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RADIOLABELING OF MONOCLONAL ANTIBODIES WITH METAL CHELATES

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Radiolabeling of Monoclonal Antibodies with Metal Chelates

Abstract: For nearly a decade, the monoclonal antibody (MoAb) technique has been regarded as an encouraging and promising approach which may lead to rapid advances in both diagnosis and therapy of human cancer. This paper deals with all the chemical aspects of the radiolabeling of MoAbs with metal chelates. It elucidates the selection of radionuclides to be used for diagnostic purpose and therapeutic use. It reviews all the bifunctional chelating agents used in labeling MoAbs with metal radionuclides, and the prime techniques related to the protein modification, i.e., the coupling of MoAb and chelator, and the subsequent process of radiolabeling. Factors affecting tumor-to-normal tissue ratios are discussed. The comparative assessment of advantages and disadvantages of In-111 and I-131 labels is made.

CONTENTS

ABBREVIATIONS AND DEFINITIONS

MoAb: Monoclonal Antibody; **RII**: Radioimmunoimaging; **RIL**: Radioimmunolocalization; **RIT**: Radioimmunotherapy; **DTPA**: Diethylenetriaminetetraacetic Acid; **EDTA**: Ethylenediaminetetraacetic Acid; **TTHA**: Triethylenetetraaminehexaacetic Acid; **TETA**: 1,4,8,11-tetraazacyclotetradecane-N,N',N",N'"-tetraacetic Acid; **DFA**: Desferroxamine; **MT**: Metallothioneine. **C/P**: The bifunctional chelating agent to protein molar ratio during the coupling reaction. **T/N**: % I.D. per gram target to % I.D. per gram normal tissue ratio.

INTRODUCTION

For nearly a decade, the monoclonal antibody (MoAb) technique has been regarded as an encouraging and promising approach which may lead to exciting advances in both diagnosis and therapy of human cancer. In principle, MoAbs combined with radionuclides can specifically target the tumor cells expressing the proper antigenicity. This technique has been termed radioimmunoimaging (RII) or radioimmunolocalization (RIL).

The pioneering work on antibody labeling and tumor localization was conducted in 1948-1949 by Pressman and his co-workers [1-3] who developed the methods for radioiodination of antisera,polyclonal antibodies against mouse kidneys. These I-131 labeled antibodies were subsequently found with radioautography and tissue counting techniques to localize in the target renal tissue. In 1953 Pressman further succeeded in using polyclonal antisera against osteogenic sarcoma to localize the animal tumor by external scanning [4]. This is the first report of real in-vivo radioimmunoimaging for tumors. The first attempt to use I-131 carrying antibodies for tumor therapy was made by Bale and Spar [5].

In 1974, after Gold and Freedman discovered carcinoembryonic antigen CEA [6], Mach and his co-workers [7] reported that in-vivo tumor localization was achieved with affinity purified antibody to CEA in nude mice grafted with human colon carcinoma tissue, and Goldenberg et al. [8] published similar experimental results using a Syrian hamster model system. During the course of 70-80 decades, numerous papers have appeared describing the use of radiolabeled polyclonal and affinity purified antibodies, as well as antibodies against diverse tumor associated antigens, CEA, alpha-fetoprotein, and human chorionic gonadotropin for tumor imaging [9-13]. All the previous studies dealt with radioiodine, mainly I-131, as the radiolabel for the antibodies.

Although many positive immunolocalizations resulted from these antibody applications, non-specific binding and cross-reactivity were often observed. The production of uniquely specific anti-tumor antibodies, therefore, was a major goal to be attained.

Two Nobel laureates, G. Kohler and C. Milstein developed the hybridoma technology in 1975, which enabled MoAb to be made in large quantities [14]. Eventually, the advent of tumor specific MoAb stimulated again a great interest in the use of radiolabeled antibodies for tumor imaging. The use of alternate radionuclides to I-131, such as In-111 and Tc-99m, minimized the problems of in-vivo dehalogenation and internal radiation exposure. Since then there has been a great expansion in the number of publications on this subject, reflecting the obvious progress in MoAb techniques and radiolabeling methods [15-22]. The better in vivo immunological specificity of MoAb over previous polyclonal antibodies has been demonstrated.

However, in practice, much of the promise, especially the expected high specificity of immunoreactivity, has not been achieved yet. Insufficient uptake ratios of tumor to normal tissue (or target to nontarget ratio, T/N) still affects the performance of radioimmunoimaging with MoAb. It will be some time before radiolabeled MoAbs are used in actual standard clinics. Many problems, such as the characterization of antigens and cell receptors, the preparation of optimal MoAbs, and the development of the chemical modification and radiolabeling method for MoAbs, remain to be solved.

Radionuclide selection, the radiolabeling methods and the concomitant chemical modifications, and the metal chelates used in this technique are reviewed in this paper. In fact, how to select an appropriate radionuclide and an effective chelate for MoAb to carry the radiolabel is a very important issue in enhancing the immunological specificity of the tumor seeking MoAb, thus improving the MoAb technique.

The previous review papers [23-28] were helpful to the present authors in writing this article.

I. SELECTION OF RADIONUCLIDES

1. Radionuclides of halogens or metals

In the past, the majority of radioimmunolocalization studies used I-131 as a radioactive label. This nuclide has some advantages: (1) Easy commercial availability based on routine production in reactors; (2) Relatively simple

methodology in labeling antibodies. Iodination of the immunoglobulin molecule is usually accomplished by utilizing effective iodination agents, such as Iodogen or Chloramine-T [29-32]. Both procedures yield antibodies with high specific activity and give high yields. The Iodogen method is milder and easier to perform than Chloramine-T; (3) It may be used for either gamma camera imaging or therapy at different administered activities.

Nevertheless, the use of I-131 has shown pronounced drawbacks for use in radiolabeling MoAbs: (1) Significant in vivo instability caused by deiodination or release of free iodine from the antibody molecule, reducing the T/N ratio, prominently tumor-to-blood ratio [33,24]. It results in a high thyroid uptake, thus increasing the localized radiation dose to this critical organ. Any administration of radioiodine labeled antibody also requires preblocking of the thyroid gland with an excess of iodine to prevent its specific uptake. (2) When it is used as a diagnostic label, its long half-life of 8 days unnecessarily prolongs in-vivo radiation exposure; (3) The emission of beta-particles, delivers a high radiation dose to the patient; (4) The high gamma energy (364 keV, 82% ; 637 keV, 7%), necessitates the use of a high energy photocollimator equipped for gamma scintigraphy.

Iodine-123, with its 13.3 h half-life and 159 keV gamma rays, is an ideal nuclide in imaging studies [34,35], but its high production expense and poor availability significantly limit the current application.

Bromine-77 decays with a 57 h half-life, and 239 keV and 521 keV gamma rays. The dehalogenation of bromine-labeled antibody is supposed to be less than that of iodine since the C-Br bond is stronger than the C-I bond [25]. But, the attachment of radiobromine onto an antibody is more difficult [36]. No study using brominated antibodies has been reported yet.

Astatine-211, a cyclotron produced nuclide with alpha-radioactivity, is potentially useful as a therapeutic nuclide. It has been used to label antibodies via an indirect method [26]. The main obstacle of the application of this nuclide for radiolabeling MoAb is its rather short half-life of only 7.21 h.

Assessment of all the advantages and disadvantages lead to the conclution that halogen radionuclides, in particular the widely used I-131, are not very satisfactory for use in radiolabeling MoAbs. On the other hand, the use of metallic nuclides has offered more chance of nuclide selection. For instance, indium-111 has been extensively tried in radioimmunolocalization studies.

Indium-111 has excellent nuclear properties for radioimmunoimaging: 68 h half-life, 171keV and 245keV gamma rays, and no beta-emission. The most common method for attaching radio-indium to protein is via a chelation reaction of a bifunctional chelate which has previously been conjugated with an antibody protein molecule. Diethylenetriaminepentaacetic acid (DTPA) and its derivatives are the most popular chelates employed for In-111, as well as some other metallic nuclides [37,38].

Several papers have revealed the advantages of using In-111 instead of I-131 labeled MoAb for radioimmunolocalization studies [39,40]. Generally, In-111 label offers greater tumor-to-blood ratios at a faster rate, higher level and longer retention time of radioactivity in the tumor, hence clearer radio-scintigrams of the tumor are obtained than with I-131 [41,42]. However, there are also some marked disadvantages associated with using In-111. The major one is the high non-specific uptake of In-111 in liver, kidneys and spleen of patients receiving the label [43,44]. The elimination of this high non-specific uptake background has been a pressing problem for improving the In-111 immunoimaging techniques.

Halpern has compared the advantages and limitations of In-111 and I-131 in labeling MoAb by listing 29 characteristic items. His conclusion was the radionuclide used in pursuit of an excellence should not be radioiodine and may well be radioindium [45].Granted that the application of In-111 seems more favorable, Larson and Carrasquillo in 1987 still claimed the superiority of I-131 to In-111, particularly when using with Fab fragments or when searching for hepatic tumors [46].

We will detail the comparison of pharmacokinetics between In-111 and I-131 labeled MoAbs in section IV.

Besides In-111, other metallic nuclides,such as Tc-99m,Ga-67, Cu-67, Y-90, Re-186, Au-199, are also potential in labeling MoAb for tumor diagnosis

or treatment. In recent years, the reports describing the applications of In-111 and other metallic nuclides in radioimmunolocalization have greatly increased. Virtually, it is an indication of the emergence of a new trend of the use of metallic nuclides.

2. Radionuclides for diagnosis [47-49]

When considering the selection of a suitable radionuclide for labeling MoAb, one may have one of two possible aims in mind: either to perform a diagnostic study, i.e. radioimmunoimaging or to destroy the tumor cell for therapeutic purposes, i.e. radioimmunotherapy. This section deals with the radionuclides for diagnosis.

On the basis of the criteria for choosing radionuclides for radioimmunoimaging suggested by Wessels and Rogus in 1984 [27], the present authors extend it to eight items to give the following complete criteria:

(1) Physical half-life of about 6-200 h. It should be sufficiently long to allow for imaging at the time when the T/N ratio reaches a maximum. The time needed for the tumor to uptake labeled MoAb is generally much longer, e.g.24-76h, than that for normal organs to uptake common radiopharmaceuticals in nuclear medicine. If the half-life is too long, the radionuclide will bring about an excess of unnecessary radiation dose to the patient.

(2) Gamma energy range of about 100-300 keV. The gamma-ray energy should match the scintigraph device. In conventional nuclear medicine the range of 100-300 keV is most appropriate for external scanning.

(3) High single energy gamma abundance per decay. A high photon density is desired for achieving the high imaging resolution.

(4) No emission of particules, or low abundance of low-energy particule radiation. Any accompanying beta-particle will contribute a considerable dose to the patient.

(5) Stable daughter product of the radionuclide. The radioactive daughter nuclide will not only deliver an additional dose, but also obscure the image.

(6) Production in carrier-free form. Since the number of binding sites of the protein or protein conjugating chelate molecule for the radionuclide is limited, only a carrier-free radionuclide can yield a labeled antibody of high specific activity which is necessary to yield a clear image.

(7) Satisfactory in-vivo chemical stability of the label or nuclide-protein complex. The in-vivo chemical stability depends upon the following variables: bond energy of radionuclide-protein or radionuclide-chelate-protein, i.e., thermodynamic properties; pharmacokinetics, i.e., kinetics of biomacro-molecules in body [24,50-54]. The ultimate consequence of the in vivo chemical stability of the label is demonstrated in terms of T/N ratio as a whole by means of the external imaging.

(8) Conventional availability and reasonable cost.

Besides the first-order parameters discussed above, however, there are variables which may affect the T/N ratio or efficacy of the radioimmunolocalization: (a) chemical impurities of the label, (b) immunoreactivity influenced by the process of radiolabeling, (c) uptakes and catabolism of protein-bound and unbound nuclides, (d) capillary and cell permeability, (e) plasma and whole body clearance, etc...[27]. In fact, a combination of numerous variables determine the performance of a nuclide in RII.

Table 1 summarizes physical properties of some diagnostic nuclides which are commonly used or recommended with regard to the selection criteria.

3. Radionuclides for therapy [47,49,56]

(1) Classification of potential radionuclides. The radionuclides selected for use in radioimmunotherapy, RIT, should have complementary properties to the radionuclides used for diagnoses. Their decays should be rather characterized and with a large component of particulate radiation with little or no accompanying gamma emission such that a high localized dose may be delivered.

Table 1. Selected radionuclides for labeling MoAbs in diagnoses

Radio-nuclide	Half-life (h)	Decay mode	γ-Energy (keV)	γ-Intensity (%)	Production method
Ga-67	78.3	EC	93 185 300	38(100) (62) (50)	Zn-67(p,n)
Br-77	57	EC,β^+	239 521	22.8(100) (97)	Se-77(p,n)
Ru-97	69.1	EC	216 325	86(100) (11.9)	Mo-94(α,n)[55]
Tc-99m	6.02	IT	140 143	89(100) (7.2)	Mo-99→
In-111	67.9	EC	171 245	(93) 94.2(100)	Cd-111(p,n) Cd-112(p,2n)
I-123	13.3	EC	159	82.9(100)	I-127(p,5n)Xe-123→ Xe-124(p,pn)Xe-123→
I-131	193	β^-	284 364 637	(7.46) 81(100) (8.94)	Te-130(n,γ)Te-131→

In 1986 Humm [57] illustrated all the possible sources of nuclear particules for the use in RIT, and classified these sources into five groups depending on the range of the predominant radiation emission:

a. Alpha emitters. The appeal of alpha-particles is their short range, about 50-90 um and high linear energy transfer, LET, about 80 keV/um. An alpha-particle traversing a diameter of a 10 um cell nucleus deposits an energy of 800 keV, equivalent to an absorbed dose of about 0.25 Gy. Only about 3-6 hits per cell nucleus are required to kill a fraction of 63% population [58].

Potential alpha-emitters for RIT are At-211 [59], and Bi-212 [60,61], the latter being produced from Pb-212 [62]. Bi-212 has too short a half-life (60.6min) to be used alone. Nevertheless, if a lead MoAb conjugation can be formed, Pb-212 will continuously generate Bi-212 acting on the tumor cells. Also,heavy alpha-emitters Es-253 and Fm-255 have been suggested for RIT [63].

b. Low-range beta sources (mean range <200 um). Nuclides of this category may provide a superior uniformity of local energy deposit over alpha-emitters. Candidates of this category include P-33, Lu-177, and Au-199 [64,65,66]. Au-199 can be produced carrier-free by a Pt-198(n, γ) reaction [67]; it produces a 42% yield of 159 keV photons which are ideal for simultaneous imaging.

c. Medium-range beta sources (200 um < mean range < 0.2-1mm). Possible medium range beta sources include Sc-47 [68], Cu-67 [69], As-77, Rh-105, Pd-109 [70], Ag-111 [71], I-131, Tb-161, and Re-186 [72].

d. Long-range beta sources (mean range > 1 mm). Candidates here include P-32, Y-90 [73-75], and Re-188 [76]. Investigations on Y-90 in RIT are underway with increasing interests [77,78].

e. Electron capture (EC) and internal conversion (IC) decaying sources [79]. In both processes gamma-rays and a copious flux of Auger electrons are released. Most of the Auger electrons emitted are of very short range (<1 um) and therefore are only of importance if the source is attached or localized very close to the target DNA. With IC, an electron of high energy may be released. EC or IC sources for consideration include Pd-103, Sb-119, and Hg-197.The potential of Auger electron emitters in RIT was discussed [80,81].

Table 2 summarizes physical properties of some potential radionuclides which are tested or suggested for RIT.

Table 2. Potential radionuclides for labeling MoAb in therapy

Radio-nuclide	Half-life (h)	Decay mode	Particle energy,Mev (intensity,%)	Main accompanying γ,keV	Production method
P-32	343	β^-	1.711	no	S-32(n,p)
Sc-47	81.8	β^-	0.439(60) 0.600(40)	159	Ca-44(α,p)
Cu-67	61.5	β^-	0.395(45) 0.484(35) 0.577(20)	184	Zn-68(p,2p)
Y-90	64.1	β^-	2.288	no	Sr-90→
Rh-105	35.4	β^-	0.568 0.249	319	Ru-104(n,γ)Ru-105→
Pd-109	13.43	β^-	1.028	88	Ag-109(n,p)
Ag-111	178.8	β^-,IT	0.087(61) 0.529(36)	658	Pd-110(n,γ)Pd-111→
I-131	193	β^-	0.336(13) 0.607(86)	364	Te-130(n,γ)Te-131→
La-140	40.3	β^-	1.150(19) 1.365(46) 1.680(18) 2.164(8)	1596	Ba-140→
Tb-161	166	β^-	0.520(55) 0.590(10)	460	Gd-160(n,γ)Gd-161→
Re-186	90.6	,EC	1.072(76.6) 0.934(23.4)	137	W-186(p,n); Re-185(n,γ)
Re-188	16.9	β^-	1.937(24) 2.128(74)	155	W-186(n,γ)W-187(n,γ) W-188→ Os-188(n,p)
Au-199	75.36	β^-	0.250(22.4) 0.296(71.6) 0.462(6)	158	Pt-198(n,γ)Pt-199→
At-211	7.21	α(42%) EC(58%)	5.866	687	Bi-209(α,2n)
Bi-212	1.01	α(36%)	5.77 6.05 6.09	727	Pb-212→
		β^-(64%)	2.25		

(2) Selection based on the absorbed dose calculations. In view of dosimetric aspects of RIT, Wessel and Rogus [27] made an absorbed dose calculation for the radiolabeled tumor associated antibodies which were assumed to be distributed in tumor location over a standard geometry. The calculation was done according to the formulation of the standard Medical Internal Radiation Dose (MIRD) [82]. The total dose to the tumor is delivered by three components: (a) the dose from nonpenetrating radiations, (b) the dose from penetrating radiations emitted within the target, (c) the dose from penetrating radiations emitted by the activity remaining outside the target in the rest of the body. In order to optimize the choice of radionuclides, nine nuclides with half-lives ranging from 6 to 200 h have been treated with these calculations (tables 3 and 4).

The total tumor dose, normal liver dose, and the total whole body dose are shown in Table 3. The dose ratio is computed by dividing the tumor dose

by the dose to the whole body. The ratio of nonpenetrating to penetrating radiation components (np/p) is also provided as an indicator of the efficiency of a radionuclide in use of RIT.

Table 4 shows total doses to the tumor, liver and whole body when 1 nmol of antibody is fully labeled with radionuclide (1 label per molecule).

Table 3. Absorbed dose calculation utilizing basic model assumptions [27]

Nuclide	D^{total}_{tumor} (cGy)	$D^{total}_{normal\ liver}$ (cGy)	$D^{total}_{whole\ body}$ (cGy)	Dose ratio	np/p ratio
Cu-67	320	50	41	7.8	1.41
Br-77	85	45	26	3.3	0.10
Br-82	320	210	130	2.5	0.06
Y-90	1700	200	200	8.5	>1000
Tc-99m	2.9	1.9	1.3	2.2	0.14
In-111	150	84	45	3.3	0.09
I-131	750	170	120	6.3	0.56
Re-186	840	100	99	8.5	17.2
At-211	680	170	170	4.0	152

Table 4. Absorbed dose per nanomole utilizing basic model assumptions [27]

Nuclide	D^{total}_{tumor} (cGy)	$D^{total}_{normal\ liver}$ (cGy)	$D^{total}_{whole\ body}$ (cGy)
Cu-67	160	25	21
Br-77	47	25	13
Br-82	280	190	110
Y-90	810	98	98
Tc-99m	15	9.8	6.9
In-111	70	39	21
I-131	120	27	19
Re-186	290	36	34
At-211	2900	750	750

Among the nine nuclides, Y-90 and Re-186 are of particular interests due to their superior properties in respect of the absorbed dose ratio (tumor dose to nontumor dose) and the high dose yield per nanomole.

From another standpoint on the absorbed dose, Humm [57] calculated the fractional energy deposition in tumor cell nuclei using a simple spheric cell model. He concluded that the type of radionuclide required for RIT depended upon the tumor morphology. For a highly cellular packed tumor, e.g., lymphomas or hepatocellular carcinoma, the crossfire from a long-range beta-source makes a positive contribution to the tumor dose. As the tumor mass decreases the gain from crossfire becomes rapidly smaller. Therefore, the choice of shorter-range emitters becomes more appropriate. Under single cell conditions, i.e., application of RIT to the elimination of circulating tumor cells, e.g., leukemic cells, the sources with emission ranges no greater than the cell diameter are recommended. Hence, the success of the therapy requires the antibody carrier to bind actually to each cell.

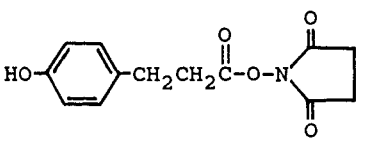
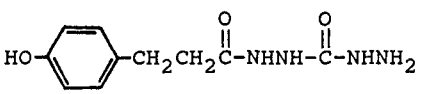
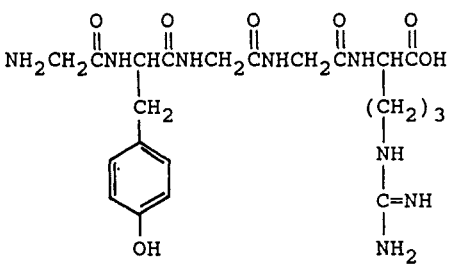
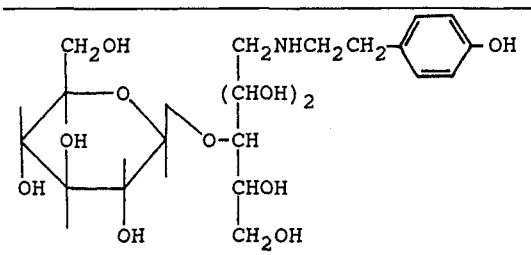
(3) Necessity of highly focused localization. For therapeutic purpose, the energy to be deposited at sites other than the tumor target must be minimal.To achieve such a goal the in-vivo distribution of MoAb should localize exclusively in the tumor site. Indeed, the issues of the final focus and the kinetic biodistribution of MoAb become much more critical when using the nuclide in RIT rather than in RII. In other words, in RII only a limited percentage of labeled MoAbs is required to localize at the tumor site, provided that the T/N ratio is high enough to obtain a good image. But,when a MoAb is used to carry a radionuclide in therapy, very high T/N ratio should be attained. Obviously, the success of therapy using any radiolabeled tumor-seeking MoAb will substantially depend upon the specificity and immuno-reactivity of the MoAb.

II. BIFUNCTIONAL CHELATING AGENTS USED IN LABELING MONOCLONAL ANTIBODIES WITH METALLIC RADIONUCLIDES

Proteins may be radiolabeled in one step through covalent bond formation between a label atom and carbon. The formation of a covalent bond of considerable strength is primarily a property of nonmetals, e.g., halogen; however many radionuclides of interest in RII and RIT are metals (Table 1 and Table 2). The direct labeling methods are used only when some metal ions can be bound by protein itself with high affinity and considerable stability. A few of metals, e.g., Tc-99m, Au-199 and Hg-197, could be directly bound to proteins, mainly via coordination binding with functional groups of proteins[26,64,66,83-88].But the stability and inertness of such links are usually in question [89]. Most of them may be labeled to proteins only via an additionally attached chelating agent, with the exception of a small number of metal binding proteins such as transferrin and metallothionein. Most proteins including monoclonal antibodies do not possess metal binding functional groups capable of forming stable bonds. One solution to this problem is to attach a metal-binding group covalently onto the protein molecule. This group, "bifunctional chelate", can be covalently conjugated to protein and will chelate a metallic nuclide, thereby forming a stable radionuclide-chelate-protein label.

In fact, in the indirect iodination of proteins the reagents used are the prototypes of the "bifunctional chelating agents" for protein labeling with metallic radionuclides. Early in 1973 "Bolton-Hunter" reagent [90] was developed (Fig.1 A). The reagent is first iodinated and then conjugated to lysine residues of protein. The "bifuctional reagents" shown in Fig.1 B and C were used in site-specific radioiodine labeling of monoclonal antibodies [91,92]. Fig.1 D shows a recently reported "bifunctional reagent" for radioiodination of monoclonal antibodies [93].

Fig.1 Bifunctional reagents for indirect iodination

	A. Succinimidyl 3-(4-hydroxyphenyl)-propionate
	B. 3-(4-hydroxyphenyl)propionyl-carbonohydrazide
	C. Glycyltyrosylglycylglycylarginine
	D. Tyramine-cellobiose (TCB)

1. Polyaminopolycarboxylic acids

The first bifunctional chelating agent pioneered by Sundberg and coworkers [94] in 1974 was a polyaminopolycarboxylic acid. The synthesized 1-(p-aminophenyl)ethylenediaminetetraacetic acid was attached to proteins via the diazonium salt (Fig.2 A1) which was instantly prepared prior to the coupling. Diazonium coupling occurs mainly with tyrosine, histidine and lysine residues. At pH 8, the attachment apparently directs to lysine residues [95]. The same research group also synthesized another bifunctional chelating agent 1-(p-bromoacetamidophenyl) EDTA (Fig.2 A2) that might react with sulfhydryl groups. It was used in labeling bleomycin and proteins with metal ions [96]. Numerous analogue derivatives have been prepared.

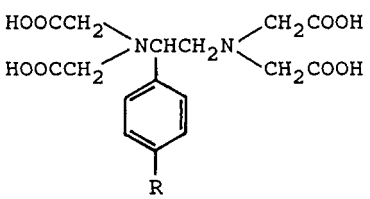
Sundberg et al. started with 1-phenylglycino-nitrile to yield azo-phenyl EDTA. One of the major problems encountered in the synthesis of multifunctional molecules is the purification of intermediates and final products. Meares' research group prepared some analogues of EDTA (Fig.2 A3-A5) using a simple procedure with amino acid derivatives [97-101]. Brechbiel et al. [102] further improved the syntheses.

The chelator A3 (Fig.2) may be coupled to proteins via a water-soluble carbodiimide involving reaction in which the acyl carboxylic acid groups are conjugated to residues with active hydrogens, such as lysine, tyrosine and cysteine. However, the remaining carboxylic groups must be prevented from participating in the conjugation and this was accomplished by forming a chelate with stable ferric ion. The iron ion was then dissociated and removed by dialysis in the last step of the conjugation. A disadvantage of carbodiimide reaction is that intra- and intermolecular coupling may occur among protein molecules [97]. The use of bromoacetamide allows rapid attachment of the chelator to protein predominantly at cysteine residues when coupling is performed at alkaline pH, although conjugation may also occur with histidine, methionine and lysine residues as well. Isothiocyanate group reacts rapidly with lysine residues at alkaline pH [103].

The bifunctional chelates shown in Fig.2 A are analogues of EDTA. Since DTPA is usually superior to EDTA in regard of chelating potentiality, considerable efforts have been devoted to develop methods for attaching DTPA to proteins. Paik et al. [104] prepared N'-(4-(2-hydroxy-5-[iminio(methoxy)-methyl]phenylazo)benzyl)diethylenetriamine-N,N,N",N"-tetraacetic acid (DTTA azo imidate, Fig.2 B1) assuming that the DTPA derivative might be more stable than the EDTA derivative. The coupling reaction is carried out at a pH less than 8.5, and the imidate is thought to be selective for the lysine residues.

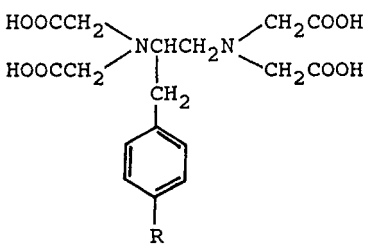
Fig.2 The chemical structures of polyaminopolycarboxylic acids used as bifunctional chelating agents

A. Derivatives of ethylenediaminetetraacetic acid (EDTA)



A1. R= $-\overset{+}{N}_2$, 1-(p-benzenediazonium) EDTA

A2. R= $-NHCOCH_2Br$, 1-(p-bromoacetamidophenyl) EDTA

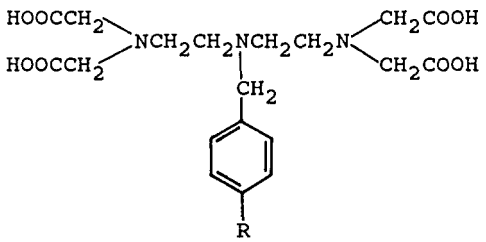


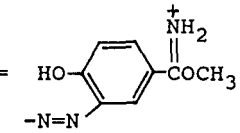
A3. R= $-OCH_2COOH$, 1-(p-carboxymethoxybenzyl) EDTA

A4. R= -NCS, 1-(p-isothiocyanatobenzyl) EDTA

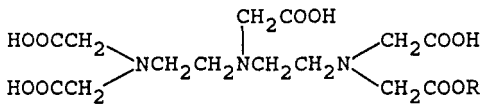
A5. R= $-NHCOCH_2Br$, 1-(p-bromoacetamidobenzyl) EDTA

Fig.2 (continue)
B. Derivatives of diethylenetriaminepentaacetic acid (DTPA)

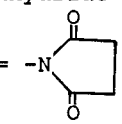


B1. R= 

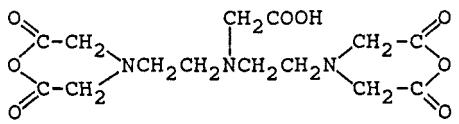
DTTA azo imidate



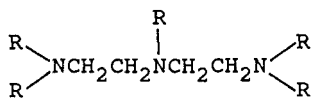
B2. R= $-COOCH_2CH(CH_3)_2$
Mixed O-isobutylcarbonic anhydride of DTPA

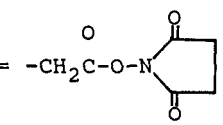
B3. R= 

N-hydroxysuccinimide monoester of DTPA

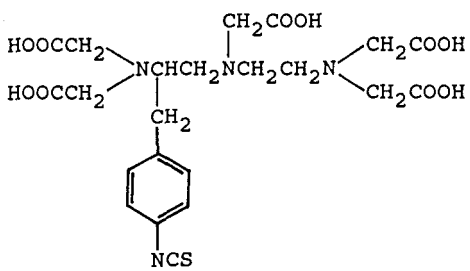


B4. Bicyclic anhydride of DTPA



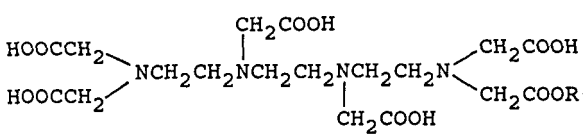
B5. R= 

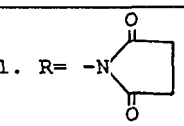
N-hydroxysuccinimide pentaester of DTPA



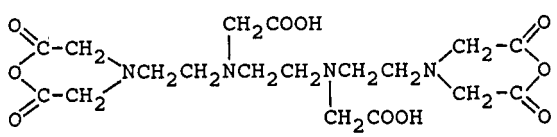
B6. 1-(p-isothiocyanatobenzyl) DTPA

C. Derivatives of triethylenetetraaminehexaacetic acid (TTHA)



C1. R= 

N-hydroxysuccinimide monoester of TTHA



C2. Bicyclic anhydride of TTHA

The de novo synthetic bifunctional chelating agents mentioned above were mainly studied over the years of 1974-1979, but did not gain popularity in radiolabeling antibodies, principally owing to the complicated synthetic procedures. In the meantime, the basic requirements of protein-chelates towards in-vivo use were explored [96,98,105]. The anhydride derivatives of DTPA (Fig.2 B2,B4) are generally easier to synthesize because the commercially available DTPA can be used as the starting material. Two methods to prepare active anhydrides for conjugating DTPA to antibodies have been developed. Krejcarek and Tucker [106] adapted the mixed anhydride technique commonly used in peptide synthesis to radiolabel antibodies. The anhydride was prepared by mixing DTPA and isobutyl chloroformate. The resulting mixed O-isobutylcarbonic anhydride of DTPA was then used without purification. Paik et al. [107] reinvestigated this technique and found that the mixed anhydride was unstable towards temperature and hydrolysis. As a consequence of the instability, the mixed anhydride cannot be isolated for identification, nor stored. Hence,the mixed anhydride was usually used immediately after preparation, and the anhydride to protein molar ratio during coupling was scarcely to be determined [107]. Two analytical methods for the anhydride were developed: a spectroscopic method using excess of benzylamine and a gravimetric method using $Ba(OH)_2$ to trap released carbon dioxide. Large differences between the theoretical and actual concentration of anhydride were observed. Anhydrides react with cysteine and tyrosine as well as lysine residues, however only the amidation product with the latter appears to be stable [108].

The second method is to prepare bicyclic anhydride of DTPA (Fig.2 B4) by mixing DTPA with acetic anhydride in the presence of pyridine. This compound was first used to attach the chelate to fatty acids and long chain hydrocarbons [109,110], but subsequently used, first by Hnatowich [38,111] and then by Paik [112] to label proteins including antibodies. Hnatowich's research group systematically investigated the coupling and the subsequent radiolabeling conditions with the cyclic anhydride,and established an optimal procedure [37,113]. Presumably, the conjugation of DTPA is to the lysine moieties in the antibody. By contrast to mixed anhydride, cyclic anhydride is quite stable. Therefore, much greater control on the conditions of coupling is possible, and, as a result, proteins may be coupled with reproducible efficiencies [37]. In our laboratory, a sample of cyclic anhydride of DTPA, which had been stored in a common desiccator at room temperature for two years, did not alter its i.r. spectrum and efficient coupling properties. Eventually, bicyclic anhydride of DTPA has been proven the most useful and popular one among many bifunctional chelating agents.

However, being multifunctional, the bicyclic anhydride of DTPA may cause intra- and intermolecular coupling of the proteins, which is the disadvantage of its use [37,114].

An alternative approach to interlink DTPA and protein is the use of N-hydroxysuccinimide ester (Fig.2 B3,B5). Najafi et al. [115,116] synthesized N-hydroxysuccinimide pentaester of DTPA (Fig.2 B5) by reacting bicyclic anhydride with N-hydroxysuccinimide and carbodiimide. The pentaester was reported to be more stable than bicyclic anhydride of DTPA. The use of the DTPA pentaester results in a higher coupling efficiency for attaching DTPA molecules to proteins compared to that of bicyclic anhydride, particularly at low protein concentrations. The short-coming of the pentaester method is the appearance of species of higher molecular weight and less stability of the label both in-vitro and in-vivo. By reacting excess molar of DTPA with N-hydroxysuccinimide and carbodiimide, Buckley et al. [117] and Paxton et al. [118,119] obtained a mono-activated species of DTPA: N-hydroxysuccinimide monoester of DTPA (Fig.2 B3). The employment of a monoester might prevent the intra- and intermolecular coupling of antibodies which often occurs when bifunctional chelating agents with more than one activated groups are used.

A de novo synthetic DTPA derivative, 1-(p-isothiocyanatobenzyl) DTPA (Fig.2 B6), has been recently presented [102,120,121]. This chelate owns one more carboxylate group to bind metal ion than does any one of the DTPA derivatives mentioned above. Animal studies have shown that antibodies labeled with In-111 through this chelate can be used for tumor imaging with nonspecific liver uptake being greatly reduced.

DTPA can also be coupled to protein with a water-soluble carbodiimide method [122]. However, the conjugation yield of this method is very low and the extent of antibody deactivation is high.

Being the dominating bifunctional chelating agents, the derivatives of DTPA, especially bicyclic anhydride of DTPA, have been widely used in labeling different proteins with a variety of metallic radionuclides, such as In-111 [37,38,102,104,107,111-128], and to a lesser extent, Tc-99m [129-131], Y-90 [74,75,77,78,132-134], Ga-67 [68,135-138], Ga-68 [139],Pd-109 [70],Sc-46 [17,68], Re-186 [72], Bi-212 [60,61], and Yb-169 [140].

The chelating agent triethylenetetraaminehexaacetic acid (TTHA) has six carboxyl groups. Like DTPA, TTHA can be readily coupled to antibodies by utilizing its N-hydroxysuccinimide monoester (Fig.2 C1) [141] or bicyclic anhydride (Fig.2 C2) [142]. It was expected that its metal binding ability would be less adversely affected than that of DTPA after conjugation with antibody due to its extra carboxyl group. However, the animal distribution of In-111-TTHA-MoAb showed that there was no superiority to DTPA, but lower T/N ratios than with DTPA [141].

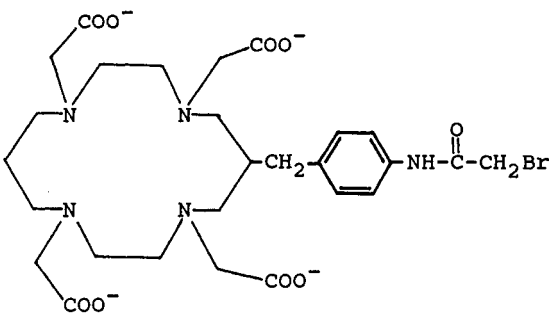
2. Macrocyclic molecules

Although polyaminopolycarboxylic acids serving as bifunctional chelating agents have given successful performances in labeling antibodies with some metallic radionuclides, especially In-111, they are not applicable to chelate many other radionuclides that are better suited for diagnostic or therapeutic applications. One interesting example is Cu-67 [143]. Copper(II) is notable for its rapid exchange of coordinated ligands [144]. It is well-known that Cu(II) binds readily and strongly to proteins, particularly albumin [145]. Neither DTPA nor EDTA reagents could prevent the binding of Cu(II) to albumin [146,147]. Therefore, it seems that stable labels cannot be formed by using derivatives of either EDTA or DTPA as bifunctional chelating agents to label antibodies with Cu(II).

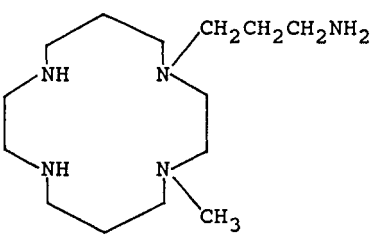
Moi et al. [146] synthesized a new bifunctional chelating agent composed of a 14-membered macrocyclic ring with four carboxymethyl-substituted nitrogens (Fig.3 A). This structure (TETA) was chosen because of the copper-sized cavity formed by the ring nitrogens, and the characteristic of slow decomposition rate of macrocyclic chelates. Even though Cu(II) TETA is less thermodynamically stable than Cu(II) EDTA or Cu(II) DTPA, it decomposes in human serum at a rate of about 1% per day which is much lower than that of Cu(II) EDTA or Cu(II) DTPA [146,147]. The crystal structure of Cu(II) TETA demonstrates that the metal is coordinatively saturated by four nitrogens and two of ligand carboxylates [148]. Indium is not complexed well by this ligand which is presumably too small for In(III) ion.

It should be stressed that metal ions should be chelated with TETA prior to coupling with antibodies. Otherwise, there may be a sterical hinderance while introducing the metal ions to the previously coupled macrocycle-antibody. If a spacer group is used to increase the isolating space between protein and chelator, the radiolabeling could follow the coupling in the general way [146].

Fig.3 The chemical structures of macrocyclic molecules used as bifunctional chelating agents



A. 6-(p-bromoacetamidobenzyl)-1,4,8,11-tetraazacyclotetradecane-N,N',N",N"'-tetraacetic acid (6-(p-bromoacetamidobenzyl)-TETA)



B. 1-(3-aminopropyl)-4-methyl-1,4,8,11-tetraazacyclotetradecane

Macrocyclic amines can also react rapidly with a variety of metal ions to form kinetically inert and thermodynamically stable complexes [149,150]. Franz et al.[151] prepared 1-(3-aminopropyl)-4-methyl-1,4,8,11-tetraazacyclotetradecane (Fig.3 B) which by treatment with 2-iminothiolane-HCl might give an active molecule containing a free sulfhydryl group for coupling.Although there is no N-acetate substitution like TETA does, the copper(II) complexes of the ligand possess a comparable serum stability to those with TETA. This bifunctional chelating agent was also used to label antibodies with Tc-99m [152].

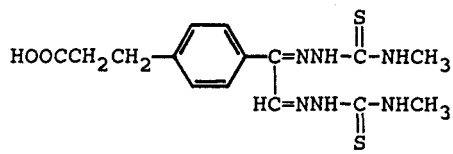
3. Sulfur containing ligands

Some metallic radionuclides, such as Tc-99m, Pd-109, Ag-111, Hg-197 and Au-199, are needed to label MoAbs. According to the Lewis acid-base theory, they are soft acids. In 1963 R.G Pearson [153] created his famous rule: hard acid prefers to bind to hard base and soft acid prefers to bind soft base. Therefore, introduction of the soft base sulfur atom to the chelating groups of bifunctional chelating agents would favor labeling antibodies with these soft acid metals.

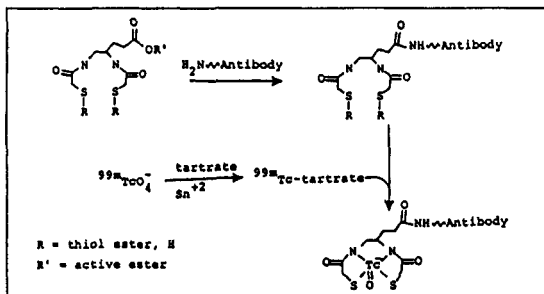
Yokoyama et al. synthesized various bifunctional chelating agents containing bisthiosemicarbazone groups for chelating technetium. The latest in the series, p-carboxylphenylglyoxal di-(N-methylthiosemicarbazone) (CE-DTS, Fig.4 A) was conjugated to human serum albumin as a model protein [154]. Further, immunoimaging of tumor bearing mice injected with Tc-99m-CE-DTS-MoAb

Fig.4 The chemical structures of bifunctional chelating agents containing sulfur atoms

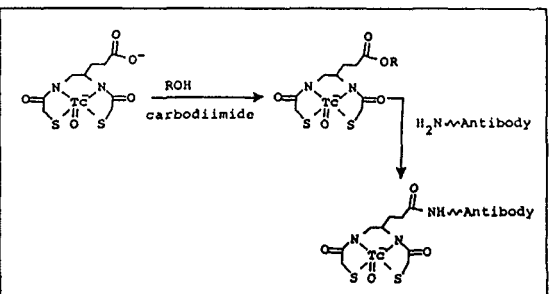
A. p-Carboxyethylphenylglyoxal di(N-methylthiosemicarbazone) (CE-DTS)



B. Diamide dimercaptide ligands and their coupling procedures [156]

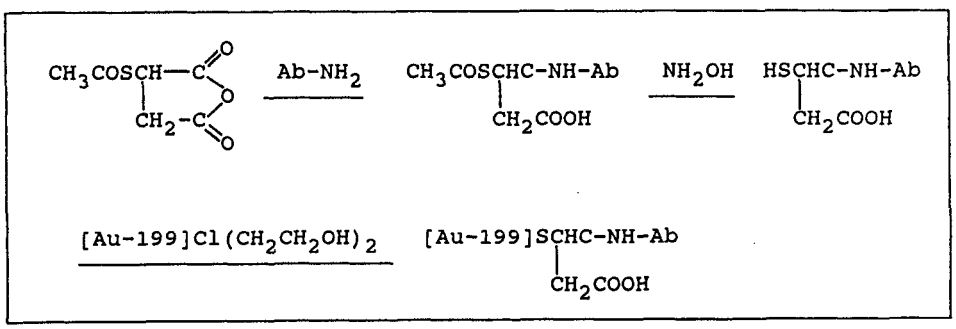


B1. Ligand conjugated to antibody prior to Tc-99m labeling



B2. Preformed chelate approach to Tc-99m-antibody labeling

C. Acetylthiosuccinic anhydride and its labeling procedure



has been made [155]. Fritzberg et al. [156] commented that some instability was seen of Tc-99m labeled CE-DTS-HSA when CE-DTS to HSA was at a molar ratio 1:1, and when the molar ratio exceeded unity, an increased liver uptake occurred.

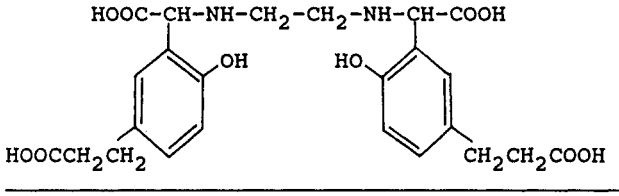
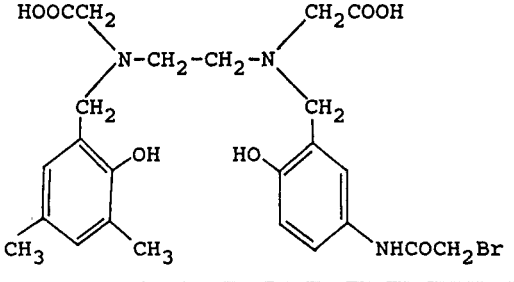
Fritzberg et al. [157] synthesized about twenty diamide dimercaptide N_2S_2 derivatives in order to develop a Tc-99m radiopharmaceutical to replace radioiodinated hippuran. In addition to radiopharmaceutical potential, N_2S_2 ligands might offer access to produce Tc-99m complexes of desirable structures and properties with high stability [158,159]. Additional carboxylate was attached for binding to proteins (Fig.4 B). Two approaches to Tc-99m-antibody labeling with the N_2S_2 ligand are possible. The "traditional" route involves conjugation of ligand to antibody and then exchange of the metal ion from a weak complex to the N_2S_2-antibody conjugate (Fig.4 B1) [160]. This procedure required incubation of the N_2S_2-antibody conjugate with Tc-99m-tartrate at pH 8 and 50°C, which is a more rigorous condition than desired. Alternatively, Tc-99m-N_2S_2 complex could contain a conjugation group that was subsequently activated and coupled to antibodies (Fig.4 B2) [161]. Further they labeled MoAb fragments $F(ab')_2$ and Fab by this means and gained successful imagings in patients [161].

We employed acetylthiosuccinic anhydride (AMSA), a typical sulfhydrylating agent, in labeling antibodies with Au-199 [65]. Its structure and labeling procedure are showed in Fig.4C. However,the stability of this Au-199 labeled antibody is in question.

4. Phenolic aminocarboxylic acids

It is well-known that the phenolic aminocarboxylic acid multidentate ligands do form quite stable complexes with trivalent metal ions such as Fe(III), Ga(III) and In(III) [162]. These ligands were demonstrated to have higher stability constants for complex formations with Ga(III) and In(III) than DTPA and transferrin [163]. Taking ethylenediamine-N,N'-bis-(o-hydroxyphenylacetic acid) (EDDHA) as a fundamental structure of the bifunctional chelating agent, Schuhmacher et al.[164] synthesized p-EDDHA (Fig.5 A). Propionic acid residues were used for covalent attachment of ligand to antibodies via carbodiimide coupling. To avoid the covalent linkage between protein and acetic acid carboxyl groups of ligand, which are essential for complex formation, Ga-67 or In-111 was chelated before coupling of ligand to proteins. The in-vivo distribution studies with Ga-67-p-EDDHA-MoAb showed that the T/N ratios of it, except the tumor-to-liver ratio, were superior to those of I-131 labeled MoAb [165]. However, the antibody labeled with In-111 via p-EDDHA appeared to be unstable in-vivo presumbly due to the rapid displacement of In(III) from the complex by Fe(III).

Fig.5 The chemical structures of phenolic aminocarboxylic acids used as bifunctional chelating agents

	A. Ethylenediamine-N,N'-bis-[o-hydroxy-m-(2-carboxyethyl) phenylacetic acid] (p-EDDHA)
	B. N-(2-hydroxy-3,5-dimethyl-benzyl)-N'-(2-hydroxy-5-bromoacetamidobenzyl)-ethylenediamine-N,N'-diacetic acid (Br-Me2HBED)

Recently, Welch et al. [166] selected N,N'-bis(2-hydroxy-3,5-dimethylbenzyl)ethylenediamine-N,N'-diacetic acid (Me_4HBED) as a fundamental structure for bifunctional chelating agent, and synthesized Br-Me_2HBED (Fig.5 B). Bromoacetamide group was added to bind to proteins. This ligand is expected to have a tremendously large stability constant with In(III), logK=40, which is much larger than that of the conventional bifunctional chelates, DTPA (logK=29) and EDTA (logK=25) [167]. However, the use of In-111-labeled antibodies through this chelating agent in RII still showed high uptake of In-111 in liver suggesting a mechanism other than simple metal transcomplexation for this retention [168].

5. Biomacromolecules

Because some biomacromolecules possess great affinities for metal ions, much attention is paid to them for antibody radiolabeling. Transferrin has been coupled to antibodies via glutaraldehyde, and the conjugates were then labeled with In-111 [169]. Gluteraldehyde reacts with proteins in a complex way involving many different residues including cysteine, histidine, tyrosine, and particularly lysine residues [170]. Schiff's base formed in this reaction must be reduced for stability; both intra- and intermolecular cross linkings probably occur. Although transferrin does form strong chelates with trivalent metals such as Fe(III) and In(III), its large size (MW=80,000) is likely to alter the labeled protein.

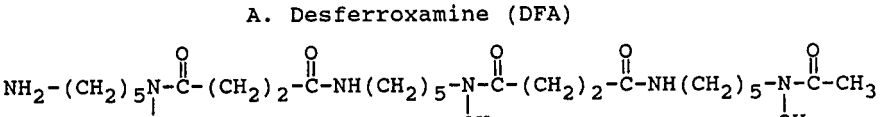
Metallothionein (MT) is characterized as a metal-binding protein consisting of 20 cysteine molecules and 41 other aminoacids with a molecular weight of 6000-7000. It has been explored as a chelate for labeling antibodies [171-173]. Metallothionein is able to form stable complexes with "soft acid" metals, e.g., Tc-99m, Ag-110m, Hg-197 and Au-199 owing to its sulfhydryl-rich nature. It may be particularly suitable for delivering a cytotoxic radiation dose to tumor cells since one metallothionein molecule can bind several metallic radionuclides.

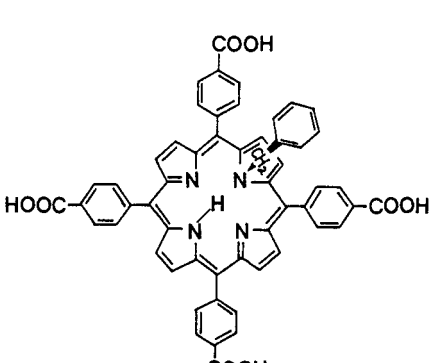
Desferroxamine (DFA, Fig.6 A) is also an effective metal chelator owning an amine group to favor the coupling [169]. Desferroxamine conjugated to proteins via glutaraldehyde [174,175] and water-soluble carbodiimide [175,176] has been labeled with Ga-67. A hemi-succinate version of desferoxamine has been proposed for conjugation with antibodies. However, practical applications of this method have not been fully explored [177].

An alternative approach to the labeling of antibodies through biomolecules is the introduction of an analogue of porphyrins: N-BzHTCCPP (Fig.6 B) for antibody labeling with Cu-67 by Mercer-Smith et al. [178,179]. Porphyrins are relatively nontoxic and their copper(II) complexes are extremely stable in human serum. N-BzHTCPP was coupled to antibodies by activating one of its four carboxylic groups.

Fig.6 The chemical structures of biomacromolecules used as bifunctional chelating agents

A. Desferroxamine (DFA)





B. N-benzyl-5,10,15,20-tetrakis(4-carboxyphenyl)porphyrin (N-Bz-HTCPP)

III. TECHNIQUES RELATED TO COUPLING AND RADIOLABELING

The process of antibody labeling utilizing bifunctional chelates generally consists of two steps: (1) antibody-chelator coupling and subsequent removal of the unconjugated ligands, (2) radiolabeling of the conjugates. The procedures used and precautions taken on the antibody modification will be discussed basically with DTPA bicyclic anhydride and In-111 that have been used and studied in great detail. These techniques and precautions described may be extended on other systems.

1. Methods of coupling

The coupling of DTPA anhydride with proteins was intensively investigated by Hnatowich's research group [37,113]. The coupling was performed by mixing protein in 0.05 M, pH 8.2 bicarbonate buffer with anhydride which was taken from its suspension in dry chloroform. Solvent chloroform was evaporated prior to mixing. The coupling reaction completed in 20 sec,and free DTPA generated during coupling through hydrolysis of anhydride was removed by Sephadex G50 column chromatography or centrifugation. Paik et al. [112] used dry dimethyl sulfoxide (DMSO) to dissolve anhydride instead. In our experience, DMSO is better than chloroform because of (1) an homogeneous solution acquired due to the excellent solubility of anhydride in DMSO, (2) an exact sampling of an aliquot of solution resulting in better reproducibility of the coupling efficiency, (3) avoidance of evaporation of organic solvent due to the good miscibility of DMSO with water.

The N-hydroxysuccinimide mono- and pentaester of DTPA could be coupled to proteins in an identical way.

An alternative approach, which deserves special attention for covalent attachment of chelator to antibodies, is the utilization of immuno-complex formations. Reardan et al. [180] first prepared monoclonal antibodies against indium labeled EDTA itself. Then Goodwin and Meares et al. [181-184] bound In-111 labeled EDTA to these anti-chelate antibodies via the specific immuno-reaction inbetween, and thereby developed two novel radioimmunoscintigraphy techniques (see Section V).

Biotin and avidin (a protein of MW 66,000) possess extremely high mutual affinity with an association constant of $10^{15} M^{-1}$. Hnatowich et al. [185,186] coupled biotinylated antibody with avidin which has previously been linked with In-111-labeled DTPA. Since avidin is multivalent, it might be conjugated with at least 15 DTPA groups without decreasing its ability to bind biotin. They have achieved a strikingly high specific activity of 300 mCi/mg of In-111 on IgG antibody using avidin conjugated with 12 DTPA groups per molecule [186]. This system has been regarded and further developed by Goodwin et al. [182].

2. Measurement of the number of chelators per protein molecule and immunoreactivity of antibody

There is no doubt that proteins may be degraded by covalent attachment, either of chelating groups or iodine atoms. The extent of degradation increases with increasing substitution. Therefore, it is usually important to determine the number of chelator groups attached to each protein molecule. To measure the number of the groups per antibody molecule is to establish whether the degree of conjugation exceeds the level at which adverse effects become apparent.

Methods to determine the number of chelating agents per antibody molecule have been studied in detail in DTPA analogues. C-14-labeled DTPA derivatives have been used to directly determine the incorporated DTPA [102,120]. However, the special need of C-14-containing reagents may restrict its wide utilization. Alternatively, the number of attached DTPA groups may be determined by titration of the chelating groups with In-111 [37,124,187]. Carrier-free In-111, at tracer activity levels, was added to the preparation coupled but unpurified from free DTPA, and the fraction of added activity on protein (labeling efficiency) could be measured by the size exclusion HPLC or Sephadex column chromatography. Because free and coupled DTPA compete equally for In-111 [37] and the direct binding of In-111 to protein (nonspecific labeling) is negligible [124], the coupling efficiency,i.e. the fraction of DTPA coupled to protein, is substantially equal to the labeling efficiency. The number of attached groups can be calculated from the coupling efficiency and the initial chelating agents to protein molar ratios (C/P). This method

can only be used in a condition of the small starting C/P ratio, by which a statistically valid high value of protein-bound fraction of In-111 is obtained. Good agreement has been reported for this determination in comparison to an involved method using stable indium [187].

Immunoreactivity of the modified antibodies has been determined by a number of methods, including affinity chromatography [54,112], competition with radioiodinated antibody for a limited number of antigenic sites bound to beads [51], cell bind assay [124, 188] and immunofluorescence assay [124].

Over-conjugation may damage the proteins, but under-conjugation may have unfortunate consequences as well, not necessarily on the protein but on the ability to label the coupled protein at high specific activity that will be discussed in the following section. In order to gain a label with both high immunoreactivity and high specific activity, it is necessary to set a suitable conjugation number by controlling the initial chelate to protein molar ratio. Many laboratories investigated the relationships between antibody immunoreactivity and the attached DTPA groups per antibody molecule [32,54,112,187,188], but quite different results were presented. From our own practice and experience, the loss of antibody immunoreactivity is acceptable if less than one DTPA group is conjugated to a antibody molecule. We would strongly recommend Hnatowich's coupling procedure [37], in which a 1:1 C/P molar ratio resulted in an about 63% coupling efficiency when the initial antibody concentration is 10 mg/ml. In such case, one antibody molecule conjugated about 0.6 DTPA groups, thereby, a label with sufficiently high specific activity could be gained. From our experience, under the condition of low C/P molar ratio, the immunoreactivity of the modified antibody remains so high, and the determination of the immunoreactivity of the final products may generally be omitted, unless other operations harmful to the antibody have been done.

Paik et al. [54] pointed out that in a modified antibody preparation, the difference between immunologically active antibodies and inactive antibodies is not due to the difference in the number of chelating agents per antibody, but more likely due to the attachment positions. Alvarez et al. [92,189] showed that monoclonal antibodies, which were modified site-specifically by attaching DTPA derivatives onto immunoglobulin carbohydrate in Fc region, gave low liver background and improved radioimmunoscintigraphy.

Finally, one should recognize that the number of chelators per protein molecule, discussed above, is only a statistically averaged number. The chelators are not shared equally in each protein molecule under the assumption that Poisson statistics apply. For example, at an average value of three per molecule, there is a small fraction of the protein molecules (5%) which are free of attached groups, whereas at a value of 0.5, approximately 60% of the protein are uncoupled [113,95]. Consequently,if the previously described immunoreactivity assays were to be performed with a preparation of low average number of groups per molecule, the assays would consist largely of uncoupled antibodies with coupled antibodies itself [113].

3. Protein radiolabeling

The radiolabeling of chelator coupled proteins is accomplished by mixing the protein with a desired amount of radionuclide which was complexed with a suitable ligand. The ligand should be strong enough to prevent hydrolysis of metal ions but too weak to prevent the donation of the ion to the conjugate. If the radionuclide is In-111 and the chelator is DTPA, citrate, acetate and oxine are the appropriate candidates of the transition ligands [37,114,124,125]. Under normal circumstances, the labeling efficiency may approach 100%, so that further purification of the product is not required.

The procedure for the radiolabeling of antibody-DTPA conjugate is rather simple. However, the contamination by even minute amounts of metallic impurities may damage the procedure. Great patience and cautions should be always maintained in this regard. Since DTPA forms strong chelates non-specifically with a variety of metallic ions, and the concentration of DTPA in the solution of coupled protein is typically low, care must be taken to avoid introducing trace metals in contents sufficient to compete with the radionuclide for the limited number of DTPA groups. In our laboratory, employment of distilled de-ionized water, ultrapure In-111 and other chemicals, acid-washed glassware and centrifugation column chromatography in experiments are all helpful to reduce the effect of trace metals. Under optimal conditions, we have achieved a specific activity of In-111-labeled antibodies up to 30 mCi/mg for IgG coupled with one DTPA group per molecule

and at protein solution of 8 mg/ml [124,190]. In addition, the introduction of trace metals is most serious after coupling and before labeling. Therefore, sample treatment in that time should be minimized.

In view of the damage caused by even minute metallic contamination, the low initial content of the protein is unfavorable to produce radiolabeled antibodies with high specific activity.A pre-concentration procedure of the protein solution is recommended to reduce such interference and to assure a reasonably high specific activity of the label [113].

The maximum theoretical specific activity of In-111 labeled antibody-DTPA conjugate containing one DTPA group per antibody molecule (MW=150,000) is 307 mCi/mg assuming the formation of a 1:1 complex of In-111 and DTPA. However, the experimentally obtainable specific activity is only in the order of 1-10% of the theoretical value. This is mainly due to the blocking of DTPA by metallic contaminants.

4. Quality control

In our laboratory, we have established the folloeing quality control standards for clinical application of In-111-labeled MoAb-DTPA conjugates [124]:

(1) radiochemical purity: The radiochemical purity, namely the labeling efficiency, of the labels is determined by Sephadex G50 chromatography, and >95% is required.

(2) Specific activity: The specific activity controlled by adjusting the relative amounts of In-111 and chelator coupled antibodies should be greater than 1 mCi/mg.

(3) Immunoreactivity: Both immunofluorescence assay and cell binding assay are used to evaluate the immunoproperty of the modified MoAbs. In immunofluorescence assay, the decreasing factor of the antibody titre should not exceed 2. With cell bind assay, the binding efficiency should be greater than 70% (nonspecific binding is below 10%).

Naturally,the preparation should be sterile and pyrogen-free.

IV. LABEL STABILITY AND PHARMACOKINETICS

1. Label stability under physiological conditions

The stability of the radiolabeled antibody under physiological conditions is an important factor which directly influences the biodistribution of the label and the performance of RII and RIT. There are three conditions relating to the instability of the labels. (1) The covalent bonds by which chelators are attached to antibodies may hydrolyze with the release of free radio-labeled chelators. (2) Radionuclides are "non-specifically" attached to weak complexing sites in the proteins in preference to the chelator groups. The weakly bound metal ions are readily transferred to other proteins and so provide nonuniform background interference. (3) Radionuclides coordinated with the chelators may be transcomplexed to serum proteins and various metal binding sites in normal tissues, or replaced by physiological and chelatable metal ions.

Generally,the covalent bond between chelator and protein is quite stable. Its breaking would result in the release of free radiolabeled chelator to the circulation, which would then be cleaned rapidly by glomerular filtration and appear in urine. However, in cases of In-111 labeled MoAb-DTPA conjugates, the accumulation of In-111 in patient urine was modest, averaging 0.26%/h [51]. Furthermore, the results of gel filtration analysis of these urines showed that In-111 did not coelute with In-111-DTPA and was therefore in a different chemical form. So this mode of instability would not contribute greatly to the loss of the label [191], unless the metabolizable linkers are used [192-194]. In addition, the non-specific labeling could be easily avoided by using a buffer with weak metal-binding properties in an appropriate concentration, or by adding of EDTA (or DTPA etc.) to the reaction mixture to scavenge unchelated metal ions [124,148]. Hence, the effects of instability due to the cleavage of covalent bonds and non-specific binding are negligible. It is more likely that dissociation of the chelates is principally responsible for the instability.

The serum stability of In-111 labeled proteins through several bifunctional chelates has been intensively investigated. The principal mode of label instability in serum is likely to be exchange of the label with transferrin. The formation constant of the chelate of indium-transferrin (logk=30.5 [52]) is larger than that of indium-DTPA (logK=29.0), and the high concentration of transferrin in serum may enhance this transcomplexation. The exchange of In-111 in serum has been investigated for several proteins with different bifunctional chelating agents. In the case of albumin coupled with DTPA using the mixed anhydride and labeled with Ga-68 and In-111, Wagner and Welch [139] reported an exchange rate to transferrin of about 9%/day for both labels. Yeh et al. [195] prepared In-111 labeled albumin via DTPA using mixed anhydride and via EDTA analogues using 1-(p-carboxymethoxybenzyl) EDTA (Fig.2 A3) and 1-(p-bromoacetamidophenyl) EDTA (Fig.2 A2). The dissociation rates of these In-111 labeled proteins in 37°C serum were determined to be 2%/day for the DTPA conjugated proteins and about 0.9%/day for the EDTA analogues. A similarly low rate of transcomplexation for proteins conjugated with EDTA analogues has been reported elsewhere [105]. The greater serum stability of In-111 labeled protein conjugated with the EDTA analogues has been explained by steric hinderance toward dissociation resulting from the presence of the bulky benzene ring in the ethylene backbone [195].

A similar 37°C serum stability study was conducted with the In-111 chelates of free DTPA and DTPA azo imidate (Fig.2 B1) [104]. The exchange rate was found to be 12%/day of the free DTPA and 10%/day of the coupled albumin.

The dissociation rates in 37°C serum of In-111 labeled antibodies coupled with DTPA via the succinimde ester and the bicyclic anhydride have been compared [115]. By affinity chromatography, the dissociation rate of the ester-coupled protein was shown to be 27%/day vs. 10%/day for the anhydride coupled protein. A similar result has been presented elsewhere [118]. The significant difference in stability was explained as possibly owing to the conjugation at residues other than lysine in case of the ester [115].

The in-vivo instability of In-111 labeled antibodies via DTPA bicyclic anhydride was measured using affinity chromatography on serum obtained from mice sacrificed at 10 min. and 24 hrs post administration [37]. The mean decrease of activity in serum binding to the column was 5.8%. Similar methods were taken to determine the degree of label instability in patients [51]. Following administration of In-111 labeled antibodies prepared via DTPA bicyclic anhydride, serum samples were analyzed by Sephadex G200 chromatography, anti-antibody and anti-transferrin affinity chromatography. The results of these separate measurements were in good consistency and showed the dissociation rate to be about 9%/day and entirely due to the transcomplexation to circulating transferrin.

Very little has been done concerning the stability of proteins labeled with metallic radionuclides other than In-111 and bifunctional chelating agents other than EDTA and DTPA analogues. Hnatowich et al. [75] measured the stability of Y-90 labeled antibodies via DTPA bicyclic anhydride in 37°C serum, and indicated that it decomposed at a similar rate as In-111 on the same protein,e.g.8-9%/day. Meares' research group investigated the serum stability of Cu-67 labeled antibodies coupled with the analogues of EDTA, DTPA and TETA [147,195]. The loss rate of copper from TETA-antibody conjugate in serum was only about 1%/day, whereas copper from DTPA- and EDTA-antibody conjugates lost about 95% in the first day of incubation. According to the published thermodynamic equilibrium constants [196], Cu-67-TETA would be expected to be less stable than either EDTA or DTPA chelates. The apparent discrepancy in the serum stability of Cu(II) chelates must lie in the kinetic rather than thermodynamic properties of the complexes.

Table 5 and Table 6 summarize the in vitro instability in 37°C serum of metallic radionuclide labeled free chelators and chelator-protein conjugates, respectively.

2. Factors affecting the accumulation of radiolabeled antibodies in tumors [198]

(1) Blood flow. Tumors grow readily from a central group of cells, so as the tumor enlarges, the dividing cells form a shell around a relatively hypoxic core. When these cells outgrow their blood supply,they die and form a necrotic central nest containing some viable cells that are very resistant to

Table 5. Dissociation rate in 37°C serum of radionuclides labeled free ligands

Chelate	Dissociation rate, %/day	Determination method	Ref.
In-111-EDTA	0.60	Electrophoresis	[195]
In-111-DTPA	2.4	Electrophoresis	[195]
In-111-DTPA	1	HPLC	[147]
In-111-BEDTA*	1	HPLC	[147]
Cu-67-DTPA	77	HPLC	[147]
Cu-67-BEDTA*	85	HPLC	[147]
Cu-67-TETA	1	HPLC	[147]
Y-90-DTPA	1.8	Sephadex G25 Chromatography	[197]

* BEDTA: p-nitrobenzyl-EDTA.

Table 6. Dissociation rate of metallic radionuclide labeled chelator-protein conjugates in 37°C serum

Chelate	Bifunctional chelating agent	Dis.Rate %/day	Detn. Meth.*	Ref.
In-111-DTPA-HSA	DTPA mixed anhydride	9	1	[139]
In-111-DTPA-HSA	DTPA mixed anhydride	1.6	1	[195]
In-111-EDTA-HSA	1-(p-bromoacetamidephenyl)-EDTA	0.060	1	[195]
In-111-EDTA-HSA	1-(p-carboxymethoxybenzyl)-EDTA	0.11	1	[195]
In-111-DTPA-HSA	DTPA azo imidate	12	1	[104]
In-111-DTPA-Ab	DTPA bicyclic anhydride	10.0	2	[115]
In-111-DTPA-Ab	Succinimide pentaester of DTPA	26.6	2	[115]
In-111-DTPA-Ab	Succinimide monoester of DTPA	11	2	[118]
Ga-68-DTPA-HSA	DTPA mixed anhydride	9	1	[139]
Y-90-DTPA-Ab	DTPA bicyclic anhydride	8-9	2	[75]
Cu-67-DTPA-Ab	DTPA bicyclic anhydride	96	HPLC	[147]
Cu-67-EDTA-Ab	1-(p-isothiocyanatobenzyl)-EDTA	95	HPLC	[147]
Cu-67-TETA-Ab	6-(p-bromoacetamidobenzyl)-TETA	1	HPLC	[147]

* 1. Electrophoresis
2. Affinity chromatography

radiation. Blood flow in this situation is very low making delivery of drugs or radiopharmaceuticals through the circulation very difficult. In contrast, in animal models such as the nude mouse, the tumor often has a good blood supply and is relatively huge. On a weight basis, such animal tumors are equivalent to 1 kg mass in a human body. This partly explains why tumors uptake 20-30% of the injected dose per gram in the mouse, compared to human studies where 1.0%/g is exceptionally high, 0.1-0.001%/g being more usual [199].

(2) Tumor size. The uptake of radiolabeled antibodies into tumor is markedly affected by tumor size. The per gram uptake of In-111-MoAb decreases as the tumor enlarges. Although the absolute uptake of the antibody in a large tumor is generally greater than in a small one [200-202]. This is consistent with the decrease in blood flow that occurs with tumor enlargement [203].

(3) Permeability. Radiopharmaceuticals must reach the tumor through the circulation, crossing the capillary wall and diffusing throughout the interstitial fluid to reach the tumor cells. The large size of the antibody molecule slows down the diffusion rate across these barriers and becomes an important factor to the uptake rate. This may be the principal reason that it takes several days to attain a significant concentration of radiolabeled antibody in human scintigraphic studies. Antibody fragments such as Fab and $F(ab')_2$ have the ability to diffuse more rapidly into the tumor.

(4) Concentration gradient. The driving force causing diffusion of labeled antibodies from the vascular compartment into the tumor is the

concentration gradient between the blood and the tumor. The higher the integral blood concentration with time, the higher the tumor concentration will be. Although the antibody fragments have a more rapid diffusion, they also have a more rapid disappearance from blood, and a lower integral blood concentration with time. Reports comparing whole antibodies with its fragments showed integral blood concentration to be more important than molecular weight since tumor concentration was highest with whole antibodies, and lowest with Fab, with $F(ab')_2$ intermediate [204,205].

(5) Antibody affinity and antigen concentration. Binding of antibodies to the tumor antigen is noncovalent. It resembles the binding of certain drugs and small molecular weight radiopharmaceuticals to receptors on the cell membrane, and the same affinity rate constants apply. Thus, the magnitude of binding of antibodies to tumor target antigen will depend on the affinity for the antigen, and on antigen amounts on tumor cells. Although in-vivo tumor accumulation of radiolabeled antibodies is influenced by many more complicated factors than simply the in-vitro binding activity (affinity), the utilization of antibodies with higher affinity constants will usually provide higher T/N ratios and better images [206,207].

3. Factors affecting radiolabeled antibody background [198]

Quantitatively over 90% of injected radiolabeled antibodies remain in the background pool and only 1% or less is fixed in the target tumor. Assuming that purity, radiolabeling and specificity of MoAbs achieve the optimum, and other factors affecting tumor uptake such as blood flow and antibody affinity are fixed, the following facts affecting the background should be taken into account.

(1) Rate of excretion. Whole antibody is only very slowly excreted and blood levels stay high even for many days. For chelate labeled antibodies the rate of elimination is even slower, creating greater background. The accumulation of In-111 in the liver may be due to the metabolic trapping or possible loss of In-111 from the chelate and the sequestration of In-111 in liver ferritin.

(2) Metabolism. Practically every preparation of radiolabeled antibodies is unique. Variables include specific activity (number of radiolabels per molecule of antibody), site of radiolabel on the antibody (hopefully not in the antigen binding site), antibody class (IgG, IgM etc.) and antibody isotype (IgG1, IgG2a etc.). Each one of these characteristics can affect the biodistribution and metabolism. For example, a heavily labeled antibody (many labels per molecule) causes rapid removal from the blood by the liver, and a completely different biodistribution. The introduction of an enzymatically cleavable linker between the chelate and the antibody results in more rapid metabolism and excretion of the chelate in the urine [192].

(3) Circulating antigen. The presence of antigen shed from tumor into serum and subsequent immune complex formation will drastically alter the pharmacokinetics of radiolabeled antibodies. This antigen-antibody complex is then removed by the liver and spleen. This results in decreased tumor uptake of the labeled MoAb. The immune complex of iodinated MoAb deiodinates at a greater rate than uncomplexed iodinated MoAb does, while In-111 is retained in the liver. Consequently, the liver concentration of radioactivity increases while using In-111-DTPA-MoAb and decreases with I-131-MoAb [208,209].

(4) Nonspecific binding. The binding of antibody to Fc receptors located on cell membranes in the liver, blood and bone marrow in most experimental mouse tumor models usually accounts for a larger total uptake than the tumor [23,210].

(5) Antibody specificity. Proper screening of monoclonals and selection of antigen will virtually eliminate cross reactions. The use of MoAbs theoretically reduces nonreacting or poorly reacting molecules to a minimum.

(6) Antibody dose. At low doses of In-111-labeled MoAb,there is a rapid removal of a large proportion of MoAb by liver, spleen and bone marrow that renders it unavailable for tumor localization [211]. The administration of higher doses of unlabeled MoAb (cold antibody) causes a prolonged circulation of the radiolabel in the plasma with less spleen and bone marrow uptake and elevated plasma levels of MoAb. Furthermore, increasing cold antibody dose will saturate nonspecific binding sites associated with nontumor tissue

[212-215]. Saturation of specific binding sites as a result of low but significant expression of tumor associated antigens on normal tissues may also occur. Many investigators have shown that administration of cold antibodies in an appropriate dose increased clinical detection rates of tumors [22,126,211,216]. However, it should be kept in mind that the possibility of saturation of nonspecific or low level specific binding sites by cold antibodies occurs at the cost of diminished tumor uptake [126].

4. Comparison of biodistribution of In-111 and radioiodine labeled MoAbs

I-131 and In-111 are the most popular nuclides in antibody radiolabeling presently, much attention is focused on the comparison studies of biodistribution of radioiodine and In-111 labeled MoAbs [42,199,217-219]. In addition to the different physical characteristics, radioiodine and In-111 have shown different biodistributions following the catabolism of the labeled MoAbs.

When expressed in fraction of the injected dose (% I.D./G), levels of In-111 accumulating in tumors are considerably higher than those of iodines There are two possible explanations for the higher tumor levels of In-111. The first possibility is that iodine is released from antibodies in tumor by a process of deiodination without degradation of antibody, whereas In-111 remains associated with the antibody in the tumor. There is, however, little formal evidence of selective removal of iodine but not indium from antibodies within tumor. The relatively rapid excretion of iodine from labeled antibodies has been cited as evidence for systemic deiodination of antibodies [199,220]. A second and more likely explanation is that both labels catabolized equally rapidly at the tumor site [221,222], but while liberated radioiodine is removed and excreted, In-111 is retained in the tumor [39,199,223]. Khaw et al. [224] reported that In-111 to I-131 ratio of an In-111 and I-131 dual-labeled MoAb in blood kept at unity, but increased gradually in the tumor. This might be ascribed to the different catabolism of free In-111 and I-131. In other words, the higher In-111 accumulation in the tumor may result from the relatively rapid decrease of I-131 in the tumor, rather than from an increase of In-111 label. The nature of tumor retention of In-111 from labeled antibodies is not understood. It is known that indium, administered simply as indium chloride, binds to serum proteins and subsequently shows some localization in tumors [225]. Whatever the explanations for levels of In-111 higher than radioiodines in tumors are, the use of In-111 results in high tumor-to-blood ratios and clearer imagings because In-111 shows a little more rapid blood clearance than that of radioiodines in 1-3 days postinjection.

The other and more important difference in biodistribution between In-111 and radioiodine labeled MoAbs is the much higher nonspecific uptake of In-111 in liver, kidneys and spleen. The high uptake of In-111 labeled MoAb in these normal organs significantly limits the utilization of this radionuclide for tumor localization. Accumulation mechanism of In-111 by liver remains poorly understood. The speculated reasons for the observed high hepatic uptake of In-111 labeled antibody conjugates are numerous and can be divided into two categories:tumor associated and nontumor associated localization. Tumor associated hepatic localization would be caused by specific antigen-antibody interactions and would be due to any or all of the following: the reticuloendothelial system (RES) uptake of immune complexes; the binding of antibody to antigen which, after shedding, was transiently expressed on the surface of cells of RES; or the presence of foci of metastatic tumor growth in the liver. Nontumor associated hepatic localization could be caused by antibody-Fc receptor interaction; the accumulation of denatured or aggregated antibody, or instability of In-111 chelate. Localization into the liver or other tissues could also be the result of expression of the antigen by normal cells [92].

The different destiny of free In-111 and radioiodine liberated from liver after the metabolism of antibodies may be mainly responsible for the difference of accumulation of them. Both of the labels dissociate in the liver, but only iodine is washed out after metabolism and/or deiodination . Indium can be substituted for iron in many iron-binding proteins. Iron is transported in the blood by means of transferrin and deposited in the liver where it is found to be ferritin [226]. Liver has a large iron binding capacity [227], and therefore, the released In-111 could be sequestrated and trapped by ferritin and other iron-binding proteins.

It is uncertain whether the labeling process itself or sequestering of

indium by the liver accounts for the high liver uptake. When the pharmacokinetics of indium labeled antibodies was compared with that of internally labeled Se-75-antibody, identical pharmacokinetics was observed suggesting that the liver uptake was not due to the labeling process but rather to the manner by which the liver handles the antibody. Slightly higher accumulation of In-111 occurred in the liver which was subsequently shown to be due to an artifact of labeling [199].

The principal mode of label instability of radioiodinated proteins is probably deiodination in tissues with the release of free iodine, whereas for In-111 labeled proteins it is likely to be exchange of the label with transferrin [24].

V. STRATEGIES TO LOWER THE BACKGROUND

Presently, tumor imaging with radiolabeled monoclonal antibodies produces a very high non-target background activity in the blood pool, liver and spleen. Since the target specificity and absolute concentration are something less than adequate, reducing the background should improve the image. Several approaches are currently undergoing investigations:

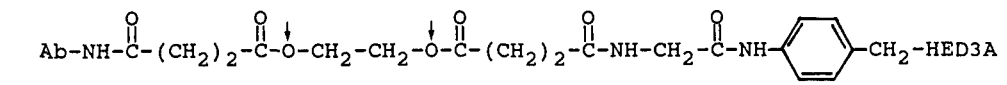
(1) Antibody fragments. Since Fab and $F(ab')_2$ fragments disappear much more rapidly from the plasma than whole antibody, being excreted more rapidly by the kidneys, while retaining at the specific binding site, they produce much higher T/N ratios. However, their use is inevitably associated with a lower target uptake, apparently due to the shorter residence time in the circulation [228]. In some studies the target uptake with whole antibody was from 3 to 24 times higher than with Fab [204,205].

(2) Second antibody. The use of a second antibody is effective in lowering the blood background [229,230]. However, this approach produced a high liver background, resulting in decreased contrast between tumor and normal liver tissue. The utilization of a second antibody may improve the images of radioiodinated antibodies since radioiodine from deiodination in the liver could be excreted effectively. However, it seems unsuitable to administer a second antibody in imagings with In-111 labeled MoAbs because radio-indium mainly deposits in the liver. It is interesting that polyclonal antibodies work better than MoAbs in transporting circulating antibodies to the liver, possibly because it is more efficient in crosslinking and lattice formation [231].

(3) Metabolizable chelate linkers [192-194,232,233]. In theory, the T/N ratio can be amplified if the labeled antibodies in normal organ and circulating blood metabolize quickly and the metabolites containing the activity excrete rapidly through the kidneys. An interposition of readily metabolizable chemical linkages between antibody and chelate is a new approach to enhance the clearance of In-111 labeled antibody in normal organs, thereby reducing the background.

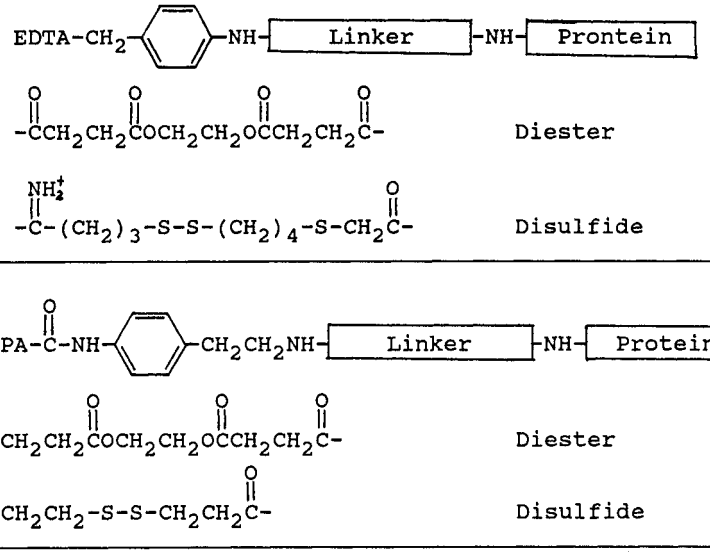
Haseman et al. [192] investigated the use of metabolizable chelates (Fig.7) for labeling MoAb. The chelator HED3A associates a linker with two enzymatically metabolizable ester bounds (arrows). Their initial results showed that the introduction of ester linkers resulted in a substantial decrease in blood background activity, a shorter biological half-life and an increase in tumor to blood ratio at the expense of a moderate decrease in absolute tumor uptake.

Fig.7 The chemical structure of antibody conjugated HED3A through *enzymatically metabolizable ester linker* HED3A: Hydroxyethyl ethylenediaminetriacetic acid



Meares et al. [193,232] and Paik et al. [194,233] compared the in-vivo behavior of In-111 labeled antibodies conjugated with EDTA or DTPA through a series of linkers (examples are shown in Fig.8). Paik reported that the disulfide and diester linkers produced significant enhancement of target-to-blood ratio. Meares found that only disulfide linker had a drastic effect on the whole-body clearance of In-111.

Fig.8 Structures of the linkers between protein and EDTA or DTPA



(4) New bifunctional chelating agents [102,120,121]. In efforts at reducing high hepatic uptake of In-111 labeled MoAbs by using new bifunctional chelating agents, the most successful instance was the introduction of 1-(p-isothiocyanatobenzyl) DTPA (SCN-Bz-DTPA, Fig.2 B6) by Esteban et al. [120]. In explaining this observation, the authors assumed that conjugation with this ligand resulted in a more stable In-111 protein label. An alternative explanation made by Hnatowich [234] suggested that in-vivo instability of the thiourea bond of the SCN-Bz-DTPA conjugate facilitates the release of the chelated In-111 to urine.

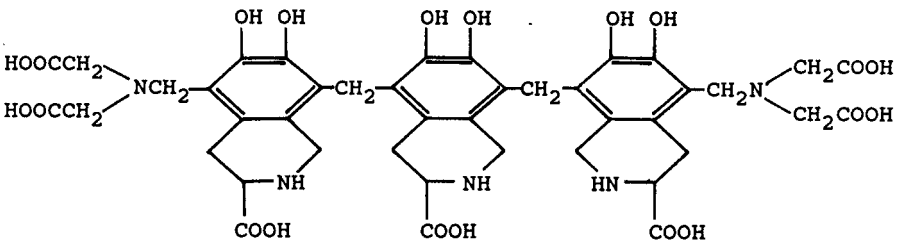
(5) Site specific antibody modification [92,189,235]. Brown et al. [235] have recently developed an alternative technique for the attachment of DTPA to the carbohydrate moieties of antibody molecules. This technique is exclusively restricted to the carbohydrate chain, unlike the somewhat random coupling in the conventional methods which involves aminoacid side chains of the antibody. Since the carbohydrate is generally present in the Fc region [236], the antigen-binding site in the Fab region may undergo minimal structural disturbance during the coupling. As a result of this derivatization procedure, the antigen-binding specificity is expected to be much improved. Rodwell et al. [92] labeled mouse monoclonal antiphosphocholine IgM with In-111 by this technique and studied the MoAb biodistribution in mice. They found significantly reduced retention of In-111 in the liver. However, Saccavini et al. [32] were not successful in using this technique to lower the liver uptake of In-111.

(6) Chelator administration. This method is based on and started from the fact that In-111 labeled free EDTA is excreted readily by kidneys. Lockshin et al. [237] and Goodwin et al. [238] observed that administration of EDTA accelerated the excretion of In-111, and the ratio of tumor to muscle increased 2-7 fold. Han et al. [239] studied the elimination of liver uptake of In-111 from nude mice bearing human colon carcinoma using a chelator "quinamic acid" (Fig.9). It is a ferric ion sequestering agent initially synthesized and clinically employed in China. Quinamic acid can be administered in relatively large dose with very low toxicity. The coordination ability of "quinamic acid" for transition metal ions lies between EDTA and DTPA. The administration of "quinamic acid" increased the T/N ratios, including the important tumor-to-liver ratio,with a cost of slight decrease of tumor uptake.

(7) Pre-targeted radioimmunoscintigraphy (see Section III.1) The pre-targeted radioimmunoscintigraphy developed recently by Goodwin and Meares et al. [181-183] was carried out in three steps (Fig.10):

The first step was i.v. injection of antibodies with slow diffusion from blood through the extracellular fluid to the tumor target, as depicted in the top of Fig.10.It may take several days to reach maximum target concentrations

Fig.9 The chemical structure of "quinamic acid" (5,8-bis-[3-carboxy-5-bis (carboxymethyl)aminomethyl-6,7-dihydroxy-1,2,3,4-tetrahydro isoquinolin-8-yl]-5,8-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid)



due to the slow pharmacokinetics of antibodies. The antibodies employed here were bifunctional antibodies, either hybrids or chimeric molecules, that could bind both a chelate and a tumor antigen.

The second step was the clearance of excess circulating antibodies (about 15-25% injected dose) by an i.v, injected "chase" hapten after maximum localization of the antibody in the target occurred. This was accomplished by using a hapten covalently bound to a slowly diffusible serum protein (human transferrin) as the "chase". The aggregates thus formed were rapidly removed by the RE cells of the liver. This process is shown in the middle of Fig.10.

In the final step, the desired radiolabel was given i.v. in the form of an epitopically derivatized bifunctional chelate which was small, rapidly diffusible and quickly cleaned, predominantly by the kidneys. This is diagramed in the bottom of Fig.10.

The pre-targeted method may achieve maximum tumor uptake of radiolabel in a rather short time, thus avoiding the need for long-lived nuclides (I-131 and In-111 etc.) and decreasing the radiation dose. Moreover, this method may reduce the high blood and liver background.

Incidentally, we should mention another novel radioimmunoscintigraphy which resembles the pre-targeted technique described above. This method is based on the reversibility of the reaction between antibody and hapten [184]. The antibody used here serves as the reversible equilibrium carrier of radiopharmaceutical. As shown in Fig.11, it involves two steps: (1) injection of the immuno-complexes of radiolabeled-chelate-based pharmaceutical, such as bleomycin conjugated radio-EDTA (BLEDTA), and anti-chelate antibody. After injection, the macromolecular complex remains in circulation for an extended period of time, during which localization of the radiopharmaceutical in its target may occur;(2) subsequent injection of an excess of competing nonradioactive chelate as a "chase" prior to imaging. The nonradioactive chelate displaces the radio-chelate-pharmaceutical attached on the antibody, leading to a rapid clearance of the non-targeted radiolabel from the circulation. This markedly improves the tumor images and reduces the radiation dose. The preliminary results seem to have potential usefulness for humans.

CONCLUSION

1. Prospects of RII and RIT

Through the great collaborative endeavours of immunologists, nuclear physician as well as radiochemists in recent years, the applications of RII for diagnosis of many common human cancers have been quite successful and will grow more satisfactory in the future. Nevertheless, the progress of RIT applications lags much behind what RII has achieved.

The realization of RIT basically lies with the actual attainments of the immunoreactivity and specificity of the radiolabeled MoAbs. RIT requires high absolute concentrations of radioactivity in the tumor and a very clean background both of which are much more critical than in RII.

However, the current development of RIT is far from being satisfactory. By our conservative prediction, the near future for the RIT is not optimistic unless some revolutionary progress emerges. Seemingly, there is still a long journey to travel before reaching the ideal destination.

Fig.10
Diagrammatic representation of three-step pre-targeted radioimmunoscintigraphy.The dotted lines represent the capillary walls with the major organs involved (liver and kidneys) on the left, and the extra cellular fluid space with target tumor cells on the right [183].

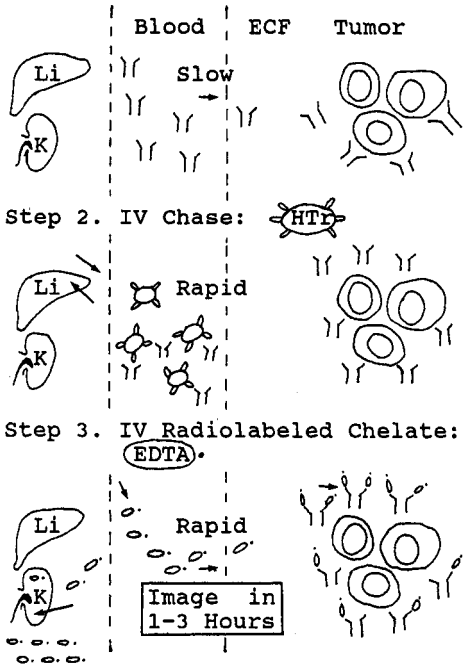
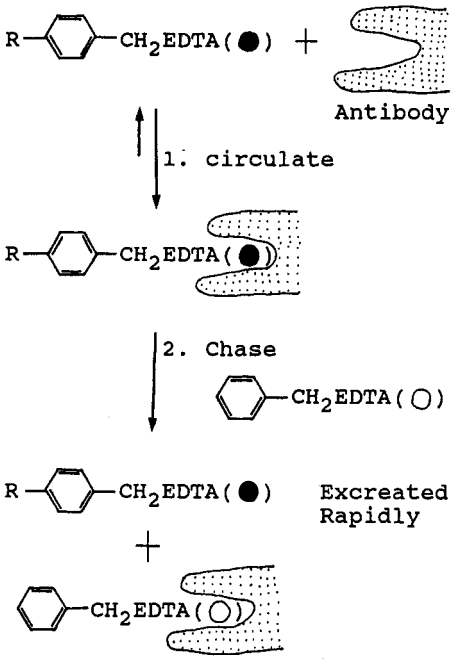


Fig.11
Diagrammatic representation of two-step radioimmunoscintigraphy with a reversible label. The radioactive metal is indicated by a solid circle; the nonradioactive isotope by an open circle. R=Bleomycin [148].



2. Employment of In-111, I-131 and other radionuclides

In this review article, we focus on mainly two radionuclides, I-131 and particularly In-111. Up to now, nuclides other than these two are very rarely dealt with in this field.

Many authors have written in regard to In-111 as a radiolabel has more favorable properties for RII than radioiodine. It is doubtless that the nuclear properties of In-111 make it prominently superior to I-131. The easy in-vivo deiodination and the low tumor-to-blood ratio are the evident disadvantages of I-131 label. On the other hand, In-111 labels always produce high retention in the liver that seriously affects the quality of imaging. Further, in view of the practical availability, particularly in the developing countries, reactor produced I-131 is certainly easier to obtain than cyclotron produced In-111.

Therefore, we would conclude that both I-131 or In-111 have their own characteristics, respective merits and shortages. In-111 is plausible to be better than I-131, but can not unconditionally replace I-131, at least not at present.

Besides, we anticipate more vigorous explorations of other potential radionuclides. For instance, the application of Tc-99m in RII is promising, supposing the difficulties with its short half-life could be overcome. The preliminary studies on the applications of Y-90, Re-186 and Cu-67 for therapeutic purpose are also very meaningful.

3. Potential chemical modifications of MoAbs

(1) Pre-targeted radioimmunoimaging (see section III). The greatest success of this method is that the time needed for the radiolabel to reach

maximum tumor concentration has been reduced from several days to few hours. Thus, it allows the utilization of short-lived radionuclides, e.g., excellent radionuclide Tc-99m. The employment of the avidin-biotin couple has shown the very promising achievement indicating a significant enhancement of T/N ratio within a short time period.

(2) Metabolizable chelates (see. Section V). These chelates contain an enzymatically cleavable linking group between protein and chelate. They provide a more rapidly excreted label for MoAb, that lowers blood and liver background. The concept of the metabolically cleavable linker should altract more attention in the future.

(3) New bifunctional agents. A promising approach to protect the label from transcomplexation and increase the T/N ratio is to synthesize and use a new variety of bifunctional chelating agents which may chelate a metallic nuclide with very high thermodynamic stability constant (logK>40) and high kinetic inertness.

(4) Site-specific antibody modification (see Section V). By a special modification towards antibody, the attachment of DTPA to the antibody molecules takes place only at the carbohydrate moieties in the Fc region. Therefore, during the coupling reaction, the antigen-binding site in the Fab region can be kept almost intact, and the immunoreactivity and T/N ratio are enhanced.

All the chemical modification methods mentioned above have enlightened people to the realization that there is potential for development of the MoAb technique. However, many challenging tasks for chemists remain before complete realization of the MoAb technique can be attained in the future.

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