates at all could be detected, and therefore the Tc(I) center, once it has been formed upon reduction, must be trapped efficiently by the three CO molecules. Even at high temperature and extended reaction time, TcCO and ReCO are stable.

While the Tc(V) precursor has to be stabilized by multidentate ligands, which remain present at high concentrations during subsequent labeling and act as competitors, only monodentate H_2O has to be displaced in the case of TcCO. Consequently, much lower concentrations of the biomolecule are required for efficient labeling, since its nucleophilic groups do not have to compete with auxiliary ligands. Furthermore, Tc(I) complexes possess a d^6 low-spin electronic configuration and are kinetically very stable, since the dissociative ligand substitution pathway is quantum mechanically forbidden. Associative substitution is strongly disfavored because of the lack of metal orbitals available for additional interaction with donating or accepting ligands. The exchange mechanism must be an interchange type (I_a or I_d) limited by the off-rate of the corresponding ligand in the inner coordination sphere. Thus, monodentate ligands such as imidazole (imz), and even more bidentate ligands, such as N- or C-terminal histidine residues, should provide sufficient stability for in vivo applications, and this was indeed observed.

Model complexes. The ideal ligand for Tc must displace coordinating water quickly, since the tolerable reaction time is limited by the short half-life of ^{99m}Tc. A high kinetic stability of the product is the prerequisite for any biological application even when challenged with a large excess of other strong ligands, such as all the nucleophiles present in human serum. We thus measured complex formation and stability for a series of model compounds.

Aliphatic amines and thioethers. Aliphatic amines by themselves did not interact with the Tc/Re(I) center. ^{1}H-NMR experiments (data not shown) with TcCO in the presence of morpholine in water revealed no coordination, even at high ligand excess and after long reaction times. Apparently, the strong σ-donating properties of aliphatic amines can hardly match the corresponding accepting properties of the Tc/Re(I) center. Furthermore, water may be a better π-donor than aliphatic amines. The ligand substitution experiments with thioethers (thioxane) yielded similar results. Although complexation was observable by ^{1}H-NMR and HPLC, the rate was found to be extremely slow even at 60°C. Only at a ligand/metal ratio of 20:1 and in organic solvent, a complex of the composition $[ReBr(thioxane)_2(CO)_3]$ was isolated from CH_3OH. The composition of the analogous ^{188}Re complex was confirmed by HPLC comparison. Slow and weak coordination tendencies were observed for several other amines and thioethers (data not shown). We conclude from these model experiments that neither the amino groups in lysine nor the thioether group in methionine will play an important role as single complexation sites in the direct labeling of proteins.



Scheme 2. (A) Complex formation of (Tc/Re) with imz. (B) Complex formation of (Tc/Re) with histamine.

Aromatic amines. The situation proved to be different in the case of imz and derivatives. ^{1}H-NMR investigations in water revealed the rapid formation of the 1:1 complex $[Re(imz)(H_2O)_2(CO)_3]^+$, the 1:2 complex $[Re(imz)_2(H_2O)(CO)_3]^+$, and finally precipitation of $[ReBr(imz)_2(CO)_3]$. When Br^- was removed by prior precipitation with Ag^+, the slow formation of a complex of the composition $[Re(imz)_3(CO)_3]^+$ was observed by ^{1}H-NMR. The structure of the latter complex was confirmed by X-ray structure analysis (Scheme 2A, Fig. 1A). For TcCO the same intermediates could be identified by HPLC comparison with the rhenium complexes mentioned above. These complexes were found to have an extraordinary high kinetic stability. While complex formation was completed after 30–60 min, decomplexation by protonation was slow, and even at pH 1 at elevated temperature (50°C) more than 97% of the original complex was still present after 24 h. Even though K_d values of most metal–imz complexes are typically in the range of 10^{-5} to 10^{-6} M (ref. 20), ligand exchange between ^{4}H-imz and ^{4}D-imz was not observed. Even a challenge with a 10^6-fold excess of phenanthroline revealed no transformation to the corresponding phenanthroline complex (data not shown). These experiments reveal the great kinetic stability even of the monodentate imz complex of TcCO and ReCO, presumably because of the high ligand field stabilization energy.

Derivatives of aromatic amines. To test whether the placement of histidine at the N-terminal position in a protein would further enhance binding by bidentate complexation of the metal, histamine was tested as a model compound. Histamine readily formed a complex of composition $[ReBr(histamine)(CO)_3]$ (Scheme 2B). X-ray analysis confirmed the structure (Fig. 1B), and comparison of the corresponding ^{99m}Tc complex with the Re complex by HPLC proved its identity. The kinetics of complex formation is similar to that of free imz. The complex $[^{99m}TcCl(histamine)(CO)_3]$ was stable in serum and resisted heating to 60°C for 12 h in phosphate buffered saline (PBS) without decomposition.

Labeling of peptides. We next tested the influence of the position of histidine on its complexation efficiency in peptides, and the poten-

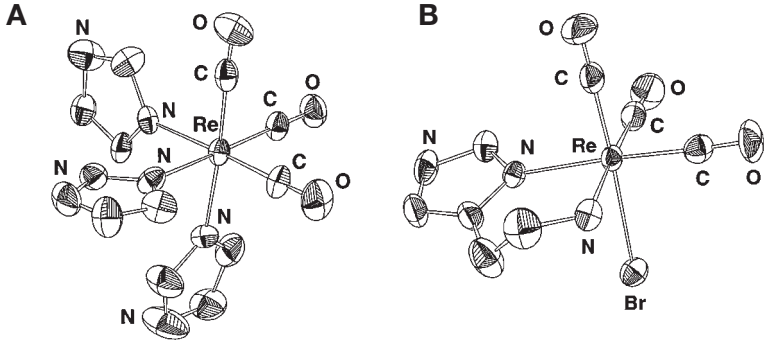


Figure 1. X-ray structure of (A) $[Re(imz)_3(CO)_3]^+$ and (B) $[ReBr(histamine)(CO)_3]$.

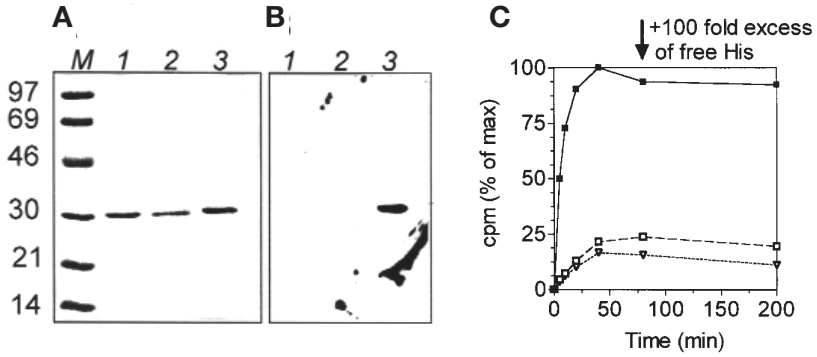


Figure 2. Site-specific incorporation of ^{99m}Tc into the scFv fragment of McPC603 with or without a His_5-tag. SDS–PAGE (A) stained with Coomassie blue and (B) detection of His-tag with anti-His-tag scFv alkaline phosphatase fusion protein. Lanes: M, molecular weight markers; 1, scFv with no histidine residue at all (H(L92)A); 2, scFv with one endogenous histidine (L92); 3, scFv with one endogenous histidine and His_5-tag. (C) The incorporation of TcCO was followed over time and challenged with free histidine at a 100-fold molar excess over scFv. ScFv with His_5-tag (■) incorporated TcCO rapidly and quantitatively, and only 1.5% of radioactivity was released after histidine challenge for 2 h at 37°C; scFv with one endogenous histidine (□) lost 18% of bound activity, and scFv without any histidine (▽) lost 29% of initial activity from the protein.

tial importance of the N-terminal amino group or the C-terminal carboxyl group, either for taking part in the complexation as a ligand or for influencing the pKa of the histidine side chain. We synthesized three short peptides: (1) His-Gly-Gly-Ala-Ala-Leu (2) Ala-Gly-His-Gly-Ala-Leu, and (3) Leu-Ala-Ala-Gly-Gly-His; and measured their complexation rates with TcCO by determining the yield obtained after a fixed incubation period by mixing TcCO with different concentrations of peptides under identical conditions. After 90 min at 37°C, 50% of the TcCO was complexed by N-terminal His-peptide (1), present at a concentration of 1.7×10^{-5} M (kinetic labeling yield EC_{50}). The second best was C-terminal peptide (3), which reached an EC_{50} at 5.4×10^{-5} M, and the most inefficiently labeled was the endo-peptide (2), which reached an EC_{50} at 9.7×10^{-5} M. The complex formation rates at 37°C are thus between 2 and 8 M^{-1} s^{-1} for these peptides, with the peptide containing the "middle" histidine being slowest.

Labeling of scFv fragments with ^{99m}Tc. We then investigated the labeling of several antibody scFv fragments, taking into account all information obtained from the model studies described above. A standard labeling protocol for scFv fragments carrying a C-terminal His-tag was devised after optimizing concentration, reaction time, and pH (see Experimental Protocol). It consists of simply mixing the scFv solution and the TcCO solution. Depending on the initial activity eluted from the generator, we obtained activities of up to 3.3 GBq/mg (90 mCi), with 90% of the label bound to the protein. Typical labeling times were 15 min at 37°C. Size exclusion chromatography on a Superose-12 FPLC column showed no protein aggregates present after labeling. Sodium dodecyl sulfate (SDS)–gel electrophoresis of the labeled scFv under reducing and nonreducing conditions showed radioactive bands at the expected molecular mass of the protein and highlights the remarkable stability of the still associated radioactive label.

Site-specific labeling of the His-tag. We also tested whether the His-tag was really the primary site of labeling by using an scFv fragment derived from the anti-phosphorylcholine antibody McPC603, as it could be conveniently purified by phosphorylcholine affinity chromatography, independently of the His tag. When this scFv fragment carried a His_5-tag, all TcCO was quantitatively incorporated after 40 min, and when challenged with a 100-fold excess of free histidine for 2 h, only 1.5% of the activity was displaced. Without the His-tag, only 25% of the radioactivity was incorporated, presumably partially at the sole histidine residue at light chain position L92, and 18% of this radioactivity was lost upon incubation with excess free histidine. When the histidine at light chain position L92 was finally mutated to alanine, total incorporation dropped further to 16%, of which 29% was lost upon incubation with excess free histidine. These data show that labeling indeed occurs predominantly at the His-tag (Fig. 2).

Stability and immunoreactivity of radiolabeled scFv in vitro and in vivo. Stability studies were performed with the anti-mucin scFv M12. After labeling and removal of the remaining pertechnetate on a Sephadex G-25 column, most of the radioactivity remained associated with the scFv after 24 h incubation at 37°C in PBS (98.9%), human serum (87.1%), or a 5,000-fold molar excess of free histidine (100 mM) in PBS (94.4%). For these measurements, labeled M12 was purified on a desalting column and adsorbed on an anti-c-myc affinity column. After washing, the bound M12 was incubated at 37°C for 24 h under the conditions of interest and the released radioactivity was determined. With a series of seven different scFv fragments, it was then shown that the immunoreactivity was well preserved after radiolabeling for all of them, ranging from 57% to 97% (Table 1). Binding assays varied (see Experimental Protocol), depending on the particular antigen for each scFv. The in vivo stability of radiolabeled scFv M12 was further analyzed after injection of ^{99m}Tc-labeled scFv fragment in mice. Gel filtration profiles of whole-mouse sera after 1 h revealed that 75% of the activity comigrated with intact scFv fragment, 14 % migrated with the albumin fraction, and 11% was of high-molecular-weight origin. The scFv is secreted through the kidney because of its small size, and in urine all of the activity was still bound to the scFv fragment after 1 h (Fig. 3A, B).

Pharmacokinetics and tumor localization. Biodistribution studies in mice were carried out by simultaneously injecting ^{99m}Tc- and ^{125}I- labeled scFv. By this method we provided an immediate internal control to determine whether the Tc label actually reflects the distribution of the scFv protein. Both labels displayed bi-exponential time/activity curves with a rapid equilibration phase and a subsequent elimination phase. The blood clearance profiles of the two different nuclides are essentially identical, both resulting in similar half-lives of 13 min for scFv M12 and 7.5 min for the anti-HER2 scFv 4D5 for the respective fast alpha phases. The organ distribution in

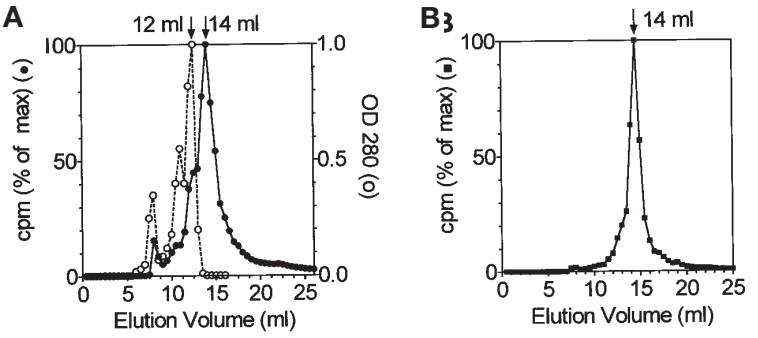


Figure 3. Elution profile of radiolabeled scFv M12 in mouse blood and mouse urine 1 h after intravenous injection. Both preparations were separated on a Superose-12 gel filtration column. (A) Mouse blood: the main radioactivity (●) elutes at 14 ml (position of scFv), the main protein peak at OD_{280} (○) elutes at 12 ml (position of albumin). (B) Mouse urine: the radioactivity (■) elutes at 14 ml (position of scFv).

Table 1. Biological activities of scFv after ^{99m}Tc labeling

ScFv	Antigen	Method	Activity[a] (%)
M12	Mucin	44-mer peptide, Dynabeads	57
MOC31	EGP2	Cell binding	90
4D5	c-erbB2	Cell binding	87
M603-H11	Phosphorylcholine	Affinity column	97
FITC-E2	FITC-albumin	Gel shift	87

[a]The biological activities were assessed after standard labeling as described in Experimental Protocol.

xenografted mice (Table 2) showed specific uptake at the tumor site for antibody 4D5, with a tumor/blood ratio of 8.9 at 24 h (1.9 for the nonspecific control scFv FITC-E2). The total accumulation at the tumor site was 1.4% injected dose per gram of tissue and is comparable to published values of other anti-c-erbB2 scFv fragments[21]. Elevated renal uptake and prolonged retention of radiolabeled antibody fragments, however, would be a problem in the therapeutic application of such agents. It is likely that this renal uptake is an inherent property of the corresponding protein molecule, which is poorly visible with ^{125}I-labeled protein because of efficient enzymatic dehalogenation[22]. Recently, encouraging results have been achieved in reducing radiation nephrotoxicity by administration of cationic amino acids, such as L-lysine[23]. Only little renal uptake was observed with the ^{99m}Tc-labeled octapeptide Lys-Gly-Gly-His$_5$-peptide alone.

Conclusions. Tc(I) is easily prepared from a Tc generator and CO gas at atmospheric pressure. It is stabilized as a tricarbonyl complex in aqueous solution, but readily exchanges its water ligand for the sp^2 nitrogen of imidazoles. The resulting complexes are very stable since the soft (easily polarized) imidazole ligands bind strongly to the soft Tc(I) center, and ligand exchange through dissociation is kinetically very slow in this d^6 system. No chelating ligands (like glucoheptonate) are required that would compete with the labeling of the proteins[24]. Therefore, $[Tc(H_2O)_3(CO)_3]^+$ is an extremely convenient reagent for labeling His-tags in recombinant proteins, which are frequently present anyway for purification purposes, by simply mixing reagents. ScFv fragments labeled with TcCO show neither trans-chelation nor loss of binding activity, and indicate the same biodistribution as iodine-labeled scFv in a tumor localization study. We believe that this technology will be useful for a wide range of in vitro diagnostics using peptides, proteins, and even small molecules, and its use may be extended to $^{186/188}$Re for radiotherapy as well.

Table 2. Biodistribution of ^{99m}Tc scFv 4D5, scFv FITC-E2, and His$_5$-peptide in nu/nu mice[a]

	Percentage of injected dose per gram of organ (24 h)		
Tissue	scFv 4D5 (anti-c-erbB2)	scFv FITC-E2 (anti-FITC control)	Octapeptide Lys-Gly$_2$-His$_5$
Blood	0.17 ± 0.03	0.20 ± 0.02	0.05 ± 0.01
Heart	0.21 ± 0.03	0.19 ± 0.02	0.07 ± 0.01
Lung	0.29 ± 0.06	0.35 ± 0.04	0.13 ± 0.02
Spleen	0.39 ± 0.06	0.73 ± 0.06	0.29 ± 0.06
Liver	1.76 ± 0.34	2.87 ± 0.22	1.51 ± 0.19
Kidney	108.60 ± 21.77	102.98 ± 11.00	2.40 ± 0.53
Stomach	0.40 ± 0.18	0.25 ± 0.11	0.42 ± 0.18
Colon	0.30 ± 0.06	0.29 ± 0.06	0.18 ± 0.08
Bone	0.16 ± 0.03	0.21 ± 0.04	0.05 ± 0.01
Muscle	0.12 ± 0.02	0.08 ± 0.03	0.02 ± 0.01
Tumor	1.41 ± 0.29	0.38 ± 0.15	N/D
Tumor/blood ratio	8.87 ± 3.27	1.9 ± 0.86	

[a]Biodistributions of ^{99m}Tc-labeled scFv and His$_5$-peptide were studied in xenografted nu/nu mice (n = 6) 24 h after injection of the radiolabel into the animals. The results express the mean ± SD. N/D, not determined.

Experimental protocol

Synthesis of $[^{99m}Tc(H_2O)_3(CO)_3]^+$. Na_2CO_3 (0.004 g, 0.038 mmol) and $NaBH_4$ (0.005 g, 0.13 mmol) were added to a Wheaton sample vial with rubber-lined screw-cap (Sigma, Buchs, Switzerland) (8 ml), which was then tightly closed and flushed for 10 min with CO. A 3 ml quantity of a generator eluate (Mallinckrodt, Chesterfield, MI) containing up to 30 GBq Na[^{99m}TcO$_4$] in 0.9% NaCl (w/v) /H_2O was added with a syringe, and the solution was heated to 75°C for 30 min. For safety reasons, the syringe should be kept in the rubber stopper during the procedure. After cooling to room temperature, 0.3 ml of a 1 M sodium phosphate solution was added (pH = 7.4). The reaction was monitored either by TLC (silica gel, MeOH/HCl, 95%/5% (v/v)), R_f = 0.35; or by gradient HPLC. The yield was greater than 95%.

Model complexes. The complexes $(NEt_4)_2[ReBr_3(CO)_3]$ and $(NEt_4)^2[TcCl_3(CO)_3]$ were prepared as described elsewhere[25]. The model complexes [ReBr(histamine)(CO)$_3$], [ReBr(thioxane)$_2$(CO)$_3$], [Re(imz)(H$_2$O)$_2$(CO)$_3$]$^+$, or [Re(imz)$_2$(H$_2$O)(CO)$_3$]$^+$ were prepared in water or in a mixture of H_2O/CH_3OH with the corresponding stoichiometric amount of ligand. After 30–60 min at room temperature the neutral complexes precipitated from solution in quantitative yield. [Re(imz)$_3$(CO)$_3$]$^+$ was prepared in aqueous solution with three equivalents of imz, after precipitation of the halides with the Ag^+ salt of a weakly coordinating anion such as PF_6^- or tosylate. Br^- or Cl^- in the model complexes resulted from the counterions in the corresponding starting materials. ^{1}H-,^{13}C–NMR spectroscopy data and elementary analyses (C, H, N; performed at the Institute of Organic Chemistry at the ETH Zürich) confirmed the structure of the described complexes.

Peptides. The peptides KGGHHHHH, HGGAAL, LAAGGH, and AGHGAL were synthesized under standard solid-phase conditions (t-Butyloxycarbonyl (BOC)-chemistry) on an Applied Biosystems (Perkin-Elmer, Foster City, CA) 433A peptide synthesizer. Labeling studies were carried out at 37°C at peptide concentrations of 10^{-4} M. The 44-mer two-tandem repeat mucin peptide (VTSAPDTRPAPGSTAPPAHG)$_2$VTSA, was synthesized in the same way and biotinylated at the N-terminal amino group with NHS-SS-Biotin (Pierce Chemical Co., Rockford, IL.

scFv Proteins. For labeling studies, seven different scFv fragments with a C-terminal His$_5$- or His$_6$-tag were used. The labeling reaction was optimized with the scFv M12, a human anti-mucin-1 antibody selected by phage display from a naive library against a 10-mer mucin tandem-repeat peptide. For comparing the labeling of scFvs with and without His-tags, we used three derivatives of a well-folding mutant of the anti-phosphorylcholine scFv McPC603-H11 (ref. 26): H11-His$_5$, H11 without a His-tag, and H11/H(L92)A, which carries no histidine at all, as the sole histidine at position L92 has been mutated to alanine. For in vivo studies, the anti-erbB2 scFv, derived from the monoclonal antibody 4D5 (ref. 27), and the anti-mucin scFv M12 were chosen. As a negative control, anti-FITC scFv FITC-E2 was used (ref. 28). Biological activity after labeling was also assessed with anti-epithelial glycoprotein-2 scFv MOC31, which was obtained by methods described elsewhere[29]. These proteins were expressed in *Escherichia coli*, purified and characterized as described[30].

Optimization of the labeling conditions. The labeling reaction was investigated between pH 4 and pH 10, which were adjusted starting with acetate buffer, switching to 2-[*N*-morpholino]ethanesulfonic acid buffer (MES) and finally using carbonate buffer. The concentration dependence of radiolabel incorporation was measured at three protein concentrations (20 μM, 35 μM, and 50 μM) over a time period of 5–80 min.

Standard ^{99m}Tc radiolabeling conditions. Usually scFv fragments were concentrated to 2 mg/ml (about 70 mM) with a 10 kDa cutoff filter

(Ultrafree-MC low protein binding; Millipore, Bedford, MA) at 1,300 g at 4°C. A 50 ml aliquot of the scFv solution was mixed with 25 ml of 0.5 M MES pH 6.2 and 25 ml TcCO. The reaction mixture was heated to 37°C for 15–30 min and separated on a fast desalting column FP10 (Pharmacia Biotech, Uppsala, Sweden). The labeling efficiency ranged from 70% to 95% of the total radioactivity incorporated.

Radioiodination of scFv. The scFv antibodies M12 and 4D5 were iodinated by the Iodo-Gen method[31] using a preactivation strategy (Pierce, protocol 28601). Iodine was preoxidized in Iodo-Gen-coated iodination tubes for 4 min and then mixed in a separate tube with the antibody for another 4 min. The sample was then passed through a gel filtration column (fast desalting) to remove any unincorporated radioiodide. Using 74 MBq/mg initial activities of NaI, typical labeling efficiencies were 70%.

In vitro stability studies. The stability of the TcCO complex binding to the scFv M12 was measured at 37°C after 24 h. Radiolabeled scFv M12 (which carries a myc-tag followed by the His_6-tag) was incubated with anti-myc-IgG1 9E10 for 90 min and then adsorbed on an anti-IgG1 agarose gel for 1 h. After washing, aliquots of the gel were incubated in PBS, human sera, or 0.1 M histidine in PBS for the time intervals indicated, and the released radioactivity was compared with the activity still bound. Nonspecific binding was determined with an unspecific IgG2a antibody instead of the anti-myc-IgG1 antibody.

In vivo stability studies. ^{99m}Tc-labeled scFvs M12 or 4D5 were injected intravenously (3 μg/0.15 MBq). Blood and urine samples after the time indicated for M12 and 4D5 were separated on a gel filtration column (Superose-12; Pharmacia Biotech, Uppsala, Sweden) and the elution profiles were compared with an aliquot of the initial antibody.

Immunoreactivity. The immunoreactivity of the labeled antibodies was assessed for the scFv 4D5 and MOC31 by the method of Lindmo *et al.*[32] on SK-Br-3 human ovary adenocarcinoma[33] cells or the SW2 human small-cell lung carcinoma cell line obtained from Dr. S.D. Bernal (Dana Farber Cancer Institute, Boston, MA), respectively. Immunoreactivity of the control anti-FITC scFv was measured by mixing an aliquot of the labeled antibody with FITC-albumin (Sigma). This mixture was applied on a gel filtration column and the radioactivity eluted at 100 kDa (scFv bound to FITC-albumin) was compared with the activity eluting at 28 kDa (scFv). Reactivity of scFv M12 remaining after Tc labeling was measured by incubating the scFv with biotinylated tandem-repeat mucin peptide for 90 min and capturing the bound activity with streptavidin-coated magnetic beads (Dynabeads; Dynal, Oslo, Norway) for 20 min. The biological activity of the anti-phosphorylcholine antibodies was assessed on a phosphorylcholine affinity column. Labeled antibody was applied on the column, the column was washed, and bound and nonbound activities were determined.

Biodistribution Studies. Blood clearance studies were performed in nu/nu nude mice. They were injected with 3 μg/0.15 MBq ^{99m}Tc and 1.2 μg/0.03 MBq ^{125}I-labeled scFv M12 or scFv 4D5 in 100 μl PBS. Blood samples were taken at 7.5, 15, 30, 60, 120, and 240 min after injection. For organ distribution experiments animals were sacrificed at 1 h, 4 h, or 24 h after injection, and tissues were collected and measured in a gamma counter. For tumor targeting studies six mice were xenografted with 10^7 SK-OV-3 cells (#HTB 77; American Type Culture Collection, Rockville, MD) on the left and right side. After two weeks, when the tumors had reached a size of about 40 mg, groups of three mice received 12 μg/0.15 MBq ^{99m}Tc-labeled scFv tumor-specific 4D5 or control scFv (anti-FITC) in 100 μl PBS. After 24 h, mice were sacrificed and tissue radioactivity measured.

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