Paper H1

A new reproducible method of introducing potentially metabolizable linking groups between ^{111}In-cyclohexyl EDTA derivates and anti-CEA F(ab')$_2$ monoclonal antibody(MAb) and their evaluation in human tumor xenografted nude mice.

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Abstract

The purpose of the present study was to synthesize and evaluate new linker-bifunctional chelating agents to enable modification of ^{111}In-MAb distribution. The approach was aimed at reproducible method with compound characterization at each synthetic step and methods that can be readily adapted to other linkers or radionuclides. (Fig.1)
Symmetrical linking groups were synthesized by reacting their free diacid terminal groups with dicyclohexyl carbodiimide(DCC) and N-hydroxysuccinimide(NHS) giving N-hydroxysuccinimide diester compounds. These diesters were then reacted in excess with N-(methyl(2-aminoethyl)carbamide)-trans-1,2-diaminocyclohexane N,N',N'-tetracetic acid(CDTAED), isolated, then conjugated to antibody.
CDTAED was synthesized in two steps as previously reported(1) by reacting CDTA with acetic anhydride and pyridine giving a monoanhydride (CDTAMA) prior to the reaction with ethylene diamine in excess.
In our study, different functional groups (ester, aliphatic, thioether and disulfide groups) which could be potentially cleaved by liver enzymes were introduced between chelate and antibody.
The linker-ligand compounds were coupled, via an amide linkage, to F(ab')$_2$ fragments of anti-carcinoembryonic antigen monoclonal antibody (anti-CEA MAb) by reacting their N-hydroxysuccinimide terminal group with amino groups present on the protein.
These immunoconjugates were then labeled with ^{111}In and subjected to biodistribution studies in tumor xenografted nude mice.
The data show(Table 1) that the aliphatic immunoconjugate seems to be the best candidate for tumor radioimmunodetection in the nude mouse model.
It offers good tumor uptake and better contrast than obtained with the use of the conventional ^{111}In-DTPA dianhydride chelate.

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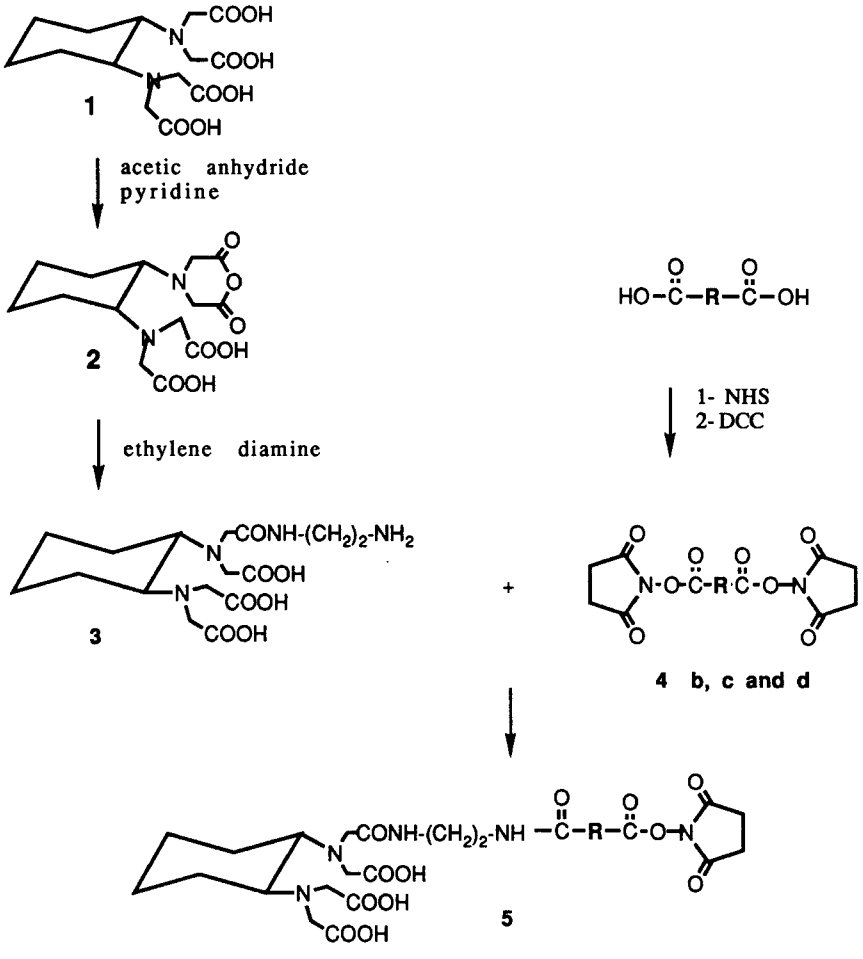
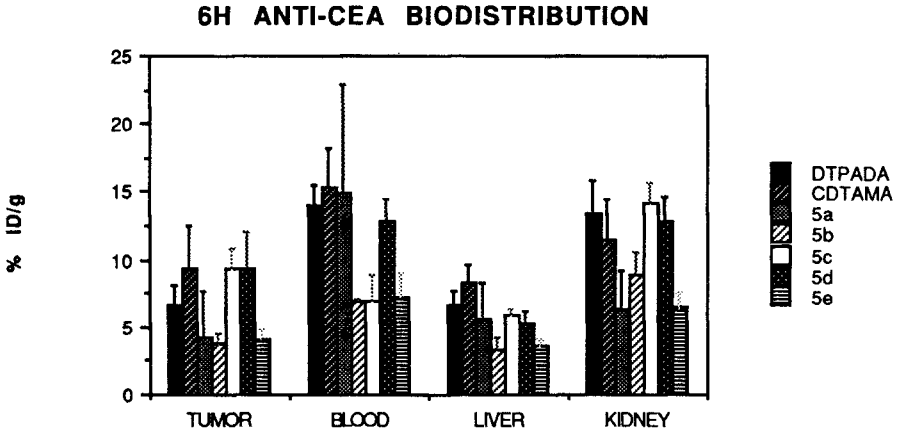
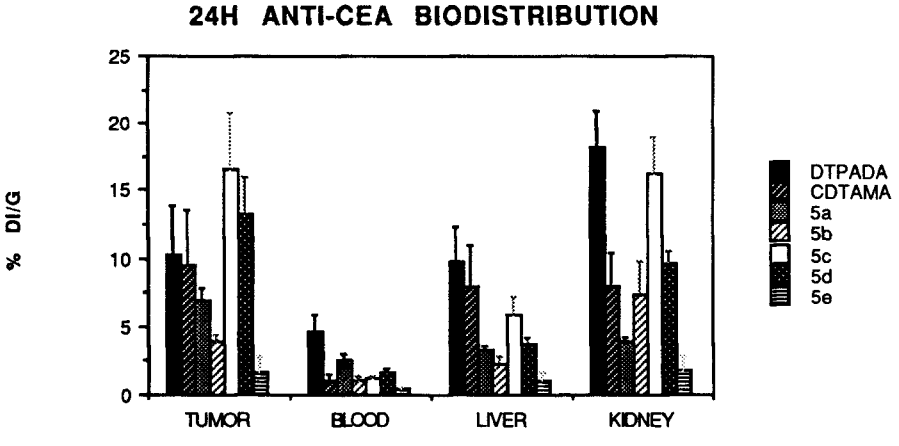
Fig.1 Synthesis of CDTA-linker-bifunctional chelating agents

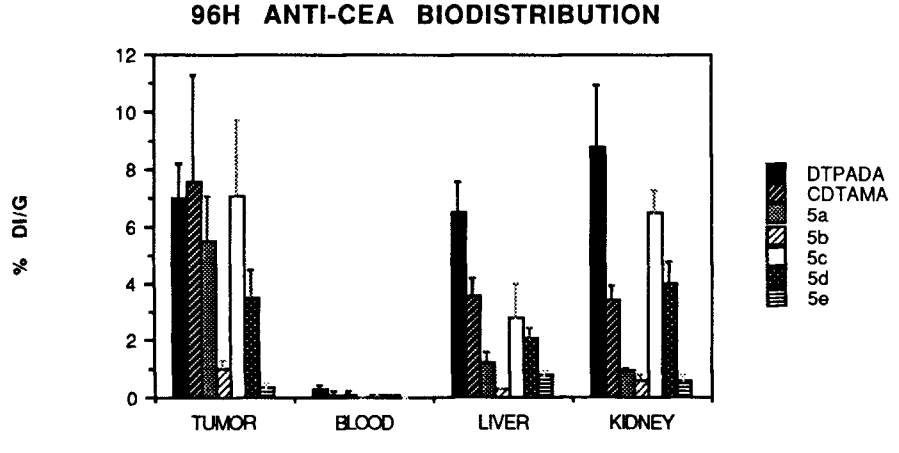


	- R -	Rdt%
a	-$(CH_2)_6$-	80
b	-$(CH_2)_2$-OCO-$(CH_2)_2$-CO_2-$(CH_2)_2$-	51
c	-CH_2-S-CH_2-	65
d	-$(CH_2)_2$-S-$(CH_2)_2$-	76
e	-$(CH_2)_2$-S-S-$(CH_2)_2$-	84

Table 1:


6H ANTI-CEA BIODISTRIBUTION
% ID/g
25
20
15
10
5
0
TUMOR
BLOOD
LIVER
KIDNEY
DTPADA
CDTAMA
5a
5b
5c
5d
5e


24H ANTI-CEA BIODISTRIBUTION
% DI/G
25
20
15
10
5
0
TUMOR
BLOOD
LIVER
KIDNEY
DTPADA
CDTAMA
5a
5b
5c
5d
5e


96H ANTI-CEA BIODISTRIBUTION
% DI/G
12
10
8
6
4
2
0
TUMOR
BLOOD
LIVER
KIDNEY
DTPADA
CDTAMA
5a
5b
5c
5d
5e

Paper H2

EFFECTS OF CHELATE RIGIDITY AND DENTICITY ON THE IN-VIVO PERFORMANCE OF ^{203}Pb AND ^{88}Y LABELED MONOCLONAL ANTIBODIES

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Based on various criteria such as imaging photons, particle emission, dosimetry, and feasibility of production, etc., a number of radiometals are considered suitable for labeling monoclonal antibodies (MAb) for radioimmunoimaging (RII) and/or radioimmunotherapy (RIT) (1). This presentation focuses on MAb labeling and evaluation with ^{203}Pb ($t_{½}$ 2.2d, γ 279 keV, 81%) and ^{90}Y ($t_{½}$ 2.7d, β^-avg. 915 keV, no γ) for RII and RIT applications, respectively. Common methods for labeling MAb using functionalized polyaminocarboxylates (PACS), such as the DTPA dianhydride (DTPADA) method, are not ideal for use with ^{203}Pb and ^{90}Y. These radiometals share a common feature in that they require bifunctional chelating agents (BCA) with a higher denticity (number of coordination sites) and greater structural rigidity in order to obtain immunoconjugates with better in-vivo kinetic stability. Although some radiometals produce improved biodistribution upon going to DTPA functionalized at the carbon backbone (2,3), non-specific uptake of radioactivity is still undesirably high in bone and kidney with ^{203}Pb and in bone and liver with ^{90}Y. In the present study, we have evaluated the following BCA with varying denticity and structural rigidity: (i) cyclohexyl EDTA monoanhydride (CDTAMA); (ii) 4-isothiocyanato-cyclohexyl EDTA (4-ICE); (iii) cyclohexyl DTPA (CDTPA); (iv) cyclohexyl TTHA (CTTHA); and (v) 1,4,7,10-tetraazacyclododecane N,N',N'',N'''-tetraacetic acid (DOTA). Cyclohexyl PACS were synthesized and functionalized using procedures developed earlier (1,4,5). Conjugation to MAb was done either through a monofunctionalized derivative (CDTAMA, CDTPA-NHS, CTTHA-NHS) or through the 4-SCN group (4-ICE). DOTA was synthesized using a new procedure utilizing cyanomethylation of commercial 1,4,7,10-tetraazacyclododecane followed by hydrolysis (Figure 1), and conjugated to MAb through one of the carboxylates that was converted to an NHS ester (6). DTPADA was used as a control ligand in all experiments.

Two antibody systems were investigated: anticolon carcinoma MAb 17-1A (Y and Pb) and an anti-CEA F(ab')$_2$ preparation (Pb only). The average number of ligands per MAb varied between 1 and 3. Lead-203 was obtained from Nordion International as a solution in HCl (sp. act. 1.0 mCi/μg), and no-carrier-added ^{88}Y (used as a stand-in for ^{90}Y for convenience) from Los Alamos National Laboratory. Centricon-purified immunoconjugates were labeled with Pb and Y at pH 5-6 (acetate or citrate buffers) and then purified further by size-exclusion HPLC. Labeling yields for the various preparations ranged between 40 and 80% (except ~10% for Pb-DOTA, Y-CDTPA, and Y-CTTHA). Serum stability was determined from in-vitro incubations up to 5 d. Biodistribution studies were performed in human tumor xenografted nude mice. In the case of ^{203}Pb immunoconjugates with 17-1A IgG (Table 1), CDTAMA produced higher blood and whole body levels and increased tumor uptake compared to DTPADA. Bone and kidney uptake, however, remained high. In the CEA-F(ab')$_2$ system, the rigid DOTA gave higher tumor to non-tumor ratios (Table 1) compared with the semi-rigid 4-ICE (T/blood, T/liver, T/bone, T/kidney at 96 hr: 87 vs 1.5; 3.3 vs 0.8; 12.0 vs 1.3; 0.8 vs 0.3, respectively). Biodistribution studies with ^{88}Y-17-1A immunoconjugates (Figure 2) showed progressive improvement as follows: DOTA $\geq$ CTTHA $\geq$ CDTPA > 4ICE >>CDTAMA >> DTPADA.

Based on these preliminary data, it is concluded that both denticity and the rigidity of the bifunctional chelating agent are important factors that contribute to higher in-vivo stability of Y and Pb radioimmunoconjugates (Table 2). Monofunctionalized DOTA, a rigid ligand with 7 coordination sites appears to provide the best combination and warrants further investigation for use with ^{203}Pb and ^{90}Y.

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Table 1. Tissue Distribution of ^{203}Pb Immunoconjugates in Nude Tumor Mice *

Conjugate**	Time, Hr	Tumor	Blood	Liver	Kidney	Bone	Whole body (% Dose)
^{203}Pb Nitrate (control)	24	0.4±0.2	7.7±1.5	6.0±0.5	27.6±1.0	12.1±0.7	58
		---	(0.05)	(0.07)	(0.01)	(0.03)	
	48	0.4±0.2	1.8±0.3	3.3±0.7	15.2±1.7	13.3±1.1	61
		---	(0.22)	(0.12)	(0.03)	(0.03)	
DTPA-DA-17-1A (10%, 1d)	24	1.1±0.2	3.6±0.1	5.3±0.2	18.6±4.3	13.0±1.6	61
		---	(0.3)	(0.2)	(0.06)	(0.08)	
	96	0.4±0.1	0.9±0.1	2.7±0.4	6.3±1.5	8.1±0.3	35
		---	(0.4)	(0.15)	(0.06)	(0.05)	
CDTA-MA-17-1A (50%, 1d)	24	6.1±0.2	8.6±0.9	6.4±0.7	16.5±0.7	12.1±0.4	77
		---	(0.7)	(1.0)	(0.4)	(0.5)	
	96	1.1±1.1	1.3±0.3	2.2±0.3	6.5±0.2	14.1±1.5	48
		---	(0.9)	(0.5)	(0.2)	(0.08)	
4-ICE-CEA $F(ab')_2$ (71%, 1d)	24	7.7±1.2	4.7±0.7	8.5±0.9	19.1±2.2	7.6±0.7	76
		---	(1.6)	(0.9)	(0.4)	(1.0)	
	96	2.3±0.5	1.6±0.2	3.0±0.4	8.1±0.8	11.0±1.3	46
		---	(1.5)	(0.8)	(0.3)	(0.2)	
DOTA-CEA $F(ab')_2$ (100%, 5d)	24	19.3±4.6	3.1±0.6	9.8±1.5	30.0±5.0	1.6±0.2	94
		---	(6.3)	(2.0)	(0.6)	(12)	
	96	12.2±2.1	0.14±0.01	3.7±0.4	14.9±1.0	1.0±0.1	48
		---	(87)	(3.3)	(0.8)	(12)	

*Data (% dose per g) are average of 3-10 animals ± 1 std. deviation. Tumor to tissue ratios are included in parentheses. All preparations were HPLC purified and monomeric fractions were injected i.v. into the mice. SW 948 colon carcinoma xenografts were used for 17-1A experiments and LS-174T xenografts for the anti-CEA experiments. **Ligand abbreviations are explained in the text. In-vitro serum stability at 37°C shown in parentheses under the conjugate.

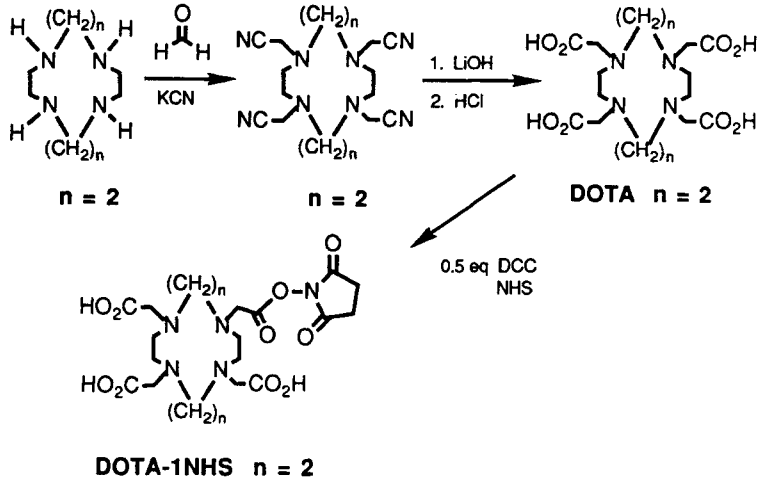

Figure 1. Synthesis of N-hydroxysuccinimidyl ester of DOTA (DOTA-NHS).

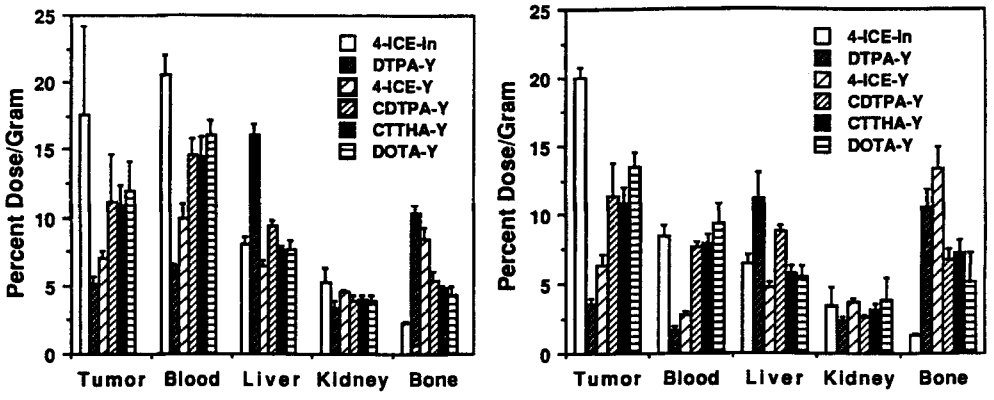

Figure 2. Tissue distribution of ^{88}Y-17-1A immunoconjugates in SW-948 human colon carcinoma xenografted nude mice at 24 hr (left) and 96 hr (right). ^{111}In-4-ICE-17-1A data are included for comparison. Ligand abbreviations are explained in the text.

Table 2. Effect of denticity (coordination sites) and structural rigidity of the ligand on the 96 hr biodistribution of ^{203}Pb and ^{88}Y immunoconjugates in nude tumor mice*

Immunoconjugate	Denticity	Rigidity	^{203}Pb			^{88}Y		
			T**	T/L	T/B	T**	T/L	T/B
DTPADA-17-1A	7	No	0.40	0.15	0.05	3.6	0.32	0.34
CDTAMA-17-1A	5	Semi-rigid	1.1	0.50	0.08	-	-	-
4-ICE-17-1A	6	Semi-rigid	-	-	-	6.4	1.4	0.48
4-ICE-CEA-F(ab')$_2$	6	Semi-rigid	2.3	0.80	0.20	-	-	-
CDTPA-NHS-17-1A	7	Semi-rigid	-	-	-	11.4	1.3	1.7
CTTHA-NHS-17-1A	9	Semi-rigid	-	-	-	10.8	1.9	1.5
DOTA-CEA-F(ab')$_2$	7	Rigid	12.2	3.3	12	13.5	2.4	2.6

*Data shown are average of 3-10 mice per experiment. T=tumor; B=bone; L=liver. Ligand abbreviations are explained in the text. Other experimental details same as in Table 1.

**Tumor uptake in % injected dose per g.

Paper H3

A New Method for Labeling Proteins with Technetium-99m

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Labeling of proteins with ^{99m}Tc has long been an area of extensive research. Most of the labeling procedures can be classified according to the following three main methods 1) direct labeling approach 2) using bifunctional chelates and 3) the preformed chelate method. Various modifications of each of these have been employed and have been extensively reviewed.[1,2] While it is quite apparent from direct labeling methods that one of the main requirements necessary for stable ^{99m}Tc labeling of proteins are native thiol groups in sufficient numbers, the main drawback of the approach are the relatively harsh reducing conditions that the protein must experience in order to expose these functionalities. To avoid this, the use of multidentate thiol containing bifunctional chelates were developed. The preformed chelate method is an important variation of this technique that is designed to achieve the same purpose.

More recently, a further category of labeling methodology has emerged that apparently obviates the need for tetra- or multi-dentate chelates.[3,4] In one of these methods, hydrazine nicotinate is linked to the protein and labeling accomplished by ligand exchange with ^{99m}Tc-glucoheptonate. The chemical nature of the resultant stable complex formed is unknown but presumably not all the coordination sites on the metal are occupied by hydrazine moieties.

We have developed a similar approach, with mild labeling conditions, involving the known affinity of technetium for thiol functionalities. The method employs the introduction of cysteine like bidentate aminothiols via a thiolactone form on to proteins and peptides, thus forming a stable amide bond between the protein and the chelating moiety (Figure 1). The protein is incubated with excess thiolactone for 8-10 hours under an inert atmosphere and is then separated from the free aminothiol, lyophilized and stored at 4 ^{0}C. ^{99m}Tc labeling of the thiolated protein is accomplished via exchange labeling with ^{99m}Tc-glucoheptonate solution for 1 hour at ambient temperature.

We have evaluated this method using various proteins such as Human serum albumin, polyclonal IgG and model polypeptides. ^{99m}Tc labeling efficiencies are high and in the order of 70-90% depending on the protein and the labeling conditions. For thiolated HSA versus native HSA the labeling efficiency was 84 $\pm$ 0.5 % and 15.5 $\pm$ 0.7 % respectively. Similarly for thiolated polyclonal IgG versus native IgG the labeling efficiencies were 74 $\pm$ 5 % and 11.6 $\pm$ 0.1 % respectively.

The extent of thiolation of the protein is significantly influenced by pH, temperature and time of incubation. These conditions as well as the factors effecting the ^{99m}Tc labeling will be described.

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Acknowledgements: Financial support provided by USPHS grant 5-RO1-CA34970 and DOE grant FG02-86ER60460.

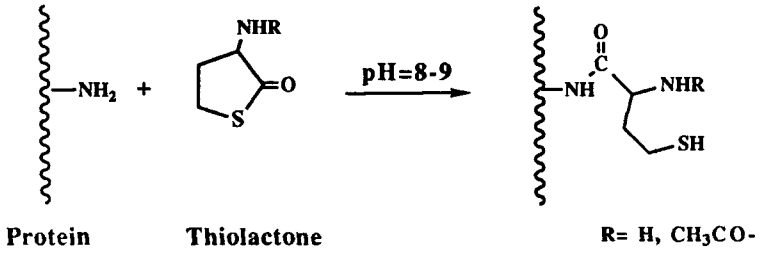

Figure 1.

A New Chelating Heterodifunctional Phosphorus-Nitrogen Ligand for Radiolabeling of Proteins

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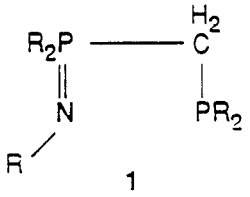
The application of monoclonal antibodies to target radionuclides to cancer sites is a subject of considerable current interest for use in cancer therapy. A variety of radionuclides have been used for preparing radioimmunotherapeutic agents via bifunctional chelating agents (BFCAs) [1]. Despite successful applications using several BFCAs for labeling monoclonal antibodies, development of new BFCAs is important for improving in vivo performances of labeled monoclonal antibodies (e.g. clearance of radioactivity from non-target tissues) and for providing versatility in labeling techniques [2]. The results of studies with a new ligand system that can form the basis to design new BFCAs is described in this report. The new ligand system developed in our laboratory are phosphiniminato-phosphine heterodifunctional ligands containing a π-acid phosphine and a sigma-donor nitrogen functionality with the general structure shown below.



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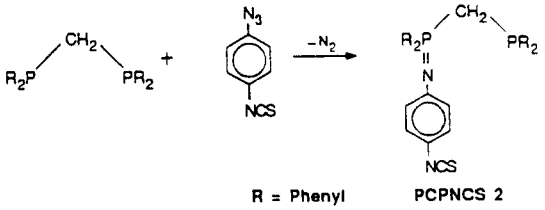
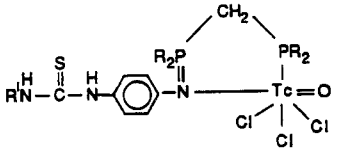
Early transition metals (e.g., Re and Tc) can be chelated through cooperative interactions of the phosphine and hard nitrogen base center. The properties of the resulting chelates with these ligands (e.g., polarity) can be readily modified by introducing a variety of substituents on the P or N atoms (R or R'). In addition, one of these substituents can be used to link these ligands or their respective preformed radiolabeled chelate to proteins or antibodies. An example of forming a BFCA and conjugation of its ^{99m}Tc-chelate to a protein is described. The BFCA used for these studies was formed by the following reaction where R = Phenyl:



R = Phenyl PCPNCS 2

Experiments to characterize the ^{99m}Tc complex with Cpd 2 were performed with a model compound in which the isothiocyanate group was reacted with t-butylamine to form the corresponding thiourea derivative (t-butyl-PCPNCS). ^{99m}Tc was complexed with the t-butyl-PCPNCS by reacting it with $^{99m}TcO_4^-$ in an HCl acidified solution obtained from a ^{99}Mo/^{99m}Tc generator (Mallinckrodt, Inc.). Typically, the ^{99m}Tc complex was prepared by adding 2-3 MBq of $^{99m}TcO_4^-$ in 0.2 mL of the eluant from a ^{99}Mo/^{99m}Tc generator which was acidified by ≈ 20 μl of Conc. HCl to generate $TcOCl_4^-$. The resulting radioactive complex was analyzed using paper, TLC and reversed phase HPLC (Figure 1). The structure of the ^{99m}Tc complex as inferred from analogous reactions of $ReOCl_4^-$ with Cpd 2, is shown below.



R' = Butyl^t

Tc-99m Butyl^t PCPNCS 3

For conjugating the ^{99m}Tc-isothiocyanate chelate (^{99m}Tc-PCPNCS) to proteins, the ^{99m}Tc-BFC formed in CH_2Cl_2 was evaporated to dryness. The residue was dissolved in 100 μl DMF and added to 1 ml of 10^{-5}M IgG in 0.05M bicarbonate buffered saline at pH 6.5-7 and incubated for 30 min. The yields of the labeled protein was determined by gel permeation chromatography using Sephadex G-100 (Figure 2).

The labeling efficiency of ^{99m}Tc-PCPNCS was 95 ±% 1.2%. Reversed phase HPLC analysis of this chelate demonstrate this to be a single chemical species (Figure 1). This complex represents the first example of a ^{99m}Tc-phosphiniminato-phosphine complex for use as a BFC. This complex exhibits excellent stability in aqueous solution over a wide pH range with no measurable decomposition for ≥ 24 hr. Decomposition begins to occur over a 24 hr period at pH of approximately 9 or higher. ^{99m}Tc-PCPNS reacted efficiently with IgG to form the ^{99m}Tc conjugate in 70-80% yields. Stability of the resulting ^{99m}Tc-PCPNCS conjugated IgG was shown to be excellent at neutral pH with no significant decomposition observed during a 24 hr incubation.

The results of this work indicate that the phosphiniminato-phosphine ligand complexed with ^{99m}Tc exhibits properties consistent with its potential use in formulating new BFCs. Even though this is a bidentate ligand, the stability of the 1:1 ligand-to-metal ratio complex is excellent. Parallel chemistry with ^{188}Re has produced identical complexation behavior with phosphiniminato-phosphine ligands. Since both the ^{99m}Tc and ^{188}Re chelates with these ligands exhibit high stability in aqueous solutions, these types of ligands should also be applicable for forming BFCs with ^{188}Re/^{186}Re for potential therapeutic applications. As with the diamido-dithiol type of BFCAs [3], this ligand system, therefore, holds the potential for preparing "matched pairs" of radioimmuno-agents for both diagnostic and therapeutic applications. Finally, the cooperative interactions between the heterofunctional chelating groups should provide stabilization for a variety of early and late transition metals [4], making these types of ligands for preparing BFCs with other transition metal radionuclides (e.g., ^{109}Pd, ^{105}Rh, etc.) highly useful.

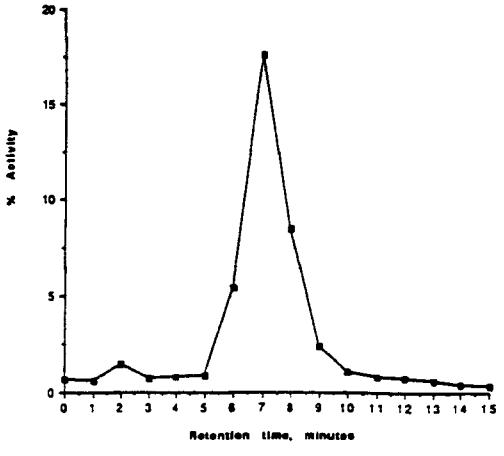


Fig-1. HPLC Profile of Cpd 3.

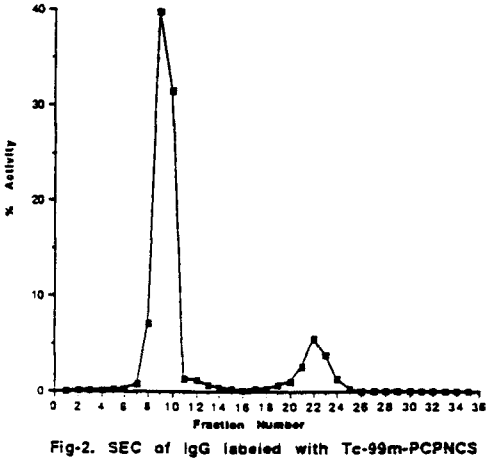


Fig-2. SEC of IgG labeled with Tc-99m-PCPNCS

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Paper H5

Tc-99m labeled Sandostatin: Preparation and Preliminary Evaluation

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Development of agents for imaging malignant tumors continues to draw considerable attention. Sandostatin (SS) is a long acting octapeptide with pharmacologic actions mimicking those of the natural hormone, somatostatin, found in 1978 to be produced by the hypothalamus and pancreas (1). SS has been labeled with I-123 (Tyr-octreotide) (2,3) and with In-111 via c-DTPA (4). The preliminary results with both agents have been highly impressive in imaging a variety of endocrine related tumors in humans, 3-24 hr following i.v. administration of 20 ug labeled SS.

We have labeled SS with Tc-99m and evaluated it in vitro for specificity and for imaging experimental tumors grown in mice. Cysteine based S-S groups were reduced to sulfhydryls using the ascorbic acid (AA) method recently developed in our laboratory (5). Typically, 10 ug SS was incubated with a 1 mg AA solution, pH 6.5, for 30 min. (22°C) and a desired quantity of Tc-99m reduced with a fresh solution of $Na_2S_2O_4$ in 0.1M acetate buffer pH 7.4. The final concentration of $Na_2S_2O_4$ was rendered 5 ug/ul reaction mixture. It was allowed to react for 1 min. and added to AA reduced SS for further incubation (22°C) for 15 min. The product was purified [Sep Pak-(3)], ethanol fractions were evaporated, taken in acetate buffer pH 7.4, and subjected to ITLC (5) and HPLC analysis (Nova Pak C-18 and 2.5% triethylamine, 17% 1-propanol in 0.1M phosphate buffer pH 6.0). A single vial kit has been prepared with yields varying between 30%-75%. (Cox et al have labeled SS with Tc-99m using Zn dust as a reducing agent (6); however, in our hands using Zn or Sn dust as a reducing agent consistently gave less than 10% yields.)

The labeled product showed saturable binding to embryonal carcinoma cells. Scatchard plot analysis showed the kd value to be 2.55×10^5 M and 85.5×10^8 SS receptors/cell (Fig 1 and 2). Approximately 10^7 cells were implanted in the right thighs of Balb/c mice and tumors were grown for 7-10 days, 0.5 to 1 cm in diameter. Several groups of five mice each received either 2 ug SS labeled with 40 uCi Tc-99m, 20 ug unlabeled SS 1 hr prior to the injection of labeled SS, 1000 i.u. of interferon 1 hr prior to the injection of 2 ug labeled SS, or 100 ug HSA labeled with Tc-99m.

At predetermined times animals were sacrificed, imaged (pinhole collimator), and dissected for tissue distribution studies. Tumor/muscle (T/M) ratios were maximal at 3 hr p.i. of labeled SS. These were 5.8 ± 0.6 compared to those of 2.3 ± 0.3 with the blood pool agent ($P=0.01$), (Fig 3). With preadministered unlabeled SS, the T/M ratios reduced to 3.8 ± 1.9 ($P=0.09$), and the % ad. dose/g of tumor reduced to 3.0 ± 0.2 from 4.8 ± 2.3 ($P=0.4$). With administration of interferon as a biological response modifier, the T/M ratios remained practically unchanged (5.7 ± 1.5 vs 5.8 ± 0.6 control) but the % ad. dose/g tumor increased from 4.8 ± 2.3 to 6.5 ± 2.9 ($P=0.5$).

In summary, SS has been labeled with Tc-99m. The yields are variable but the product shows saturable binding to embryonal carcinoma. The experimental tumors can be clearly visualized in 3 hr p.i. The tumor uptake can be reduced with cold SS, although not completely because of the high number of tumor cell receptors that may require a toxic dose of SS for saturation. The tumor uptake can be enhanced by the use of biological response modifiers (7).

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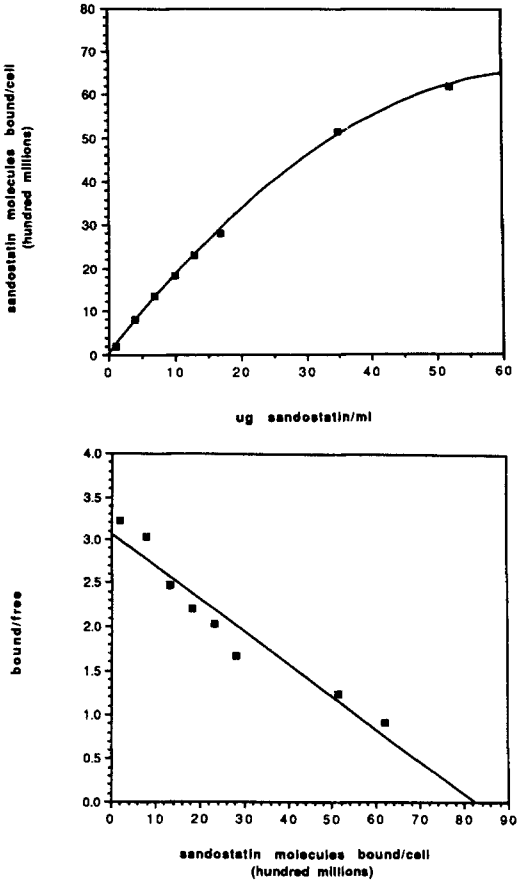

Fig 1 and 2: Scatchard plot determining the kd value of the Tc-99m SS for embryonal carcinoma cells and the number of SS specific receptors/cell.

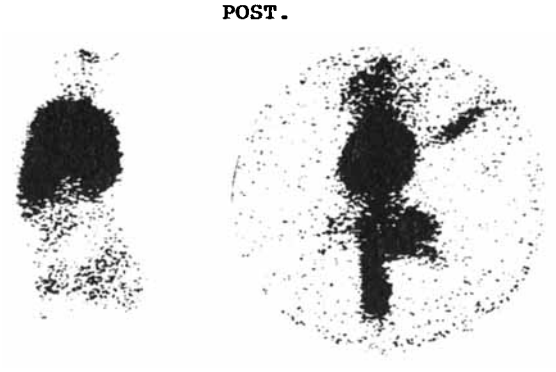

Fig 3: 3 hr posterior images of two separate mice bearing embryonal carcinoma. With Tc-99m SS, the tumor is clearly visible.

Paper H6

COPPER-LABELED ANTIBODIES FOR PET IMAGING. C.J. Anderson, S.W. Schwarz, P.A. Rocque, J.M. Connett*, L.W. Guo*, G.W. Philpott*, K.R. Zinn†, C.F. Meares‡, M.J. Welch. Mallinckdrodt Institute of Radiology, Washington University School of Medicine, St. Louis, MO 63110, *Department of Surgery, Jewish Hospital, St. Louis, MO 63110, †University of Missouri Research Reactor, Columbia, MO 65211, and ‡Department of Chemistry, University of California, Davis, CA 95616.

Positron-emitting antibodies have advantages over antibodies labeled with single-photon emitters as a result of the improved sensitivity and specificity of PET. This is particularly true in light of recent advances in PET imaging techniques including whole body imaging (1) and 3-dimensional imaging techniques (2) that increase the sensitivity of PET when used with low levels of radioactivity. Of the long-lived PET radionuclides available for antibody labeling, we have chosen to investigate ^{64}Cu for reasons which include its availability from the University of Missouri Research Reactor (MURR) in Columbia, MO, and our experience in labeling proteins with copper radionuclides (3). Antibodies labeled with ^{64}Cu could also have uses in therapy, since Apelgot and co-workers have shown it be as effective in damaging DNA as ^{67}Cu (4).

Utilizing ^{64}Cu obtained from MURR, we have evaluated copper-labeled 1A3, an anti-colorectal carcinoma monoclonal antibody, in the Golden Syrian Hamster Model (5). Antibodies were labeled using the bifunctional chelate 6-[*p*-(bromoacetamido)benzyl]-1,4,8,11-tetraazacyclo-tetradecane-*N,N',N'',N'''*-tetraacetic acid (Br-benzyl-TETA) and the spacing moiety 2-iminothiolane by the techniques described by McCall, et. al. (6). Several types of studies have been carried out utilizing this antibody and in all cases, the immunoreactivity (IR) was greater than 85% for intact antibody and approximately 70% for F(ab')$_2$ fragments. Both intact 1A3 and 1A3-F(ab')$_2$ have been shown to be virtually 100% stable in rat plasma as determined by FPLC. The absolute uptake (%ID/g) of copper-labeled intact 1A3 antibody or fragments were higher in tumor tissue than with either ^{111}In-BrΦHBED-1A3, or ^{125}I-labeled 1A3 antibodies which we have investigated (Table 1). Tumor to nontumor ratios for the antibody and its fragments are shown in Table 2. These ratios are as high or higher than those we have obtained for indium- or iodine-labeled 1A3 antibodies.

Human absorbed radiation doses for intact 1A3 and 1A3-F(ab')$_2$ were calculated from rat biodistribution data. The dose to the kidney from the fragments was extremely high, indicating that only a very small dose of ^{64}Cu-labeled 1A3 fragments could be administered to humans. For this reason we will carry out clinical trials utilizing ^{64}Cu-benzyl-TETA-1A3. If

improvements in the conjugation and purification of benzyl-TETA-1A3-F(ab')$_2$ can be made which will reduce the kidney dose, we will utilize the fragments.

Acknowledgements: This work was supported by NIH grant # CA44728, and DOE grant # DE-FG02-87-ER60512

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TABLE 1

The data below shows the mean ± standard deviation, where N = 5-30 for each MAb. Tumors were 2 days old when the radiopharmaceutical was injected, and animals were sacrificed one day post injection of labeled antibody.

LABEL	%ID/g TUMOR Intact 1A3	%ID/g TUMOR 1A3-F(ab')$_2$
copper-64	14.40 ± 4.80	10.11 ± 1.89
iodine-125	10.37 ± 4.41	4.26 ± 2.01
indium-111	7.45 ± 3.57	2.39 ± 0.76

TABLE 2

The data given below shows the mean ± standard deviation. The tumors were 2 days old when labeled antibody was injected, and the animals were sacrificed 1 day post injection.

ORGAN	Tumor/Non-tumor Ratio ^{64}Cu-TETA-1A3	Tumor/Non-tumor Ratio ^{64}Cu-TETA-1A3-F(ab')$_2$
blood	3.21 ± 0.94	7.47 ± 1.96
muscle	60.4 ± 23.5	43.9 ± 9.81
liver	9.67 ± 3.52	7.70 ± 1.72
kidney	13.01 ± 4.69	1.13 ± 0.33

Paper H7

Hydrolytic NHS-Ring Opening During BAT-Ligand Conjugation with IgG

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N-Hydroxysuccinimide (NHS) is a frequently used leaving group for the conjugation of activated esters to amino groups of peptides or proteins. Accordingly, we synthesized the NHS-BAT ester 1 (BAT = bis(aminoethanethiol)) and reacted it with IgG or IgG fragments at pH 8-8.5[1-3] (Figure 1).

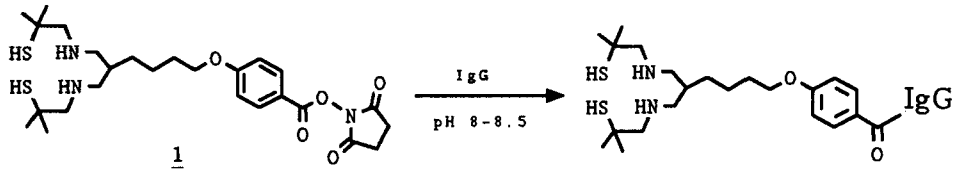


Figure 1. Reaction Scheme of NHS-BAT ester conjugation with IgG

The conjugation yield of this reaction ranged between 40-60%. The other fraction appeared as a single side product not associated with or bound to the protein. Figure 2 demonstrates the chemical appearance of the side product in a model reaction. Since HPLC

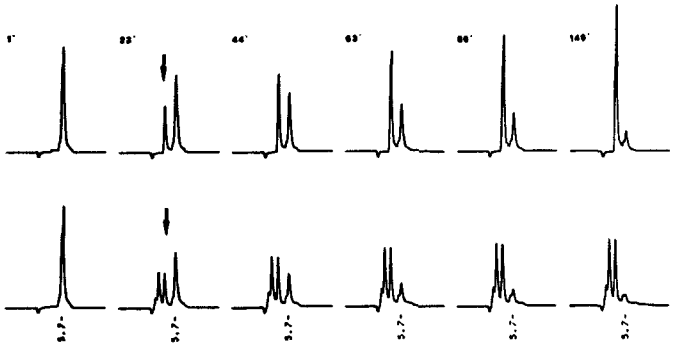


Figure 2. HPLC of NHS-BAT ester 1 in presence (lower) and absence of lysine (upper panel) at pH 8.3 as a function of reaction time; Arrows indicate the appearance of the side product.

analysis excluded the hydrolytic cleavage of the NHS group from the BAT ligand, we interpreted its appearance with an intra- or inter-molecular reaction, because the NHS-BAT ester comprises

nucleophilic heteroatoms. However, FAB-mass spectrometry of this compound surprisingly proved a mass of M+18 (Figure 3).

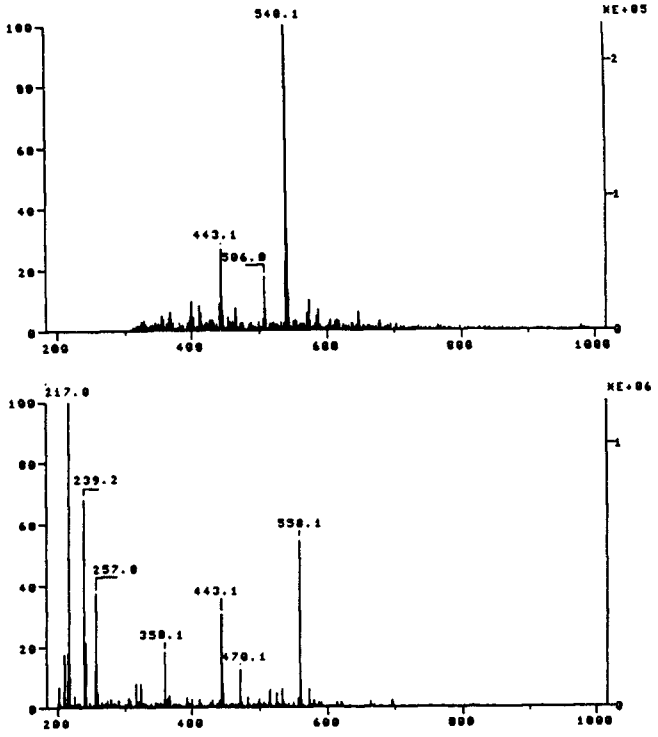
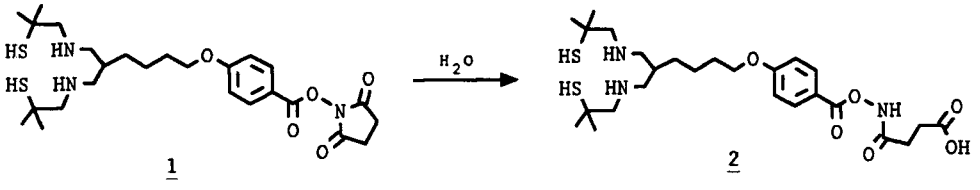


Figure 3. FAB mass spectra of the NHS-BAT ester 1 (upper panel, $[M+H]^+$ 540) and NHS-BAT ester side product (lower panel, $[M+H]^+$ 558)

This finding can only be interpreted with the addition of H_2O and NHS-ring opening. Although NHS-ring opening by aminolysis has been described before in peptide synthesis[4], it is the first time that hydrolysis of the NHS ring is described. The side reaction depicted in Figure 4 proved to be pH independent within the range of pH 6.5 - 9.2.



1 2

Figure 4. NHS hydrolysis during NHS-BAT ester conjugation

In respect to one of our currently pursued aims, namely the development of a ^{99m}Tc-"Bolton Hunter" kit for antibody labeling using the precomplexation route, this side reaction represents a limitation. The application of "inert" perfluorinated phenols on the other side is of little value for BAT-type bifunctional ligands since the corresponding esters including the ^{99m}Tc complex show only little water solubility. The preconjugation route as depicted in Figure 1 is, however, not influenced by the side product formation of this type. Low molecular weight contaminants are easily separated during workup of the antibody.

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Paper H8

Receptor Imaging with Radiolabelled Peptides - Successful Imaging of Kidney and Lung Receptors with ^{123}I & ^{111}In - Labelled Atrial Natriuretic Factor.

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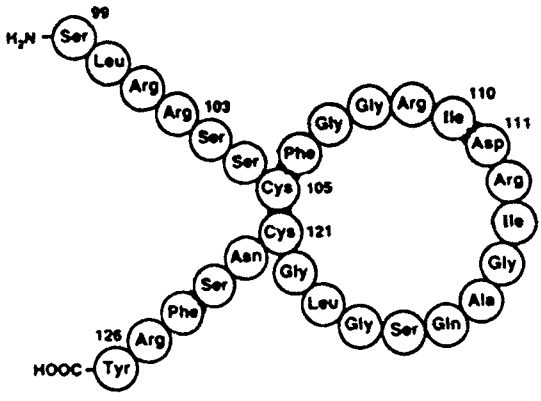
Atrial Natriuretic Factor (ANF) is a twenty eight amino-acid peptide with very high affinity for receptors found primarily in kidney and lung. The *in vivo* half-life of ANF is very short, typically less than two minutes. Despite this short half-life radiolabelled ANF can be successfully used to image receptors. We have found that ANF, labelled in very high specific activity with iodine at the terminal tyrosine residue, will rapidly accumulate in receptors in the kidney and lung tissues in rats, rabbits, dogs and monkeys. Clearance of the radiopharmaceutical from the blood is essentially complete by 3 minutes. That the basis for uptake in the target organs is receptor mediated is confirmed by the co-injection of receptor antagonists which dramatically alter the biodistribution. The key to this successful imaging is extremely high specific activity, typically 30 kCi/mmole. Using ^{123}I-ANF it is possible to selectively image the different ANF receptor sub-classes by the co-injection of selective receptor antagonists.

The need to provide radiopharmaceuticals labelled with ^{111}In or ^{99m}Tc prompted the development of derivatives of ANF containing a chelator. It is well known that the bioactivities of small peptides are very sensitive to changes in amino-acid sequence. It was therefore unclear if the attachment of a chelator to ANF would violate the "tracer principle". ANF is a twenty eight amino-acid peptide which contains no lysine residues. The only feasible site for attachment of a chelator by nucleophilic reaction is the terminal nitrogen at position 99. This was hopefully sufficiently removed from the active site at residues 109-113 to permit radiolabelling without loss of activity.

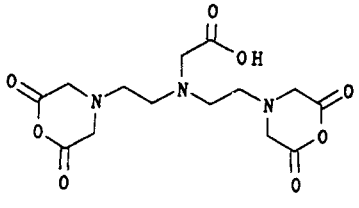
Reaction of a modified ANF derivative(r-ANF$_{101\text{-}126}$) with DTPA-anhydride **(1)** gave good yields of a chelate molecule which could be readily labelled with ^{111}In. Binding studies on this new molecule showed that it had an affinity for the ANF receptor which was in the range 1-10 less potent than ANF itself. Imaging with this ^{111}In-DTPA-ANF molecule provided rapid visualization of ANF receptors *in vivo.* The biodistribution was altered by the co-injection of ANF antagonists indicative of true receptor uptake. The images were of better quality by comparison with ^{123}I-Tyr-ANF, due to the greater *in vivo* stability of ^{111}In-DTPA-ANF.

r-ANF$_{101\text{-}126}$ has also been coupled with N-(2-hydroxy-5-isothiocyanatobenzyl),N'-(2-hydroxybenzyl)-ethylenediamine-N,N'-diacetic acid **(2)**. This molecule allows gentler reaction conditions than required for DTPA-anhydride and offers greater *in vivo* stability of the chelator against transferrin when compared to ANF-DTPA.

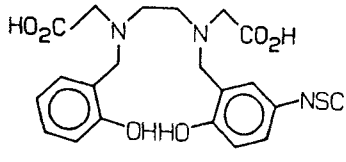
Images of ANF receptors in rats, dogs and monkeys will be presented. This promises to be a powerful technique for simple non-invasive *in vivo* receptor imaging. These experiments show the ease with which peptides can be used as substrates for the imaging of peripheral receptors with metal chelates. It seems likely that many other peptides are amenable to this type of chemical modification.



Structure of Rat Atrial Natriuretic Factor



DTPA (1)



HBED (2)

Acknowledgments: The donation of a Sophy 20P-256 computer for image acquisition and analysis by Sopha Médical, France is gratefully acknowledged.

Paper H9

Synthesis of 2-(p-SCN-benzyl)-*trans*-cyclohexyl-DTPA for α-Particle Mediated Radioimmunotherapy

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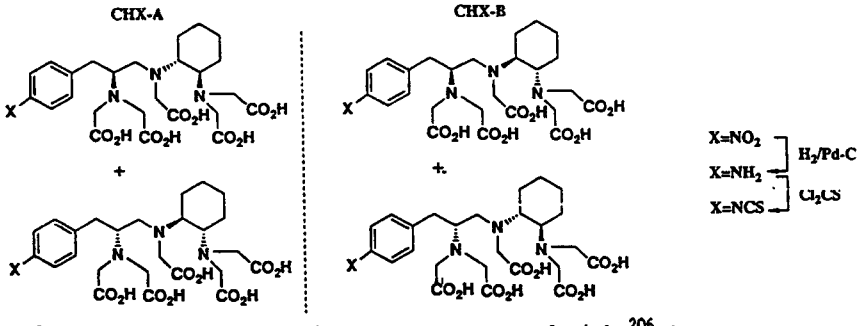
Chemistry Section, Radiation Oncology Branch and Laboratory of Tumor Immunology and Biology, NCI, NIH, Bethesda, MD and Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD.

Several bifunctional derivatives of DTPA (2-(p-SCN-Bz- (1B-DTPA), 2-(p-SCN-Bz)-6-Me-(1B4M-DTPA), 2-(p-SCN-Bz-)-*trans*-cyclohexyl-DTPA (CHX-DTPA) were synthesized in order to provide a chelate for antibody linkage of the α-emitter ^{212}Bi. Of these, only the most sterically rigid DOTA and CHX-DTPA were stable *in vivo*.

The CHX-DTPA was synthesized by converting BOC-p-nitrophenylalanine to the mono-amide of (±)-*trans*-1,2-diaminocyclohexane. The carbamate was removed and the amide reduced to generate the triamine. Akylation with t-butyl bromoacetate led to the penta-ester which after deprotection gave two isolable products which were separated by preparative HPLC. The two products were determined to be the two pairs of enantiomers possible from the reaction sequence and were labeled CHX-A and CHX-B. Synthesis of a single enantiomer from BOC-(l)-p-nitrophenylalanine was performed to ascertain the absolute configurations of the ligands. The acid was coupled to CBZ-(R,R)-1,2-diamino-cyclohexane by diimide to produce an amide, which after deprotection and reduction gave a single triamine. The triamine was alkylated as above, and after deprotection found to correspond to CHX-A by HPLC. Thus, the stereochemistries of CHX-A were determined to be (S,R,R) and the enantiomer, and that CHX-B by necessity to be the corresponding pair of diastereomers. The nitro groups were hydrogenated and the anilines were treated with thiophosgene to provide an isothiocyanate for protein conjugation.



In vivo usefulness of the DTPA conjugates was assessed with ^{206}Bi labeled mAbs as well as in a serum stability experiment. The (CHX-A)-103A conjugate was comparable to ^{35}S labeled 103A, with 40%ID/g to neoplastic spleens 1 hr p.i. and with < 0.10%ID/g in other tissues. The (CHX-A,B)-B72.3 conjugates had levels of activity in the kidney comparable to ^{125}I labeled B72.3, ca. 8%ID/g after 2 hr. In contrast, the kidney level for the 1B4M-DTPA B72.3 ^{206}Bi conjugate was 18%ID/g. However, serum stability studies showed that 1B4M-, CHX-DTPA and DOTA lligands lost only a few percent of radiobismuth over 6 hrs. This result contradicts the *in vivo* results leading to the conclusion that *in vitro* serum stability studies are not a reliable predictor for *in vivo* stability.

The efficacy and toxicity of α-particle radioimmunotherapy of Rauscher murine erythroleukemia was then assessed with mAb 103A labeled with ^{212}Bi conjugated with CHX-A DTPA. Median survival of the mice treated with 150 μCi ^{212}Bi-(CHX-A)-103A was 118 d versus 63 d for untreated animals. No histologic evidence for toxicity was seen.

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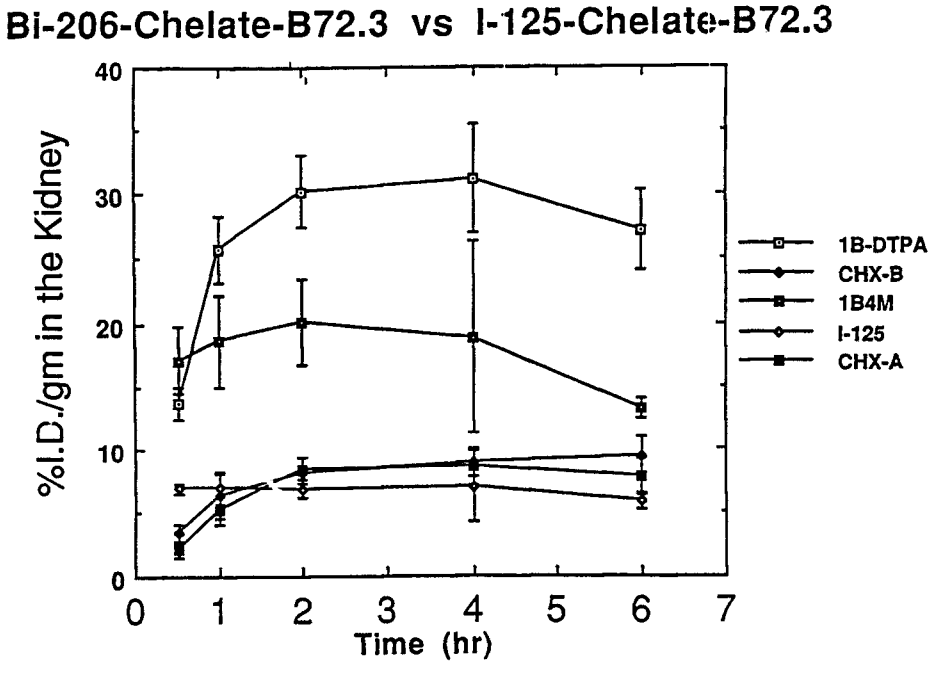
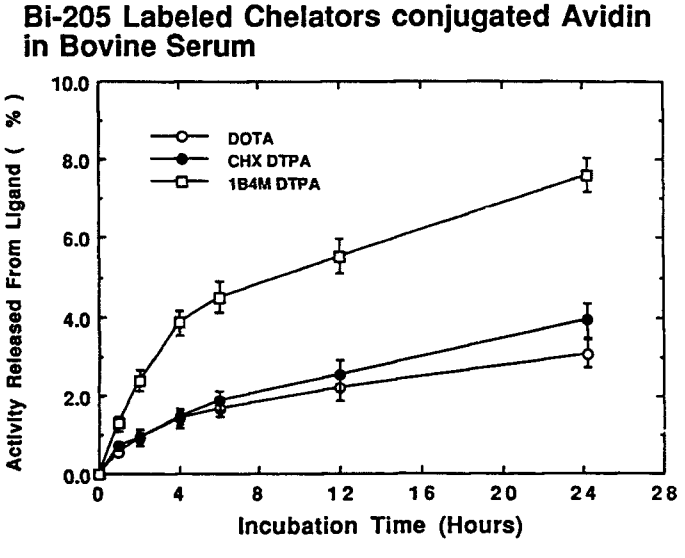
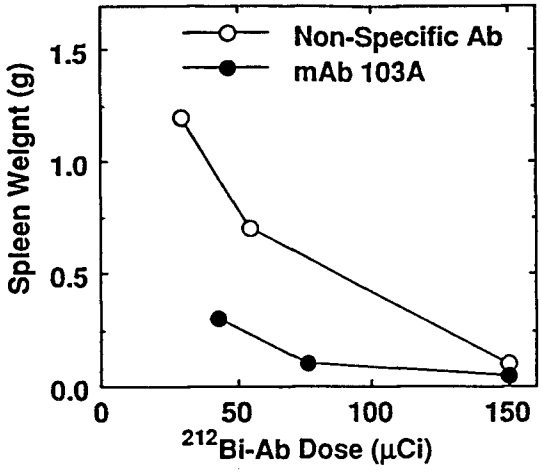
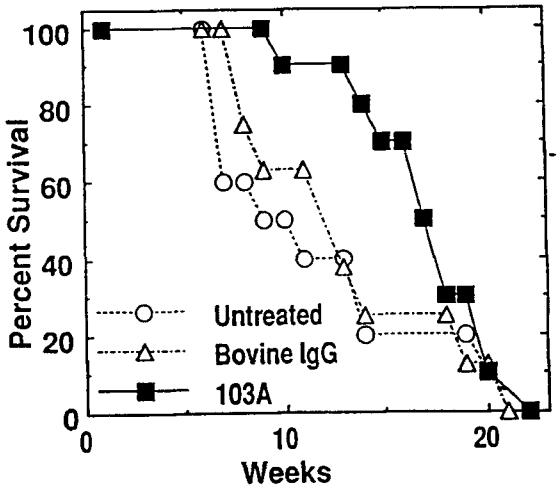

Bi-206-Chelate-B72.3 vs I-125-Chelate-B72.3
%I.D./gm in the Kidney
Time (hr)
1B-DTPA
CHX-B
1B4M
I-125
CHX-A


Bi-205 Labeled Chelators conjugated Avidin in Bovine Serum
Activity Released From Ligand (%)
Incubation Time (Hours)
DOTA
CHX DTPA
1B4M DTPA

Tumor therapy assessed by measuring the decrease of tumor bulk in Leukemic spleens of mice with Rauscher erythroleukemia.





RADIOIODINATION OF PROTEINS USING N-SUCCINIMIDYL-4-HYDROXY-3-IODOBENZOATE.

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Direct radioiodination of monoclonal antibodies (MAbs) and other proteins using oxidants such as Iodogen or Chloramine-T generally results in the formation of iodinated tyrosine residues (Figure 1). It is generally believed that the extensive deiodination of proteins observed *in vivo* is due to the structural similarity of these labeled amino acids to thyroid hormones such as iodotyrosine for which there are many deiodinases. To circumvent this problem, we and others have developed acylation agents such as N-succinimidyl- 3-iodobenzoate (SIB) and have shown that avoiding iodination *ortho* to a hydroxyl group reduced thyroid uptake (an *in vivo* indicator of dehalogenation) from labeled MAbs by as much as 100-fold (1). However, the thyroid uptake of a MAb labeled using the Bolton-Hunter reagent, an acylation agent which has a hydroxyl group *ortho* to the radioiodine, was only twice that observed for the same MAb labeled using SIB (2).

In order to determine whether the two-carbon spacer between the aromatic ring and the active ester present in the Bolton-Hunter reagent was responsible for reducing deiodination, N-succinimidyl-4-hydroxy-3-iodobenzoate (SHIB) was synthesized (Figure 1). This was accomplished in two steps, starting with labeling of 4-hydroxybenzoic acid with ^{131}I using Chloramine-T. After purification by reverse-phase HPLC, 4-hydroxy-3-[^{131}I]iodobenzoic acid was isolated in greater than 80% radiochemical yield. The title compound was then prepared after a 30 min reaction at room temperature by the dicyclohexylcarbodiimide-mediated esterification of 4-hydroxy-3-[^{131}I]iodobenzoic acid with N-hydroxysuccinimide. The product was isolated by HPLC over silica gel in 50-70% yield.

Radioiodination of MAbs by reaction with SHIB was much more difficult than labeling using other N-succinimidyl ester acylation agents. Using protein concentrations of 5 mg/ml in pH 9.0 borate buffer, only a 10-15% coupling yield was observed after a 60 min reaction. In comparison, after a only a 20 min reaction, coupling efficiencies are generally greater than 65% for SIB. We speculate that the lower coupling yield for SHIB may be due to the presence of the *para* hydroxyl group which could make the ester carbonyl less succeptible to nucleophilic attack by both +I and +M effects.

MAb 81C6 reactive with human gliomas was labeled by reaction with both [^{131}I]SHIB and [^{125}I]SIB. Specific *in vitro* binding to D-54 MG glioma homogenates was 78 ± 3% for 81C6-[^{131}I]SHIB compared to 84 ± 3% for 81C6-[^{125}I]SIB. The tissue distribution of the two labeled 81C6 preparations were compared in normal mice. No difference was observed in the thyroid uptake of ^{131}I and ^{125}I at day 1 (Table 1); however, with time, thyroid accumulation of ^{131}I became as much as 3-fold that seen for ^{125}I. These results suggest that MAbs labeled with SHIB are more succeptible to deiodination than those labeled using SIB and provide further elucidation of the structural requirements necessary for minimizing protein deiodination.

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IODINATION SITES ON PROTEINS

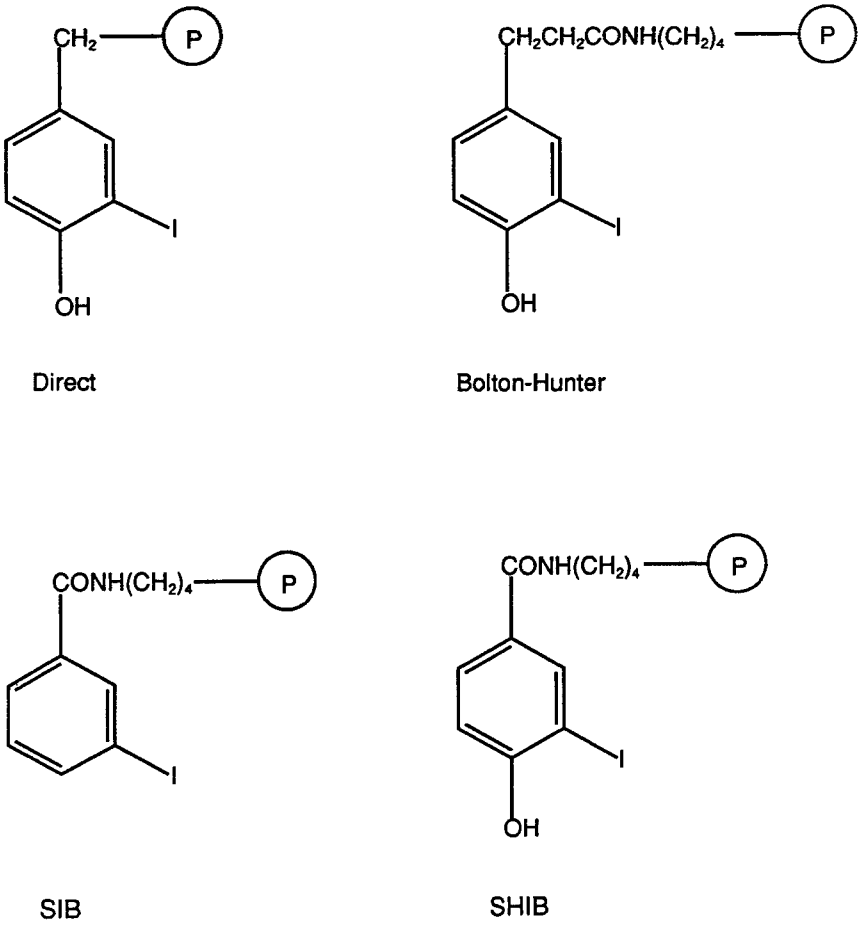

Figure-1

Table 1. Uptake of radioiodine in thyroid of normal mice injected with MAb 81C6 labeled by reaction with [^{131}I]SHIB and [^{125}I]SIB

Time (days)	% Injected dose per Organ	
	SHIB	SIB
1	0.44 ± 0.08	0.44 ± 0.08
2	0.43 ± 0.11	0.34 ± 0.03
5	0.45 ± 0.08	0.24 ± 0.01
6	0.53 ± 0.14	0.16 ± 0.10
7	0.24 ± 0.04	0.15 ± 0.03

Paper H11

Synthesis, Characterization and Evaluation of Two New Polyaminocarboxylates for Linking Indium-111 to Monoclonal Antibodies

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Our aim was to develop new chelation structures that could be coupled to monoclonal antibodies without compromising the antibody activity, and that would bind indium-111 to antibody under in vivo conditions with appreciable kinetic stability. In this report we describe the synthesis, characterization and evaluation of two new polyaminocarboxylates, LiLo (1,3-bis{N-[N'-(2-aminoethyl)-2-aminoethyl]-2-aminoacetamido}-2-(4-isothiocyanatobenzyl) propane-N,N,N',N'',N''',N'''',N''''',N'''''-octaacetic acid, and HETA (3[4-isothiocyanatobenzyl],6,16-dioxo-1,5,8,11,14-pentaazacyclohexadecane-N,N',N''-triacetic acid) useful for attaching indium-111 to monoclonal antibodies.

The synthesis involved the condensation between p-nitrobenzyl bromide and diethyl malonate. The (p-nitrobenzyl)diethyl malonate obtained was converted to a diamine, and the diamine was coupled to DTPA and esterified. The product mixture was purified by silica gel chromatography to obtain LiLo ester and HETA ester. These compounds were further converted to isothiocyanate derivatives and hydrolyzed in the presence of acid to obtain LiLo and HETA (Fig.1).

The chelating agents LiLo and HETA were coupled to an anticolorectal human monoclonal antibody of IgM isotype, 16.88 (1). The immunoconjugate was purified by gel filtraiton chromatography and analyzed by SDS/PAGE gel electrophoresis and HPLC. Competitive binding analysis showed that the immunoactivity of the antibody was not affected by the presence of LiLo or HETA or the conjugation procedures used.

The immunoconjugates were labeled by incubating with indium-111 in a buffer solution containing acetate/citrate, and purified by gel filtration chromatography. The kinetic stabilities of the radiolabeled conjugates were determined in phosphate buffered saline solution, pH 7.2, at 37C, 7% CO2, by competition in the presence of excess DTPA (LiLo:DTPA - 1:>5000). At intervals an aliquot of the solution was removed and analyzed by thin layer chromatography and/or HPLC. Indium-111 not bound to 16.88 migrated as In-DTPA (Rf= 0.7), whereas indium bound to 16.88 stayed at the origin. Stability studies in normal human serum were performed as described by Deshpande et al (2). More than 90% of indium-111 remained attached to the antibody even after incubation at 37C over a period of 10 days.

These results demonstrate that the new bifunctional chelating agents LiLo and HETA are suitable for attaching indium-111 to monoclonal antibodies. Presence of eight carboxyl groups in LiLo and the macrocylic structure of HETA may be responsible for the kinetic stability.

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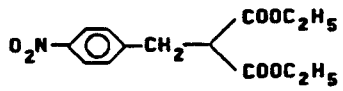
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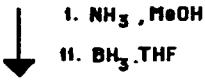
$COOC_2H_5$
$COOC_2H_5$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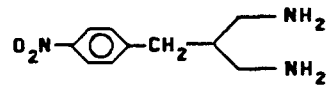
11. BH_3.THF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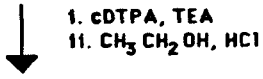
NH_2
NH_2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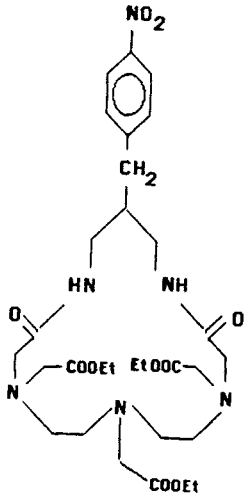
11. CH_3CH_2OH, HCl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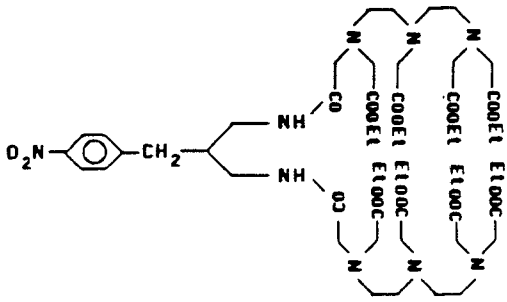
CH_2
HN
NH
O
O
N
COOEt
EtOOC
N
N
COOEt

+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NH
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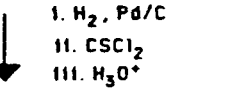
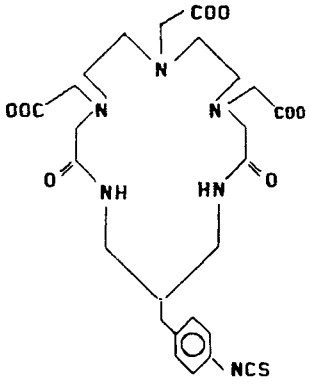

1. H_2 , Pd/C
11. $CSCl_2$
111. H_3O^+


COO
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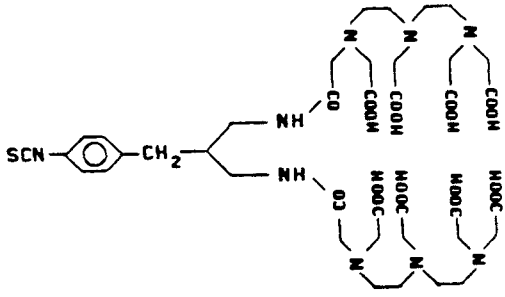
OOC
N
N
COO
O
NH
HN
O
NCS

HETA

+


SCN
CH_2
NH
NH
CO
CO
N
COOH
HOOC

LiLo

Paper H12

Re-186 labeled antibodies : Preparation and evaluation.

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Re-186 has been regarded as an ideal radionuclide for radioimmunotherapy because of its physical half life of 90 hours and 1.07 MeV beta emission.

Fritzberg et al (1, 2) have labeled monoclonal antibodies with Re-186 by chelation to diaminodithiolate ligands. We have developed a method to directly label IgG, IgM and fragmented antibodies with Re-186. The labeling yields as determined by ITLC, molecular filtration and gel filtration were greater than 95% and the colloid formation was less than 5%. The labeled antibodies were stable under physiological conditions.

The labeling method involves reduction of antibodies with ascorbic acid pH 6.5 (3) ,and then incubation with perrhenate reduced with stannous chloride in citric acid for 3 hrs at 37°C. SDS-PAGE and autoradiography of labeled IgM, IgG and $F(ab')_2$ antibodies indicated uniform labeling and that no fragmentation of the monoclonal antibodies had taken place during the labeling procedure. Immunospecificity of Re-186 labeled human neutrophil specific IgM, as determined by in vitro antigen excess assay, was comparable to that of Tc-99m-IgM. A nuclear histone specific Re-186-TNT-1-$F(ab')_2$ was evaluated in mice bearing experimental tumors. The tumor/muscle ratios at 4 and 24 hr were 5.9 ± 0.21 and 13.8 ± 6.7 respectively compared to that of 2.4 ± 0.3 at 4 hr p.i. with a nonspecific protein (Fig. 1).

The metabolic fate of these Re-186 labeled antibodies in plasma, urine and liver is being investigated.

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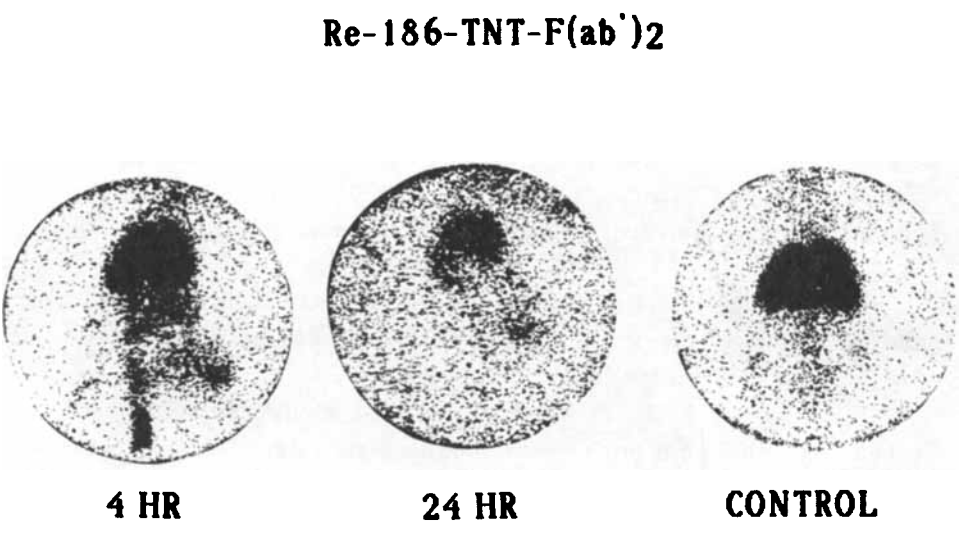

Fig 1: Posterior gamma camera images of mice bearing embroyonal carcinoma. With Re-186-TNT-1-F(ab')2 the tumor is clearly visible. The control animal received Tc-99m-HSA as a nonspecific protein.

Paper H13

Synthesis and Characterization of Radiolabelled Conjugates of Human Transferrin and IgG and TNT-1-F(ab')$_2$ Fragments

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Transferrin has been chemically modified to serve as a novel bifunctional chelating agent for antibody labelling in order to resist loss of the radiolabel *in vivo*. Transferrin can bind a variety of metal ions (1) and one of the most strongly bound is the In(III) ion having stability constants, K_1 and K_2, of the same approximate order of magnitude as Fe(III) and Ga(III) (2,3). Utilizing the favorable metal ion binding properties of this protein, an immunoconjugate complex can be constructed which can bind the radionuclide at least as strongly as other advantageous serum molecules. The synthetic strategy is first to prepare the Zn(II) analogue of transferrin, Zn_2Tf, followed by modification at pH 8 with a moderate excess of the heterobifunctional cross-linker sulfosuccinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (sulfo-SMCC) (4) as shown in Scheme 1. Zn_2Tf is preferred as a synthetic starting material as the protein is oriented in its metal-binding conformation prior to modification with sulfo-SMCC. Apotransferrin which has been modified by reaction with sulfo-SMCC exhibits an extensive loss of its metal-binding capabilites. Human polyclonal IgG and TNT-1-F(ab')$_2$ possess a number of cysteine disulfide linkages (approximately 35 and 13, respectively) that can be activated *via* reduction with dithiothreitol (DTT). A 1000-fold excess of DTT : antibody in pH 8 phosphate buffer yields reactive thiol groups on both the IgG and the resulting TNT-1-Fab' fragment (F(ab')$_2$ is cleaved into Fab' fragments under these conditions) (5) as shown in Scheme 2. These thiol groups are then reacted with the maleimide-activated Zn_2Tf species yielding [IgG]-X-[Zn_2Tf] or [Fab']-X-[Zn_2Tf] species, Schemes 3 and 4, respectively. HPLC analysis and SDS-PAGE indicate that [IgG]-X-[Zn_2Tf] is produced in a 1:1 stoichiometry along with higher molecular weight conjugates; [Fab']-X-[Zn_2Tf] is synthesized predominatly as a 1:1 stoichiometric conjugate along with more substituted conjugates as shown in Table I. Gel chromatography is used to separate and purify the conjugate complexes. The introduction of $^{111}In(C_9H_6NO)_3$ to these immunoconjugate complexes in a pH 8.2, 0.1M sodium bicarbonate buffer results in the transferrin binding the In(III) ions while displacing the Zn(II) ions (log K_1 is 8.0 and log K_2 is 6.6 for Zn(II) ions binding to Tf (1)) as demonstrated by HPLC. Murine models are being used to evaluate the advantages these agents can offer *vs.* ^{111}In labelled DTPA-antibody complexes.

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$$[Zn_2\,Tf]\text{-}NH_2 + 50\ \text{Sulfo-SMCC} \xrightarrow{2^{\circ}C,\ 20\ min,\ pH\ 8} [Zn_2\,Tf]\text{-}NHC(O)\text{-}R\text{-}Maleimide$$

Scheme 1.

$$IgG + 1000\ DTT \xrightarrow{37^{\circ}C,\ 60\ min,\ pH\ 8} [IgG]\text{-}SH$$

$$F(ab')_2 + 1000\ DTT \xrightarrow{37^{\circ}C,\ 60\ min,\ pH\ 8} 2\,[Fab']\text{-}SH$$

Scheme 2.

$$[Zn_2\,Tf]\text{-}NHC(O)\text{-}R\text{-}Maleimide + [IgG]\text{-}SH \xrightarrow{8^{\circ}C,\ 10\ h,\ pH\ 7} [Zn_2\,Tf]\text{-}NHC(O)\text{-}R\text{-}Maleimide\text{-}S\text{-}[IgG]$$

Scheme 3.

$$[Zn_2\,Tf]\text{-}NHC(O)\text{-}R\text{-}Maleimide + [Fab']\text{-}SH \xrightarrow{8^{\circ}C,\ 10\ h,\ pH\ 7} [Zn_2\,Tf]\text{-}NHC(O)\text{-}R\text{-}Maleimide\text{-}S\text{-}[Fab']$$

Scheme 4.

Table I. HPLC Results of Conjugation Reactions

R*	Approx. MW, kD	Species**
1.89	50	F
1.72	80	Tf
1.67	100	F2
1.53	150	IgG
1.53	130	F-Tf
1.41	180	F-Tf-F or F2-Tf
1.37	210	Tf-F-Tf
1.35	230	IgG-Tf
1.00	>300	>300kD MW conjugates

*R is calculated as (peak elution time)/(void volume elution time)

**F is Fab'; Tf is Zn2Tf; F2 is F(ab')2

Paper H14

PRODUCTION AND EVALUATION OF Sc-47 FOR RADIOIMMUNOTHERAPY

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The half-life (3.35d), intermediate energy beta emissions [441.1 keV_{max}(68%), 600.5 keV_{max}(32%)] and imageable gamma ray [159.4 keV [68%] of ^{47}Sc make it an attractive radionuclide for radioimmunotherapy. Sc also has favorable coordination chemistry similar to ^{111}In and ^{90}Y for chelation and attachment to antibodies (MoAb). We report here the production and purification of no-carrier-added ^{47}Sc, as well as preliminary MoAb labeling studies.

We have investigated the production of ^{47}Sc by irradiating natural titanium targets with protons at the Brookhaven Linac Isotope Producer (BLIP), as well as with the fast neutron reaction $^{47}Ti(n,p)$ at the High Flux Beam Reactor (HFBR). Nuclear cross-sections for $^{44m,46,47,48}Sc$ were measured at proton energies of 49.1, 79.0, 107.3, 131.6, 150.2 and 191.8 MeV (Figure 1). These data were combined with published data to calculate optimal thick target yields for ^{47}Sc with minimal contamination from other Sc isotopes (of comparable or longer $t_{\frac{1}{2}}$) also produced. We calculate that 224 mCi of ^{47}Sc could be produced from a 3.35 d, 35 MeV proton irradiation (50 μA beam current) of a 0.29 cm thick Ti target with 4.1% ^{48}Sc, 4.7% ^{46}Sc, and 83.2% ^{44m}Sc contamination. The presence of ^{44m}Sc may not be a drawback in therapy applications. Thicker targets could produce more ^{47}Sc but with some degradation of radiopurity. The end of bombardment yield from a 3 d irradiation of ^{47}Ti at HFBR was 396 mCi/g ^{47}Ti. Radioimpurities were all less than 0.1%. Recovery and reuse of the enriched ^{47}Ti target will be required for large scale production with this route.

Four radiochemical separation procedures were investigated to isolate Sc from the Ti target: (a) cation exchange (AG-MP50 resin) in HCl/ammonium acetate media; (b) solvent extraction of Ti cupferrate in 1 N HCl into chloroform; (c) solvent extraction of Sc with tri-n-butyl phosphate (TBP) in 10 N HCl; and (d) extraction chromatography with TBP fixed on silica gel. Table 1 compares these procedures for Sc recoveries, Ti separation factor and separation time. Although cation exchange and extraction chromatography with TBP gave comparable separation results, traces of TBP eluted with Sc and caused subsequent labeling problems. Thus, cation exchange has been adopted for use in production runs. The Ti is eluted with 1 N and 4 N HCl, followed by water, and Sc is eluted with 1 M ammonium acetate at pH 4.7.

Several bifunctional chelating agents have been used to attach ^{47}Sc to anticolon ca MoAb 17-1A. These are DTPA dianhydride (DTPADA), cyclohexyl EDTA monoanhydride (CDTAMA), and 4-isothiocyanato cyclohexyl EDTA (4-ICE). In normal mice the DTPADA and CDTAMA immunoconjugates demonstrated high liver uptake, indicative of poor in-vivo stability. In-vitro serum stability for DTPADA and 4-ICE-17-1A preparations was determined with incubations at 1 and 4 d. The loss of label from 4-ICE at 1 and 4 d was between 1-4%, compared to 53% and 100% loss, respectively, with DTPADA. Biodistribution in human tumor xenografted nude mice for 17-1A conjugates is shown in Figure 2 for Sc-4-ICE, Sc-DTPA and In-4-ICE. Good tumor uptake, prolonged blood retention and low bone and liver uptake of 4-ICE are consistent with its high serum stability. Conversely, the poor distribution of DTPADA is not surprising due its low serum stability.

In conclusion, we have demonstrated two practical routes to produce therapeutic quantities of ^{47}Sc. Preliminary MoAb labeling studies with the semi-rigid polyaminocarboxylate 4-ICE show promise.

Work supported by the US Department of Energy under contract #DE-AC02-76CH00016.

Table 1. RESULTS OF Ti/Sc RADIOCHEMICAL SEPARATIONS

METHOD	%Sc RECOVERY	Ti SEPARATION FACTOR*	SEPARATION TIME (h)
CATION EXCHANGE AG-MP50	97 (CARRIER) 80 (NCA)	1×10^{-6}	2 (at 1 mL/min)
Ti CUPFERRATE EXTRACTION	98	1.4×10^{-4}	1.5 (5 extractions)
Sc EXTRACTION into TBP	90	7×10^{-1}	0.3 (1 extraction)
EXTRACTION CHROMATOGRAPHY with TBP	98 (CARRIER) 90 (NCA)	1×10^{-6}	4 (at 0.7 mL/min)

*Ti measured by spectrophotometric analysis of Ti-H_2O_2 complex (detection limit <0.1 μg/mL)

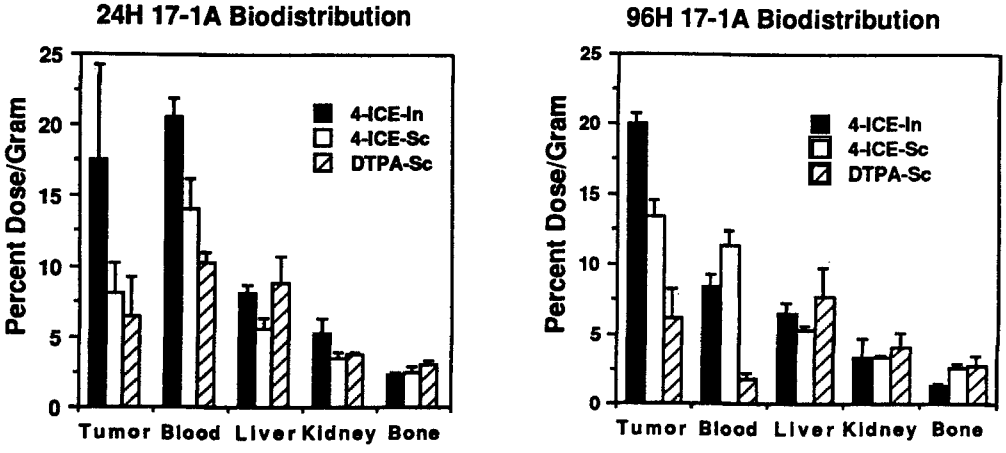

Figure 2. Tissue distribution of ^{47}Sc and ^{111}In 17-1A IgG immunoconjugates in human colon ca xenografted (SW948 cells) nude mice.

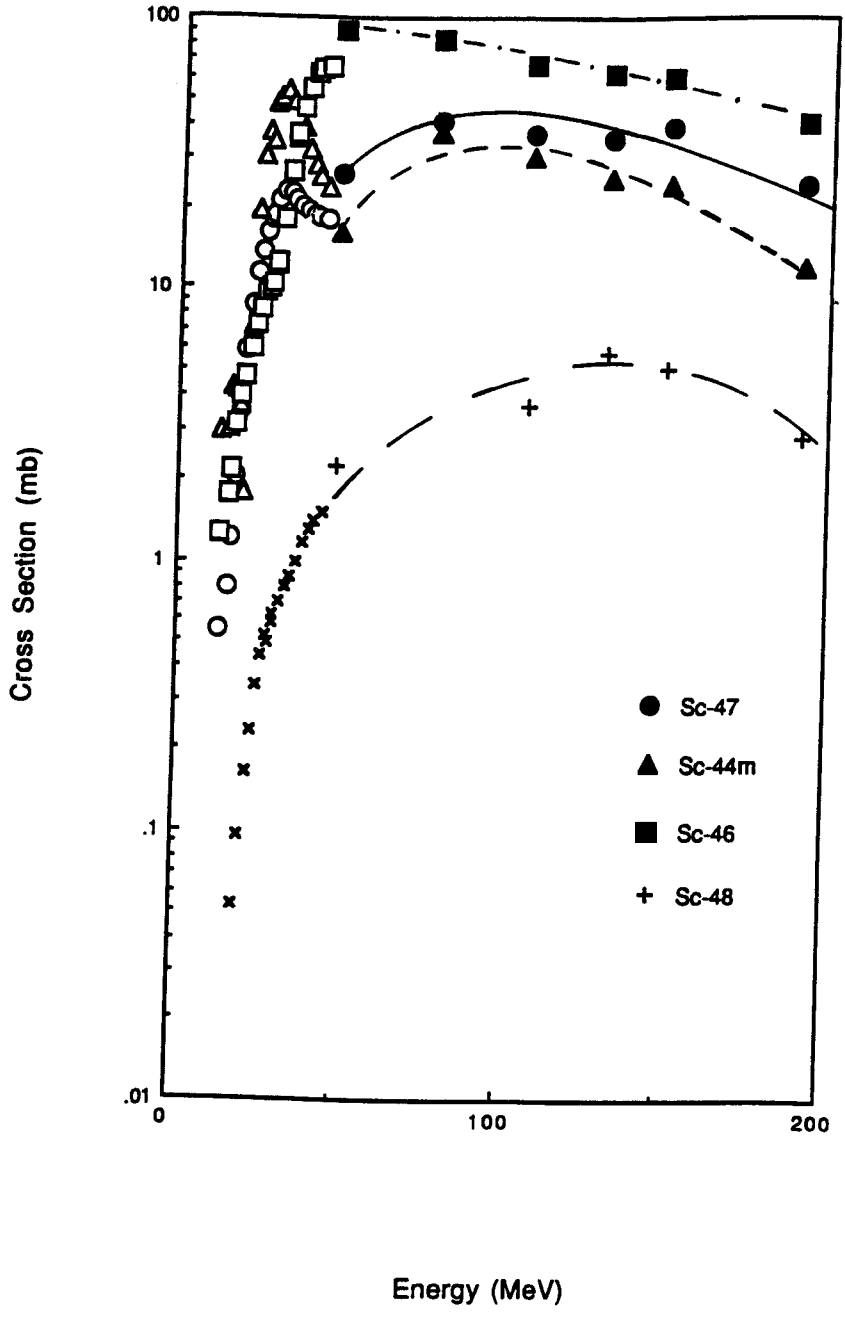

Figure 1. Nuclear excitation functions for ^{44m,46,47,48}Sc from proton irradiation of Ti. Data form 49 MeV and higher energies from this study. Lower energy data taken from R. Michel, G. Brinkman, H. Weigel, W. Herr. J. Inorg. Nucl. Chem. 40, 1845-1851 (1978). Lines drawn only as a visual aid.

Paper H15

Stabilization of Ester Bond in Plasma to Enhance Target Radioactivity Delivery by Antibody Conjugated with Metallic Radionuclide through Metabolizable Ester Linkage.

Y. Arano[§], T. Inoue[§], T. Mukai[§], Y. Taoka[§], M. Hosono[¶], H. Sakahara[¶], J. Konishi[¶], and A. Yokoyama[§]. Faculty of Pharmaceutical Sciences[§], and School of Medicine[¶], Kyoto University, Sakyo-ku, Kyoto 606, Japan.

High radioactivity localization in nonspecific organs and low radioactivity delivery to the target tissue constitute some of the major problems in diagnosis and therapy using monoclonal antibodies (MoAbs) labeled with metallic radionuclides. One rational radiochemical approach to the problems has been the conjugation of the MoAb with a radiochelate via a metabolizable linkage. We have designed, synthesized and radiolabeled MoAbs holding various cleavable linkages (1, 2). An easy cleavage of the linkage has induced fast radioactivity clearance from not only nonspecific organs but from circulation, due to the rapid generation of a stable radiochelate with fast urinary excretion. On the other hand, it has been observed that radiolabeled MoAb of high likage stability while in the plasma favors high target radioactivity delivery. Therefore, it was considered of interest to search for some approach to prevent the ester bond from the enzyme attack while in plasma, but keeping its cleavable ability in the nonspecific organs.

In this study, monoclonal antibody against osteogenic sarcoma (OST7, IgG_1) was used as a model. This antibody was treated with 2-mercaptoethanol to reduce disulfide bonds (3), and to this freshly thiolated antibody, the reaction product of N-[[4-(maleimidoethoxy)succinyl]oxy]succinimide (MESS) (2) and deferoxamine (DF) was added, followed by the addition of iodoacetamide to alkylate unreacted thiol groups, as shown in Fig. 1. After purification of the reaction mixture by column chromatography, the conjugate (DF-MESS-OST7) was adjusted to 1 mg/ml in PBS (0.1M, pH 6.0). The ^{67}Ga labeling was performed using ^{67}Ga-citrate. For comparison, ^{67}Ga-labeled OST7 via a nonmetabolizable linkage was also prepared according to the same procedure using N-(E-maleimidocaproloxy)succinimide (EMCS) (DF-EMCS-OST7) instead of MESS .

Size-exclusion HPLC analysis of each ^{67}Ga-labeled OST7 showed the same retention time as that of the original OST7. Cell binding assay of the two ^{67}Ga-labeled OST7 demonstrated the full retention of the original immunoreactivity. In mice biodistribution studies, the two ^{67}Ga-labeled OST7 presented similar radioactivity clearance from the circulation (Fig. 2). On the other hand, difference was detected in the organ radioactivity distribution of the two ^{67}Ga-OST7; while persistent hepatic and renal radioactivity was observed with ^{67}Ga-DF-EMCS-OST7, the radioactivity clearance from those organs was notified with ^{67}Ga-DF-MESS-OST7 (Fig. 2). These results demonstrated the release of ^{67}Ga chelate of succinyldeferoxamine from ^{67}Ga-DF-MESS-OST7 occurred in the liver and kidney while its release was prohibited in plasma. Thus, the present conjugation design of using antibody molecule to induce a steric interference against the enzyme access to its substrate in plasma but to facilitate the cleavage of the ester bond in the liver and kidney appears as a plausible approach to increase target selective radioactivity delivery by the MoAb using metallic radionuclides.

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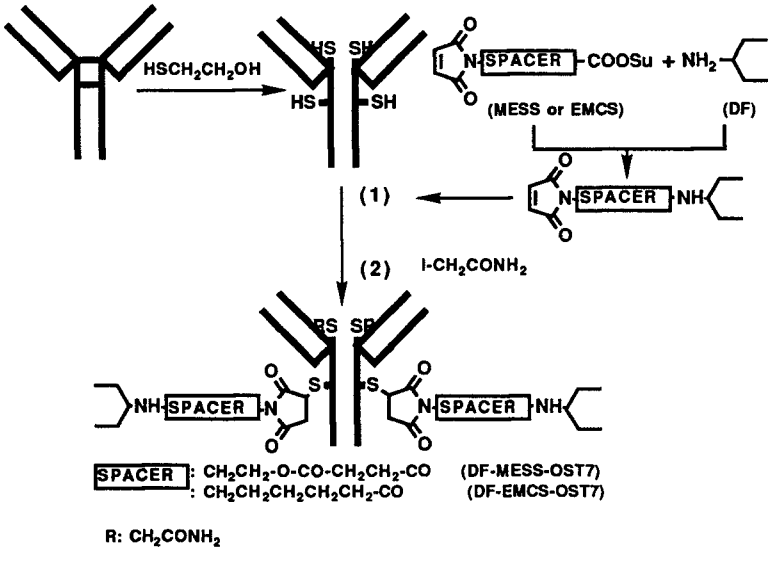

Figure 1. Scheme for the preparation of DF-MESS-OST7 and DF-EMCS-OST7.

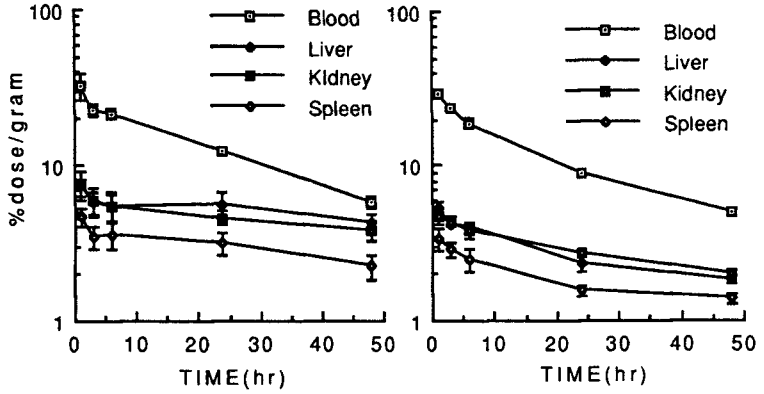

Figure 2. Biodistribution of radioactivity after intravenous injection of ^{67}Ga-DF-EMCS-OST7 (left) and ^{67}Ga-DF-MESS-OST7 (right) in mice. Data are expressed mean and s.d. of five mice each point.

Paper H16

RADIOSYNTHESIS OF TECHNETIUM-99m LABELED HUMAN VERY-LOW DENSITY LIPOPROTEIN FOR SCINTIGRAPHIC STUDY OF LIPOPROTEIN RECEPTOR ACTIVITY IN VIVO.

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Alterations in the saturable, receptor-mediated metabolism of low-density lipoprotein are thought to modify metabolism of the lipoprotein and predispose to atherogenesis and coronary heart disease. Scintigraphic study of lipoprotein catabolism *in vivo* is thus of value for investigation of human pathophysiology as well as evaluation of dietary and pharmacologic therapies.

Approaches to radiopharmaceuticals for this goal include analogues of LDL, whereby the lipoprotein has been labeled with residualizing labels such as [^{125}I]tyramine-cellobiose (1), [^{111}In]DTPA (2,3) and ^{99m}Tc (4). Of the radionuclides employed as a radiolabel, technetium-99m has the most widespread use in clinical nuclear medicine.

A disadvantage of radiolabeled LDL as a radiopharmaceutical is the relatively slow biological clearance of the lipoprotein (plasma $t_{1/2}$ = 22 h), necessitating long intervals between tracer injection and sufficient accumulation of radioactivity in lipoprotein receptor-rich tissues. We have reported the advantage of gallium-68 labeled very-low density lipoprotein (VLDL) over the corresponding LDL tracer for PET study of lipoprotein receptor activity (5). Because the more rapid clearance of VLDL may make clinical investigations with ^{99m}Tc more convenient, we report here the radiosynthesis and preliminary imaging results using [^{99m}Tc]VLDL.

Based on reports of [^{99m}Tc]LDL preparation (4), [^{99m}Tc]VLDL was prepared via *in situ* reduction of [^{99m}Tc]pertechnetate by sodium dithionite. The general labeling procedure was to add [^{99m}Tc]pertechnetate (10 mCi in 0.5 mL) to human VLDL, addition of $Na_2S_2O_4$, and incubation at ambient temperature for a selected interval. The labeled product was separated via gel permeation chromatography (Sephadex G-50/150). For *in vivo* application, the [^{99m}Tc]VLDL was passed through a 0.45 μ Millex-GV filter unit. Quality assurance for radiochemical purity was accomplished using radio-HPLC (TSK-GEL G5000-PW; 0.15 M NaCl; 0.5 mL/min).

Figure 1 shows the effect of reaction time on the production of [^{99m}Tc]VLDL. The illustrated data represents the mean from 2-3 experiments. Reaction conditions were 0.46 mg VLDL, 9.9-10.0 mCi [^{99m}Tc]pertechnetate, 10 mg $Na_2S_2O_4$, total volume 2.1 mL. The reaction interval begins with the addition of reducing agent to the mixture of lipoprotein and [^{99m}Tc]pertechnetate. Radiochemical yields of about 40 percent were achieved within 30 minutes, corresponding to an overall preparation time of 40 minutes.

The minimum amount of reducing agent needed for radiolabeling is shown in Figure 2. Reaction conditions were 0.71 mg VLDL, 0.6 mCi [^{99m}Tc]pertechnetate, total volume 0.6 mL. For masses less than 1 mg, sodium dithionite was added as a solution in 100 μL glycine buffer (pH 10.0), whereas for greater masses the reducing agent was added as a solid. As seen from these results, radiochemical yields were greatest when 2 mg or more of $Na_2S_2O_4$ were employed.

Preliminary experiments using rabbits are encouraging. For the control case, [^{99m}Tc]VLDL rapidly localized within the lipoprotein receptor-rich liver (at 130 min post injection, liver/blood ratio R = 18). For the hypercholesterolemic case, in which lipoprotein receptors were saturated, R = 2 at 130 min post injection. This rapid discrimination between the two physiological states is an improvement over radiolabeled LDL, which even after 24 hours achieves much lower R values *in vivo*.

This work was supported in part by DOE grant DE-FG02-87 ER60512.

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FIGURE 1

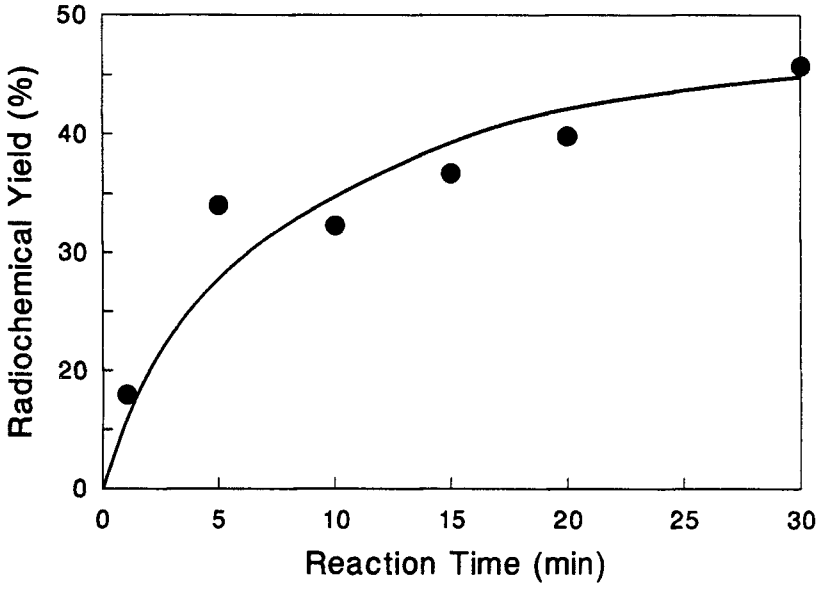
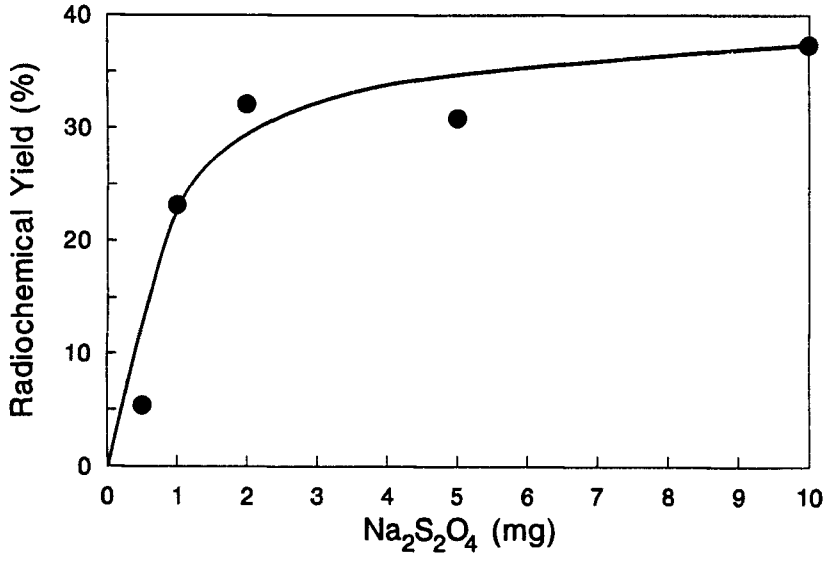


FIGURE 2



Paper H17

Approaches to Monoclonal Antibody Labeling by Use of Metallic Radionuclides

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Hybridoma technology offers the possibility to produce monoclonal antibodies with specificity for tumor associated antigens. This allows their use in targeting cancer cells with diagnostic and therapeutic radionuclides. A sussessful tumor targeting depends to a great extent on labeling techniques which preserve the integrity of the antibodies and guarantee label stability under physiological conditions. We report on the evaluation of a series of open chain and macrocyclic polyaminopolycarboxylates in terms of their ability to coordinate Y^{3+} (Y-90) and their possible use for antibody labeling. Thermodynamic versus kinetic stability is discussed. Potentiometric titration allowed the determination of stability constants. It is shown that backbone-substituted DTPA ligands (octadentate) have higher stability constants compared to N-substituted ones (DTTA derivatives, heptadentate). The difference being between 10^3-10^6 depending on the availability of amido groups. The thermodynamically most stable complex is formed by 1,4,7,10-tetraazacyclododecane - 1,4,7,10-tetraacetic acid and its bifunctional dervative ($K^{ML} > 10^{25}$). The corresponding tetraazacyclotridecane tetraacetic acid is less stable by a factor of 10^7 compared to DOTA and 10^3-10^4 compared to the DTPA derivatives. But kinetically it exhibits higher stability than the open chain ligands as determined by transchelation of Y-88 to transferrin and yttrium exchange in aqueous solution.
Furthermore we present data on the synthesis and evaluation of two new biotin-bifunctional ligand conjugates which can be labeled with Tc-99m and Re-188. Their use in tumor pretargeting with the avidin-biotin system proved to be successful in clinical trial, with glioma patients and uveal melanoma patients.

Paper H18

SITE-SPECIFIC MODIFICATION OF ANTI-CEA Fab'-FRAGMENTS WITH CHAIN-TERMINAL CHELATING POLYMERS: FORMATION OF REDUCIBLE AND NON-REDUCIBLE BONDS

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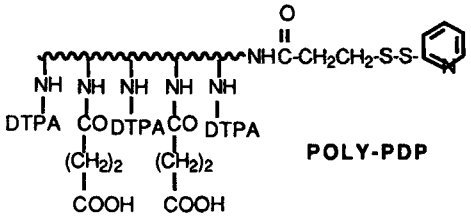
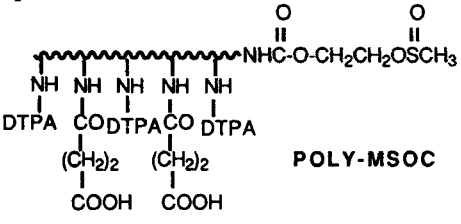
Polylysine-based chain-terminal chelating polymers have been used for site-specific modification of anti-CEA Fab'-fragments via their SH groups situated near the hinge region of the antibody molecule. The polymer chains with MW of 10000 contained ~30 DTPA residues. Two different chemical functions were introduced at the terminus of the polymer chain - 3-(2-pyridyldithio)propionyl (PDP) and 2-(methylsulfonyl)ethyloxycarbonyl (MSOC) (see below). Both of the polymers were prepared starting from poly-D,L- $\mathcal{E}$ -N-CBZ-lysine containing the single terminal NH_2 group.



POLY-PDP



POLY-MSOC

Poly-PDP was further converted into Poly-SH by the use of dithiothreitol and then allowed to react with Fab'-TNB (thionitrobenzoic moiety, protecting SH groups); coupling efficiency was 100%. The reaction resulted in the formation of a reducible S-S bond. Poly-MSOC was firstly deprotected in the basic medium with the formation of chain-terminal Poly-NH_2, which was subsequently converted into Poly-MPB upon its reaction with N-succinimidyl-(2-maleimidophenyl)-butyrate (SMPB). Then Poly-MPB was allowed to react with Fab'-SH which resulted in a non-reducible thioether bond; coupling efficiency was 30-40%.

After their labelling with ^{111}In, both of the conjugates prepared had a specific radioactivity of about 70-90 uCi/ug, i.e. 20-30 times more than the same Fab'-fragments modified with low-molecular weight chelating agents. Immunoreactivity of the conjugates was as great as 85-95%, as shown in the test with CEA immobilized on Sepharose CL-4B.

Thus, the methods described enable the binding of as many as 40-60 metal ions to an antibody molecule whilst preserving its immunoreactivity. Such polymeric conjugates after labelling with Gd^{3+} ions could be useful for MRI as immunospecific contrast agents. They could also be used in the preparation of immunoconjugates labelled with fluorescent ions, such as Eu(3+) and Tb(3+).

RADIOACTIVE LABELED PHOTSENSITIZERS FOR TUMOR DIAGNOSTIC AND PHOTODYNAMIC THERAPY (PDT)

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Aim: Today the most applied photosensitizers are of small molecular weight, moderately soluble in water (pH range 6.8-7.5), highly lipophilic and show alltogether a tendency to form aggregates. 24 hours after an intravenous injection high rates of the applied photosen-sitizers can be detected in the liver and urin, or are distributed diffusely all over the body included the skin. Only 0.02-0.3% of the applied dose are accumulated in the malignant tissue. The determination of the photosensitizers distribution in tissues via the fluoreszenz measurements is difficult and can be used only for superficial tumors. The determination of the optimal time point of maximal photosensitizer uptake in tumors and for the laser light irradiation is untill today an unsolved clinical problem. Theoretically a radioactive labeling of photosensitizers with ^{99m}Tc, ^{111}In or ^{72}Ga offers the possibility to use non invasive nuclear medical techniques to determine the distribution, but most of these radiactive compounds are not stabil in vivo. Since a couple of years we investigate small molecular weight compounds radioactively labeled with ^{131}J and covalently linked to macromolecular carrier systems. These new compounds show the following properties: a) a long circulation time; b) a high in vivo stability; c) a low clearence rate; d) a low trapping by the liver; e) a high water solubility in the pH range 6.8-7.5; f) a high accumulation in the neoplastic tissue.

Techniques: We used serum albumin (SA) and the synthetic polymer PEOM as macromolecular carrier systems for the photosensitizers derived from porphyrine and phthalocyanine. The compounds are covalently bound to the carrier systems and radioactively labeled with ^{131}J to enable a non invasive follow up of the total body distribution at different time intervals post administration. Tumors investigated: human tumors CX1, LX1, MX1, HS1 s.c. xenotransplanted in nude mice; DPT and O-348 s.c. transplanted in nude mice. Ar^+-laser (488nm-514 nm) and Ar^+-dye light (630nm, 650 nm, dose rate 10 mW/cm^2) was used for fluorescence determination of the photosensitizers content in the tissues. A gamma camera was

used for scintigraphic investigations at different time after the drug administration. Ar^+-dye and Ar^+-Ti:Saphir laser (630nm, 656nm and 690 nm, dose rate 150 mW/cm^2, dose applied 6-9 days after the drug administration: 60-80 J/cm^2) was used for PDT.

Results: 1. Depending on the tumor species, eight days after the drug administration 10% to 30% of the total applied dose were scintigraphicaly detected in the tumor regions. 2. The trapping in the liver was below 15%. 3. The clearance rate via kidneys was below 5%. 4. The macromolecular bound photosensitizers showed no detectable accumulation of ^{131}J in the thyroid gland. 6. The loss of ^{131}J from the non macromolecular bound photosensi-tizers was observed within 12-24 h after the drug administration. 7. The measured radioactivity ratio between tumor and normal tissue was better than 20:1. 8. 24-48 h after PDT destruction of the tumor tissues a rapid loss of the accumulated radioactivity in this region was observed. 9. The macromolecular derivatized photosensitizers showed a remarkable stability against day and laser light.

Conclusions: 1. The macromolecular carrier systems can be used to channel radioiodine labeled photosensitizers on porphyrine and phthalocyanine basis into tumor tissues.
2. The measurement of the radioiodine labeled photosensitizers can be used for clinical non invasive determination of the optimal time point for laser induced fluorescence diagnostic and PDT.
3. In patients the circulation time of the new radioiodine labeled and carrier bound photosensitizers was longer than 6 days and the excretion in the urin not higher as 5% per day of the applied dose.
4. The accumulation in the tumor tissue (metastatic mamma carcinom and malignant melanoma) was 8-15% of the dose applied (single clinical investigations).

Paper H20

Direct 99mTc labeling of Monoclonal Antibodies (MoAb): Chemistry and Biodistribution studies of two Technetium cores: 99mTechnetium-Oxo-MoAb (99mTcO-MoAb) 99mTc-Nitrido-MoAb (99mTcN-MoAb)

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Among the radionuclides currently used in nuclear medicine,^{99m}Tc is the most suitable element for imaging applications,due to its nuclear properties and its availability from a generator.
The most studied technetium core,to date,is TcO and almost all the aspects of its chemistry are known.Recently Baldas and Bonnyman[1] proposed a method for preparing a TcN core based on substitution reaction on $TcNCl_4^-$,but Baldas procedure does not appear to be completely applicable to diagnostic nuclear medicine because it does not permit careful control of the sterility and apyrogenecity of injectable radiopharmaceutical preparations.In our laboratory we have developped a new procedure based on the reaction scheme[2].

$$[^{99m}TcO_4]^- + HCl + [P(m\text{-}C_6H_4SO_3)]Na_3 + H_2N\text{-}N(CH_3)\text{-}C(=S)SCH_3 \longrightarrow [^{99m}TcN]_{int} \rightarrow {}^{99m}Tc\text{-}L$$

The procedure involves the previous formation of a technetium intermediate complex.After this step,a ^{99m}TcN radiopharmaceutical is formed by simply substituting the ligands onto the intermediate substrate,without changing the metal oxidation state.
We have optimised the labeling reaction to give the highest antibody ^{99m}TcN-MoAb labeling yield and compared it to ^{99m}TcO-MoAb prepared by using the conventional $SnCl_2$ reduction of pertechnetate.
Stable direct antibody labeling with ^{99m}Tc requires an activation step based on generation of sulfhydryl groups,which show high affinity binding sites for ^{99m}Tc.This reaction is performed with no structural deterioration or any loss of immunobiological activity of the MoAb.The direct 99mTc antibody labeling was explored using intact anti-CEA antibodies (IgG),activated antibody (IgGa) and Fab' fragments .
The labeling yield obtained for TcO core was 31.6% for IgG,98.1% for IgGa and 98.2% for Fab'.These results are higher than those obtained for TcN core.Labeling yield was only 3% for IgG,50% for IgGa and 50% for Fab'.The non bound activity was due to non reacting intermediate and was eliminated before stability and biodistribution studies.
The radioimmunoreactivity is not affected by ^{99m}Tc labeling.The stability of 99mTc-antibody bond ,determined by competition with iminodiacetate groups (chelex 100) was very good for IgGa and Fab' carring TcO or TcN core .

We examined the biodistribution of ^{99m}TcN-MoAb and ^{99m}TcO-MoAb complexes injected in athymic nude mice.3 animals were sacrified 2,6,24 hours after the injection.Biodistribution data were expressed as percent of injected dose per gram of organ.The results obtained 24 hours post injection(Table 1) showed that Tc core did'nt influence the "in vivo" antibody kinetic and organ distribution and that TcN core shows the capacity to react only with sulfhydryl groups giving stable and specific bond on antibody.

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Table I
Biodistribution of ^{99m}Tc radiolabeled antibodies in nude mice 24 hours p.i.

	% Injected dose/g			
Tissue	**^{99m}TcN-IgGa**	**^{99m}TcO-IgGa**	**^{99m}TcN-Fab'**	**^{99m}TcO-Fab'**
Blood	7.2 ± 0.33	8.6 ± 0.95	0.7 ± 0.05	0.5 ± 0.06
Liver	8.8 ±.0.39	5.3 ± 1.35	3.5 ± 0.61	2.0 ± 0.50
Kidneys	4.2 ± 0.33	6.6 ± 0.90	45,7 ± 6.46	20.0 ± 2.72
Tumor	6.3 ± 1.28	6.6 ± 2.50	5.2 ± 1.40	4.5 ± 1.69

Mean ± SD for 3 animals

Paper H21

A SIMPLE CHROMATOGRAPHIC METHOD TO DETERMINE BY-PRODUCTS IN LABELING DTPA CHELATE MONOCLONAL ANTIBODIES WITH (^{111} In) INDIUM

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Labeled monoclonal antibodies are widely tested in clinical research and some of then are routinary used as approved radiopharmaceuticals.
The most useful form is the $F(ab')_2$ fragment labeled with ^{111} In.

Since the antibody does not contain sufficently strong complexing groups, it is chemically transformed by adding an extra chelating group.
This transformation is performed by reacting the $F(ab')_2$ fragment with a 5 to 10 fold molar excess of bicyclic DTPA (1). After the conjugation, the unreacted bicyclic DTPA, hydrolysed to DTPA, is removed by gel filtration chromatography.

The modified $F(ab')_2$ usually contains 1 to 2 moles of chelate per mole of fragment i.e. 10 to 20 nmoles of chelating group per mg of antibody. This is a very small quantity corresponding to the theorical complexing ability of 600 to 1000 ng of a first transition row metal.

So all the reactions carried out on antibodies must be carefully checked for the possibility to introduce metal contaminants.
In general, low radiochemical yields in labeling DTPA modified antibodies are discribed as extra-metal contamination.(2).

In our experience of preparations of several batches of $F(ab')_2$ - DTPA antibodies from laboratory to industrial size it appeared that the major source of non antibody bounded ^{111} In was ionic ^{111} In - DTPA complex.
This release appears several days after the conjugation and the the purification step of the modified antibody.

We developed a simple TLC method to obtain informations about the chemical forms of ^{111} In in radioactive preparations namely ionic non chelated ^{111} In, ^{111} In $F(ab')_2$ - DTPA, and ionic ^{111} In - DTPA.

EXPERIMENTAL

OC 125 $F(ab')_2$ fragments of murine origine were obtained from CENTOCOR. The conjugation was carried out in HCO_3/CO_3 buffer at pH 8 with bicyclic DTPA in a molar exces of 5. The reaction was stopped by passing the reacting mixture through a Sephacryl column eluted with a sodium acetate buffer pH 5.
The fractions showing more than 95 % of radiochemical purity with conventional chromatography (ITLC Gelman SG , sodium citrate 0,1M pH 4 as eluent) were pooled and stored at 4°C and at room temperature
Then a chromatographic control using cellulose layers and CH3CN/CITRATE buffer 0,1M pH 5/WATER : 30/20/3 as eluent.

In this medium, antibody bounded ^{111}In remains at the start, ionic free ^{111}In runs (with tail) as citrate at Rf = 0,35 and ^{111}In DTPA complex (reference sample) runs at Rf = 0,50.

RESULTS

When we applied this control to the labeling of several antibody - DTPA preparations, we found that the major radiochemical contaminant was the free ^{111}In DTPA (Table 1).

	J 0	J 30	J 60	J 75
1	3	5	5	9
2	10	12	16	20
3	35	42	50	55

% of free ^{111}In DTPA in antibody-DTPA preparation / interval between antibody-DTPA preparation and labeling studies.

The ionic DTPA was easily removed by gel filtration or ultrafiltration and radiochemical radiolabeling was then greater than 95 %.
This delayed form of DTPA contaminant was believed to come from ester linked DTPA wich would be more rapidily hydrolized than normal amide linked DTPA.

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Paper H22

EVALUATION OF A DIRECT METHOD FOR TECHNETIUM LABELING INTACT AND F(ab')$_2$ 1A3 AN ANTICOLORECTAL MONOCLONAL ANTIBODY. S.W. Schwarz, P.A. Rocque, C.J. Anderson, M.J. Welch, J.M. Connett,* G.W. Philpott.* Mallinckrodt Institute of Radiology, Washington University School of Medicine, St. Louis, MO 63110, *Department of Surgery, Jewish Hospital, St. Louis, MO 63110

We have investigated the Thakur[1,2] method for technetium labeling of proteins, using an anticolorectal monoclonal antibody (MAb) 1A3[3]. We have used the method for labeling intact 1A3 and F(ab')$_2$ fragments. The method enploys 3500 molar excess ascorbic acid:Ab to reduce a fraction of the available disulfide bonds to free sulfhydryl groups. The MAb is incubated for 60 minutes at 25° C, using 1.0 M ascorbic acid. The technetium-99m is reduced using 5.0ug/ul sodium dithionite, then added immediately to the reduced antibody.

It was necessary to optimize labeling conditions since radiolabeling yields were less than 90%, and immunoreactive (IR)[4] values varied from 73-93% for intact 1A3, and 50-70% for 1A3 F(ab')$_2$ fragments. After a 30 minute labeling reaction at 25° C, the reaction mixture was purified using Sephadex G-50/50 spin columns equilibrated in 0.9% NaCl to remove unbound technetium. Fast protein liquid chromatography (FPLC) analysis indicated the IR values were inversely correlated to the presence of a low molecular weight (3,000-4,000 daltons) radiolabeled peak. With both the intact 1A3 and F(ab')$_2$ 1A3, removal of this peak was accomplished by spin column purification, and the IR increased to 93% and 70% respectively.

Biodistribution of the Tc-labeled antibodies was performed in Golden Syrian hamsters implanted with GW39 human colon carcinoma[5]. The %ID/g tumor, tumor/blood and tumor/muscle ratios were obtained at 4 and 18 hours for ^{99m}Tc-1A3 intact, and at 4,8, and 19 hours post injection for the ^{99m}Tc-1A3-F(ab')$_2$ (See Table 1). The hamster biodistribution results indicate a low tumor uptake of 99mTc-1A3-F(ab')$_2$. This was unexpected since ^{99m}Tc-

1A3-F(ab')$_2$ was determined to be 70% immunoreactive. The hamster biodistribution data suggests that ^{99m}Tc-1A3-F(ab')$_2$ prepared by this direct method of labeling is not stable *in vivo*.

Serum stability was performed in mature female Sprague Dawley rats. Each animal was injected with 700uCi/45ug of ^{99m}Tc-1A3-F(ab')$_2$. The animals were anesthetized with ether. Blood samples were withdrawn via cardiac puncture at 5, 15, 30, 60, and 180 minutes post injection. Plasma samples were analyzed via quantitative FPLC using a Superose 12 column, eluted with 0.02 M NaH_2PO_4 and 0.05 M Na_2SO_4, pH 6.9. Counts per minute (cpm) injected on the column and the total cpm eluted were measured and the percentage of cpm under each protein peak was calculated. For each time point, starting material and plasma samples were aditionally analyzed using silica gel instant thin layer chromatography (ITLC-SG) with 2 M Urea as the solvent to determine the amount of $^{99m}TcO_4^-$ in these samples. ITLC-SG strips, soaked in 5% HSA, using the solvent ethanol: ammonium hydroxide: water (2:1:5), was used to analyze for free reduced technetium species. Additionally, the blood samples were analyzed for %ID/g at the same time points. Analysis of the plasma samples indicats that by 5 minutes post injection there is a significant amount of $^{99m}TcO_4^{-4}$ present in the plasma while starting material remained intact.

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TABLE 1.

Comparison of ^{99m}Tc-1A3 and ^{99m}Tc-1A3-F(ab')$_2$ in Golden Syrian hamster model.

MAb	Time	%ID/g Tumor	Tumor/Blood	Tumor/Muscle
^{99m}Tc-1A3	4 h	3.24 +/-0.16	0.57 +/- 0.05	14.07 +/- 3.61
	18 h	6.19 +/-0.85	2.56 +/- 0.39	26.5 +/- 6.86
^{99m}Tc-1A3-F(ab')$_2$	4 h	2.68 +/- 0.62	0.89 +/- 0.25	11.65 +/- 3.89
	8 h	2.66 +/- 0.51	2.02 +/- 0.76	13.04 +/- 4.77
	19 h	2.61 +/- 0.40	4.25 +/- 1.24	22.2 +/- 5.67

N = 5 for each group.
Data are given as $\bar{x}$ +/- standard deviation.
Tumors were transplanted 3 days before MAb injection.
Ratios of tumor to non-tumor tissue and the % immunoreactive injected dose bound (%ID)/g are presented.