Preparation, Characterization And Biological Evaluation Of Cellobiose And Dextran Carriers Of Prosthetically Labeled Iodine-125 For Use In Radioimmunotherapy Of Tumors Of Glial Origin.

Preparation, Characterization and Biological Evaluation of Cellobiose and Dextran Carriers of Prosthetically Labeled Iodine-125 for Use in Radioimmunotherapy of Tumors of Glial Origin

by

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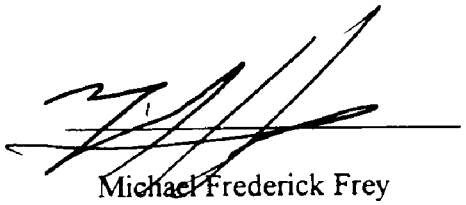
This dissertation is respectfully submitted to the Graduate Faculty of Lehigh University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.



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Dedication

This work is dedicated in Loving Memory to Frederick Phillip Frey Jr.

Acknowledgements

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ABSTRACT

Prosthetic carriers of radioiodine were prepared and conjugated to the anti-epidermal growth factor receptor (anti-EGFr) monoclonal antibody (MAb) 425. Three classes of prosthetic carrier were produced: tyraminyl-cellobiose originally developed by Pittman, tyramine conjugates, and small molecule labels including N-succinimidyl p-iodobenzoate.

Tyraminyl-cellobiose was prepared via two methods. Direct conjugation in phosphate buffer, pH = 7.4, in the presence of $NaCNBH_3$ yielded a product in 3-6 weeks. Conjugation in DMSO yielded a product in 48 hours which could not be readily purified. ^{125}I-tyraminyl-cellobiose was attached to anti-EGFr MAb 425 via either cyanuric chloride ($C_3H_3N_3Cl_3$) or via carbonyl diimidazole (CDI). The cyanuric chloride method of radioiodination lead to high specific activity protein as was demonstrated by elution of the protein construct through Sephadex G-25 gel exclusion columns. All cyanuric chloride constructs eluted with a single high activity peak at the void volume (MW >> 25 kDa). Conjugates having high specific activity were tested for their ability to bind to A1207 (EGFr +) glioma cell and SW707 (EGFr-) colorectal carcinoma cells as compared to ^{125}I-MAb 425. Binding to the A1207 positive controls varied between 1 and 3% of the total binding of directly iodinated MAb 425. Neither the direct labeled or ^{125}I-tyraminyl-cellobiose labeled 425 showed any significant binding to the SW707 EGFr negative controls. Uptake of a ^{125}I-tyraminyl-cellobiose-MAb 425 construct having a 2% binding into A1207 was studied. Approximately 50% of all bound radioiodine was taken up into the glioma cells. The constructs were not capable of delivering a cytoplasmic level of ^{125}I higher than that of direct

labeled antibody.

Derivatives of dextran, polyaldehyde dextran (PAD) and carboxymethyl dextran lactone (CMDL) were loaded with tyramine and iodinated to high specific activity. The ^{125}I-tyramine-PAD conjugates were not sufficiently soluble to produce IgG constructs. The ^{125}I-tyramine-CMDL conjugates were attached in the presence of CDI to antibody. These conjugates bound with reduced avidity (<2% of ^{125}I-MAb 425) not unlike the tyraminyl-cellobiose labeled antibody.

Labeling of MAb 425 with *p*-^{125}I-iodobenzoate demonstrated a loss of approximately 80 % of total binding to A1207 cells compared to ^{125}I-MAb 425 alone. This has been attributed to covalent attachment to a necessary lysine ϵ amino group.

Specific Aims

The specific objectives of this research were as follows.

1. **To** prepare tyraminyl-cellobiose via the method of Pittman and via alternate routes.
2. **To** radioiodinate the tyraminyl-cellobiose produced and then conjugate the "hot" construct to anti-EGFr monoclonal antibody 425.
3. **To** examine the ^{125}I-tyraminyl-cellobiose-MAb 425 construct as an agent for radioimmunotherapy by studying its uptake and retention into tumor cells of glial origin, specifically U87 and A1207 cells.
4. **To** produce derivatives of dextran which are capable of being radioiodinated through the incorporation of suitable aryl functionality.
5. **To** examine the direct uptake of those dextran derivatives into tumor cells of glial origin.
6. **To** conjugate the "hot" iodinated dextran derivatives of dextran onto MAb 425 and examine uptake of these immunoconjugates into A1207 cells.
7. **To** examine the use of radioiodinated uridine as a potential nuclear targeting agent in A1207 cells.

Objectives

The overall objective of this project was to examine methods for increasing the cytotoxicity of radioiodinated derivatives of monoclonal antibody 425. Direct labeled antibody had been shown to be useful in *in vivo* radioimmunotherapy of human glial tumors when it was combined with surgery and external beam irradiation.[1,2]

Initial *in vitro* experiments with ^{125}I-MAb 425 demonstrated that the direct labeled antibody was not capable of accumulating within the cytoplasm to a level high enough to effect cell kill.[3] These experiments did demonstrate, however, that the antibody was internalized very rapidly and that some processing of the antibody was occurring resulting in the loss of radioactivity in a form other than whole intact IgG. We therefore proposed to examine a known labeling method shown to increase cellular retention in fibroblast cells using low density lipoprotein as a carrier. The label (^{125}I-tyraminyl-cellobiose) was developed by Pittman in 1983 for use in increasing residence time of radioiodine and was used with relative success.[4] Our initial objective was to produce the label and conjugate that label to MAb 425. The conjugate would then be evaluated for its ability to both bind and internalize into cells of glial origin. The hope was that the label would demonstrate superior retention within the cells with minimum loss of the antibody's ability to bind glial cells.

The premise behind the tyraminyl-cellobiose trap was that the pendant sugar moieties were becoming involved in glycolysis and being trapped as phosphorylated

intermediates. Other carbohydrates could potentially exhibit similar behavior *in vivo* and our second objective was to examine the use of polymeric carbohydrates as backbone carriers of prosthetic labels. While not possessing free C_6 hydroxyls, dextran (α 1,6 polyglucose) does possess several advantages as a backbone carrier. First and foremost unlike most other polymeric carbohydrates, dextran is relatively non-immunogenic.[5] Since any immunotherapeutic system will require multiple injections active agent avoiding an immune response is critical. Secondly, dextran is readily modified to include both nucleophilic and electrophilic molecules. This would allow for the incorporation of a variety of small molecules onto the dextran which would themselves be carrying ioiodine-125. Third and finally, the dextran would provide a means of incorporating more than one radioiodine per molecule of IgG. Increasing the amount of radiolabel delivered per internalized antibody would increase the cellular concentration assuming the mechanism of iodine release was dependent on the degradation of the antibody.

An additional objective of this project was to examine the antibody itself in ways other than the direct study of its conjugates. To this end the effect of labeled MAb 425 on irradiated cells would be examined. In addition the ability of A1207 cells to internalize ^{125}I-deoxyuridine and the uptake of the DNA precusor by the nuclear components of the cell would also be studied.

PART I

INTRODUCTION
and
HISTORICAL BACKGROUND

I. INTRODUCTION and HISTORICAL BACKGROUND

I.A. General Introduction

This year there will be an estimated 17,500 new cases of cancer of the CNS which will result in approximately 12,500 deaths. Of these deaths roughly 80% will have been caused by gliomas, tumors which arose from glial cells within the brain. These diseases represent the fourth leading cause of cancer death among middle age males and are the 2nd leading cause of cancer death in young children.[6]

The standard treatment for such disease is surgical extirpation followed by a dual course of external beam irradiation of the tumor site and chemotherapy. The patient is often subjected to up to two or three treatments of external beam irradiation with doses in the range of 55-70 Gy.[7] Even with this rigorous treatment the disease is almost always lethal with a 50% survival rate at six months and a maximum survival time of around two years. Chemotherapy has been shown to do little for enhancing the survival time of these patients.[8]

The problem of treating these tumors is quite different from those of liver, lung, colon, or other organs. In general, brain tumors , including gliomas, do not metastasize to other tissue. The malignancy tends to remain localized in the brain. Surgical removal is almost always incomplete and herein lies the problem. It is not a new tumor or tumor site which results in patient death but the regrowth of the original tumor which is ultimately

fatal. In theory, a therapeutic system which could clean-up the residual tumor left after surgery and radiation therapy would leave the patient disease free and allow that patient to return to a normal life.

Thus the difficulty in treating glial tumors ,while substantial, is limited to a need to obliterate malignant tissue missed by current therapeutic strategies. Radioimmunotherapy (RAIT) is ideally suited to treat these tumor remnants. Combining the cytotoxic properties of a radionuclide with the cellular targeting ability of a monoclonal antibody, such systems are theoretically capable of selectively destroying diseased tissue over that which is healthy. Since tumors of glial origin do not generally metastasize it is possible to use such an agent in a variety of ways; systematically through *intravenous* injection, via multiple direct injections to the site of tumor removal, or through irrigation of the tumor site following surgery.

The challenge of RAIT is multifaceted. Initially a tumor marker must be identified on the surface of the desired target. This marker must either be unique to the malignant tissue or significantly overexpressed in tumor cells as compared to healthy tissues. The marker must then be used to raise a monoclonal antibody which both recognizes this marker and has suitable biological characteristics. A radionuclide must then be selected for use with the antibody and finally, nuclide-antibody conjugates must be developed, tested and optimized. The research presented herein deals with a RAIT system for which a suitable antigen/antibody combination had already been produced and thus examines the development of radioimmunoconjugates for use in this system.

The type of conjugates which were examined are essentially of two types one

based on a disaccharide, cellobiose, and others based on a glucose polymer, dextran. The production of these "carrier" molecules is discussed in some detail as well as the methods employed to attach these carriers to the antibody , anti-epidermal growth factor receptor monoclonal antibody 425 (anti-EGFr MAb 425). Several of these radioimmunoconjugates were tested using *in vitro* cell based assays for their ability both to bind and internalize into tumor cells of glial origin. In addition to the examination of these conjugates other studies involving anti-EGFr MAb 425 were undertaken. These include uptake of radioiodinated DNA precursors, the effect irradiating tumor cells on the uptake of electrophilically labeled antibody, and the effect of covalent modifications of MAb 425 on binding avidity.

I.B. Historical (General)

I.B.1. Radiotherapy

In the years which followed the Manhattan project the effect of ionizing radiation on biological matter was studied with great interest. One of the most important findings of these early studies was that rapidly dividing tissues are far more sensitive to exposure to x- and gamma irradiation then are tissues which divide more slowly.[9] The meaning of this information was realized almost immediately, neoplastic disease can be treated by selectively exposing the malignant tissue to a relatively large dose of x-irradiation. This

discovery lead to the development of a new field of study, radiotherapy. Since the 1950's exposing tumor masses to external beam irradiation has been an accepted form of therapy especially for post-surgical eradication of malignant remnants.[10]

The sensitivity of rapidly dividing tissue to radiation is postulated to be due to chromosomal damage induced by one of two mechanisms. The first mechanism involves direct cleavage of the chromosomal DNA through interaction with particulate radiation. In this case a high energy particle such as an alpha (α)or beta (β) particle collides with the DNA causing strand cleavage.[11,12] The other mechanism involves the formation of reactive chemical intermediates such as hydroxide radicals, super oxide anions and others (see figure I.1).[13] In this case, the water or oxygen found within the cell is transformed into a destructive species which then interacts with the DNA causing chemical cleavage of the DNA through a host of processes. These chemical species do not have long half-lives in water and must be produced in regions proximate to the nuclear DNA. In order to effect cell kill upwards of 50 strand cleavages are required.[14] Normally dividing tissue is equally subject to the damaging effects of the irradiation. However, the slower dividing tissues have time to repair damage to DNA before entering the mitotic cycle. It is at the point of cellular division that excessively damaged DNA will result in cell death.[15]

The problem inherent to the use of external beam irradiation is that the beam must pass through healthy tissue in order to reach the target tissue. This leads to collateral damage which often results in unwanted side effects. Even with computer guided tomography to accurately target tumor sites, this problem remains serious.

One solution to this problem is to localize the source of ionizing radiation at or in

the tumor site. Sources for such radiation were another of the rewards of early experiments into nuclear physics. The ability to generate usable quantities of radioactive nuclides through the use of particle accelerators produced a wide variety of radioactive elements with varying decay and emission properties. Some of these emit only low energy γ particles while other release extremely high energy β and α particles. The higher energy particles were and are still employed for use in radiotherapy. Generally the nuclides such as ^{198}Au, ^{32}P, or ^{137}Cs are surgically implanted as radioactive "seeds" at

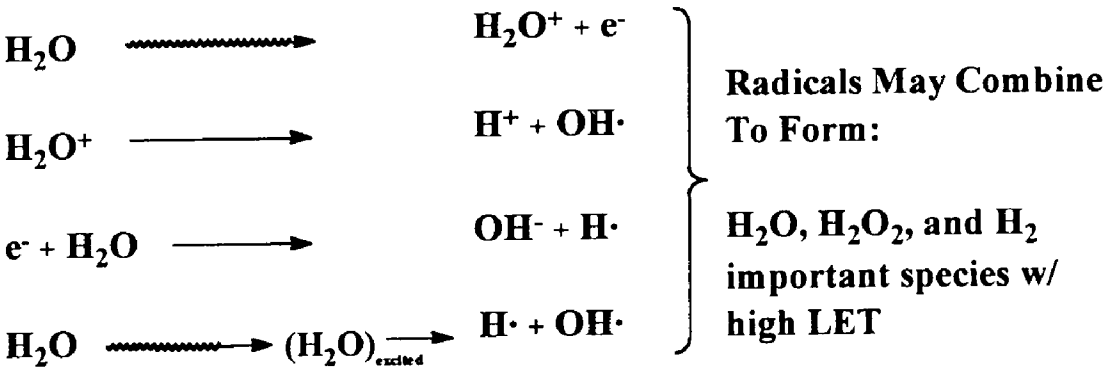


Figure I.1 Formation of reactive chemical species from the interaction of ionizing radiation and water. Squiggly arrows represent interactions with radioactive particles. High LET (Linear Energy Transfer) radiation can produce these species from water molecules which are at great distance from the actual radionuclide.

the tumor site. In doing so only tissue which is very near to the tumor site is subject to collateral damage.[16] Even so, these nuclides often are often capable of doing damage over

many cell diameters and side effects such as nausea, appetite loss, or loss of hair are often experienced by the patient. In addition the procedures are invasive and the radiation damage can impair the healing process.

One therapeutic nuclide which does not suffer from this drawback is radioiodine-131 when used to treat diseases of the thyroid.[17,18,19] The reason for this is the natural affinity of the thyroid for iodine. The nuclide is taken up rapidly and specifically into the thyroid cells and thus is localized at the target site. Mimicking this specificity of a toxic agent for a given tissue has been a long time goal of medicinal chemists and others trying to minimize the effects of such agents on normal cells. The most promising system for imparting such specificity involves the use of monoclonal antibodies and their derivatives.

I.B.2. Immunotherapy, Radioimmunodiagnostics (RID) and Radioimmunotherapy (RAIT)

Antibodies are proteins, having an approximate mass of 150 kDa which are capable of binding specifically to target antigens with a dissociation constant K_D on the order of 10^{-7}.[20] Before 1975, antibodies were raised as polyclonal sera.These antibodies all of bind the same antigen but not necessarily in the same manner or the same strength. While these polyclonal antibodies are useful in detecting antigens they are composed of a variety of proteins with varying chemical and biological characteristics and are therefore not useful for the development of therapeutic systems which need to be rigidly characterized. The development in 1975 by Kohler and Milstein of monoclonal antibodies

made such therapeutic systems possible.[21] The production of a cell line which originated with one (1) hybridoma (spleen cell + carcinoma) ancestor meant that all antibody produced from that cell line would be of identical composition and therefore have identical chemical properties as well as identical specificity and binding avidity for a target antigen.

It was originally thought that these antibodies themselves would be therapeutically useful on their own. This was not the case, however, and the research rapidly turned toward conjugation of drugs, toxins, and radionuclides. The earliest of these studies involved the attachment, through chemical means, of toxins such as diptheria toxin, ricin, and the ricin A chain to the antibody through chemical means to produce immunotoxins.[22] Other studies focused on the delivery of well known anti-tumor agents such as adriamycin, methotrexate and others generally called immunoconjugates[23,24]. These studies demonstrated some of the inherent difficulties in using an antibody as a carrier of drugs. The injection of immunotoxins into the system almost invariably lead to hepatotoxicity and severe side effects. Conjugation of drugs directly to the antibody caused a loss of binding avidity.[25] At a low load of drug (1-5 molecules of drug / antibody) binding avidity was retained, but the antibody was not capable of delivering an effective dose of the drug to its target cell. The conjugation of therapeutically useful radionuclides to antibodies was being studied concurrently with the development of immunoconjugates and immunotoxins.

Radioimmunoconjugates have been developed for two distinct purposes, detection of antigens and destruction of malignant tissue. Radioimmunoconjugates developed for

detection of an antigen fall into a class known as radioimmunodiagnostics. These conjugates involve the attachment of γ or $\beta+$ (positron) emitting particles to an antibody which recognizes a surface epitope of a particular malignancy. The conjugate is injected into the patient and after an allowed clearance time the patient is "imaged" by gamma cameras to reveal those areas where antibody has accumulated. One of the more successful radionuclides to be used for such purposes is technetium 99m.[26] This nuclide has been used successfully in conjunction with a variety of antibodies for imaging a wide range of tumors. Indeed of all classes of immunoconjugates, those used for RID can be considered the most successful. The reason for this is that the immundiagnostic need only have a minimal specificity for the tumor site. Any concentration of the radionuclide will be detected by the imaging cameras and a relatively small amount of conjugate will suffice for these purposes.[27]

The second class of radioimmunoconjugates are those which are used to treat disease and fall under the heading radioimmunotherapy (RAIT). Unlike RID, RAIT systems require that a lethal dose of radionuclide be delivered to the tumor or disease site and further accumulation in other tissues must be avoided at all cost. The requirements for RAIT are as follows: An antibody raised to a suitable antigen (one which is unique to, or overexpressed on, the target tissue) which is not shed and preferably can be internalized, a radionuclide which decays with high enough energy to effect cell kill and a suitable half-life and the means to attach the radionuclide to the antibody in high specific activity.

Several nuclides have been used in conjunction with monoclonal antibodies for

Nuclides Used In Radioimmunotherapy (RAIT)

NUCLIDE	ΔE, Type/Yield/Avg. length in H_2O	Tumor	Reference
Iodine-125	e^-/30 keV/5-10 nm	gliomas, colorectal	1,2,35
Iodine-131	β^-/606 keV/2.4 mm	colorectal	33,35
Yttrium-90	β^-/2.3 MeV/12.0 mm	colon,lymphoma	29, 30
Rhenium-186	β^-/1.1MeV/11.0 mm	pancreas	28
Astatine-211	α /5.9 MeV/65 um	lymphoma	40,42
Bismuth-212	α /6.1 MeV/70 um	lymphoma	31

Table I.1

therapeutic purposes and many have shown promise. Table **I.1** lists a few nuclides, their important decay properties as well as some tumor types in which they have been therapeutically useful. These nuclides can be separated into essentially two groups: radioactive metals which must be attached to an antibody via a chelating prosthetic group,including ^{90}Y, ^{186}Re, and ^{212}Bi, and radiohalogens,^{131}I, ^{125}I, and ^{211}At, which can be covalently attached either via direct electrophilic halogenation of aromatic groups present on the antibody or through prosthetic carriers of the radionuclide.

The most important properties of a radionuclide to be used for RAIT are the half-life ($t_{1/2}$), the energy of decay, and the range over which that energy is deposited. The half-life of a suitable nuclide should be long enough to allow that nuclide to be attached to the antibody and short enough that once it is delivered it will provided a significant

number of decays within the cell during a single cell cycle. The optimum length is about eight days or about the half-life of iodine 131. The energy of decay is also important. All the nuclides shown in Table **I.1** are of high enough energy to effect cell kill. The major difference between these nuclides is the distance over which the decay energy is deposited. Notice that the high energy β emitters deposit their energies over distances of millimeters while the α emitters only deposit their energy over a range of tens of microns. The average cell radius is only on the order of 20-100μm so that a β particle localized at the surface of a tumor cell is still capable of damaging tissue 100 cell diameters away. This can lead to collateral damage. The α particles are significantly better in this respect depositing their energy over only one or two cell diameters. Iodine-125 which is an Auger electron emitter will be discussed in some detail later in this chapter.

These nuclides have all been used with some success to treat malignancies. The two ß-emitting radiometals ^{186}Re and ^{90}Y have demonstrated success against solid tumors, colon and pancreas respectively, but again this success is due in large part to the high energy of the emitted ß particle as well as the 11-12 mm average radius over which this particulate energy is deposited.[28,29,30]

Attachment of the radiometals, ^{212}Bi included, to the antibody is not trivial from a chemical perspective. Early studies showed that classic organic ligands did not produce stable chelates when subjected to physiological conditions and these conjugates often had reduced avidity for the target cell. The problems have been minimized by the development of special chelating protheses which maximize the *in vivo* stability of radiometal-immunoconjugates and minimize the deleterious effects of metalation on the

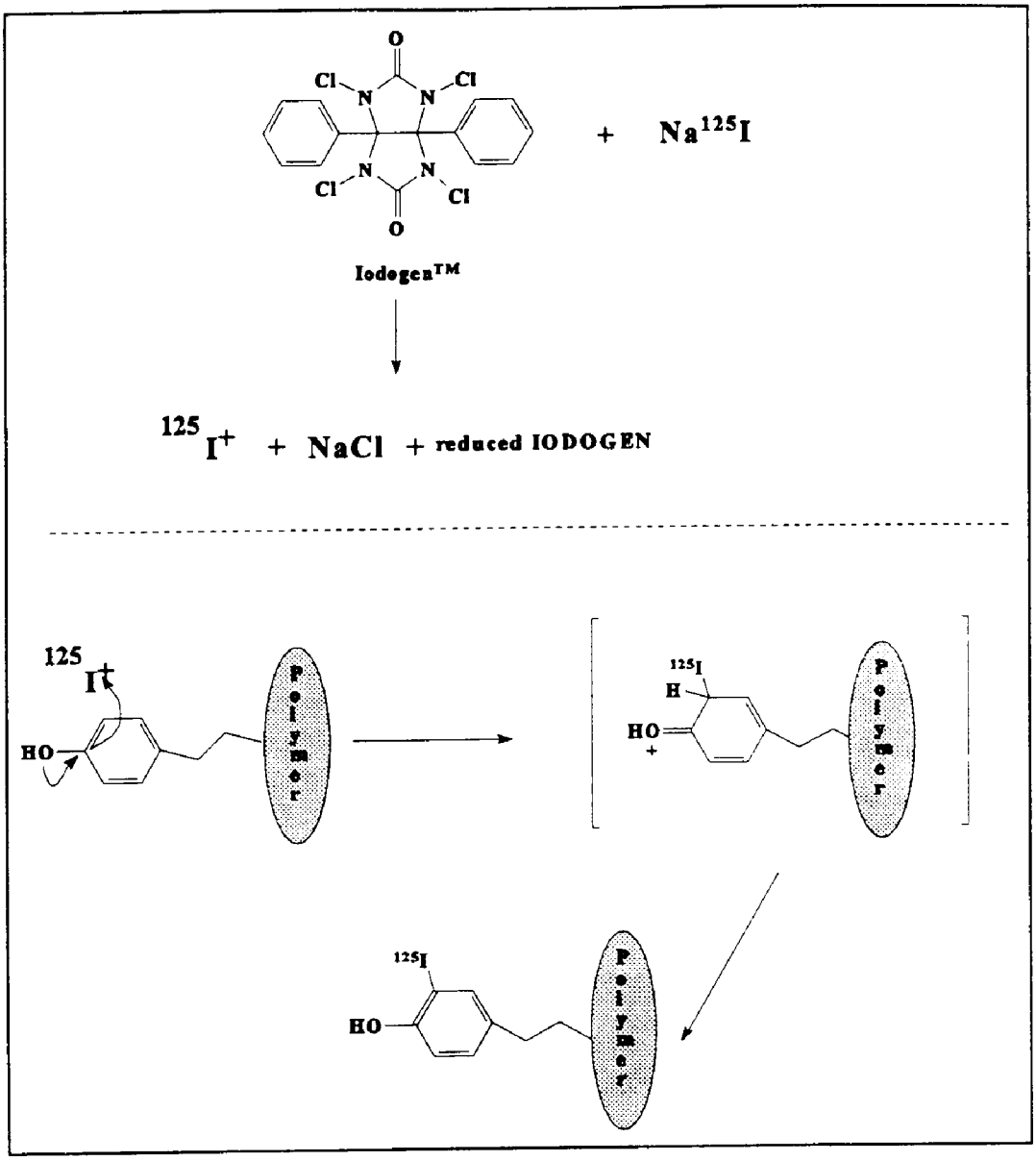


Figure I.2 The Iodogen™ method for iodinating proteins or polymers carrying iodinatable groups. Iodogen converts iodide anion to the +1 cation which can then electrophilically attack aryl functionalities. In proteins the group most often substituted is tyrosine.

antibodies binding avidity and specificity.[31,32,33] Even so, of the metals only bismuth-212 has decay properties which are acceptable in terms of limiting collateral damage, due to its

low LET.

While radiometals are being pursued vigorously as nuclides for RAIT the radiohalogens, especially the radioiodines, have not been forgotten. Iodine-131 in particular has received significant attention over the last five years .[34,35,36] This is due to its ready availability and ease of incorporation into protein molecules.

Radioiodination of proteins, ^{131}I,^{125}I and ^{124}I, for purposes of studying their biodistribution has been known for years and today kits are available to electrophilically iodinate proteins on their aromatic amino acids (most often tyrosine, see figure I.2) These direct labeled proteins are often subject to dehalogenase enzymes.[37] These scavengers remove iodide from the labeled protein for eventual transport to the thyroid. This can be overcome by using prosthetic labels designed to be poor substrates for dehalogenase enzymes.[38,39] These labels are often active-esters of para-substituted benzoic acids which are both easily iodinated and readily conjugated to a therapeutic antibody. Iodine-131 has demonstrated systematic toxicity which is often attributed to its high linear energy transfer (LET). This high LET has produced some acute toxic side effects in patients being treated for solid tumors with ^{131}I-RAIT [40].

Astatine-211, in contrast, demonstrates a low LET compared to ß emitters. This halogen α emitter deposits an incredible 5.9 MeV of ionizing radiation over a distance equal to approximately one cell diameter. Astatine chemistry should parallel that of iodine, since they are both members of the halogen family, this is not the case. Direct labeling of proteins with ^{211}At yields radioconjugates which are not stable *in vivo*, presumably due to reactions with free thiol found in plasma.[41,42] Prosthetic carriers similar

to those developed for use with ^{125}I and ^{131}I have been prepared and used to form stable bioconjugates of ^{211}At.[43] Research into the effectiveness of these ^{211}At conjugates is in the very early stages but seems to have some potential. The major difficulty in using this nuclide is the need for massive shielding to protect the user.

While all these nuclides have demonstrated potential, the remainder of this document deals with a very specific radionuclide, namely iodine-125 and its use in conjunction with an antibody capable of recognizing tumors of glial origin.

I.C. Historical (^{125}I-MAb 425 RAIT)

I.C.1. Epidermal growth factor receptor expression in tumors of glial origin and anti-EGFr MAb 425.

Epidermal growth factor receptor (EGFr) is a transmembrane glycoprotein of 170 kDa molecular weight.[44] Upon binding of epidermal growth factor to its receptor, a tyrosine kinase is activated which sets in motion a signal transduction;this leads eventually to mitosis. In addition, the binding of EGF to its receptor leads to down regulation of the receptor and portions of the EGF/EGFr complex have been shown to be taken up specifically into the nucleus.[45] Normal brain tissue does not express a significant amount of EGFr, however, malignant tumors of glial origin often demonstrate a significantly

enhanced expression of the receptor.

Waterfield, Schlessinger and co-workers studied 37 brain tumors and determined that glioblastomas and astrocytomas showed higher than normal expression of the EGF receptor. Furthermore, more than forty percent of the glioblastomas studied had developed multiple copies of the gene for the EGF receptor.[46] In one case, the number of copies was as high as 50 compared with (1) one copy for normal brain tissue. A related study was conducted by Torp and co-workers who examined expression of EGFr in brain tumors using two different biotinylated monoclonal antibodies raised against the epidermal growth factor receptor and a strepavidin-fluorescein marker.[47] The results showed that all malignant gliomas showed positive fluorescent staining, while 18 of 29 highly malignant gliomas (glioblastoma multiforme and astrocytoma) stained strongly. These results indicate that EGFr is overexpressed in brain tumors of glial origin making it a suitable candidate for radioimmunotherapy.

Several monoclonal antibodies have been raised against the epidermal growth factor receptor. Many of these bind specifically to epitopes either on the carboxy terminus of the protein (an intracellular domain) or to surface carbohydrates. The former is not useful because the carboxy terminus is not accessible from the extracellular fluid and the latter lacks specificity since the tumor cells express nearly identical protein cores but differ in manner and type of glycosylation. One antibody which does target an extracellular protein epitope of the EGFr molecule is monoclonal antibody 425 (MAb 425) produced by Steplewski and co-workers at the Wistar institute.[48] MAb 425 is murine monoclonal antibody which was raised against A431 human carcinoma cells which overexpress the

EGF receptor approximately 100 fold. The antibody binds equally well to both glycosylated and deglycosylated EGFr. In sharp contrast to other antibodies raised against this receptor , MAb 425 is capable of inhibiting the binding of epidermal growth factor to both high and low affinity EGFr, the converse however is not true. EGF has no effect on the binding of MAb 425 to EGFr. Importantly, MAb 425 does not activate the tyrosine kinase activity of EGFr as would EGF but it does seem to be able to downregulate the receptor. There is no indication that MAb 425 is capable of activating the mitotic cycle.

Several studies have been undertaken with unlabeled MAb 425 to determine its effect on the growth of cells which overexpress EGFr. The results of one study undertaken by Bender and co-workers at the Wistar institute demonstrated that MAb 425 is capable of suppressing growth of xenografted tumors in nude mice.[49] In mice with untreated F39 human glioma xenografts, tumors tripled in size in a matter of six weeks while those in the treated group increased only about 50% in size in the same amount of time. In addition, it has been shown that MAb 425 is rapidly internalized (40 % internalization within 6 hours) into both A1207 and U87-MG glioma cell lines (See section I.C.3) . All of the aforementioned properties of this antibody made it an ideal candidate for use in a RAIT system designed to treat glioma tumors.

I.C.2. Choice of iodine-125 as the nuclide for use in MAb 425 RAIT.

Re-examining Table I.1 one nuclide stands out from the rest both in energy and half-life, iodine-125. The half-life of this nuclide is fairly long at 62 days especially

compared to the other nuclides whose half-lives can be measured in hours. The other distinguishing feature of iodine-125 is the very short path over which it deposits its energy. At 10 nm the linear energy transfer is 3 to 4 orders of magnitude smaller than alpha emitters and greater than 6 orders of magnitude shorter than the beta emitters.[50] The reason for this range of energy deposition is the mechanism by which iodine-125 decays. The nucleus of iodine-125 captures an electron to generate a tellurium-125 daughter. This capture causes the release of a low energy γ particle and results in the loss of an excited outer shell electron. This Auger electron is of high enough energy to break bonds (highly reactive) but it lacks the momentum of a β particle.

The extremely low LET of iodine-125 led scientists to originally believe that this nuclide was not capable of causing any significant damage to biological materials. Since its gamma component could be detected by use of a gamma scintillation counter, the nuclide was employed in many biological studies especially those involving proteins and was generally considered extremely safe.[51] In the late 1970's, Adelstein and Kassis began to look at the potential health effects of iodine-125.[52] They suggested that the reason there was no apparent effect from the nuclide was not that it was incapable of inflicting damage but that it was not able to interact with matter at or near the nucleus which would result in the necessary DNA strand cleavage. An experiment performed by Kassis and co-workers demonstrates clearly that iodine-125, under the proper conditions, can be extremely lethal. Table I.2 shows the results of a study which examine the effect of iodine 125 when it was localized to one of three cellular compartments. Using aqueous $Na^{125}I$, ^{125}I-rhodamine, and ^{125}I-deoxyuridine, Kassis was able to detect localization of the

Cellular Compartment	Lethal Dose D_{37}	Carrier of 125-Iodine
Extracellular	10,000 mBq/Cell	$Na^{125}I$
Cytoplasm	109 mBq/cell	^{125}I-dihydrorhodamine
Nucleus	1.3 mBq/cell	^{125}I-deoxyuridine

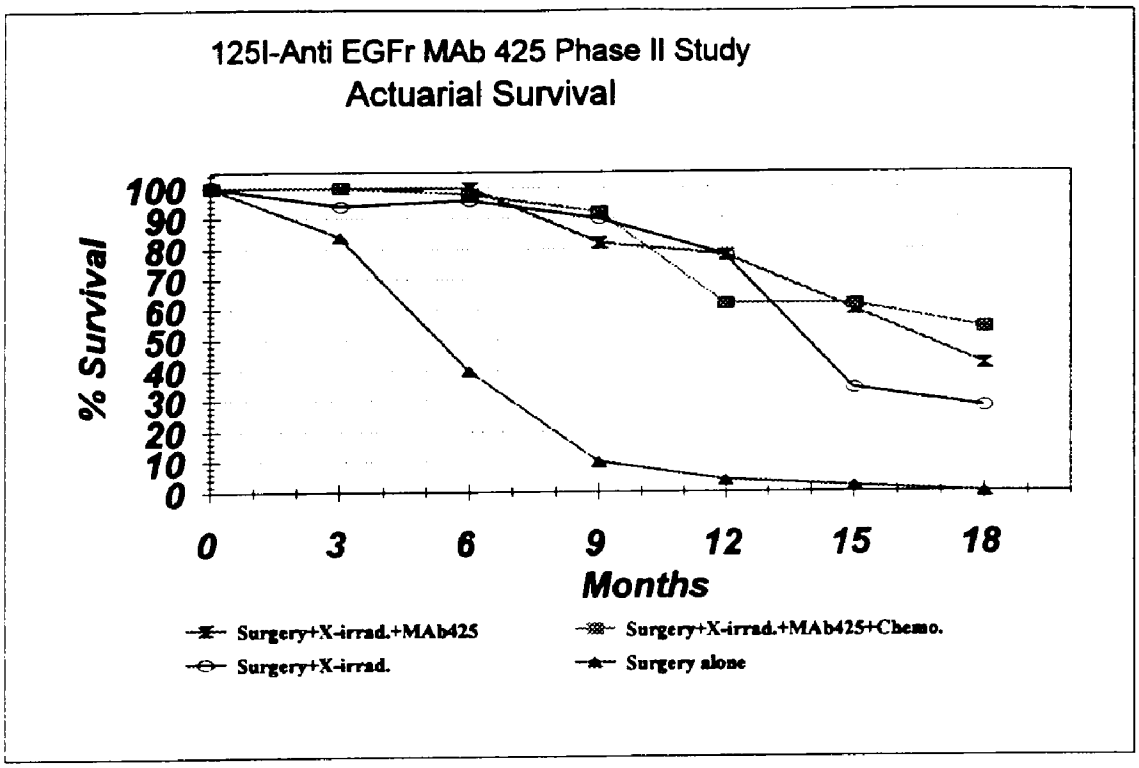
Table I.2
Lethality of ^{125}I in
Various Cellular Compartments

radionuclide in the extracellular, cytoplasmic, and nuclear compartments respectively.[53] The cytotoxicity of iodine-125 in each of these compartments was determined and, as Table I.2 shows, there is a 100 fold increase in cytotoxicity for radionuclide taken up into the cytoplasm versus iodine-125 which is outside the cell and a 10,000 fold increase if the iodine-125 is taken up into the nucleus.

This data suggests that iodine-125 is capable of effecting cell kill and could therefore be used therapeutically. The requirement for such a therapy would be the ability to deliver the nuclide to the cytoplasmic or nuclear compartments of the target cell. MAb 425 antibody is capable of binding to and internalizing into glial tumors making it a suitable choice as a carrier for iodine-125 RAIT of brain tumors. Moreover, initial studies indicate that upon internalization the antibody/receptor complex may be endosomally transported to the nucleus which would provide an additional benefit in such a system.[54]

I.C.3 Clinical studies and Biological Properties of ^{125}I-MAb 425

For these reasons the MAb 425 antibody was used in Phase I and Phase II clinical studies aimed at suppressing regrowth of tumor following surgery and external beam radiation. These studies involved the use of MAb 425 which was electrophilically labeled with iodine-125 via the Iodogen™ method (see figure I.2). Figure I.3 shows the results of a Phase II clinical study carried out by Brady and co-workers at Hahnemann Hospital in Philadelphia. In this study 50 patients having Grade IV (glioblastoma multiforme) or Grade III (anaplastic astrocytoma) gliomas were treated by surgical removal of the tumor



(Note: surgery+x-irrad.+chemo. is not shown but survival is nearly identical to that of surgery+x-irrad. no real benefit is gained through chemotherapeutic regimens.)

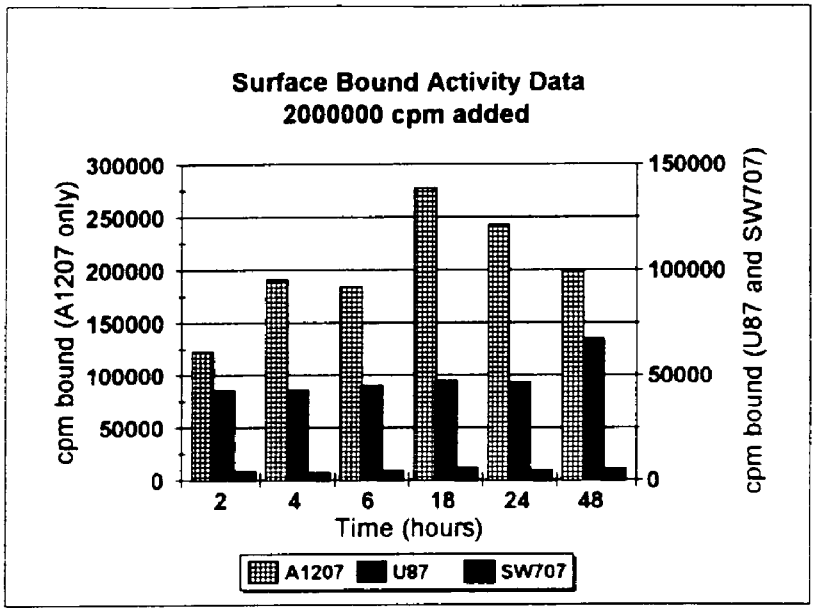
Figure I.3 Survival of Glioblastoma Multiforme or Astrocytoma patients following therapy. ^{125}I-MAb 425 RAIT increased survival in patients following surgery and x-irradiation.

followed by 50 Gy of external beam irradiation. The patients were then given three 50 mCi injections of ^{125}I-MAb 425 at three week intervals with or without a concurrent course of chemotherapy. The survival of these patients is compared in figure I.3 to patients receiving one of three different treatments surgery alone , surgery + external beam irradiation, surgery + external beam irradiation+ chemotherapy (not shown, the data

is nearly identical to the surgery+irradiation curve). As figure I.3 shows, survival in those patients who had no radiation therapy was extremely poor, with 50% surviving at just under six months. External beam therapy increases the 50% survival time to about 14 months. The use of the ^{125}I-MAb 425 had a significant effect on survival both with and without chemotherapy. This indicated that the radioimmunoconjugate (RIC) was getting to the tumor site and having a positive therapeutic effect.

This *in vivo* work was done in the absence of any fundamental study of the ^{125}I-MAb 425 construct on glial tumors *in vitro*. While it was known that the radioimmunoconjugate bound and internalized into these cells and that unlabeled antibody was capable of suppressing the growth of these tumors in animal models, no data concerning the fate of the iodine-125 had been collected.

A. Binding of Direct Labeled 425 to the Surface of A1207 and U87 EGFr(+) glioma cells and SW707 EGFr(-) colorectal cells following various incubation times.



B. Internalization of direct labeled MAb 425 following various incubation times into A1207 and U87 EGFr(+) cells.

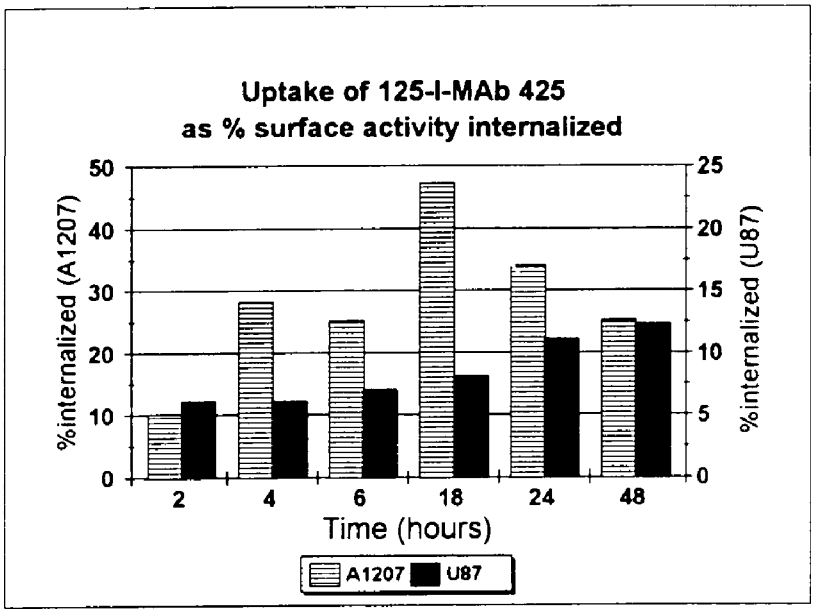


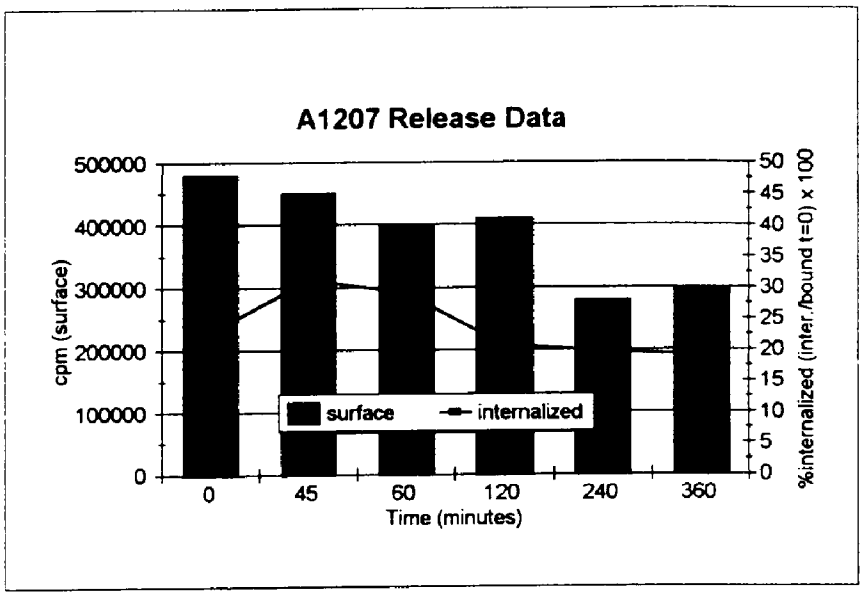
Figure I.4 Binding and Internalization of ^{125}I-MAb 425 into A1207 and U87 cell lines. Graph A represents binding to the surface of the cells following incubation with $2x10^6$ cpm of direct labeled antibody. Graph B is representative of the surface to internalized activity ratio for the radioiodine. Note: both graphs have two Y-axes. In both graphs the data from A1207 cells is plotted with respect to the left Y axis and all other data plotted with respect to the right axis.

Following the Phase II study, new experiments were carried out in order to determine what improvements, if any, could be made to the RIC to enhance the already demonstrated therapeutic potential. To this end studies of the uptake and release rate of the ^{125}I-MAb 425 RIC were carried out in several glioma cell lines including the A1207 and U87 cell lines and the SW707 human colorectal cell line was used as a negative control (SW707 show no overexpression of the epidermal growth factor receptor). As can be seen in Figure I.4 B uptake into both the A1207 and U87 cell lines is extremely rapid reaching a maximum after approximately 12 - 18 hours. Figure I.5 A and B demonstrate that this rapid uptake is accompanied by a rapid release of the internalized activity to the cytoplasm.[55] This rapid loss of activity was determined to be a major obstacle to effectively using the ^{125}I-MAb 425 RIC for radioimmunotherapeutic purposes. Additional studies have shown that the iodinated material which is being released from the cytoplasm is not capable of binding to EGFr suggesting further that the antibody is degraded during or after internalization and that the released material is a peptide fragment , free iodotyrosine, or free radioiodine.[56]

The results of these later studies indicated a need for a cytoplasmic trap. A carrier for the radioiodine which would not be readily released from the cytoplasm of the glioma cells. In addition, it was determined that due to the long $t_{1/2}$ of iodine-125 a dose greater than one iodine nuclide / IgG would be necessary to guarantee a therapeutically suitable number of decays within a target cell. Parts II and III of this document deal with two different types of prosthetic carriers each designed to

address one of the two aforementioned concerns.

A.



B.

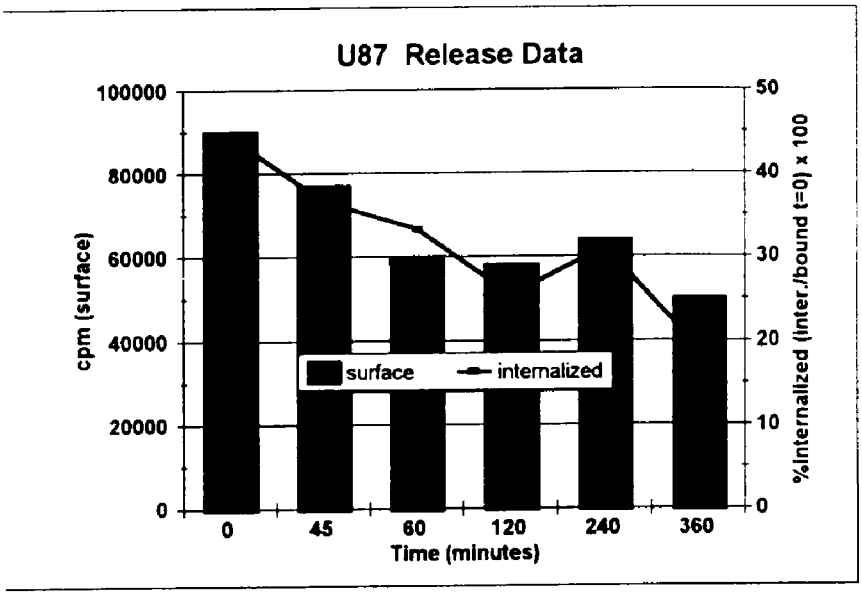


Figure I.5 Release of radioiodine-125 from the surface and cytoplasm of A1207 (A) and U87 (B) human glioma cell lines. Surface activity plotted vs. left Y axis and internalized activity plotted vs. right Y axis.

PART II

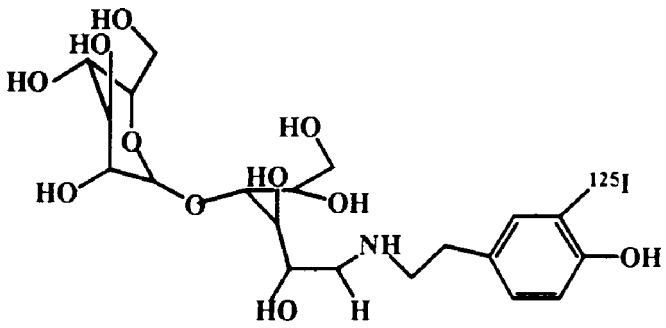
TYRAMINYL-CELLOBIOSE SYNTHESIS AND BIOLOGICAL EVALUATION AS A CYTOPLASMIC TRAP OF RADIOIODINE FOR USE IN MAb 425 BASED RAIT

II. Tyraminyl-cellobiose Synthesis and Biological Evaluation.

II.A. Background and Rationale

Early studies of the uptake of ^{125}I-labeled monoclonal antibody 425 into glial tumors overexpressing the EGF receptor demonstrated that the labeled antibody was being taken up specifically into the tumor and was rapidly being released into the supernatant surrounding the cells. (See section I.C.3) Uptake of iodine-125 was therefore limited to a certain cytoplasmic maximum in the presence of hot antibody and this cytoplasmic activity was rapidly lost once the hot antibody was removed from the cell suspension. One of the specific objectives of this project was to use carbohydrate carriers to increase the cytoplasmic residence time of the iodine-125 in the hope that a higher level of radioactivity could be incorporated and maintained.

Pittman and co-workers, in studying the uptake of radioiodinated low density lipoprotein (LDL) into cultured fibroblast cells of human origin, encountered a similar problem of cytoplasmic release.[4] Pittman began his studies using LDL which had been electrophilically iodinated on aromatic amino acid residues. These preliminary studies showed that nearly 94% of the label was being released from the cell following uptake of the labeled protein. The problem was resolved with the use of a metabolic trap, tyraminyl



^{125}I-tyraminyl-cellobiose

Figure II.1 tyraminyl-cellobiose used by Pittman to trap iodine-125 in fibroblasts following internalization on LDL.

cellobiose, which would hold the radioiodine within the cytoplasm.

The structure of tyraminyl-cellobiose is shown in figure II.1 The label is the reductive amination product of tyramine (*des*carboxytyrosine) with the open aldehyde form of cellobiose (glcβ1,4glc). Radioiodine, either ^{125}I or ^{131}I, is incorporated via

common electrophilic methods leading to ortho substitution on the phenolic portion of the molecule prior to attachment to the protein (e.g. the Iodogen method see figure I.2).

The presumed mechanism by which such a trap works is through the phosphorylation of the glucose residues by enzymes in the glycolytic pathway such as hexokinase . Phosphorylation by this enzyme would presumably occur at either one or both of the free C6 hydroxyls on the disaccharide portion of the label. Using this label and cyanuric chloride as a coupling agent, Pittman was able to essentially eliminate the loss of hot label from the human fibroblast cells having trapped 95% of the radioiodinated material.[4]

The rationale behind the use of this label for the ^{125}I-MAb 425 system was that this label could potentially overcome the rapid loss of cytoplasmic radioactivity which was shown to occur with direct labeled MAb 425. The principle was to incorporate the ^{125}I-tyraminyl-cellobiose label at a specific activity equal to or just slightly less than 1 (1 radioiodine/ molecule of IgG). This would produced a RIC which differed from direct labeled antibody only in the disaccharide moiety and would hopefully behave similarly to direct labeled antibody in terms of EGFr binding and cellular uptake.

II.B. Synthesis of tyraminyl-cellobiose (ii.1)

We examined the use of this label as a potential method for increasing the cytoplasmic concentration of iodine-125 in U87-MG and A1207 cells to levels which are capable of effecting significant cell kill. In order to do so, we began by synthesizing the

label as per the method of Pittman outlined in figure II.2 .* The primary amine of tyramine adds to the aldehyde of the reducing end of cellobiose resulting in the formation of a

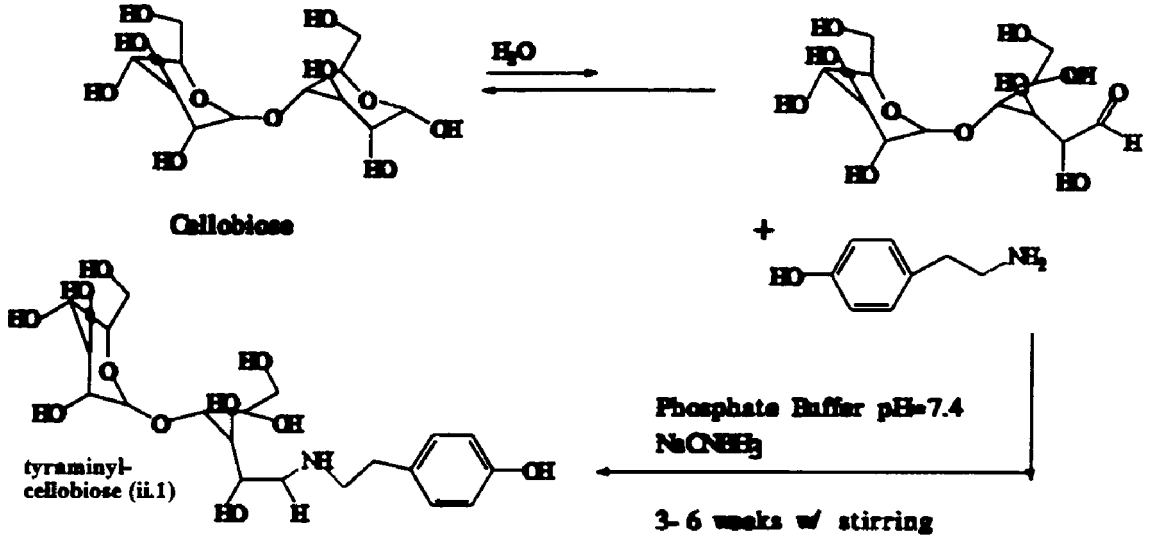


Figure II.2 Synthesis of tyraminyl-cellobiose (ii.1)

Schiff's base. The coupling is made irreversible by the *in situ* reduction of the imine by $NaCNBH_3$.

In a buffered (pH=7.4) aqueous system the label can be synthesized in no less than 3 weeks due to the hemi-acetal-aldehyde equilibrium greatly favoring the closed hemi-acetal form of the sugar . Studies were undertaken to optimize the yield and rate of production of the tyraminyl-cellobiose label. Four reactions were run simultaneously and

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* A note on compound numbering. All compounds in this dissertation including protein conjugates are numbered using small roman numerals which signify the chapter in which the material is first described, followed by a number which indicates the order in which the item appeared. For example, the third compound of chapter 3 would be **iii.3**

followed by thin layer chromatography . Two of the four reactions were kept at 25°C and the other two were held at 55°C for the duration of the experiment. The reaction progress was monitored by thin layer chromatography on silica gel plates using a 50:50 mixture of toluene and methanol as the mobile phase. The plates were analyzed by detection of ultraviolet active chromophores and by burning in an iodine chamber. No significant difference was observed in the rate of product formation or disappearance as evidenced by I_2 staining or by UV analysis. Essentially no improvement was evidenced by TLC when the reaction was carried out at higher temperature.

Other possibilities for decreasing the total reaction time in aqueous buffer were ruled out. Stoichiometric control of the rate (increasing the concentration of tyramine) was not feasible due to the difficulty in separating the starting materials from the final product. Purification is best carried out by cation-exchange chromatography at low pH. This method will bind both tyramine and the newly formed tyraminyl-cellobiose adduct.

A second method of synthesizing the tyraminyl-cellobiose label was employed. This method used anhydrous DMSO as the solvent for conjugation as per the method of Smesrød.[57] DMSO is known to stabilize the aldehyde form of the reducing sugar over the hemiacetal form. The DMSO allowed for a much more rapid formation of the adduct but even following ion-exchange chromatography a significant amount (approximately 5 moles DMSO: 1 mole adduct) of DMSO remained associated with the adduct as evidenced by NMR and elemental analysis. Further experiments were not performed with this material. For all experiments involving radiolabeling or conjugation of tyraminyl-cellobiose to antibody tyraminyl-cellobiose produced from aqueous buffer was used.

II.C. Labeling of tyraminyl-cellobiose (ii.1) with iodine-125.

Following preparation of a stock quantity of the tyraminyl-cellobiose label a series of labeling and conjugation experiments were carried out on this single reference material. The tyraminyl-cellobiose was iodinated using nonradioactive iodine-127 using the Iodogen™ method. A noticeable shift in the UV chromophore of the phenolic ring was observed consistent with an ortho-iodination (see figure B.4 in Appendix B). The tyraminyl-cellobiose was readily iodinated by $Na^{125}I$ in tubes which had been coated with Iodogen to produce compound **ii.2** .

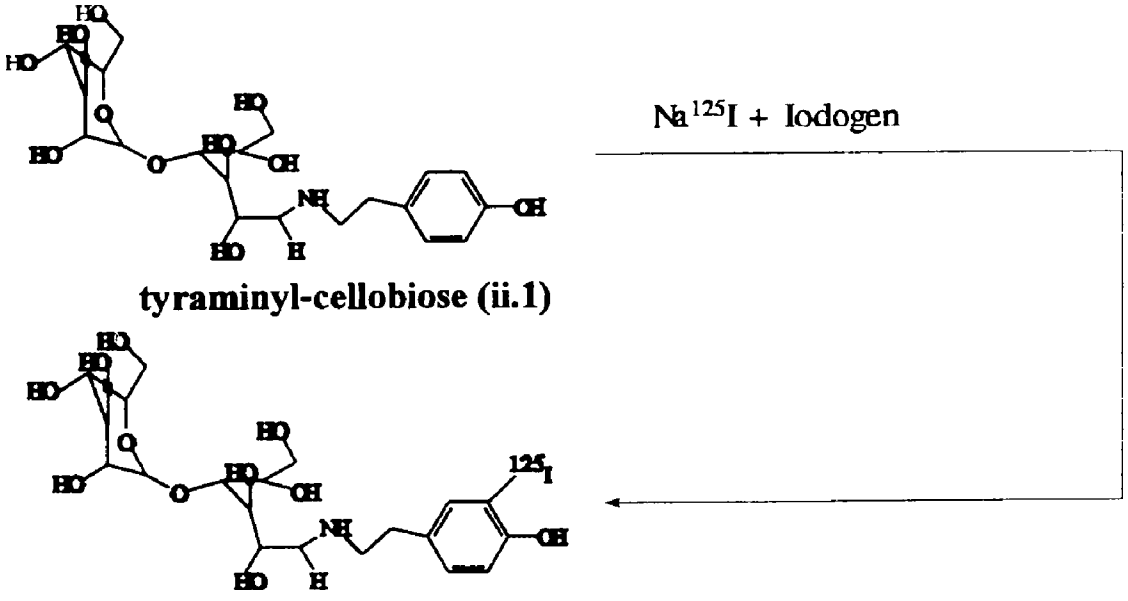
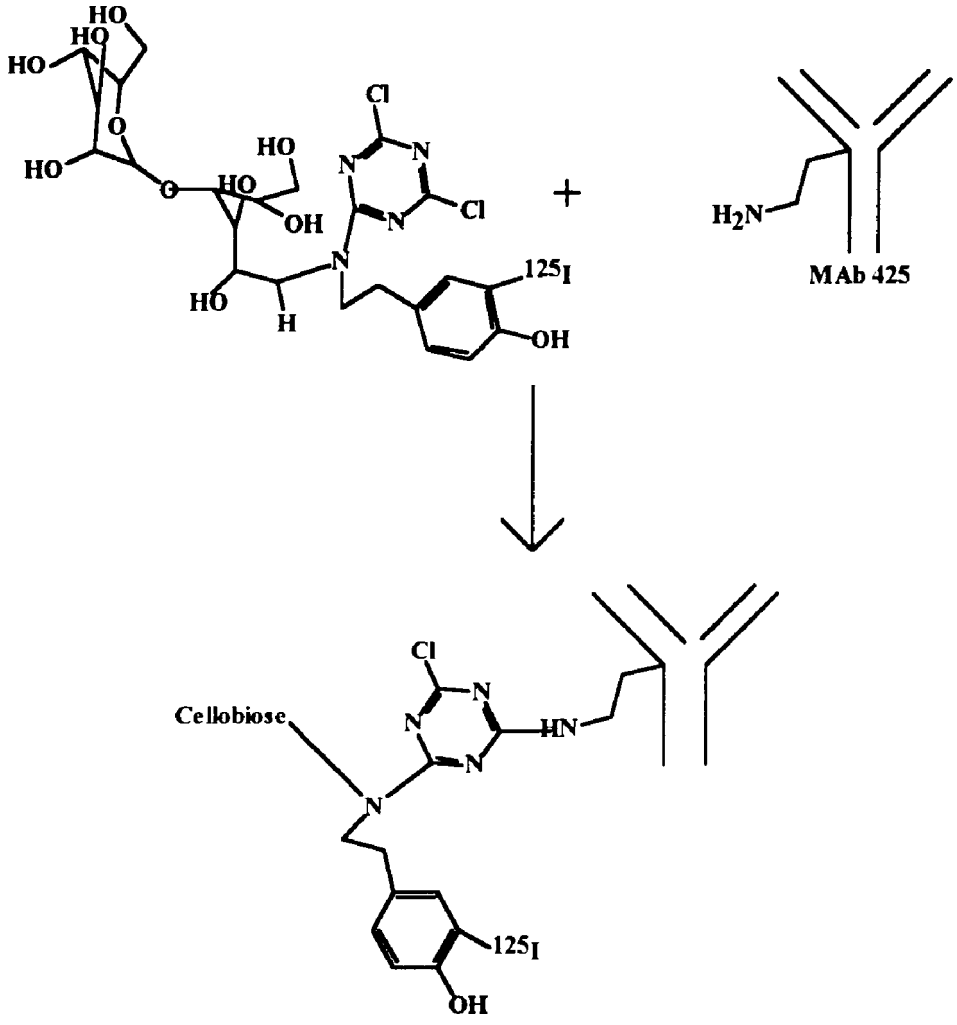


Figure II.3 Labeling of tyraminyl-cellobiose with iodine-125.

The formation of the "hot" iodo-tyraminyl-cellobiose was evidenced by the formation of a new spot in ^{125}I-thin layer chromatography (^{125}I-tlc). The hot labeled tyraminyl-cellobiose was then used to prosthetically label MAb 425.

II.D. Conjugation of ^{125}I-tyraminyl-cellobiose (ii.2) to MAb 425

II.D.1 Formation of the ^{125}I-tyraminyl-cellobiose-MAb 425 construct via cyanuric chloride (ii.3a)*



^{125}I-tyraminylcellobiose-MAb 425 ii.3a

Figure II.4 Conjugation of ^{125}I-tyraminyl-cellobiose (ii.2) to MAb 425 to form the immunoconjugate ii.3a.

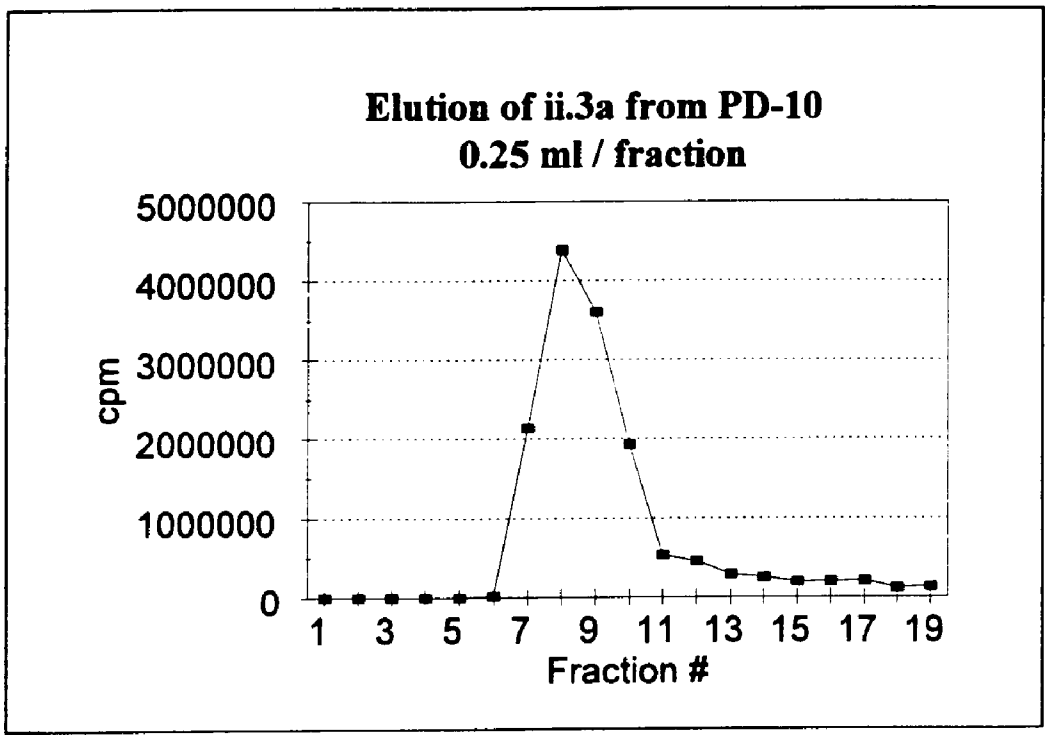


Figure II.5 Elution Profile of ^{125}I-tyraminyl cellobiose-MAb 425 from Sephadex G25 in a pre-packed PD-10 column. (void volume = 2 mL)

Initial experiments aimed at coupling the ^{125}I-tyraminyl cellobiose to the antibody followed procedures based on the original method of Pittman.[4] The method initially employed was to couple the ^{125}I-tyraminyl cellobiose to LDL via the use of cyanuric chloride (2,4,6,-trichlorotriazine) (see figure II.4). The first several attempts at labeling the protein were unsuccessful due to the use of ascorbic acid as a quenching agent intended to block further oxidation of $Na^{125}I$ to $^{125}I^{+}$. The pH of the ascorbic acid solution was significantly below 7 (pH<5.5) inhibiting the necessary nucleophilic attack by the secondary amine of **ii.2** at one of the chlorinated sp^2 carbon sites. In later experiments

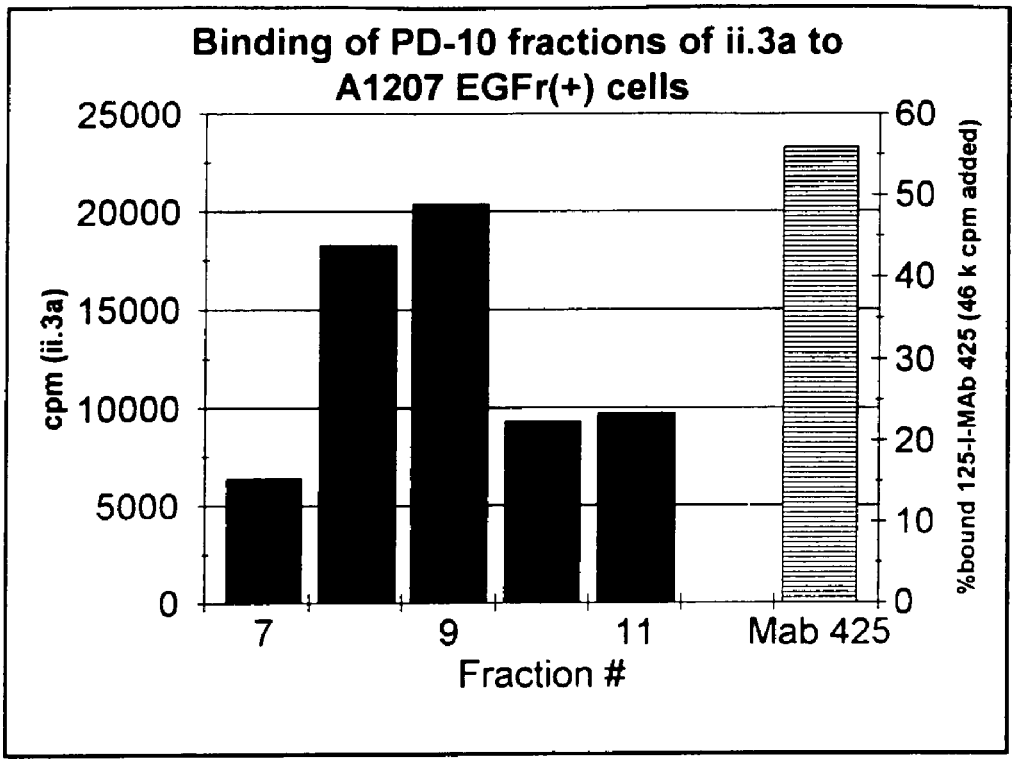


Figure II.6 Binding of eluted ii.3a fractions from PD-10 purification. Note only 20,000 cpm bound when between 2,000,000 and 5,000,000 cpm were added. This is a marked difference from 425 which binds about 56% of 46,000 cpm added counts of iodine-125.

sodium bisulfite was used as the quenching agent and the pH was adjusted to between 9 and 10 pH units. The entire iodination reaction mixture was then added to a solution containing one equivalent of cyanuric chloride dissolved in dry acetone. Following a short incubation period 333 ug of Mab 425 was added and the entire mixture was allowed to incubate for one hour. Purification of the ^{125}I-tyraminyl cellobiose-cyanuric chloride-Mab 425 constructs (**ii.3a**)** was accomplished through the use of a PD-10 column which was packed with Sephadex G25. Figure II.5 shows the elution profile of one of the cyanuric

** The ^{125}I-tyraminyl-cellobiose conjugates of anti-EGFr MAb 425 are designated by two different numbers. Those which were produced via a cyanuric chloride crosslinking are designated **ii.3a** and those which were produced via a carbonyl diimidazole crosslinking are designated **ii.3b**.

chloride conjugated constructs. Further conjugations using cyanuric chloride resulted in elution profiles similar to those shown in figure II.5. The single elution peak is representative of the void volume of the G-25 PD-10 column and consistent with high molecular weight (greater than 25 kDa) iodinated material. It is also consistent with the MAb 425 elution profile (see figure B.1). All fractions under the peak were analyzed separately. Each fraction was analyzed for its ability to bind to the surface of the A1207 EGFr (+) cell line as well as the SW707 colorectal EGFr(-) cell line. Binding experiments were carried out at 4°C to prevent any internalization. The binding of fractions within the elution peak for **ii.3a** is shown in figure II.6. The best binding fractions were found, as expected, toward the center of the peak. These fractions bound upward of 1% of the total added activity which is extremely low when compared to direct labeled 425 for which approximately 50% all added activity is bound at similar concentrations (cpm/cell). No appreciable binding of SW707 cells by **ii.3a** was observed. Although the binding of **ii.3a** to A1207 cells was modest it was deemed high enough to use the **ii.3a** conjugates in further experiments to determine the uptake and release characteristics in the A1207 and U87 EGFr (+) cell lines.

II.D.2 Formation of the ^{125}I-tyraminyl-cellobiose-MAb 425 construct via carbonyl-diimidazole (CDI) (ii.3b)*

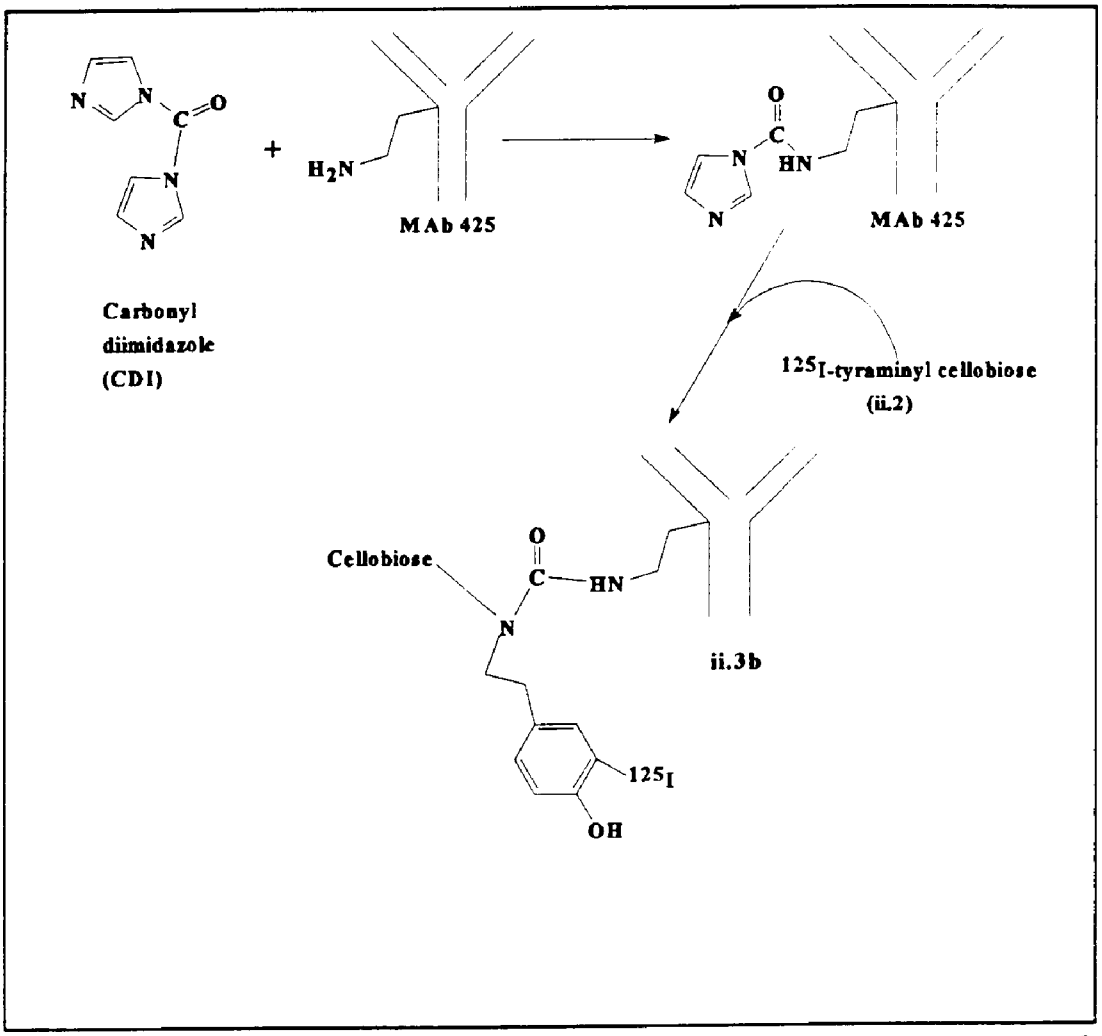


Figure II.7 Preparation of ^{125}I-tyraminyl-cellobiose-MAb 425 RIC through use of carbonyl diimidazole. Ratio of CDI to IgG in first step varied between 1:1 and 15:1 .

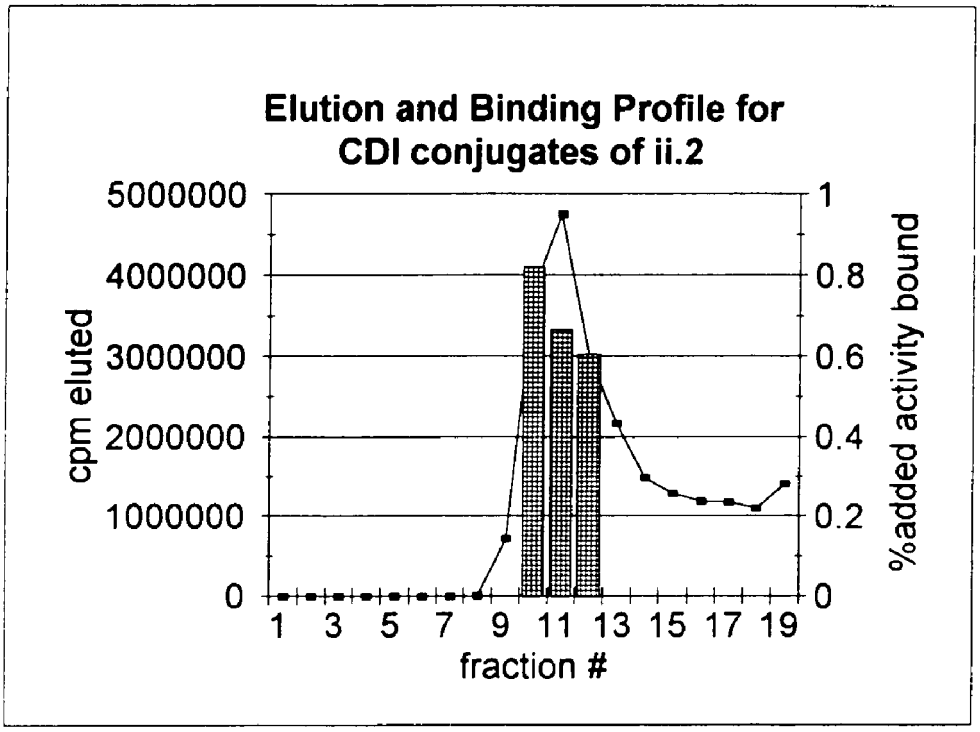


Figure II.8 Elution of ii.3b from Sephadex G25 and binding of selected fractions to A1207 human glioma cells. The conjugate shown here was produced from a 10 mole excess of CDI:IgG. Binding is comparable to cyanuric chloride conjugation. Below 3:1 mole excess CDI no radioactive peak is observable in the elution profile.

A second method employed for conjugating **ii.2** to MAb 425 was to activate the antibody with carbonyl diimidazole (CDI) (see figure II.7).*** Several ratios of CDI: IgG were used in these conjugations to try and maximize the specific activity of the final product. At low stoichiometric ratios of CDI:IgG (1-2 moles CDI/mole IgG), very poor conjugations resulted as evidenced by PD-10 purification. No activity eluted in the void volume of the column, and instead, a baseline level of radioactivity was observed at greater than 3 ml eluent. At higher CDI:IgG ratios (3-15 moles CDI/ mole IgG) a high specific activity peak was observed in the void volume as shown in figure II.8 . Again individual fractions under this elution curve were analyzed for the ability to bind to A1207 cultured human glioma cells. The results of one such examination are shown under the elution peak in figure II.7 . It can be readily observed that the binding of these fractions is comparable to **ii.3a** binding. No binding was observed for the **ii.3b** conjugates with SW707 as expected. The binding to A1207 by **ii.3b** was not high enough to select it over **ii.3a** conjugates for use in internalization and release studies. In order to be consistent therefore with the method developed by Pittman, the **ii.3a** conjugates were used exclusively in the internalization and release studies which follow.

*** CDI coupling chemistry is described in Wong, S.S. (1991) Chemistry of Protein Conjugation and Cross-Linking, CRC Press, Boca Raton, FL

II.E Uptake and Release of the ii.3a RIC in A1207 EGFr(+) cells.

II.E.1 Internalization of ii.3a into A1207 cells.

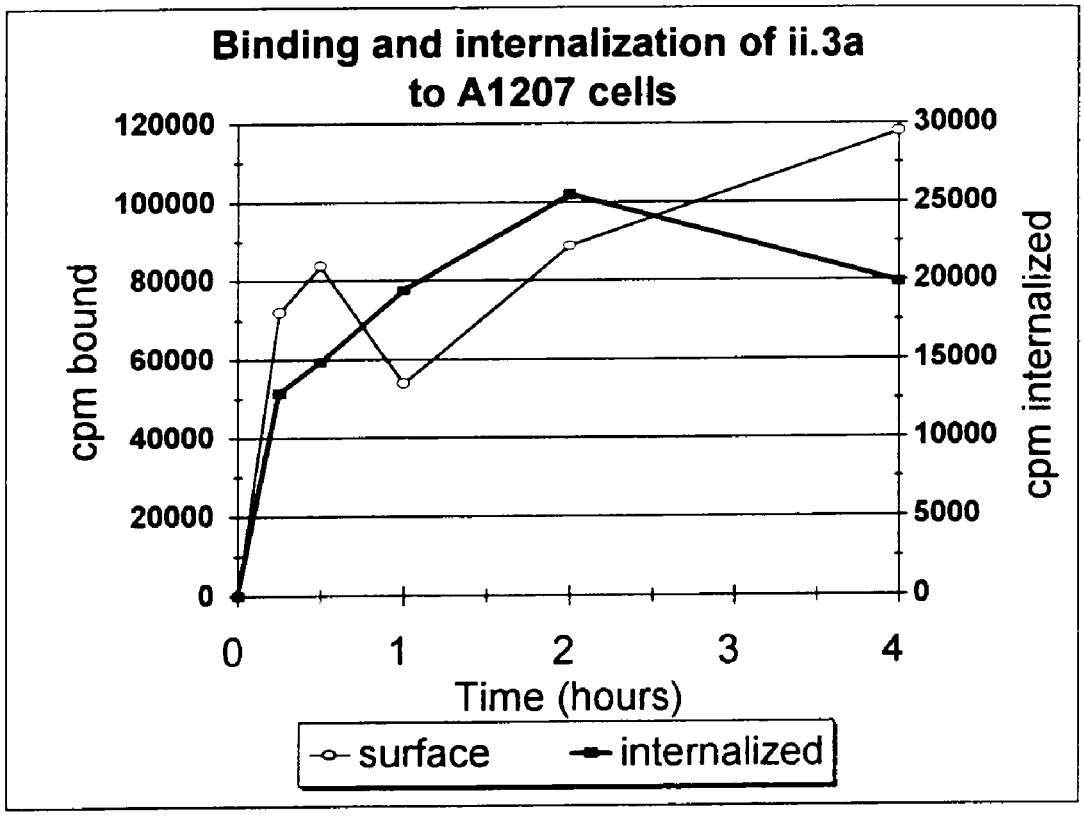


Figure II.9 Internalization of ii.3a into A1207 cells.

The internalization of MAb 425 **ii.3a** conjugates was monitored over a period of 24 hours using a method which allowed for the determination of both surface bound and internalized activity. Using this method, cells were incubated for the indicated amount of

time with **ii.3a.** Following the incubation period the radioactive supernatant was removed and discarded. The cells were thoroughly washed with PBS and then incubated in an acetic acid wash at 4°C. The cells were re-pelleted and the acid wash collected. This cells were then washed twice with PBS. The radioactivity present in the acid wash is due only to that radioactivity which was on the exterior membrane of the cell and is thus a measure of antibody binding.[3] Following the acid wash the cells were digested completely in hot (>90°C) NaOH and the saponified cells were then counted. The activity found in the saponified samples is due to internalized (cytoplasmic) activity.

Since the rate of internalization is dependent on the quantity of antibody bound at the surface of the cell and since the **ii.3a** conjugates have a binding avidity < 2% of that of direct labeled MAb 425, the surface and internalized activities were co-plotted in order that comparisons can be drawn. Figure II.9 shows both the surface bound activity (plotted with respect to the left Y axis) and internalized activity (plotted vs. the right Y axis) for various incubation times up to 4 hours. The input activity for these cells was approximately one mCi.

The internalization experiments seemed to indicate that in spite of marginal binding avidity, the uptake of **ii.3a** into the A1207 cells was occurring. Further experiments were done to determine whether there was any noticeable effect of the cellobiose on release of radioiodine from the cytoplasm of the EGFr(+) cells.

II.E.2 Release of ii.3a internalized activity from EGFr(+) cells.

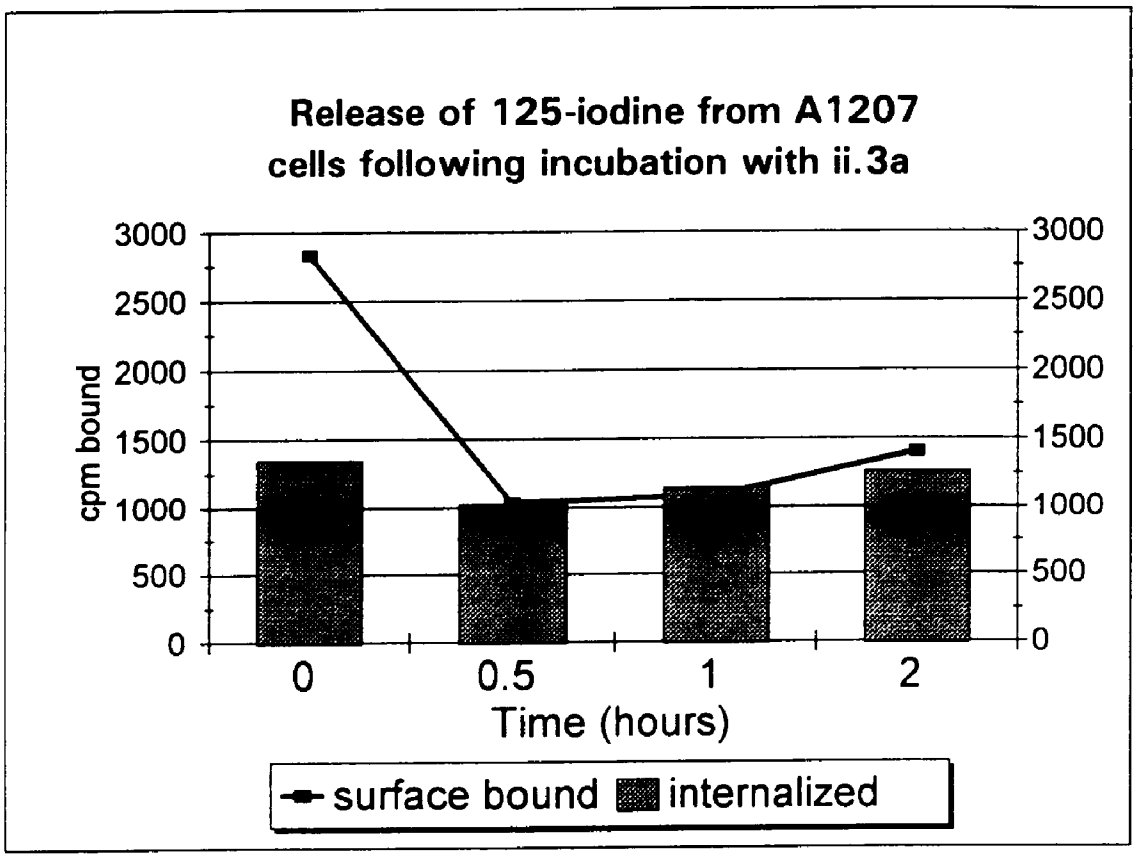


Figure II.10 Release of ii.3a internalized activity

In a manner similar to the uptake studies discussed in section II.E.1 the rate of release of internalized **ii.3a** activity was examined. In performing these experiments A1207 cells were incubated with **ii.3a** for 24 hours to be assured of maximum internalization. The added activity was removed and fresh media was added to the cells .

At varying times following the removal of the activity the newer media was removed and the cells washed . The cells were then acid washed and saponified as previously discussed. Figure II.10 shows the results of one typical release study, followed for only 2 hours. The surface bound activity dropped precipitously in a similar manner to direct labeled antibody. The internalized activity appears to remain rather constant indicating a slower release than would be expected. However, this does not necessarily infer that the tyraminyl-cellobiose is having an effect on the release rate. While the internalized activity is rather flat over the course of these experiments, it is also very low. The quantity of radioactivity required to internalize a quantifiable amount of radioiodine from **ii.3a** is extremely high. Exposure to this level of activity (1mCi) for 24 hours was certainly toxic to at least a portion of the cells. The remaining cells may have been damaged and therefore unable to process the internalized material in a normal fashion. The internalized activity therefore may not be due to the same process as the rapid uptake of 425.

II.F Section II Conclusions

Several conclusions can be drawn from the work presented in this section. First, tyraminyl-cellobiose can be produced in fairly reasonable quantity even though the length of time involved is somewhat inhibitory. It is probably possible to use the DMSO methodology to improve reaction time though better methods of purification than those employed herein will be necessary.

The primary test of a prosthetic is its ability to be attached to the carrier protein. Cyanuric chloride and carbonyl diimidazole are both suitable means for conjugating the ^{125}I-tyraminyl cellobiose to MAb 425. In both cases high activity protein peaks were

observed in the void volume of the G25 gel exclusion columns used to purify conjugates **ii.3a** and **ii.3b**. Unfortunately neither conjugate retained any significant ability to bind to the EGFr receptors on the surface of the target glial cells. In both cases maximum binding was at about 1% of total added activity which is not high enough for clinical use in RAIT. As will be discussed later in section IV this may be an antibody specific problem rather than an inherent problem in the chemistry employed .

The low binding **ii.3a** conjugates did demonstrate some ability to internalize into EGFr(+) cells but considering the relatively low amount which was bound initially the quantity of radioiodine which does end up in the cytoplasm is not sufficient for purposes of ^{125}I-RAIT. Release of the radiolabel does appear to be slower but is not significantly (or statistically) different from the normally observed release when direct labeled MAb 425 is the carrier.

II.G Experimental

II.G. 1a Synthesis of tyraminyl-cellobiose (ii.1):

Tyraminyl-cellobiose was prepared as per the method of Pittman and co-workers[4] Briefly; 1.0 g tyramine (7.3 mmol), 2.5 g cellobiose (7.3 mmol) and 0.49 g of $NaCNBH_3$ (7.8 mmol) were dissolved in 60 mL of 20 mM phosphate buffer pH = 7.4. The solution was placed in a 150 mL Erlenmeyer flask equipped with a stirring bar and a ground glass stopper. The solution was allowed to stir for 3 weeks at room temperature. The solution was evaporated to dryness *in vacuo* leaving a light yellow solid. The solid was dissolved in a minimal amount (~ 5 mL) of DMSO. The DMSO solution was precipitated by the slow addition of 15 mL of acetone. The dull white precipitate was filtered, washed with acetone and allowed to dry. The resulting white solid was dissolved in 10 ml of distilled water and applied to a 25 x 5 cm column packed with Rohm & Haas IR-118H cation exchange resin. The column was washed with 2 L of distilled H_20 and was then eluted with 0.5 M NH_4OH. The effluent was sampled for pH with indicator paper and all effluent with pH greater than 9 was collected. The solution was evaporated to dryness *in vacuo*. The resulting light yellow solid was suspended in acetone, filtered, washed with acetone and allowed to dry. 0.51 g **ii.1.** Rf 1:1 toluene/MeOH 0.77 . Analysis for $C_{20}H_{33}N0_{11}$ calcd. C 49.8%, H 7.27%, N 2.91% found C 47.8%, H 7.13% , N 2.80%

UV absorption max. @ 274 nm. ϵ = 1126 cm^{-1} $moles^{-1}$.

^1H NMR ($DMSO_{d6}$) δ(ppm); 6.8 (AB quartet, 4p), 5.2 (m, 8p), 3.5 (dd, 4p) see Fig B.3

II.G.1b Alternative synthesis of tyraminyl-cellobiose (ii.1):[57]

To a 100mL Erlenmeyer flask having a ground glass joint was added 80 mL of anhydrous DMSO, 6.85 grams cellobiose and 2.74 grams of tyramine. The slurry was allowed to stir for 1 hour. A solution of 1.52 grams of $NaCNBH_3$ in 35 mL of anhydrous DMSO was prepared and was added dropwise over 1.5 hours using a pressure equalizing addition funnel. The clear solution was allowed to stir at RT for 24 hours. The solution was then reduced *in vacuo* with heat to 70% of the original volume. 300 mL of 100% EtOH was added slowly to the reaction solution causing a yellow precipitate to form. The sticky solid was filtered out of the DMSO and washed several times with acetone. The resulting solid was dissolved in 400 mL H_2O and applied to an Amberlite IR-118H strong acid cation exchange column. The column was flushed with 1000ml of H_2O. The column was then eluted with 0.5 M NH_4OH. A 600 mL fraction was collected and reduced *in vacuo* to an off white solid. The solid was dissolved in minimal DMSO and then re-precipitated by addition of 250 mL of acetone. The resulting oil was decanted from the solution and was crystallized by the addition of THF/methylene chloride 1:1. The resulting solid was filtered off and dried. mp 98-100^{0}C. NMR see figure B.3 note large DMSO peak.

II.G.2 Cold labeling of tyraminyl-cellobiose with Iodine-127.

100 uL of 0.01 M **ii.1** and 100 uL of 0.0246 M NaI were added to a test tube coated with

10 milligrams of Iodogen™ and allowed to react for 3.5 minutes. The reaction was quenched by the addition of 100 uL of 1% sodium bisulfite. The reaction mixture was diluted to 5.0 mL and a UV spectrum was taken. The absorption maximum was 300 nm vs. 280 nm for **ii.1.** (see figure B.4)

II.G.3 Elution of MAb 425 from Sephadex G-25 pre-packed PD-10 column

A pre-packed Sephadex PD-10 column was washed with 20 mM phosphate buffer pH = 7.4. The column was then charged with 300 µL of a 10 mg/mL solution of monoclonal antibody 425 and .3 mL fractions were collected to a total volume of 8 mL (25 fractions). The individual fractions were diluted to 1 mL. The fractions were then analyzed by UV spectroscopy for absorbance at 280 nm using 20 mM phosphate buffer as a blank. A plot of fraction number vs. absorbance shows the antibody eluting within the void volume of the PD-10 column (~2mL, see Figure B.1). The experiment was repeated with similar results and further purifications using the PD-10 column were modelled on this protocol.

II.G.4 Labeling of tyraminyl-cellobiose with Iodine-125.

Iodogen was coated onto the wall of a test tube by evaporation of 1 mL of $CHCl_3$ containing 10 milligrams of Iodogen with swirling. A 0.2 M solution of tyraminyl-cellobiose in H_2O was prepared and 100 ul was placed in the bottom of the Iodogen

coated tube. To this tube was added 50 mCi of $Na^{125}I$ in NaOH. The tube was held at room temperature for 20 minutes. The reaction was then quenched with 0.5 mL of 1M ascorbic acid or with 0.5 mL of 1% sodium bisulfite.

II.G.5 Conjugation of ^{125}I-tyraminyl-cellobiose (ii.2) to MAb 425 via Cyanuric Chloride

The entire contents of one tyraminyl-cellobiose reaction vial was added to a vial containing 100 ul 0.2 M cyanuric chloride. To this was added 100 ul 0.4 M NaOH and the reaction allowed to proceed for 30 seconds. To this was added 100 uL 0.4 M acetic acid followed by 300 ul of a 10 mg/mL solution of monoclonal antibody 425 in phosphate buffer containing 0.1% NaN_3. The reaction was allowed to proceed at RT for 1 hour. The entire reaction mixture was charged to a Sephadex PD-10 prepacked G25 gel exclusion column which had been pre-equilibrated with 10 mL of 20mM PBS at pH = 7.4. The column was eluted with 10 mL of PBS and 0.25 mL fractions were collected and counted in a gamma scintillation counter to determine the activity of each fraction.

II.G.6 Conjugation of ii.2 to MAb 425 via carbonyl diimidazole ;ii.3b

Into each of 5 1.5 ml microcentrifuge tubes was placed 25 µL of MAb 425 (10.0 g/mL) to this was added 25 µL PBS pH = 7.2 . Volumes of 5,10,15,20,or 25 µL of 0.2 M CDI

were added to tubes # 1-5 respectively and the reactions allowed to proceed for 5 minutes. The CDI reaction was followed immediately by 5 mCi of **ii.2** The reaction was allowed to proceed for 1 hour after which the entire reaction volume was charged to a PD-10 column and eluted. The high activity fractions (**ii.3b**) were collected and tested for their ability to bind to A1207 cells.

II.G.7 Binding of PD-10 fractions to A1207 cells.

For each fraction to be analyzed two microtiter wells (96 well plate) are coated with a suspension ($5x10^6$ cells/mL) of approximately 250,000 A1207 cells in PBS. To each of these is added 50 uL of the conjugate to be tested (activity added is generally between 200,000-500,000 cpm for **ii.3a** and **ii.3b** or between 40,000-60,000 cpm for direct labeled MAb 425). The cells are allowed to incubate for 1 hour at O^0C with shaking , to prevent internalization. The cells are then spun at 2500 rpm to repellet. The radioactive supernatant is removed and the cells are washed 3x with repeated centrifugation with RIA buffer (see Appendix for RIA buffer formulation). The cells are then entirely removed from the microtiter well using a cotton swab and the swabs are individually counted for activity. This method is used generally for all direct binding assays presented herein.

II.G.8 Internalization of ii.3a into A1207 cells.

Petri dishes were seeded with 1.25×10^6 A1207 human glioma cells. At 4,3,2,1, and 0.5 hours before the experiment was to end 50 uL of the undiluted solution iv.3

(approximately 3.1 x 10^6 cpm) was added to one of the petri dishes. At the termination of the experiment all six dishes were placed on ice for one hour to halt internalization. Each petri dish was then trypsinized for 5 minutes and then stripped of its cells with PBS and mild shaking. . The cells were transferred to a plastic 15 mL centrifuge tube and were centrifuged for 6 minutes at 1200 rpm for 10 minutes. The supernatant was removed completely and set aside. The cells were subsequently resuspended in 5 mL of a 25 mM solution of AcOH in 300 mM NaCl to remove any activity bound to the surface of the cells. The cells were centrifuged again a 1200 rpm for 10 minutes, the acid wash was removed and set aside. The cells were then washed twice with 5 mL of PBS and the washes combined with the acid wash. The combined washes were counted. The cells were then saponified by addition of 5 mL of hot 20% $NaOH_{(aq)}$. The NaOH was removed and counted. The entire process was repeated for each of the six petri dishes. Thus for each time point there are three samples which generate data points; supernatant (added activity), acid wash (bound activity) and homogenizing buffer (internalized activity).

II.G.9 Release of internalized ii.3a activity.

Release studies were carried out in an identical manner to internalization studies from II.G.7 above with the following exceptions. All cells were incubated for 24 hours with $3x10^6$ cpm of **ii.3a**.. Following the 24 hour incubation the supernatant was removed from the cells and the cells washed three times with 5 mL of growth media. New media was applied to the surface of the cells. At 0, 0.5 ,1, and 2 hours following the addition of the new media the cells were analyzed for surface and internalized activity as in section II.G.7.

PART III

DERIVATIVES OF DEXTRAN

(α 1,6 POLYGLUCOSE)

AS CARRIERS OF ^{125}I-TYRAMINE

FOR INCREASED RADIOIODINE

DELIVERY IN MAb 425 BASED

RAIT

III. Derivatives of Dextran for Increased Delivery of Radioiodine-125.

III.A Background and Rationale

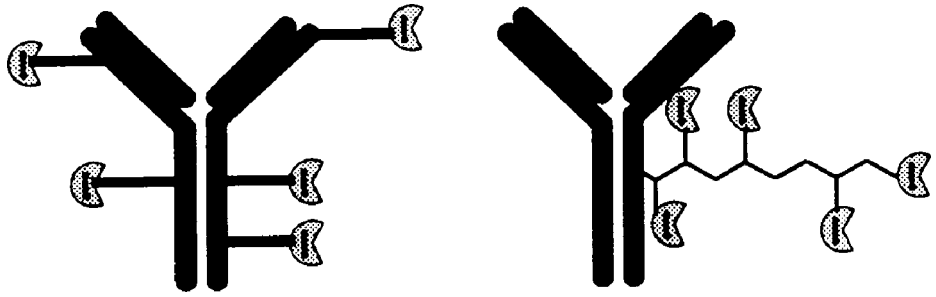


Figure III.1 Direct vs Polymer Drug/Nuclide Conjugation

In any radioimmunotherapeutic system the ability of the individual radionuclide to effect cell kill is highly dependent on the number of chromosomal damaging radioactive

disintegrations which occur within one cell lifetime. For many of the nuclides listed in Table I.1 the $t_{1/2}$ of the nuclear decay is short in other words there is a high number of decays/minute. Iodine-125 has the longest half-life of any of the nuclides listed in Table I.1 and therefore has the least number of decays/mole minute. In addition, the decays which do occur must be in extremely close proximity (10 nanometers) to the nucleus of the target cell. If ^{125}I-RAIT is to be successful then a high level of the radionuclide must be delivered to each targeted cell. Increasing the direct load of radioiodine onto an antibody is not feasible due to the high concentration of $Na^{125}I$ required as well as the inherent loss of binding avidity when multiple covalent bonds are formed with the IgG.[58] One possible solution is demonstrated in figure III.1. Instead of trying to attach multiple iodines to one

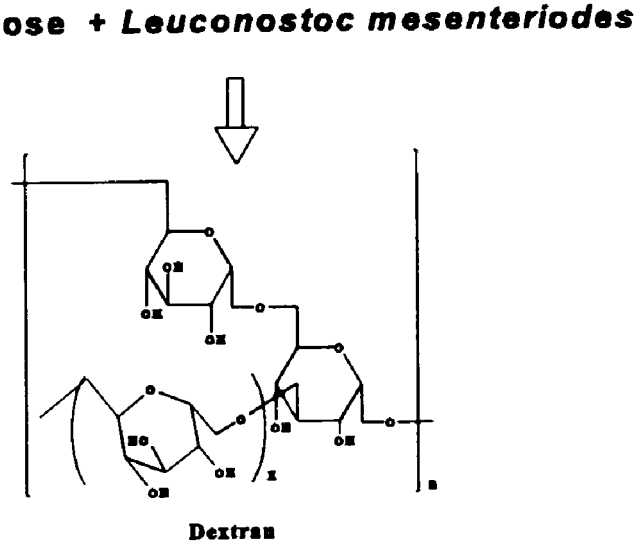


Figure III.2 Dextran when produced by *leuconostoc mesenteriodes* has a molecular weight on the order of 2000 kDa.

IgG a polymeric carrier is employed which can hold many radioiodines. The radioconjugate is then attached to the IgG forming , under ideal situations, a single

covalent bond.

There are many polymers which could be used to "piggy-back" radioactive molecules and further be conjugated to IgG. The polymer chosen for use in the MAb 425 ^{125}I-RAIT system studied herein is dextran. Dextran is a homopolymer of glucose synthesized by the *Leuconostoc Mesenteroides* bacterium.[59] The primary backbone of dextran is α 1,6 linked and has α 1,3 branches (see figure III.2). The bacterial product has a molecular weight on the order of 2,000 kDa. This product is enzymatically digested to remove all branches, hydrolytically cleaved to smaller fragments and a 40 kDa fraction isolated. This 40 kDa fraction is suitable for use in polymeric delivery.

Dextran has certain characteristics which make it especially suitable for use in immunotherapeutic systems . Dextran has been shown to be non-immunogenic which is critical since these RAIT systems require multiple injections and allergic responses have been shown to be problematic in some antibody based therapies.[5] In addition dextran is easily modified to contain both aldehyde and amino functionality allowing for the rapid incorporation of both electrophilic and nucleophilic small molecules. In addition as a polysaccharide dextran can impart water solubility to molecules which may otherwise be insoluble. This section discusses the preparation of four derivatives of dextran and the use of three of these as potential carriers of radioiodine for use in RAIT.

III.B. Preparation of dextran derivatives.

Dextran Derivative	Compound Number	Functional Group	Load groups mole dextran	Molecular Weight
Polyaldehyde (PAD)	iii.1	-CHO	280-310	40 kDa
Polyamino ($DexNH_2$)	iii.2	R_2NH, RNH_2	2^0; 50-60 1^0; 17-25	18-35 kDa
Carboxymethyl (CMD)	iii.3	-COOH	200-220	50 kDa
poly-lactone (CMDL)	iii.4	-COOR Cyclic	100-230	50 kDa
poly-hydrazide ($CMDNHNH_{2)}$	iii.5	-COOH $-CONHNH_2$	~100	60 kDa

TABLE III.1
Derivatives of Dextran Prepared for Use in RAIT
Molecular Weights are approximate. Load is based on titration or elemental analysis.

Several derivatives of 40 kDa dextran were prepared as potential carriers of radioiodine. These derivatives and some of their properties are listed in table III.1.

III.B.1 Polyaldehyde Dextran and Polyamino Dextran

The oxidative cleavage of vicinal diols to aldehydes is well established. This reaction can be used with glucose and glucose derivatives including dextran to form a

dialdehyde. Polyaldehyde dextran **iii.1** is produced by oxidation of a dextran in a solution of KIO_4 (see figure III.3). Purification of the modified polymer is readily accomplished by dialysis against H_2O. The oxidation can be controlled to some extant to introduce between 100 and 350 aldehydes per 40 kDa polymer. The PAD produced for this work was oxidized to contain between 270-310 aldehydes per 40 kDa polymer. This polymer was used to produce a readily iodinated polymer.

PAD can be conjugated to any primary amine, hydrazine or hydrazide. Primary amine conjugation is generally accomplished via *in situ* reduction of a Schiff's base. Through a similar reaction the aldehydes of the PAD can be converted to primary amines by condensation of ammonia followed again with *in situ* reduction with $NaCNBH_3$. In this manner one can convert the electrophilic PAD into nucleophilic polyamino dextran ($DexNH_2$) **iii.2**. The major obstacle to the use of $DexNH_2$ is the inevitable formation of intra- and intermolecular crosslinks. The reduction of imines by $NaCNBH_3$ creates primary amines which can react further with the non-reduced aldehydes which remain on the polymer. These crosslinks are reduced to form secondary amines which are not available for reaction with electrophilic drugs such as active esters. In general no more than 15 free primary amino groups could be incorporated per molecule of 40 kDa dextran even though a significant amount of nitrogen could be incorporated into the polymer. [60] Since the character of $DexNH_2$ could not be guaranteed it was not used for further production of material suitable for use in the MAb 425 RAIT system.

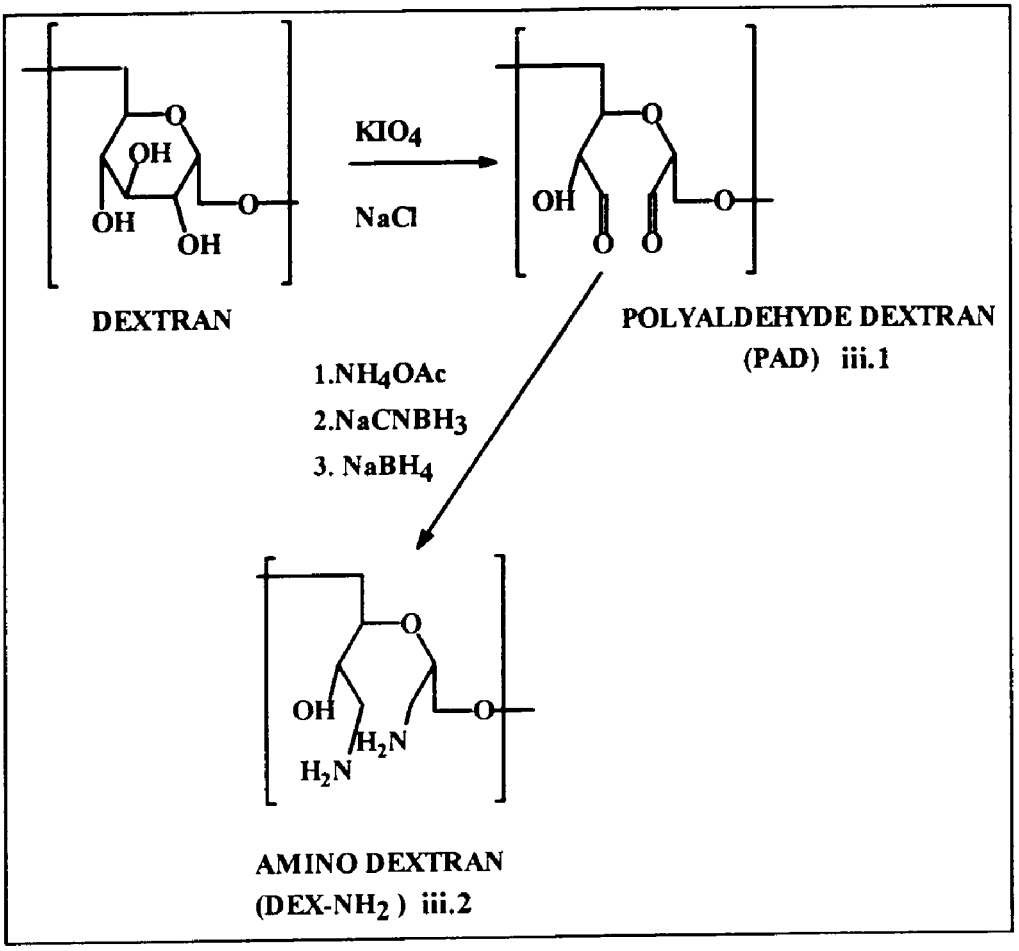


Figure III.3 Synthesis of Polyaldehyde Dextran (PAD, iii.1) and and Amino Dextran ($DexNH_2$, iii.2).

III.B.2 Carboxymethyl Dextran (CMD) , Carboxymethyl Dextran Lactone (CMDL) and Carboxymethyl Hydrazide ($CMDNHNH_2$).

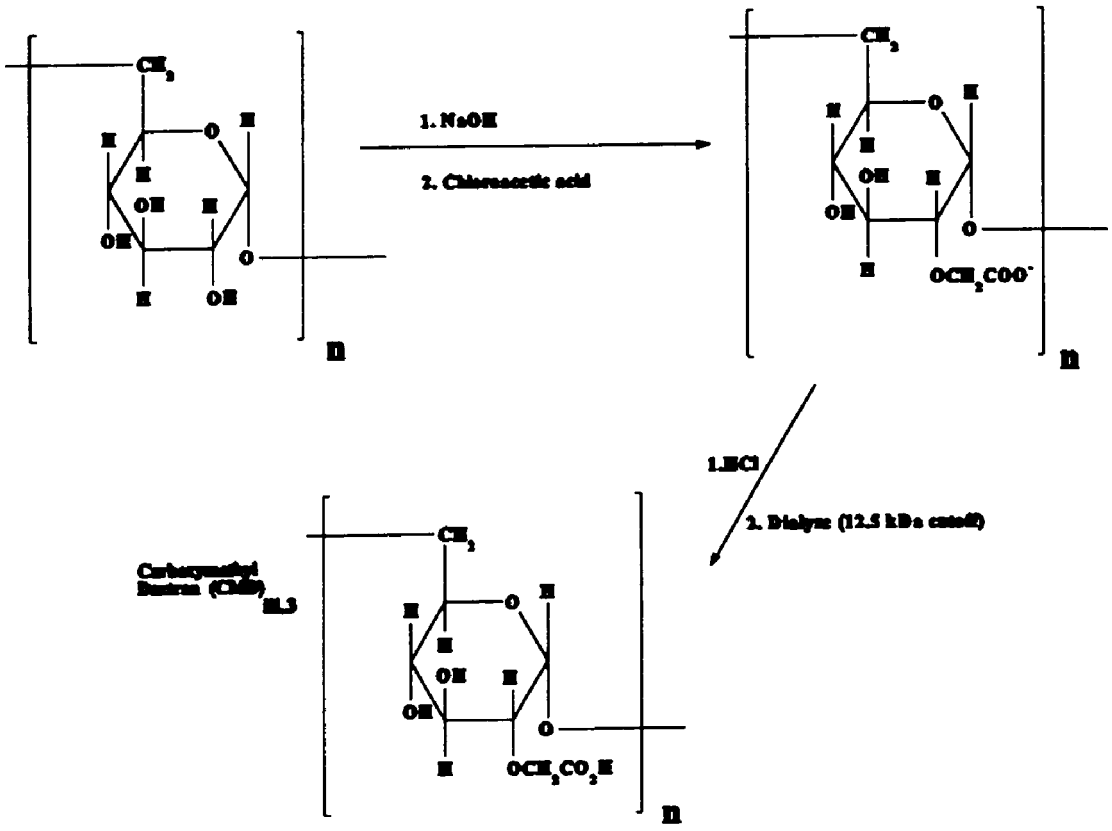


Figure III.4 Preparation of Carboxymethl Dextran CMD iii.3

Reaction of dextran with chloroacetic acid in base leads to one of the most useful drug carriers, carboxymethyl dextran (CMD) **iii.3** (See figure III.4). Carboxymethyl dextran for this work was produced with loads having 0.5 or 1 ($-0CH_2COOH$ per glucose residue).[61] CMD alone is a useful polymer for conjugation. It is readily activated through a variety of means. Some of the more common methods for activation include

formation of active esters via carbodiimides or carbonyl diimidazole and N-hydroxysuccinimide or p-nitrophenol. These methods have been used with success for conjugation of a variety of materials including anti-tumor agents, proteins, and fluorescent markers.[62] The presence of the ionizable -COOH groups increases the solubility of the polymer even following conjugation of highly aliphatic or lipophilic drugs.

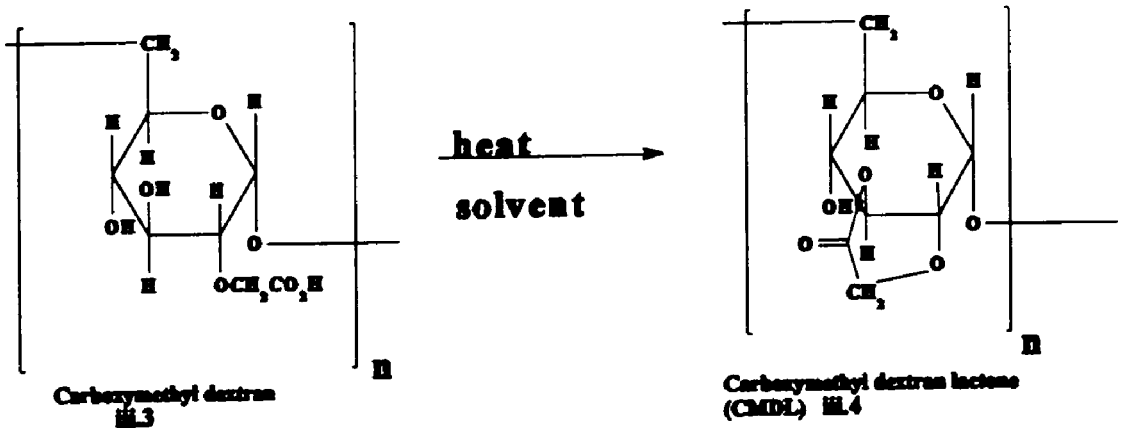


Figure III.5 Preparation of Carboxymethyl dextran lactone (CMDL) iii.4 Solvent used can be xylene, toluene, or acetonitrile.

One recently developed method for activating CMD is through the formation of intramolecular lactones.[63] CMD which is refluxed in xylene or toluene will dehydrate to form 6 member lactone rings as shown in figure III.5. This lactone CMDL **iii.4** is shelf stable and is pre-activated for conjugation to primary amines, hydrazides, or hydrazines. While lactonization does cause some loss of water solubility , saponification or hydrolysis of the lactone restores the water solubility of the polymer. Conjugation can be carried

out either *in situ* as the lactone is being formed or separately in organic solvents or in H_2O. Significantly when primary amines are conjugated to CMD through CMDL the product is an amide rather than a secondary amine. This bond while robust is certainly more labile than a secondary amine linkage, especially in the presence of proteases .

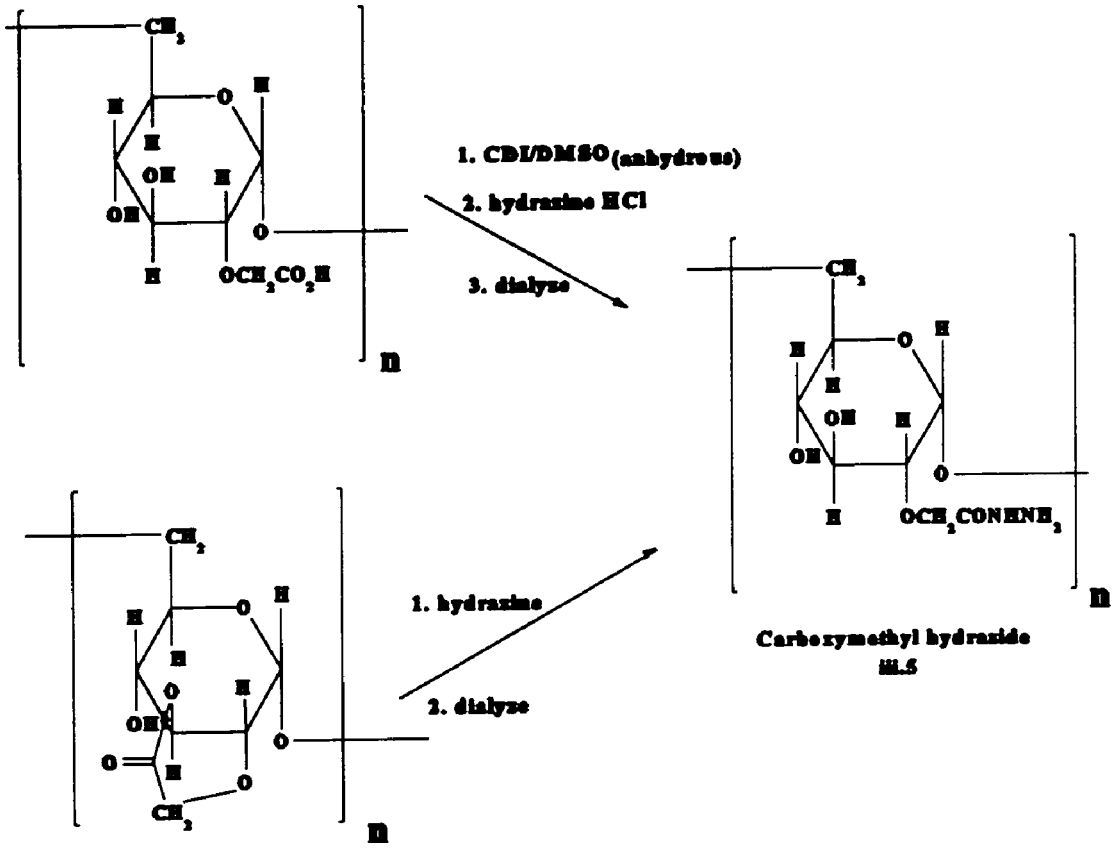


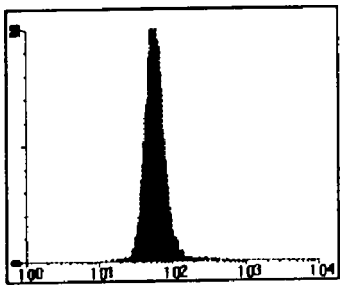
Figure III.6 Preparation of Carboxymethyl hydrazide iii.5

A second derivative of CMD which can be produced either via CDI activation or lactonization of the carboxyl groups is dextran hydrazide $CMDNHNH_2$ **iii.5** . Addition of the activated CMD to an excess of hydrazine in solution will readily form the hydrazide while avoiding the formation of extensive crosslinks. This polymer is similar to $DexNH_2$

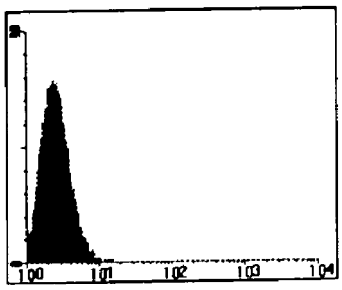
in that it is capable of reacting with electrophilic drugs and further the load of reactive hydrazides is far higher than the load of primary amine available in amino dextran.

III.C Binding of Dextrans to A1207 human glioma cells.

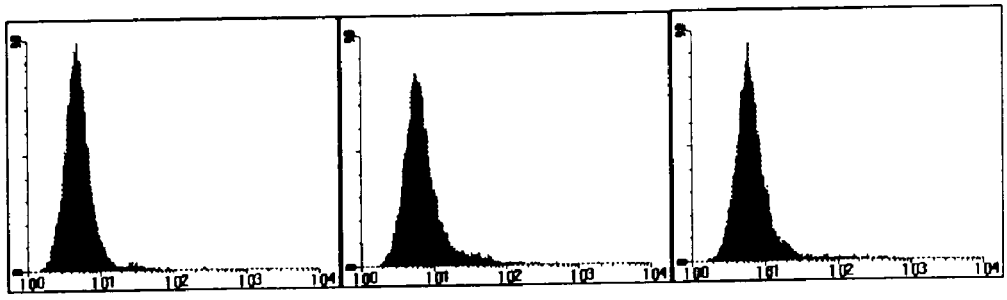
To determine the ability of dextrans to bind to A1207 cells unassisted by antibody three dextran derivatives were used. Two commercial FITC-labeled dextrans having



MAb 425 - FITC (positive control)



unlabeled MAb 425 (-) control



CMDNHNH-FITC **20 kDa Dextran-FITC** **40kDa Dextran-FITC**

Figure III.7 **Flow cytometry results for A1207 cell which were incubated with 20 uL of 300µg/mL solutions of FITC labeled dextrans. Top;(+) and (-) controls. Bottom; Dextrans, hydrazide dextran-FITC and two different molecular weight FITC-dextrans. Y-axes are number of cells and X-axes are fluorescent intensities.**

molecular weights equal to 19.6 kDa and 39.8 kDa respectively and a prepared CMDNHNH-FITC **iii.6** derived from the hydrazide dextran were dissolved in phosphate buffered saline. These solutions were serially diluted and added to microtiter wells containing 500,000 A1207 cells. Following a 90 minute incubation and three washes the cells were sorted using a flow cytometer which measured fluorescence at 519 nm (corresponding to FITC). Figure **III.7** shows the results of this experiment. The cells showed minimal increased fluorescence after incubation with the three FITC-labeled dextrans at extremely high concentration (300μg/mL). Below this concentration the FACS profile is identical to that of the negative control (No FITC). This is in direct comparison to FITC-MAb 425 which shows strong absorbance after incubation for 90 minutes with the A1207 cell line.

This experiment serves to show that dextrans do not bind specifically or non-specifically to A1207 cells at concentrations comparable to those which were used in RAIT experiments. While uptake of the dextran into tumor cells would not necessarily be a detrimental effect, any non-specific binding is highly undesirable as such binding would presumably occur equally on both normal and malignant tissues.

III.D Tyramine conjugates of PAD and CMDL synthesis and radiolabeling.

In order to use dextran as a carrier of radioiodine, a group capable of being iodinated must be introduced into the polymer backbone. Tyramine was chosen because of its relative simplicity and availability. As a phenol tyramine is readily iodinated. As a primary amine, tyramine can be readily conjugated to either PAD or CMDL.

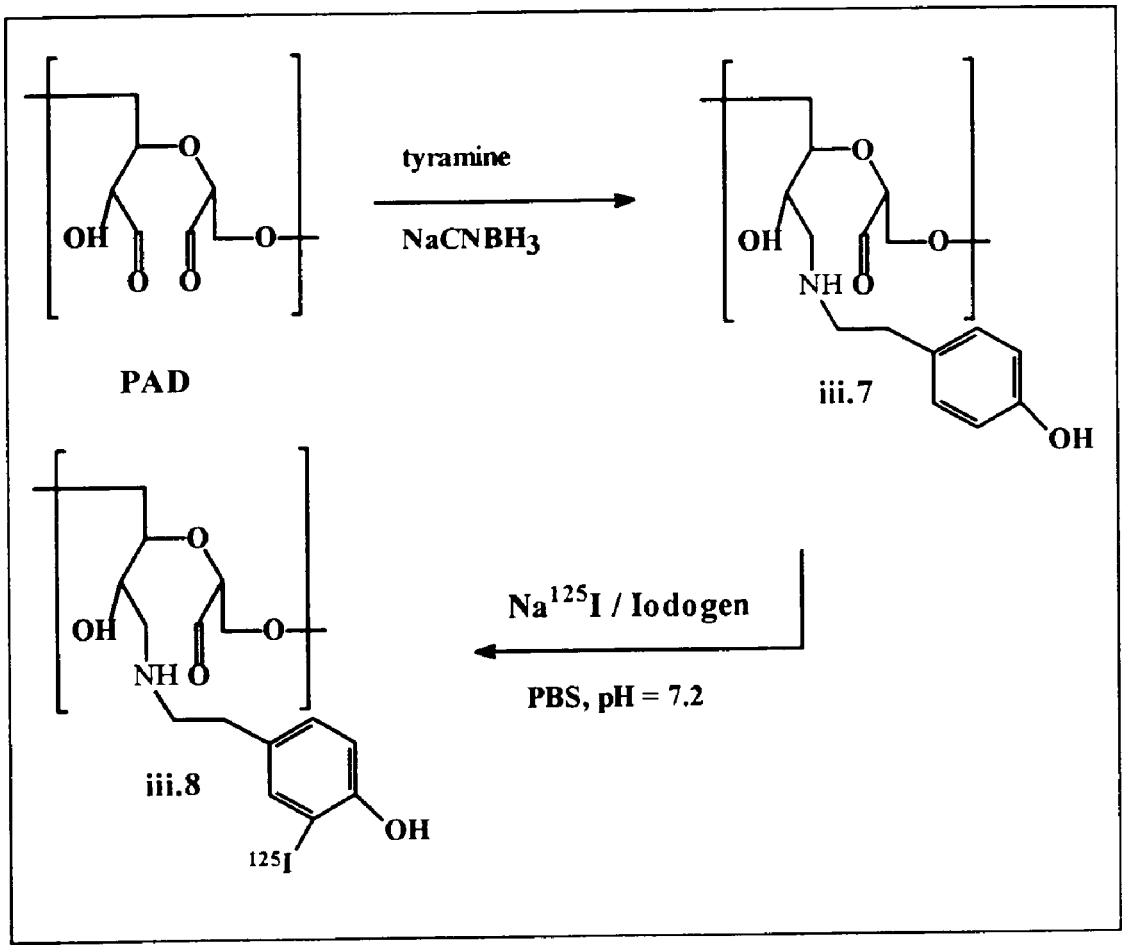


Figure III.8 Preparation of PAD-tyramine conjugates and subsequent radioiodination

III.D.1 Preparation of PAD-tyramine iii.7 and PAD-tyramine-^{125}I iii.8.

Tyramine was conjugated to polyaldehyde dextran in manner similar to the formation of the tyraminyl-cellobiose label discussed in part II of this document. The tyramine was allowed to react with the PAD in the presence of sodium cyanoborohydride. The reaction proceeded through the formation of an imine which was reduced by the borohydride to a secondary amine. The PAD which was used for this conjugation had approximately 300 aldehyde groups per 40 kDa polymer and was reacted with enough tyramine to achieve a load of approximately 100 tyramine molecules / dextran polymer. This level of conjugation was not achievable due to the precipitation of the PAD-tyramine conjugate **iii.7**. Dilution of **iii.7** in acetate buffer pH = 4.5 followed by exhaustive dialysis first against acidic acetate buffer and then against water returned the polymer to solution. Following lyophilization, the polymer was shown to have approximately 50 molecules of tyramine loaded per molecule of 40 kDa dextran by both elemental analysis and by UV spectroscopy (using the ϵ value for tyraminyl-cellobiose).

The PAD-tyramine conjugate **iii.7** was radioiodinated by the Iodogen^{TM} method to high specific activity (10:1 mole ratio of radioiodine to dextran polymer was used as in comparison of a 1:1 mole ratio for the direct labeling of MAb 425). Upon iodination however the polymer once again precipitated from solution and as such was not suitable for conjugation to MAb 425. The iodinated PAD-tyramine **iii.8** was not used in further studies.

III.D.2 Preparation of CMDL-tyramine iii.9 and CMDL-tyramine-^{125}I iii.10.

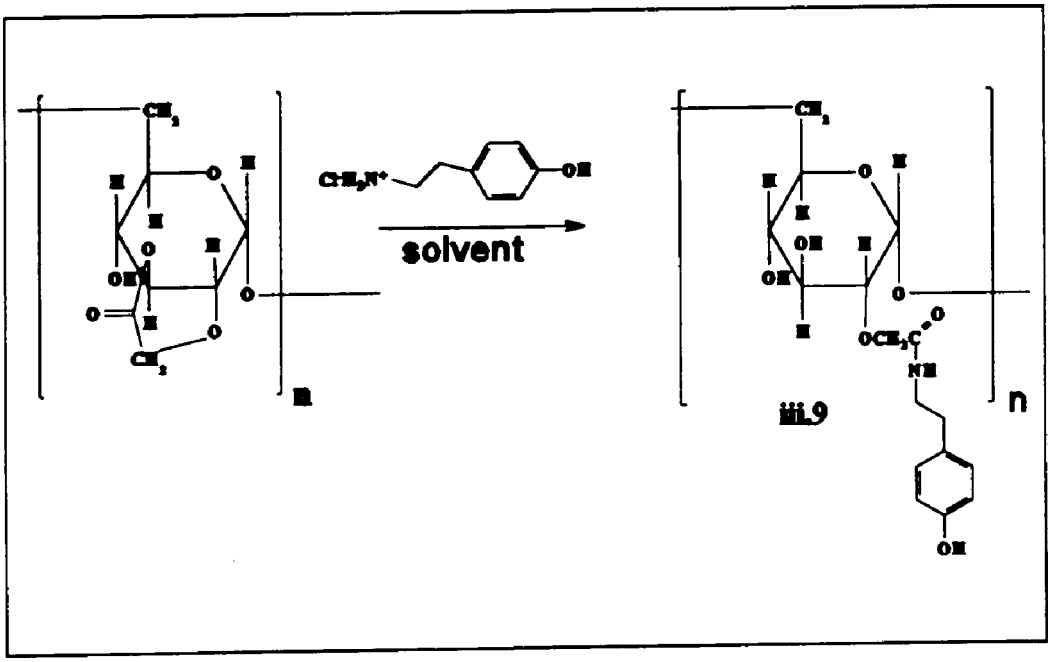


Figure III.9 Preparation of tyramine-CMDL conjugates iii.9

Tyramine was conjugated to CMDL via one of two methods. The tyramine was either coupled *in situ* during the formation of the lactone in toluene or via a separate coupling procedure in water using CMDL which had been isolated. In either case a very high load of tyramine was incorporated both having loads in the range of 200-220 tyramines per molecule of dextran **iii.9**. The reason such high loads were possible in the

very high solubility of CMD in water.

This high load and high solubility made for facile radioiodination of **iii.9** . As with the tyramine-PAD conjugates, the polymer was reacted with a 10 fold excess of $Na^{125}I$ in the presence of iodogen. Following quenching of the reaction with sodium bisulfite the dextran was purified via elution over a PD-10 column. A radioactive peak corresponding to the void volume of the column was seen, demonstrating the presence of a high molecular weight radioactive peak. The iodinated CMDL-tyramine **iii.10** (not shown) was presumed to have a load of iodine-125 in the range of 8-10 iodines per 40 kDa dextran. Unfortunately, no method was available to determine that value empirically.

A Note on Load of Radioiodine-125.

It is important to note that high loads (specific activities) of radioiodine are possible with iodine-125 while not being possible with iodine-131. The reason for this is that iodine-125 can be produced as a "carrier free" solution. This means that all the iodine present in the $Na^{125}I$ solution used to label the protein or polymer is iodine-125.[64]

$$\frac{dN}{dt} = \lambda N = A \qquad \text{eqn. III.1}$$

Equation III.1 describes the relationship between the number of molecules of a radionuclide N to the measurable activity A. The value of lambda is $\lambda = \cdot\frac{693}{T_{1/2}}$ which allows for a rapid conversion of activity to moles of radionuclide as long as the half life of the nuclide is known using equation III.2.

$$\text{moles} = T_{1/2} \times \left(\frac{\text{activity (counts/s)}}{\text{Avogadro's \# x 0.693}} \right) \qquad \text{eqn. III.2}$$

Using this equation 25 mCi (9.25×10^8 counts/sec.) of radioiodine-125, whose half life in seconds is 5.36×10^6 sec., has 1.18×10^{-8} moles of radioactive iodine. Since the material is carrier free this is the total amount of iodine present. This is comparable to the number of mole of protein found in 1.77 mg of IgG. This amount of 40kDa dextran has a mass of 400µg. These quantities are easy to manipulate in the laboratory. In comparison to iodine-131 this activity is quite high. Only 4% of all iodine in iodine-131 solutions is the desired nuclide making actual loads of 1 (one)^{131}I/IgG impossible.[65]

III.E ^{125}I-tyramine-CMDL-MAb 425 iii.11 preparation and Biological Evaluation.

III.E.1 Conjugation of iii.10 to MAb 425.

^{125}I-tyramine-CMDL **iii.10** was conjugated to MAb 425 in the presence of carbonyl diimidazole. No reaction had been performed to specifically open the lactone rings of the CMDL which were unreacted after the conjugation of the tyramine or during the radioiodination procedure. Even so, it is reasonable to expect that a large number of these remaining lactones were hydrolyzed during one of the two procedures. For this reason it could not be assumed that the dextran would still be activated after

radioiodination. CDI provided the most reasonable means for coupling of the dextran to the monoclonal antibody. CDI coupling, when effective, is quick and quantitative (from the standpoint of the two molecules being coupled) moreover it can activate both carboxylic acids as well as amines so it could functionalize both the IgG and the iodinated CMDL **iii.10**. Other coupling agents such as carbodiimides while suitable for coupling of non-radioactive materials simply are two slow to use with a 40-50mCi solution of iodinated polymer. Following conjugation the reaction mixture was purified on a PD-10 column to remove any coupling agent which might remain as well as on a Sephadex G-75 column to remove any unconjugated **iii.10**. The entire void volume (5 mL) of the G-75 column was collected as one fraction and used for binding experiments **iii.11**.

III.E.2 Binding of iii.11 to A1207 cells.

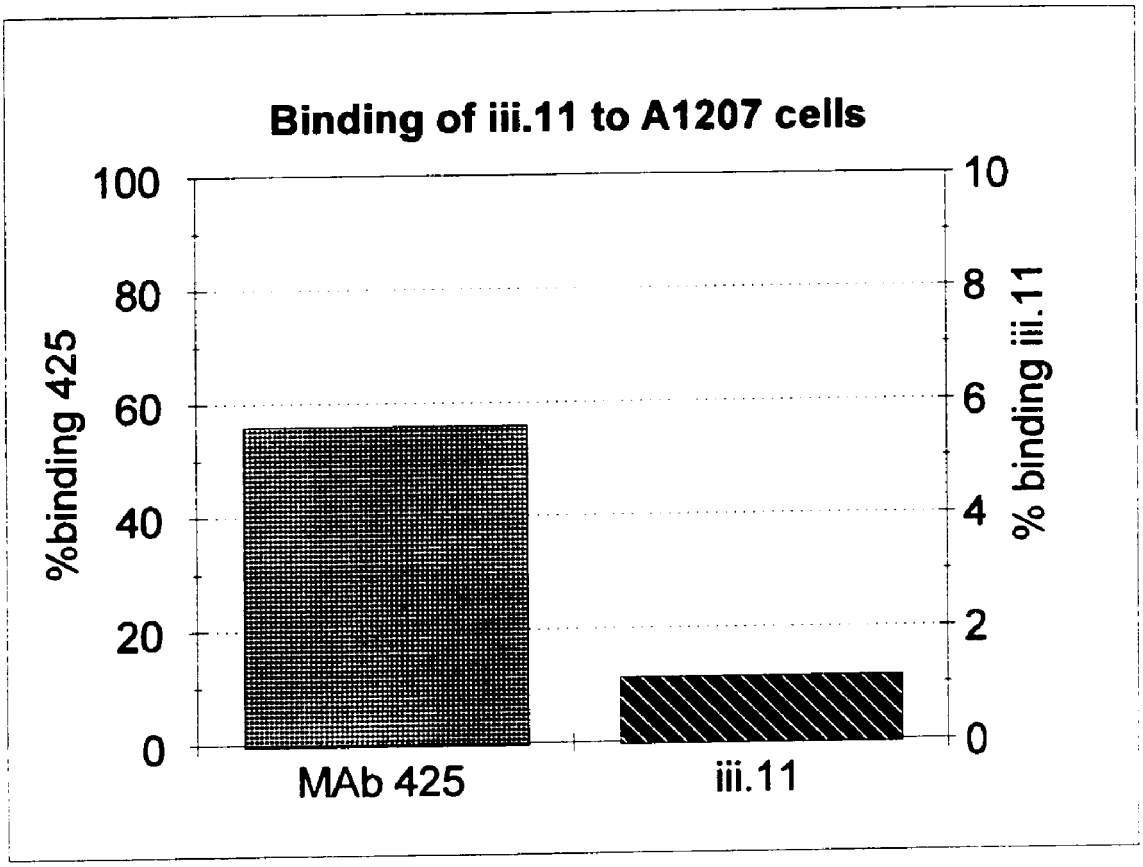


Figure III.10 Direct Binding of iii.11(^{125}I-tyramine-CMDL-MAb 425 conjugate) to A1207 cells as compared to ^{125}I-MAb 425 direct binding.

A binding study was performed in an identical manner to those done in section II with the PD-10 fraction of the tyraminyl-cellobiose labeled antibody. The results of the study are shown in figure II.10 The values for **iii.11** are the average of a minimum of six

repeats of the same experiment (S.D. +/- 0.1%). As can be noted the binding of this material to the A1207 cells is roughly identical to those radioimmunoconjugates formed from the conjugation of tyraminyl-cellobiose to MAb 425. This modest binding avidity could not justify further experiments with the **iii.11** label.

III.F Part III CONCLUSIONS

Dextrans have been synthesized and modified so to permit facile radioiodination. Gel exclusion studies seem to indicate that a high level of activity can be incorporated into the dextran backbone but no specific determinations of the activity were made.

Given its solubility and these ease with which nucleophiles can be attached to it, carboxymethyl dextran is a viable carrier for radioiodine. It is capable of carrying very high loads of tyramine and can be iodinated using standard protein methods. The hydrazide derivative of this molecule shows no non-specific binding to A1207 cells indicating that it would not be taken up into healthy tissue. Indeed recent work with liposomes has shown that some polymers can prevent uptake into macrophages by sterically blocking access to the liposome. Unfortunately, the MAb 425 conjugates of this polymer seem to suffer a similar fate as the tyraminyl-cellobiose conjugates in that the binding avidity of the protein seems to be greatly reduced. Such low avidity would not allow for a sufficient load of radioiodine to be delivered to tumor. This polymer may prove useful however with other antibodies.

III.G Experimental.

III.G.1 Preparation of Polyaldehyde Dextran (iii.1)

Polyaldehyde Dextran was prepared as previously described with minimal modification.[66] An example is as follows; 10.0 grams of 40 kDa dextran was exhaustively dialyzed (membrane cutoff = 12,000 Daltons) against distilled water. The dialysate was lyophilized to yield 5.2 grams of a fluffy white solid. 5.0 grams of this solid were dissolved in a solution of 6.4 grams $NaIO_4$ (30 mmol) and 1.0 gram NaCl (17mmol) in 500 mL of distilled H_2O. The reaction is allowed to proceed overnight at room temperature with stirring. The solution was reduced to 100 mL *in vacuo* and exhuastively dialyzed (membrane cutoff = 12,000 Daltons) against distilled H_2O. The dialysed material was reduced to 25 mL *in vacuo* and lyophilized to yield 2.6 grams of a fluffy white solid. IR 1727 cm^{-1}-Aldehyde content was determined by the method of Zhao, using hydroxylamine hydrochloride and back titrating liberated acid.[67] A typical preparation involved the production of between 5 and 15 grams of PAD with aldehyde content between 285 and 310 moles of -CHO per mole 40 kDa dextran.

III.G.2 Preparation of an Amino Dextran (iii.2)

200 milligrams of PAD (1.5 mmoles CHO) were dissolved in 50 mL of 8mM phosphate buffer pH= 7.4. To this solution was added 3.0 grams NH_4OAc (32 mmoles). The solution was allowed to stir and equilibrate for 6 hours at RT. Following this period 3 x 75 mg portions of $NaBH_4$ were added and the solution stirred for 1 hour. The solution was

dialyzed exhaustively (membrane cutoff = 12,000 daltons) against distilled H_2O. The contents of the dialysis tubing were placed in a 100 mL round bottom flask and reduced *in vacuo* to 15 mL and then lyophilized to yield 80 milligrams of a white solid. Analysis of amine content; C 42.6% H 7.80%, N 4.04% (d.s. = 1.8)

III.G.3 Preparation of Carboxymethyl Dextran (iii.3).

A solution of 10 grams of dextran (40 kDa) in 70 mL of water was prepared. This was added to a suspension of 15 grams of NaOH in 15 mL of distilled water. A separate suspension of 22.0 grams 2-chloroacetic acid in 25% sodium carbonate was prepared. The dextran/NaOH solution was added to this suspension and allowed to stir with heating for 1 hour. The NaOH was neutralized and the pH was adjusted to 4. The sodium salt of CMD was precipitated by slow addition of 900 mL of a 3:2 mixture of methanol and 2-propanol. The resulting precipitate was redissolved in minimal water and dialyzed against H_2O. The dialyzed material was lyophilized and a small amount of resulting fluffy material was used to make IR measurements. This material showed a distinct absorption band at 1600 cm^{-1}. The remaining material was redissolved in 100 mL of 1% HCl. After allowing the solution to stir for 1 hour the material was again dialyzed exhaustively and then lyophilized. The resulting white fluffy solid was anaylzed by IR and no trace of the 1600 cm^{-1} band was present but a new band at 1734 cm^{-1} had been formed. The load was determined by titration analysis with NaOH to be 0.56 moles COOH / mole glucose (see figure B.8). Other preparations had a higher load up to 1.2 moles COOH/ mole glucose.

III.G.4 Lactonization of CMD to form CMDL (iii.4).

Carboxymethyldextran lactone was prepared from carboyxmethyldextran by the method of Heindel.[63] Briefly, 1.0 gram of CMD is placed in a 250 mL RBF followed by 100 mL of mixed xylenes (b.p. 140 °C) The flask is equipped with a stirring bar and a reflux condeser. The suspension of CMD is stirred at reflux for 15 hours. Following the reflux the flask is allowed to cool and the xylene is removed *in vacuo*. The solid material was then analyzed by IR showing a new abosorption peak at 1741 cm^{-1} **(iii.4)**

III.G.5 Preparation of hydrazide dextran $CMDNHNH_2$ via CDI.

A quantity of 0.101 grams of carboxymethyldextran (d.s. = 0.5) and 1.67 g CDI were placed in a 4 dram vial to which was added 5.0 mL anhydrous DMSO. The vial was capped and shaken and was then heated on a steam bath until all solid was dissolved. This entire contents of the vial was added dropwise with stirring to a solution of 600 mg $NH_2NH_3^+Cl^-$ in 40 mL H_2O. The reaction volume was reduced and dialyzed against H_2O exhaustively over 3 days.

III.G.6 Preparation of PAD-tyramine conjugates (iii.7)

A suspension of 1.0 grams (6.25 mmoles) polyaldehyde dextran and 0.5 g tryamine·HCl (3.0 mmoles) in 50 mL 20mM phosphate buffer, pH = 7.4 was prepared. The mixture was allowed to stir for 15 mintes until the polymer was thoroughly swollen. To this suspension was added 0.226 (3.5 mmoles) $NaCNBH_3$ and the mixture was again allowed to stir at ambient temperature. As the reaction progressed the suspension was approaching a uniform solution (after 10 minutes) but within an additional 15 minutes a white

precipitate forms. The reaction was diluted to 200 mL with 22 mM PBS. The reaction was allowed to proceed for approximately one hour. The pH was raised above 9 and the volume reduced slightly *in vacuo* causing a precipitate to form again. The solid was filtered off, dissolved in acetate buffer pH=3 (50 mL) and the acidic solution dialyzed against acetate buffer (ionic strength = 0.03, pH = 4.75). The acetate buffer was exchanged with H_2O and again dialyzed . The dialysate was lypohilized to a fluffy white powder. This material was analyzed by elemental analysis and UV absorption analysis using the method of Zhao.[68]

Combustion analysis; C 41.19%, H 6.05%, N 1.49%, by Zhao's method the load is 0.2 moles tyramine /mole glucose, by UV calculation the load is 0.24 moles tyramine / mole glucose or 50 and 60 moles tyramine / mole dextran respectively.

III.G.7 Preparation of FITC labeled dextran hydrazide (iii.6).

Hydrazide dextran (**iii.5**, 100 mg 2 micromoles) was added to a 4 dram vial followed by 8.0 ml of a carbonate buffer (pH = 9.1) to which was added 10 mg FITC (25 micromoles). The reaction proceeded overnight after which the entire reaction mixture was divided and added to two Centricon 10 centrifugal filtration devices. The solution was centrifuged at 1500 rpm for 2 hours. A subsequent 2 mL of distilled water were added to the pre-filter portion of the Centricon and the devices centrifuged until the volume was below 0.5 mL . The wash step was repeated twice after which the pre-filter volume was brought to one (1) mL in each of the two Centricon devices. The pre-filter solution was removed and

lyophilized into a bright yellow solid (**iii.6**).

III.G.8 Conjugation of tyramine to CMDL (iii.9).

Into a 100 ml Erlenmeyer flask with a ground glass joint were added 100 mg CMDL (iii.4, 5×10^{-4} moles lactone) and 30 mL distilled water. To this was added 205 mg tyramine (1.47×10^{-3}moles), and the reaction was allowed to stir for 36 hours at which time all solid was in solution. The volume was reduced to 10 mL and dialyzed exhuastively against distilled water. The dialyzed solution was lyophilized resulting in 200 mg of a fluffy white solid. The solid was analyzed by nitrogen analysis to determine load. iii.9 combustion analysis C:49.7% H:6.61% N:3.76%. Using the method of Zhao, the load was determined to be 0.91 moles tyramine/mole CMDL or about 225 moles tyramine /mole 40 kDa dextran polymer.

III.G.9 Radioiodination of (iii.9) CMDL-tyramine to form iii.10.

In a 1.5 mL vial was place 375 µL of a 3 µM solution of iii..9 (1.15×10^{-9} mole tyramine-CMDL) followed by 25 mCi of $Na^{125}I$ (1.2×10^{-8} moles $^{125}I^-$) and the mixture was allowed to react for 25 minutes. The reaction mixture was quenched by addition of 100 µL of 0.1% sodium bisulfite. The reaction mixture was then applied to a PD-10 column. Fractions were collected and those containing > 60,000 cpm were pooled. The pooled fractions were concentrated on a Centricon 10 centrifugal filtration device. The material was then used immediately for conjugation to MAb 425.

III.G.10 Conjugation of iii.10(^{125}I-tyraminyl-CMDL) to MAb 425; formation of iii.11

A fraction (1/10th) of the concentrated solution of iii.10 above was added to 15 μg of MAb 425 (10.0 mg/mL IgG, 15μg = 1 x 10^{-10} moles IgG) followed by 10 additions of 20μL of 1x10^{-4} M carbonyl diimidazole in acetone. The CDI was added at 10 minute intervals and the reaction was stopped by addition to a PD-10 column 10 minutes after the final addition. The conjugate was purified on the PD-10 column and the high activity fractions were collected and individually tested for their ability to bind A1207 cells.

III.G.11Binding of iii.11 PD-10 fractions to A1207 cells.

Binding studies were performed identically to those described in section II.G.7.

III.G.12 Binding of FITC-dextrans to A1207 cells.

Stock solutions of 3 mg/mL CMDNHNH-FITC , 3mg/mL 19 kDa FITC-dextran and 3.8 mg/mL 39 kDa FITC-dextran in phosphate buffer were prepared. To each of 20 wells were added 1x 10^5 A1207 cells in PBS. Dilutions of each of the three FITC-dextran stock solutions of 1:10, 1:20, 1:40, 1:80 and 1:160 in phosphate buffer were prepared. For each of the FITC-dextrans 20 μL of each of the dilutions (not the stock solution) were added to one of the microtiter wells containing the A1207 cells. Three of the remaining wells had 50 μL of FITC-MAb 425 added to them and three had 50 μL of unlabeled 425

added to them. The cells were allowed to incubate at 4°C for 20 minutes. The cells were centrifuged and washed with washing/blocking buffer (see Appendix A for a description of buffers). The wash step was repeated three times. The cells were then taken into FACS buffer and analyzed by FACS data shown in discussion section.

PART IV

USE OF

N-SUCCINIMIDYL *p*- TRI-(N-BUTYL STANNYL) BENZOATE TO DETERMINE THE EFFECT OF COVALENT MODIFICATION OF MAb 425 ON BINDING TO A1207 CELLS

IV.A Background and Rationale

The previous sections of this document discussed studies involving small (tyraminyl-cellobiose) and large (dextran derivatives) prosthetic carriers of iodine-125 as labelling agents for attachment to anti-EGFr monoclonal antibody 425. Those studies demonstrated that using various crosslinking reagents Mab 425 could be prosthetically labelled to a high specific activity but not without a significant loss of binding avidity.

An inherent problem in the use of homo-bifunctional crosslinking reagents is their lack of specific conjugation chemistry. Indiscriminate reactions with nucleophiles, especially primary amino groups, can lead to crosslinking and possible dimerization or oligomerization of protein moieties or polymeric carrier molecules. The crosslinking agents in these studies, either cyanuric chloride or carbonyl diimidazole, were used in molar excess of IgG in order to obtain a protein with high specific activity of radioiodine. The combination of excess agent as well as non-productive bond formations could be a possible explanation for the loss of binding avidity. Another cause for loss of binding could be the presence of highly reactive amino acids within the binding pocket of Mab 425 which are being covalently modified by the coupling agent. Loss of this critical amino acid leads to a proportionate loss of antibody binding.

As discussed in section I.C.2, iodine-125 has been used extensively to study the biodistribution of a variety of proteins *in vitro* and *in vivo*. A wide range of prosthetic labels have been developed to assist in the attachment of radioiodine to these proteins. Many of

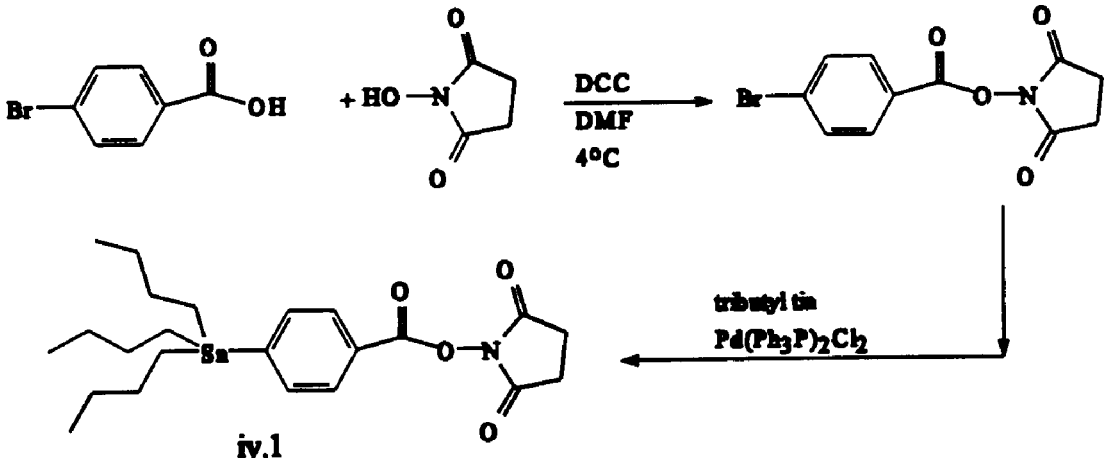
these labels are modified N-succinimidyl benzoate esters. An early example of this type of label is the "Bolton-Hunter" reagent which has gained wide popularity and is now commercially available. Such labels do not suffer from the disadvantages of the homo-bifunctional crosslinking reagents. They are incapable of forming covalent protein dimers and since their coupling to protein occurs stoichiometrically a minimal excess is all that is required for high levels of radioiodine to be incorporated into a target protein. We studied the effect of using such a label to produce a high specific activity Mab 425 on the binding of that radioactive antibody to A1207 cell lines.

Given the wide choice of labels available in the literature, we chose to use *p*-(tri-n-butyl stannyl)benzoic acid *N*-succinimidyl ester developed by Wilbur and co-workers.[68] This label was developed for use in radioimmunotherapy to deliver iodine-131 or iodine 125. Radioiodine is attached via an *ipso* substitution of I^+ onto 4 position of the benzene ring displacing the tributyl tin group. There are two advantages of using this label over many of the others known in the literature. First the presence of the tributyl tin group in the unlabeled compound but not in the labeled compound allows for easy separation either by conventional chromatography or by HPLC. Secondly the compound is labeled at the 4 position instead of the 3 or 5 position and it is not ortho to a phenolic -OH. This makes the label far less susceptible to any dehalogenase enzymes that may be present in the *in vivo* applications.

IV.B Synthesis and Conjugation of the Wilbur Label.

IV.B.1 Preparation of the Wilbur Label iv.1.

The tributylstannyl based label was produced as described by Wilbur and co-workers with the single exception that a needless protection-deprotection step was eliminated. The original synthesis incorporates the tributyl tin group into ethyl p-bromobenzoate which is subsequently saponified to the benzoic acid derivative. The acid moiety is then activated by formation of the N-succinimidyl ester. In our case, the tributyl tin was incorporated directly into N-succinimidyl p bromobenzoate and subsequently purified (See figure IV.1). The purified label had identical physical characteristics to those reported by Wilbur as well as matching the published TLC and NMR data (see figures B.12 and B.13).[68]



iv.1

Figure IV.1 Synthesis of the Wilbur label.

IV.B.2 Radioiodination of iv.1.

The method of iodinating was tested with non-radioactive NaI prior to use of any radioactive material. Reaction **iv.1** of with NaI in a solution of Iodogen™ in ethyl acetate formed the cold labelled compound **iv.2a** as evidenced by monitoring the reaction by TLC using 3:2 hexane:ethyl acetate as a mobile phase. This method was then used to label **iv.1**

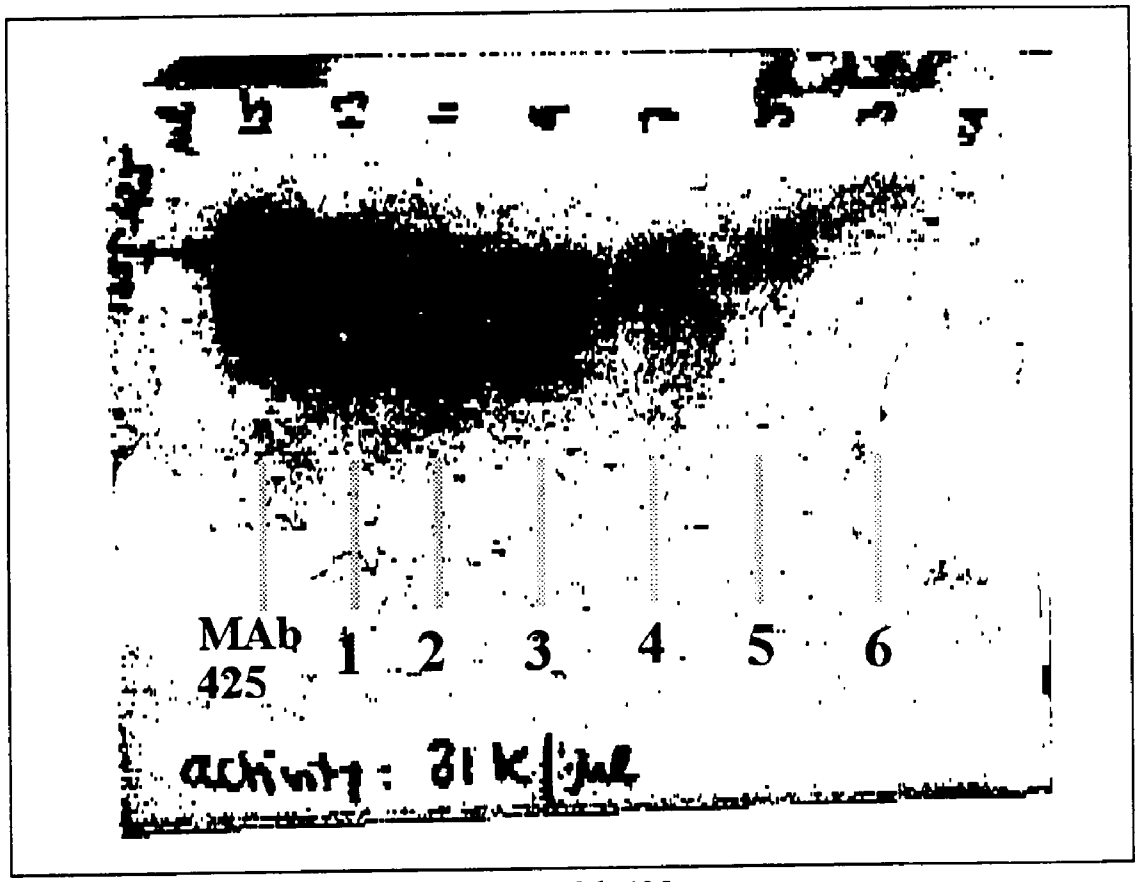


Figure IV.2 7.5% SDS- PAGE of **iv.3** Mab 425 construct.
Lane 1 contains direct labeled ^{125}I- Mab 425
Lanes 2-7 contain serially diluted 1:2^{n} **iv.3**
with the highest activity being 31,000 cpm

with iodine-125. The hot labeled material was purified by standard chromatography on silica gel using 3:2 hexane:ethyl acetate. After elution of the unlabeled material, any free alkyl tin species fractions were collected and then analyzed by gamma scintillation. The three fractions containing the most activity were pooled and used to prosthetically label monoclonal antibody 425. Identification of the radiolabel as **iv.2b** was achieved through the use of ^{125}I-TLC using either a "cut and count" method or autoradiography.

IV.B.3 Conjugation of IV.2b to MAb 425

Conjugation to Mab 425 was achieved through use of a bi-phasic reaction between a solution of IgG in phosphate buffered saline and iv.2b still in an ethyl acetate/hexane organic phase. Purification of the radioiodinated antibody was done by ultrafiltration of the aqueous layer (following evaporation of residual organic solvent) through a Centricon 100 filtering device (nominal molecular weight cutoff of 100 kDa). The hot antibody was identified by migration on a 7.51% SDS-PAGE electrophoresis gel (Figure IV.2) showing the protein band to co-migrate with direct labeled Mab 425.

IV.C Binding and Uptake Studies of iv.3 into A1207 EGFr(+) Cells

IV.C.1 Binding of iv.3 to A1207 and SW707 Cells

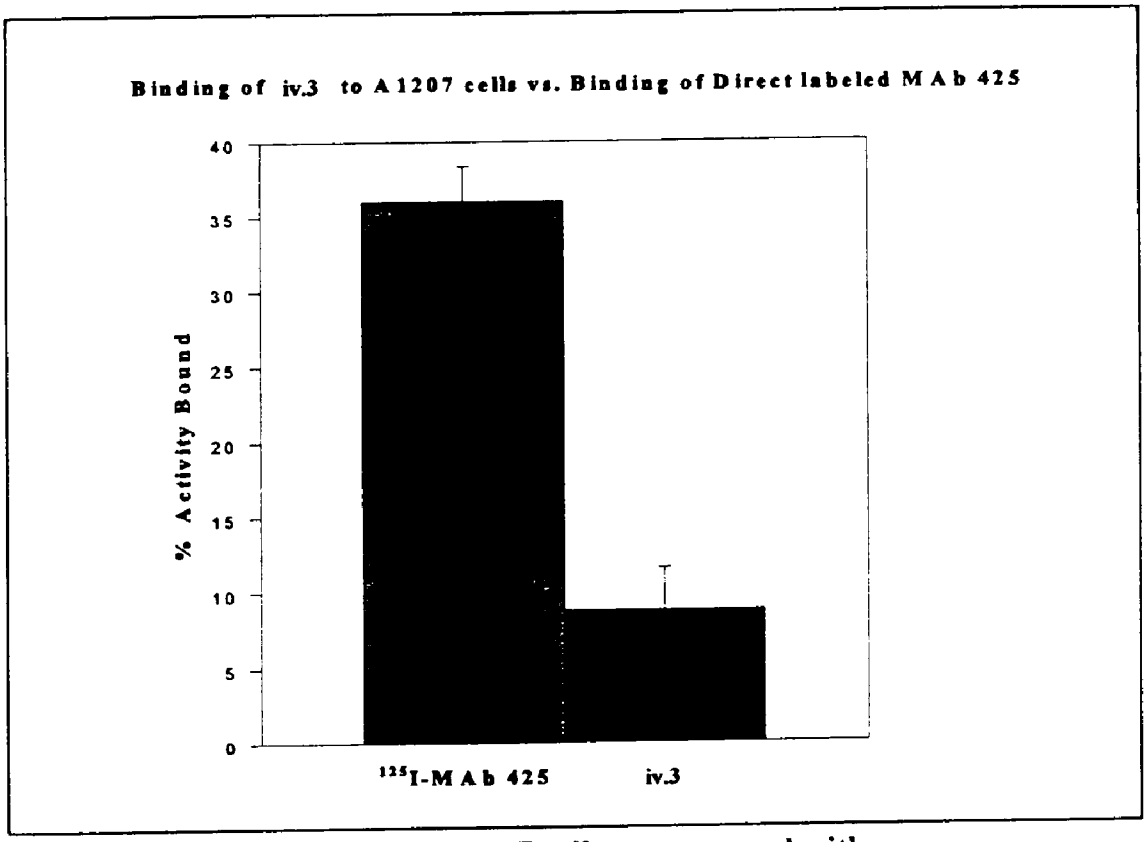


Figure IV.3 Binding of iv.3 to A1207 cells as compared with binding of direct labeled ^{125}I-MAb 425

Three experiments were run with **iv.3** in order to determine its characteristics. The first experiment was a binding assay against the A1207 and SW707 cell line with results compared to those obtained from direct labeled Mab 425. The results of that study are shown in figure IV.3.

As can be seen the direct labeled 425 binds at about 60% of the added counts while

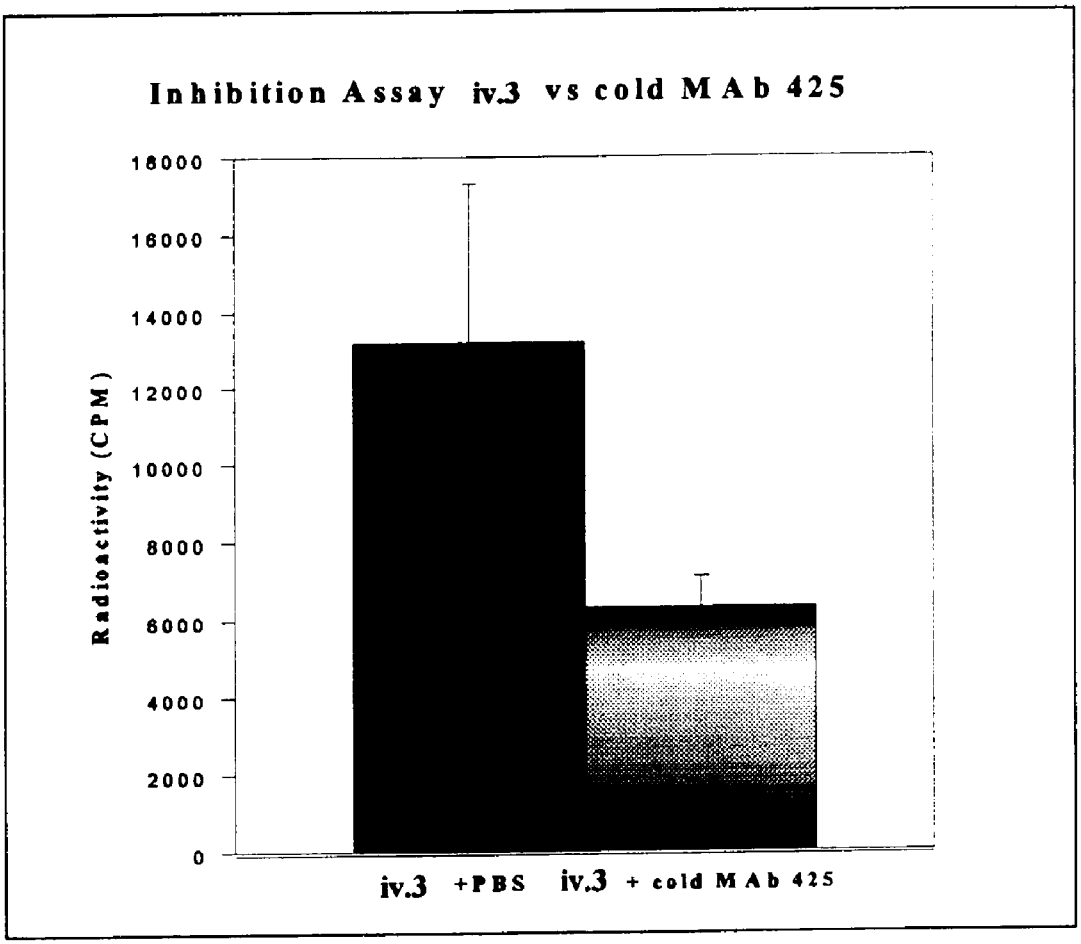


Figure IV.4 Competition Assay iv.3 vs cold MAb 425

the binding of iv.3 is reduced to less than 20% . Neither the direct labeled 425 or **iv.3** bind significantly to the EGFr(-) SW707 cell lines. A further comparison was perforomed with

iv.3 to guarantee that the binding which was observed was specific. A competition assay was run between iv.3 and cold MAb 425. An excess of cold MAb 425 was added to 500,000 cells followed by 34,000 cpm of iv.3. The presence of the cold MAb 425 suppressed 50% of the binding of iv.3 to the target EGFr(+) cells. This is indicative of specfic binding (see Figure IV.4).

IV.C.2 Internalization of iv.3 into A1207 cells

As was done with the tyraminyl-cellobiose conjugate ii.3a the ability of iv.3 to internalize into A1207 cells was studied. To this end 1 million cpm of iv.3 was added to several petri dishes containing 1 million cells each and the iv.3 radioimmunoconjugate was allowed to incubate with these cells over varying time point (see figure IV.5). The net result was that no significant internalization was observed.

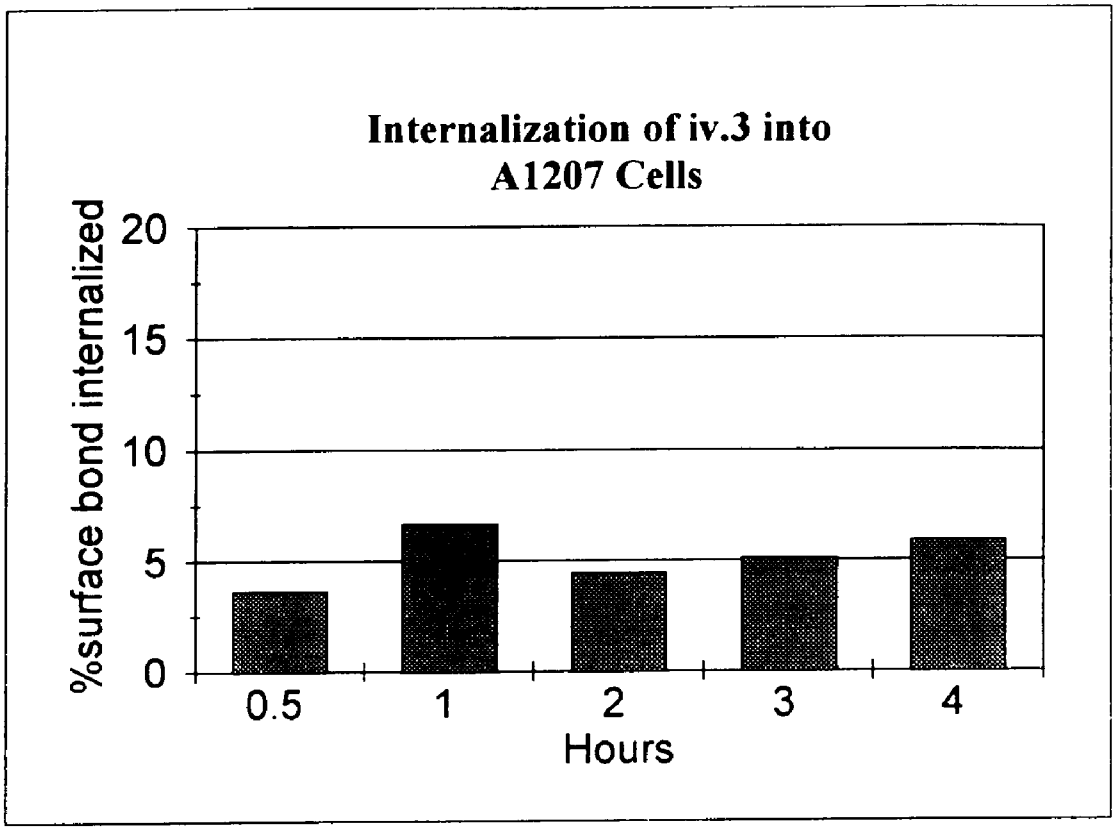


Figure IV.5 Internalization of iv.3 into A1207 cells.

IV.D Part IV Conclusions

The experiments discussed herein demonstrate that while the MAb 425 IgG can be radiolabeled using electrophilic protheses such as the Wilbur label such conjugation is detrimental to the antibody's ability to bind EGFr(+) cells. The Wilbur label represents one of the best possible electrophilic attachments possible, one in which the ratio of label/antibody can be kept at a minimum while still achieving high specific activity. The failure of this label to produce a radioimmunoconjugate with favorable binding properties is surprising and speaks to the limitations of using antibody for such conjugations. It may very well be that MAb 425 has a reactive amino group within its binding pocket and that covalent modifications by electrophiles inevitably react with that amino acid first. This results in a pronounced loss of binding avidity.

IV.E Experimental

IV.E.1 Synthesis of N-succinimidyl *p*-bromobenzoate

The N-hydroxysuccinimidyl ester of *p*-bromobenzoic acid was prepared via a carbodiimide coupling reagent. To a 125 mL Erlenmeyer flask having a ground glass joint and fitted with a stirring bar was added 10.0 grams of p-bromobenzoic acid (50 mmoles). The flask was sealed with a neoprene septa and purged with nitrogen for 30 minutes. Following the nitrogen purge 80 mL of anhydrous DMF was added to the flask with a cannula needle. To an 8 dram vial containing 11.1 grams (50.0 mmole) of dicyclohexyl carbodiimide (DCC) was added ,via syringe, 10 mL of anhydrous DMF. The vial was shaken until all the DCC was in solution. Both the 125 mL Erlenmeyer containing the bromobenzoic acid solution and the 8 dram vial containing the DCC solution were chilled in ice water to 0°C. The DCC/DMF solution was removed from the flask via a syringe and added in its entirety to the bromobenzoic acid solution. The reaction was allowed to proceed in a nitrogen atmosphere at 4°C for 24 hours. After 24 hours a precipitate had formed. The precipitate was filtered off and washed with 3x10 ml portions of anhydrous DMF. The combined DMF supernatant and washes were reduced *in vacuo* to a light brown gum. The gum was triturated with anhydrous diethyl ether to produce 13.5 grams of an off white crystalline solid. The off white solid was suspended and stirred in boiling 100% EtOH and subsequently filtered. The resulting white solid was dried *in vacuo* with heat (60°C) to yield a final mass of B.1 of 9.13 grams. TLC (3:2 hexane/EtOAc Rf = 0.44; 100%

hexane Rf = 0) ^{1}H NMR ;δ 8.0 ppm (AB quartet , 4p), 3.0 ppm (s, 4 p).(see figure B.10)

IV.E.2 Synthesis of N-succinimidyl p-iodobenzoate

N-succinimidyl p-iodobenzoate was synthesized via DCC coupling in a manner identical to the synthesis of the bromo analog above. ^{1}NMR δ ppm; 7.9 (AB quartet, 4p), 3.0 (s, 4p) (see figure B.11)

IV.E.3 Synthesis of N-succinimidyl p-(tri-n-butyl stannyl) benzoate.

The synthesis of N-succinimidyl p-(tri-n-butyl stannyl) benzoate was accomplished via the method of Wilbur with minor modification.[68] To a three neck round bottom flask was added 6.8 grams (23 mmoles) of the p-bromobenzoate from above. The flask was equipped with a stirring bar, a reflux condenser and two neoprene septa. The flask was flushed with nitrogen for 15 minutes after which time the reflux condenser was fitted with a gas inlet and the entire apparatus placed under a head of nitrogen. Anhydrous toluene (100 mL) was added via a cannula and the mixture stirred for 10 minutes. The mixture was then heated to reflux at which time 25 mL (45 mmoles) of *bis*-tributyl tin was added via syringe. A suspension of 30 mg of $(\phi_3P)_4Pd$ in 5 mL of toluene was added via syringe. The addition of the palladium catalyst resulted in the formation of a clear yellow solution. The reaction was allowed to proceed at reflux for 4.5 hours after which time a brown precipitate of Pd^0 formed. The heat source was then removed and the flask allowed to cool under nitrogen. The flask was then attached to a Büchi rotary evaporator and the reaction volume was reduced *in vacuo* to 75 mL. The evacuated flask was refilled with

nitrogen removed from the evaporator and sealed with a third septum. An aliquot was removed and analyzed by TLC (3:2 hexane/EtOAc) which showed 5 spots under UV Rf's: 0.15, .7, .85, and 1. The entire reaction mixture was charged to a 40x5 cm flash chromatography column packed under nitrogen with Merck high grade silica gel suspended in 100% hexanes. Approximately 1 liter of 100% hexanes was passed over the column to wash off the unreacted *bis*-tributyl tin (Rf in 3:2 hexane:EtOAc ~1). The column was then eluted with 3:2 hexanes:ethyl acetate under Flash conditions. Solvent (300 mL) was allowed to pass over the column after which time TLC analysis of the eluent began to show a UV active spot with an Rf = 0.7 in 3:2 (hexane:EtOAc). Fractions (5 mL) were collected until the TLC analysis no longer showed one spot at Rf= 0.7. All fractions showing a single spot were pooled (total volume ~ 350 mL) and reduced in vacuo to form 4.72 grams of a yellow oil (**iv.1**). ^{1}H NMR δ(ppm); 7.9 (AB quartet, 4p), 2.9 (s, 4p), 1.3 (m, 8p), 0.7 (t, 9p). ^{13}C NMR. δ(ppm); 169,162,153,137,129,125,28,27,25,14,10.

IV.E. 4 Cold labelling of N-succinimidyl p-(tri-n-butylstannyl) benzoate iv.1.

To an eight (8) dram vial was added 5 milligrams of iv.1 above. The oil was dissolved in 10 mL of Ethyl Acetate. To this was added 2 milligrams of Iodogen™ followed immediately by 1 mL of a 5 mg/ mL solution of NaI (cold) in H_2O. The vial was then shaken and the reaction allowed to proceed at room temperature. A color change was immediately apparent in the aqueous layer which had become yellow/brown characteristic

of iodine (I_2) solutions. The reaction was tracked for 2 hours by removing 5 μL aliquots from the organic layer and spotting 1-3 μL on a silica gel TLC plate. The plate was also spotted in a separate lane with N-succinimidyl p-iodobenzoate B.2 as a migration marker. A spot which co-migrated on the plate with the cold iodo derivative at Rf= 0.45 in 3:2 hexane /EtOAc was immediately apparent. Another spot at Rf = 0.7 was also present which was consistent with the unreacted **iv.1** The intensity of the spot at Rf = 0.45 grew with disappearance of the spot at Rf = 0.7 until by 1.5 hours into the reaction all of the starting material was consumed and only the material co-migrating with the iodo-benzoate derivative was present in the organic layer.

IV.E.5 Radioiodination of p-tributyl tin benzoate N-succinimidyl ester.

iv.1 12 mg , 28 umoles was dissolved in 2 mL of ethyl acetate (14 mM). This solution was diluted to 7 uM in EtOAc. A 2 mL (14 nanomoles iv.1) portion of this solution was placed in a test tube which had previously been coated with 10 milligrams of Iodogen™. To this tube was added 25 mCi of $Na^{125}I$ (corresponds to approximately 11 nanomoles of $^{125}I^-$) in approximately 100 μL of NaOH , pH = 8.5 to 10. The reaction was allowed to proceed for 10 minutes after which the entire reaction mixture was charged to a 30 X 5 cm column which had been previously packed with Silica gel in hexane. The column was primed by allowing 10 mL of hexane to pass over the column and then eluted with 3:2 hexane:EtOAc. A 20 mL fraction was collected to wash off any unreacted $Na^{125}I$, $^{125}I^-$, unlabeled iv.1, and any free alkyl tin species. Following this wash 4 mL fractions were

collected to a total of 36 mL. A 20 μL aliquot was removed from each of the ten fractions (1 from the 20 mL wash and 9, 4mL fractions) and the amount of radioactivity determined by gamma scintillation. It was determined that fractions 1-4 contained the majority of the radioiodinated material and TLC's were run of each of these. Three separate TLC experiments were run. First a sample of fraction #2 was applied to a plastic backed silica gel plate and the plate run in 3:2 hexane:EtOAc. The plate was cut into 8, 1 cm slices which were subsequently analyzed for radioiodine-125 by gamma scintillation. Following development in 3:2 hexane:EtOAc the plate was autoradiographed. Since only the correctly labeled active ester derivative was capable of attachment to IgG fractions 1-4 were combined and a TLC of this combination was run as previously done on a glass backed plate. Following the combination of fractions 1-4 the organic solvent was evaporated to one fourth of its original volume to a final volume of 4 mL.

IV.E.6 Conjugation of iv.2 to Mab 425

The conjugation of iv.2 to anti-epidermal growth factor monoclonal antibody 425 was accomplished in a bi-phasic system. 100 μL of a 5 mg/mL solution of Mab 425 was added to 1.9 mL of PBS (pH = 7.4) making a solution whose final concentration of IgG was 250 ug/mL. The entire contents of this material was placed in a 10 mL test tube. To this tube was added 2.0 mL of the solution of iv.2 from section from above. The tube was capped and thoroughly shaken. The tube was then attached to a vertical spinning device and allowed to tumble for 30 minutes. The aqueous layer was removed from the

tube and any remaining organic was allowed to evaporate (approximately 35 minutes). The aqueous solution was then placed in a Centricon™ 10 ultrafiltration device (nominal MW cutoff = 10 kDa) and the solution centrifuged at 2200 rpm until the volume was less than 200 μL. The solution was diluted to approximately 2 mL and ultrafiltered a second time. The volume was adjusted to 1mL. A 10 μL aliquot of this solution was drawn and placed in a gamma scintillator and it was found to have an activity of 62,000 cpm/ μL. A 50 μL aliquot was drawn and diluted to 100 μL. A 7.5% SDS-PAGE of this solution was run using 13,11,9,7,5, and 3 μL of the solution in each of 6 wells respectively with a sample of directly labeled ^{125}I-MAb 425 as a control. The gel is shown in figure IV.2 The result being that the sample of the prosthetically labeled 425 co-migrates with the directly lab labeled 425.

IV.E.7 Binding of iv.3 to A1207 glial and SW707 colorectal cell lines

A1207 and SW707 cells were harvested from a confluent culture. Both cell lines were centrifuged to form 1-2 gram cell pellets and then resuspended in PBS containing 0.1% NaN_3. The cells were washed twice with the PBS/azide buffer to a final volume of $5x10^6$ cells /mL. A 96 well microtiter plate was prepared as follows 6 wells of column **3** and 6 wells of column **4** had 50 μL of the $5x10^6$ cells/mL suspension of A1207 cells placed in each. Into each of 4 of the wells of column **7** and 4 of the wells in column **8** was placed 50 μL of the 5×10^6 cells/mL suspension of SW707 cells. Prior to the experiment both direct labeled Mab 425 and iv.3 were diluted to 400 cpm/ μL. To each of the wells in

columns **3** and **7** was placed 40,000 cpm of direct labeled Mab 425 (specific activity 0.8 moles ^{125}I / mole IgG) in 100 µL of PBS and to each of the wells in columns **4** and **8** was placed 40,000 cpm of iv.3 The microtiter plate was mechanically shaken for 90 minutes at room temperature. The microtiter plate was then centrifuged at 1200 rpm for 7 minutes to pellet the cells. The supernatant was removed from the cells. 100 µL of 0.2% NaN_3 in PBS was added to each well that contained a cell pellet. The cells were resuspended in the PBS/azide buffer and re-pelleted as before with centrifugation. The PBS/azide solution was removed and the process repeated. Following removal of the second PBS/azide supernatant the cells were physically and completely removed from individual wells by a cotton swab (one clean swab/ well). The swabs were placed in sample holders and counted in a gamma scintillator to determine the amount of activity bound to each cell sample.

IV.E.8 Uptake of iv.3 into A1207 cells.

Six petri dishes were seeded with $1.25x10^6$ A1207 human glioma cells. At 21,4,3,2,1, and 0.5 hours before the experiment was to end, 50 µL of the undiluted solution iv.3 (approximately $3.1x10^6$ cpm) were added to one of the petri dishes. At the termination of the experiment all six dishes were placed in a refrigerator at 4°C for one hour to halt internalization. Each petri dish was then stripped of its cells with PBS and mild shaking. Note: no trypsin or EDTA buffer was required. The cells were transferred to a plastic 15

mL centrifuge tube and were centrifuged for 6 minutes at 1200 rpm for 10 minutes. The supernatant was removed completely and set aside. The cells were subsequently resuspended in 5 mL of a 25mM solution of HOAc in 300 mM $NaCl_{(aq)}$ to remove any activity bound to the surface of the cells. The cells were centrifuged again a 1200 rpm for 10 minutes, the acid wash was removed and set aside and the pellet was re-suspended in 1 mL of homogenizing buffer. After suspension in the homogenizing buffer the cells were frozen at -85°C to completely rupture the cell. The cells were then defrosted and centrifuged at 3000 rpm for 5 minutes. The homogenizing buffer was removed and another 1 mL of fresh homogenizing buffer added to the pellet. The pellet was resuspended and centrifuged at 3000 rpm for 5 minutes. The second 1 mL of homogenizing buffer was removed and combined with the first. The entire process was repeated for each of the six petri dishes. Thus for each time point there are three samples which generate data points; supernatant (added activity), acid wash (bound activity) and homogenizing buffer (internalized activity).

IV.E.9 Competitive binding to A421 human glioma cells iv.3 vs. cold Mab 425.

A431 cells were harvested by trypsinization of confluent culture and a pellet was prepared by centrifugation. The pellet was resuspended in 5% γ- globulin free horse serum in PBS (RIA buffer). The pellet was washed twice with RIA buffer to remove any leftover growth media and brought to a final concentration of $5x10^6$ cells/mL. A 96 well

round bottom microtiter plate was used to support the cells for the assay. To each of 6 wells in columns 3 and 4 was added 50 μL of the A431 cell suspension. The entire plate was centrifuged at 2000 rpm for 10 minutes at RT. The supernatant was removed from the cells and the pellet subsequently broken apart. The radioactively labeled antibody **iv.3** was diluted 1:2 with either PBS or a 5 mg/mL solution of cold Mab 425. A 50 μL aliquot of the PBS diluted sample was added to each of the cell containing wells of column 3 and likewise 50uL aliquot of the cold 425 diluted sample was added to each of the cell containing wells of column 4. The plate was allowed to incubate with shaking for 90 minutes. The plate was then centrifuged at 2000 rpm for 10 minutes and the supernatant removed. The cells were washed twice in this manner with RIA buffer. Following removal of the second RIA buffer supernatant the cells were individually removed with cotton swabs and the swabs counted for gamma activity.

PART V

ADDITIONAL STUDIES

V. Additional Studies

Two additional studies were undertaken which relate to ^{125}I-RAIT of human glioma tumors using MAb 425. The first study was an examination of the uptake of ^{125}I-deoxyuridine into the cytoplasm and nucleus of A1207 cultured human cell lines. The second study was to determine what effect (if any) external beam irradiation had on

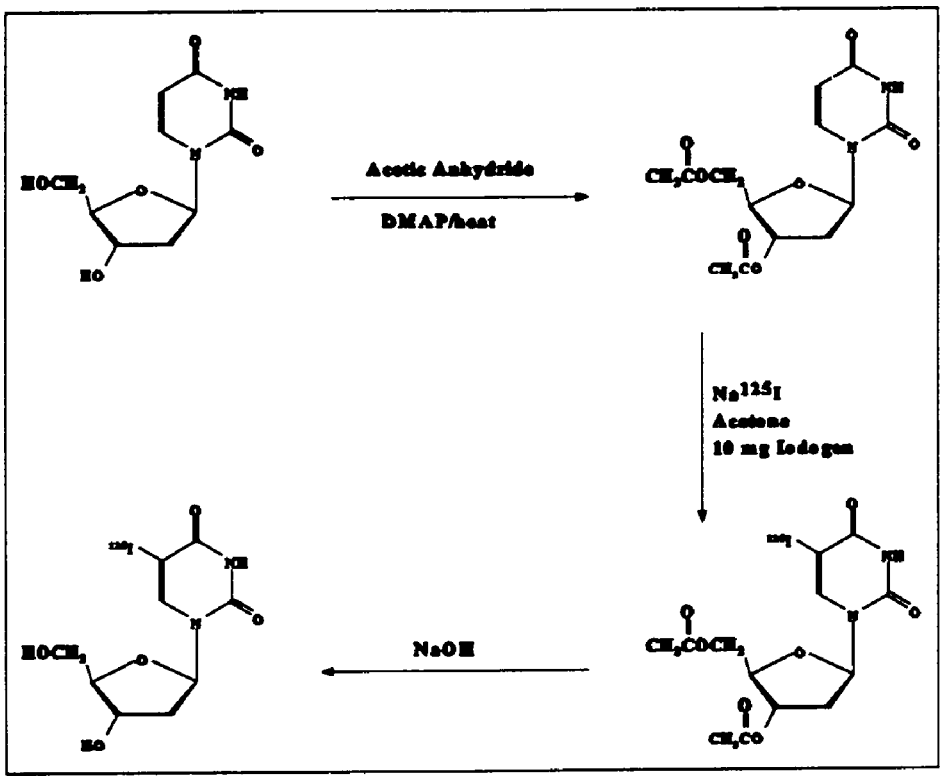


Figure V.I Acetylation, iodination and deprotection of deoxyuridine.

binding and uptake of ^{125}I-MAb 425.

V.A Uptake of ^{125}I-deoxyuridine into A1207 cells.

Since iodine-125 shows a maximum therapeutic benefit when it is localized to the nucleus of the target cell a prosthetic carrier which is capable of specifically targetting the nucleus would be benefitial. Toward the end goal of eventually preparing radio-immunoconjugates of MAb 425 which would carry such prosthetic labels we examined the uptake of a DNA precursor deoxyuridine which had been radioiodinated in the 5 position of the pyrimidine ring.

In order to facilitate radiolabeling of the deoxyuridine the 5' and 3' -OH's groups of

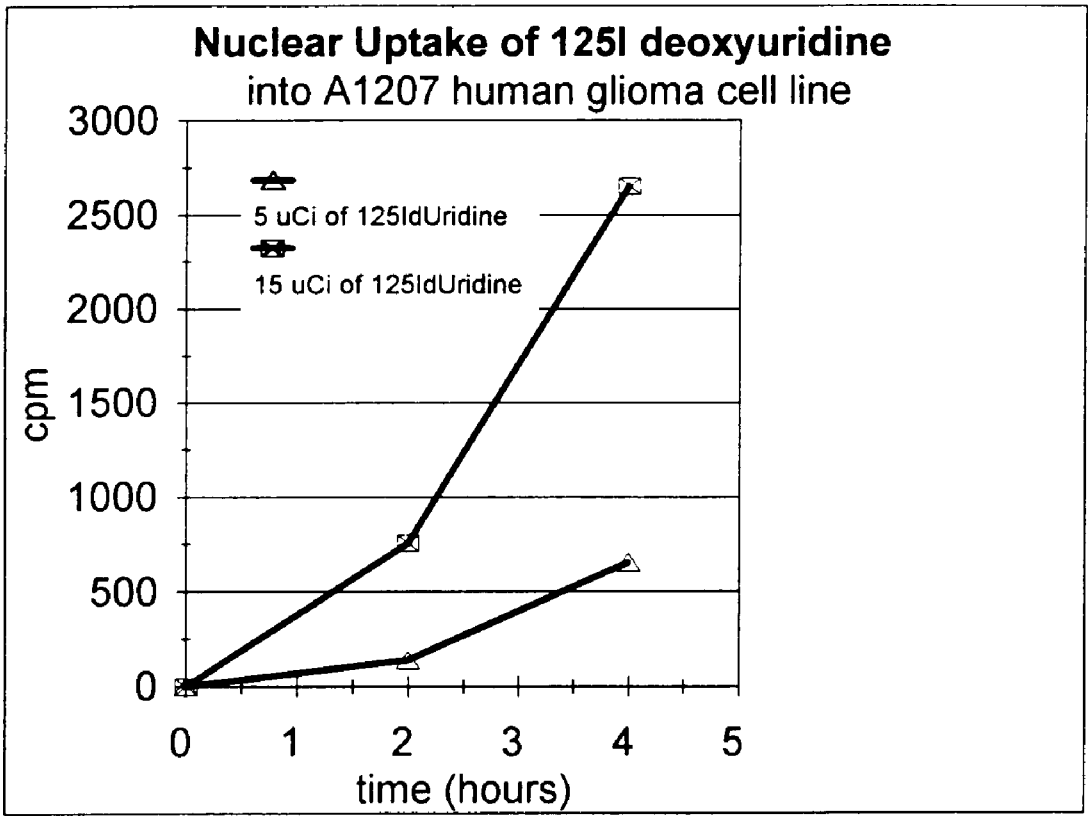


Figure V.2 Uptake of ^{125}I-2'deoxyuridine into the nuclear fraction of A1207 cells

the ribose sugar were acetylated in acetic anhydride. The acetylated compound **v.1** was soluble in a variety of organic solvents including ethyl acetate. The acetylated material **v.1** was radioiodinated in a solution of $Na^{125}I$ and Iodogen™ in ethyl acetate to form the 5-^{125}I-iodo-2'-deoxy uridine **v.2**. This material was hydrolyzed *in situ* by the addition of 10% NaOH to remove the acetyl groups. The deacetylated **v.2** was extracted into the basic aqueous phase and hydrolysis was monitored by ^{125}I-tlc in 9:1 chloroform:EtOAc. When the tlc system indicated that the reaction was finished the radiolabled 5'iododeoxyuridine **v.3** was added at either 5 μCi or 15 μCi to petri dishes containing $1x10^6$ cells the cells were allowed to incubate with **v.3** for 0,2 and 4 hours figure V.2 shows the results of this experiment. As can be clearly seen the labeled uridine interalizes and accumulates into the nucleus very effectively. This data is completely consistent with the work done by Kassis and others in tumor models other than glioma.[53]

V.B Effect of External Beam Irradiation on Internalization of ^{125}I-MAb 425 into U87 EGFr(+) cells.

In section I.2 the Phase II clinical studies which were undertaken with the ^{125}I-MAb 425 antibody were discussed. These studies showed an enhanced survival when

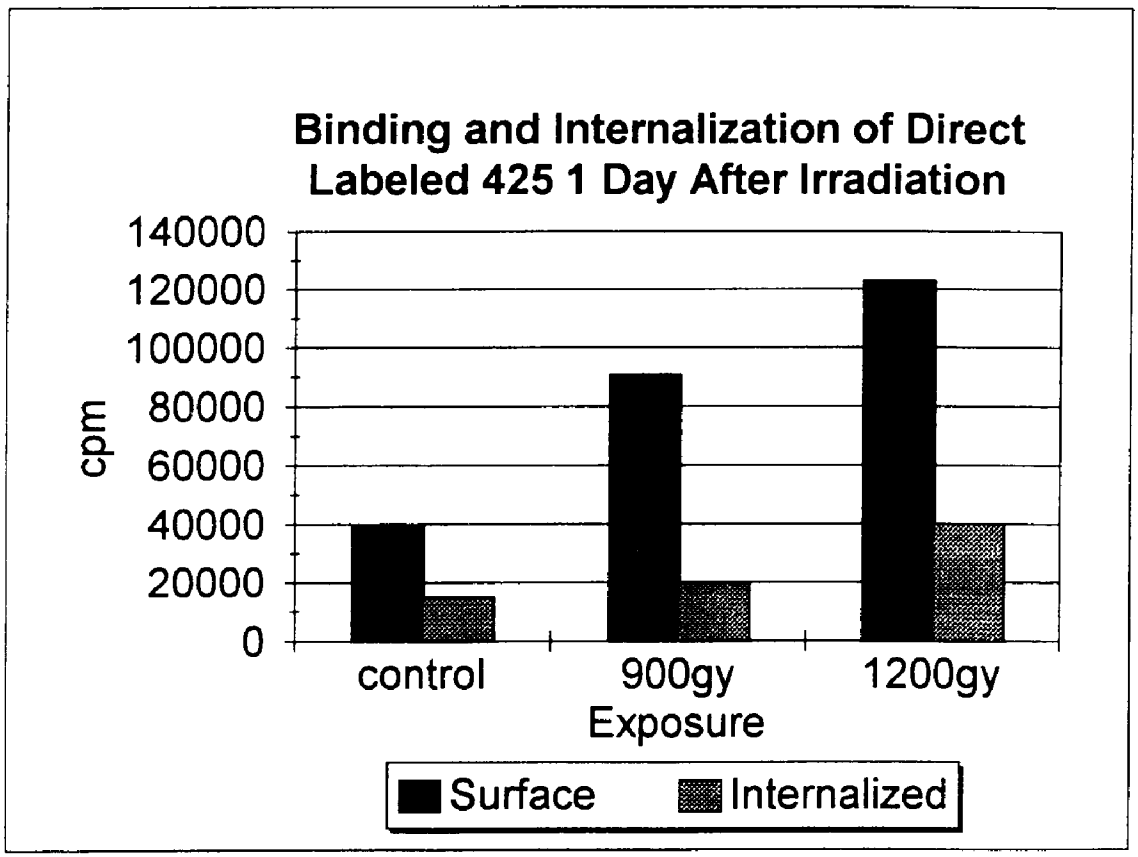


Figure V.3 Binding and internalization of ^{125}I-MAb 425 into U87 cells 24 hours after irradiation. Binding and internalization data are determined at 5 hours incubation with direct labeled antibody.

patients were treated with the ^{125}I-MAb 425 following surgery and radiation therapy. A study was undertaken as part of this research to look at the effect of external beam irradiation on the internalization of the direct labeled antibody into U87 EGFr(+) cells. Petri dishes having confluent U87 cells were irradiated with either 0, 900 or 1200 centigray of external beam x-rays. At 1, 4, and 7 days post-irradiation the ability of the cells to internalize direct labeled MAb 425 was examined. The day 1 results are shown in figure V.3. In all cases the internalization was highest at one day post irradiation with a linear increase proportionate to the dose of x-irradiation received by the cells. At day 4 total internalized activity is reduced for all cells but a trend toward higher uptake for higher irradiation levels remains. At day 7 the control and 900 cGy cells have internalize at about the same level while the 1200 cGy cells have significantly reduced internalization vs. day 1. This effect can be attributed to the cell killing caused by the combination of the external beam irradiation with the rapid uptake of ^{125}I-MAb 425.

V.C Experimental

V.C.1 Synthesis of 3',5'diacetyl-2'-deoxyuridine (v.1).

1.0 gram of 2'deoxyuridine was dissolved in 70 mL of acetic anhydride and to which was added 20 milligrams of dimethylaminopyridine (DMAP) as an acylation catalyst. The solution was refluxed overnight and then allowed to cool. The acetic anhydride was removed by three additions of 100 mL of ethanol followed by *in vacuo* evaporation . The gummy solid which resulted still had a scent of acetic anhydride. The material was recrystallized in EtOH/H_2O (80:20). The resulting white solid was dissolved and re-evaporated 10x in anhydrous EtOH. The result was 1.37 grams of a white crystalline solid **v.1**. TLC (9:1 chloroform/ methanol) showed one spot Rf = 0.75. mp = 107-108^{0}C.

^{1}H NMR;δ(ppm) 11.4 (s, 1p), 7.7 (d, 1p), 6.2 (t, 1p), 5.7 (dt, 1p), 5.2 (m,1p), 4.2 (s, 4p), 2.0 (s, 6p)

V.C.2 Labeling of v.1 with iodine-125.

10 mg of **ii.1** were dissolved in 100mL $CHCl_3$. 1 mL of this chloroform solution was removed and diluted to 10 mL with additional chloroform. 1 mL of this diluted solution was added to a test tube coated with 10 mg of Iodogen™ . An additional 1

milliliter of chloroform was added to bring the final reaction volume to 2 mL. One (1) mL of this final solution was added to a 1 mL solution of 25mCi of $Na^{125}I$. The mixture was shaken and allowed to react for 1 hour. The reaction was analyzed by ^{125}I-tlc (9:1 $CHCl_3$/ MeOH) at 0, 10, 25 and 35 minutes after the addition of the radioiodine. At time = 0 all activity was located at the origin but by time = 25 minutes a definitive peak at Rf = 0.6 was detected. Following quenching of this reaction the pH of the aqueous layer was brought to 10 with NaOH and the acetyl groups hydrolyzed to form **v.2** 5-^{125}I-iodo2'deoxyuridine . This material was used without further purification.

V.C.3 Uptake of 5-^{125}I-iodo2'deoxyuridine (v.2) into the nucleus of A1207 cells.

Petri dishes were prepared which contained 1 x 10^6 cells. To six of these dishes was added 5 uCi of **v.2** and to an additional six dishes was added 15 uCi of **v.2**. Two dishes for each activity of **v.2** were incubated for 0, 2 and 4 hours (2 dishes for each time point for each concentration). Following incubation, the cells in each petri dish were washed and the supernatant removed and collected. The cells were incubated in cold acetic acid for 20 minutes at 4°C followed by three washes with PBS the acetic acid and all PBS washes were combined and counted. The cells were then lysed in lysis buffer and spun at 1500 rpm. The nuclear fractions were separated and counted.

V.C.4 Effect of irradiation on the uptake of direct labeled MAb 425.

Confluent T-flasks of U87 cells were irradiated with 0, 90, or 120 gy of x-rays. The cells were allowed to grow for 1, 4, or 7 days after which they were harvested, pelleted and split into petri dishes containing 1 x 10^6 cells each. To each petri dish was added 1x10^6 cpm of ^{125}I-MAb 425. The dishes were allowed to incubate for 1-6 hours after which the cells were removed, pelleted and washed twice with RIA buffer. The cells were then allowed to incubate in cold acetic acid for 15 minutes, the acetic acid was removed and the cells washed twice with RIA buffer. The acetic acid and two washes were pooled and counted. The cells were then saponified in boiling 20% NaOH. The NaOH solution was collected and counted.

PART VI

GENERAL CONCLUSIONS

VI. General Conclusions

This document has dealt with several different materials which were intended for or actually used in iodine-125 based radioimmunotherapy (RAIT). From these studies several general conclusions can be drawn.

The use of tyraminyl-cellobiose labeling discussed in Section II proved to be unsuccessful. The label was difficult to prepare and to synthesize and failed to have the purported effect upon rate of release of radiolabel from the cytoplasm. While Pittman and co-workers had significant success using this label in cultured fibroblasts with LDL no parallel result wa found with monoclonal antibodies. The probability is that the uptake up LDL into human tissues is less dependent on fine molecular structure than is an antibody. IgG receptor recognition requires that amino acids in the binding domain of the protein remain unmodified through conjugations procedures. Modification of this domain almost certainly occurred during the cyanuric chloride and carbonyl diimidazole conjugations of tyraminyl-cellobiose to MAb 425.

Dextran has demonstrated potential for use in RAIT systems. The work presented herein demonstrates that the polymer can be easily and successfully modified to carry a high load of raidioiodine/molecule dextran. Further the dextran derivative tyramine-CMDL was successfully conjugated to MAb 425 following iodination of the polymer . This conjugate demonstrated minimal binding ability when compared to direct labeled 425. Internalization of ioiodine-125 is essential for an *in vivo* therapy to be benefitial.

Poor binding results in low delivery and would not be useful in such systems. It may be possible to employ newer conjugation chemistries to improve delivery on MAb 425. Dextran may be useful with other antibody systems though none were explored herein.

Monoclonal antibody 425 has proved to be a reluctant participant in the formation of high recognition radioimmunoconjugates. For every successful conjugation which was presented in this document several unsuccessful conjugations were attempted. Moreover the use of the Wilbur label as a carrier of iodine-125 with MAb 425 demonstrated that the 425 protein does not respond well to electrophilic covalent modification, especially using those reagents which will likely couple to lysine ϵ-amino groups. These findings are unfortunate since the single greatest obstacle to the development of a successful iodine-125 radioimmunoconjugate for use in RAIT is the production of a monoclonal antibody which not only targets a well defined cellular marker but which internalizes rapidly following binding to the surface epitope. MAb 425 has what could be characterized as almost ideal binding properties and uptake properties with the single exception that the internalized activity is rapidly released from the EGFr(+) cells which 425 targets. Certainly this antibody should not be abandoned but future immunoconjugates should avoid using lysine modifying reagents. This can be accomplished through the use of water soluble maleimide derivatives which contain active esters for conjugation to nucleophilic drugs or polymers. Loss of reactive amino acids is avoided by partial reduction of hinge region disulfides to form free thiols which can combine with the maleimide end of the crosslinking reagent. Such conjugations have been shown to reduce the loss of binding avidity.[69]

Another possibility is to attempt to clone the MAb 425 gene and place the hypervariable amino acid sequences which make up the binding pocket of the IgG into a more suitable antibody framework. Such manipulations of antibody fragments have become commonplace in molecular biology labs worldwide and provide perhaps the best solution. Genetically modified proteins could be produced which not only are capable of conjugation but which are inherently susceptible to electrophilic iodination and are designed to degrade slowly in cytoplasmic compartments.

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APPENDIX A

MATERIALS AND METHODS

A.1 Materials and Methods.

All organic chemicals and solvents unless otherwise specified were purchased from Aldrich chemical, Milwaukee, WI and were of reagent or higher grade. Proteins including BSA, HSA and anti-mouse IgG's were purchased from Sigma Chemical Company St. Louis, MO. Microtiter plates were purchased through Sigma Chemical, all were 96 well polystyrene single cast plates with unmodified surfaces. $Na^{125}I$ was supplied by the NEN Division of Dupont Inc, DE in 25 mCi quantities in a solution of NaOH pH= 9.0-10.0. Iodogen was purchased from Pierce, Rockford, IL. The dextran used in these studies was high-purity, Clinical Grade 40 kD dextran produced by Pharmachem Corp., Bethlehem, PA. Sephadex PD-10 prepacked columns were produced by Pharmacia Inc. and supplied by Sigma Co. Growth media and trypsin were purchased from Gibco Laboratories, Grand Island, NY. All volumetric additions less than 1.0 mL were accomplished using Eppendorf 10, 20, 200, or 1000 μL volumetric pipettes using the appropriate Eppendorf pipette tips. ^{1}H and ^{13}C NMR spectra were taken on a JEOL FX-90 spectrophotometer. All melting points were taken on either a Thomas-Hoover capillary or Fisher-Johns melting point apparatus and are reported uncorrected. Infrared spectra were obtained as 1% disks in potassium bromide on a Mattson Polaris High Resolution FTIR. Ultraviolet spectra were obtained in water on a Perkin Elmer Lambda 5 UV-Vis Spectrophotometer. Combustion analyses were performed on a Perkin Elmer Model 2400 Combustion Analyzer by QTI Corporation, Bound Brook, NJ with pre- and post-run calibration for %N (using high purity acetamide standard) and a standard deviation from the calculated value of $\pm$ 0.02%

on the calibration standard. SDS-PAGE studies were performed using either 7.5 or 10.0% gels and either a Sigma Brand or Bio-Rad mini-gel system. All activities were determined using a 1193 GammaTrac gamma counter from TmAnalytic, Inc. Elk Grove Village, IL. Centrifugation was done using one of the following devices a Fisher Centrific Model 228 or a Silencer H-130N Series, Model H-103NB, Rupp & Bowman Co., MI. Flow cytometry studies were done using a Becton Dickinson FACSort flow cytometer analyzing for cell surface fluoresence using the LYSIS 2 software package produced by Becton-Dickinson. All text was produced with Word Perfect for Windows 6.0a and all graphs are from Quattro Pro For Windows 5.0 .

Cell lines and culture methods.

Cell Line	cell type	EGFr density
A1207	glioblastoma	5×10^5/cell
U87	astrocytoma	5×10^4/cell
F39	glioblastoma	3×10^4/ cell
SW707	colorectal	$<<5 \times 10^3$/cell

Data from Bender, et al., *Cancer Research, 52,* 1992, calculated by Scatchard analysis of RIA-data using MAb 425 which had been directly iodinated.

Cells were harvested by the following method. Confluent cultures were removed from incubation and all media removed from the surface of the cells with a 5 mL pipette. 5 mL of trypsin (1%) was added and the cells incubated for 5 minutes. 5 mL of media were added and the cells forced away from the growth surface using either a tapping method or

a cell scraper. The cells were then transferred to a 25 mL polystyrene centrifuge tube and pelleted. The supernatant was removed and the cells resuspended in growth media. This was repeated twice. The cells were then resuspended in 10 mL of media and counted under a microscope. The cells were then diluted to a concentration of $5x10^6$ cells/ mL and used as such for binding and internalization experiments.

Buffers and Solutions

Phosphate buffer; Phosphate buffer and PBS were prepared from pre-made powders supplied by Sigma. Phosphate buffer (no saline) was composed of a mixture of sodium and potassium phosphates and had a pH of 7.2. It was prepared as a 0.03 M stock solution and diluted accordingly. PBS solutions had the following composition 0.12 M NaCl, 2.7 mM KCl, and 10 mM phosphate, the pH = 7.4

Homogenizing Buffer (pH 8.0) composition; 15 mM TRIS, 2 mM EDTA, 0.5 mM EGTA, 60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 0.5% Nonident P-40, 15 mM 2-mercaptoethanol, 1 mM PMSF.

Stripping Buffer; acetic acid /NaCl 1%/0.1M

Washing Buffer; 1% BSA,0.1% tween, 0.01% NaN_3 in 20 mM PBS

Blocking Buffer; 20 mM PBS, 1% HSA, and 0.01% NaN_3

RIA Buffer; 5% γ-globulin free horse serum in 20 mM PBS.

FACS Buffer, 8.12 g/L NaCl, 0.28 g/L KCl, 0.36 g/L disodium EDTA, 0.26 g/L KH_2PO_4, 2.35 g/L Na_2HPO_4, 0.43 g/L LiCl.

APPENDIX B

ADDITIONAL FIGURES

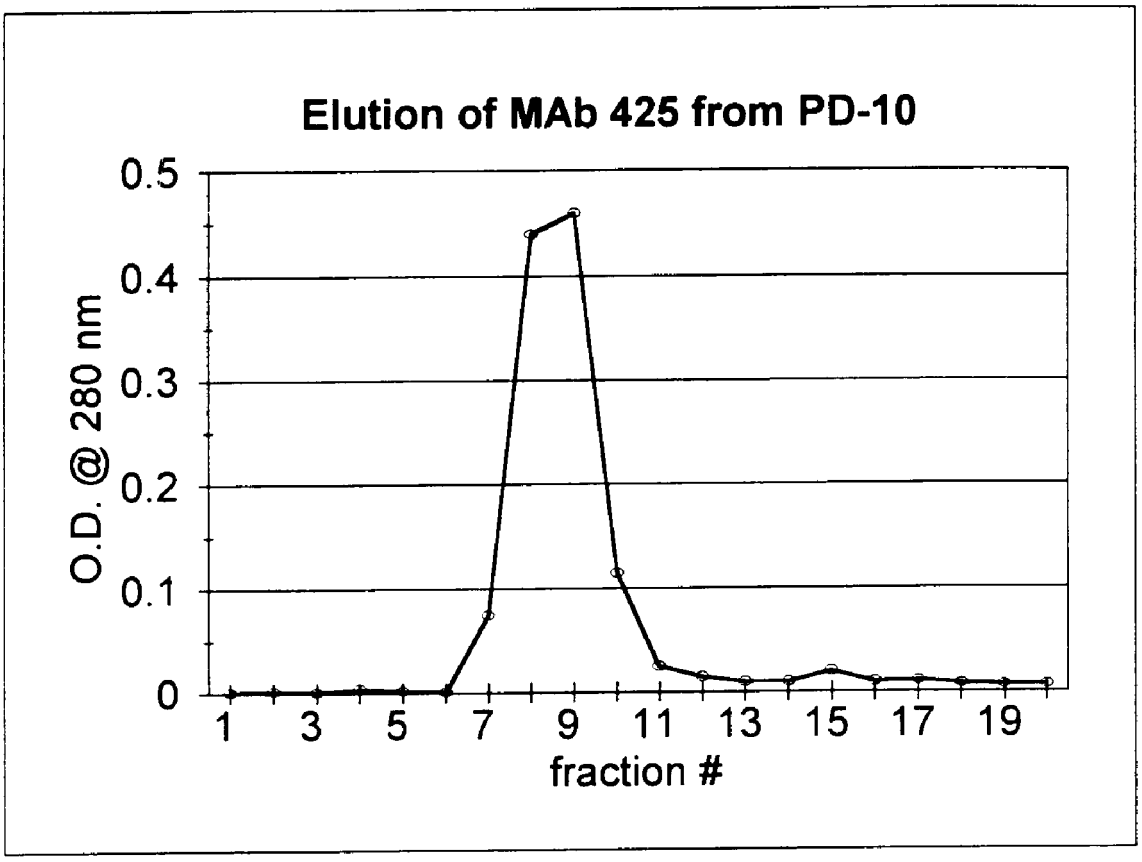


Figure B.1 **Elution of MAb 425 from PD-10 pre-packed Sephadex G25 column. Total mass charged to the column = 5 mg. Each fraction is 0.25 mL approximately.**

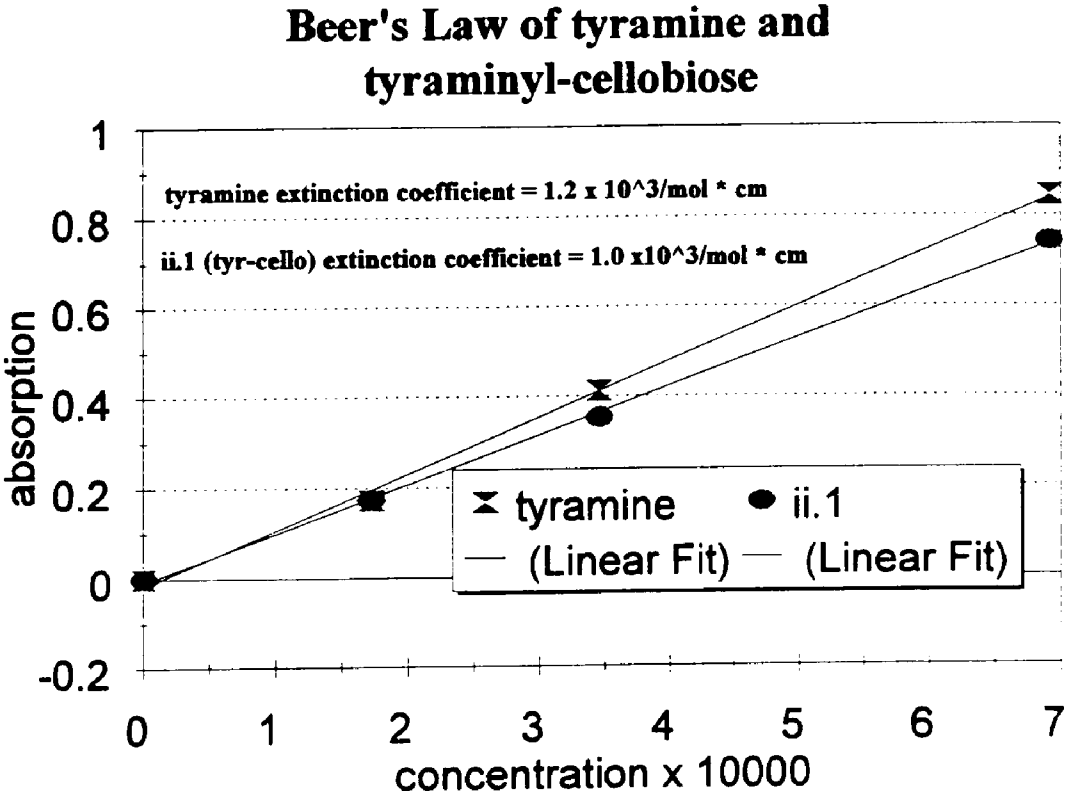


Figure B.2 Beer's Law Analysis of tyramine and tyraminyl-cellobiose ii.1.

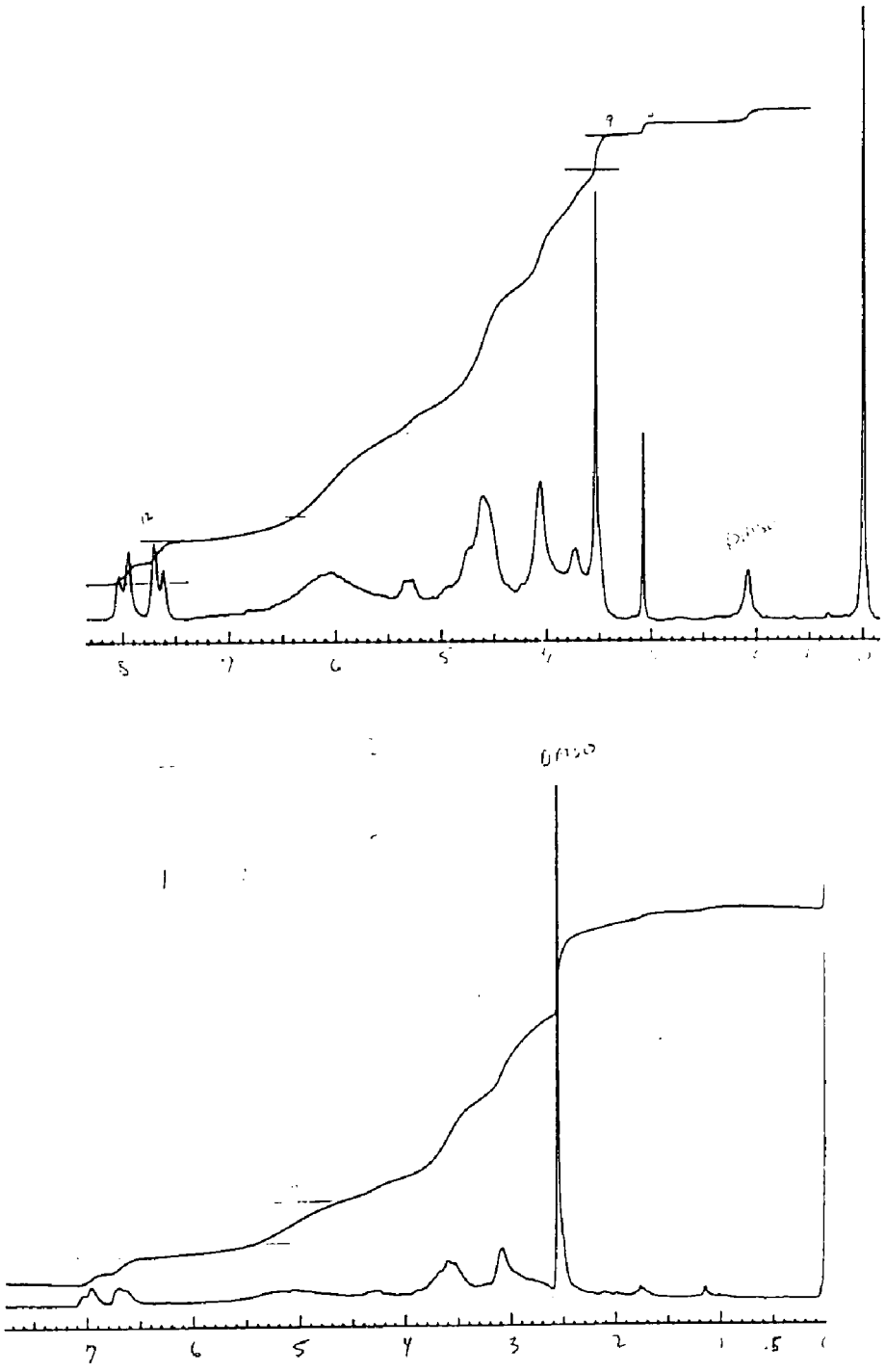


Figure B.3 ^{1}H **NMR of ii.1 Top; spectrum of ii.1 prepared in aqueous buffer Bottom; spectrum of ii.1 prepared from DMSO.**

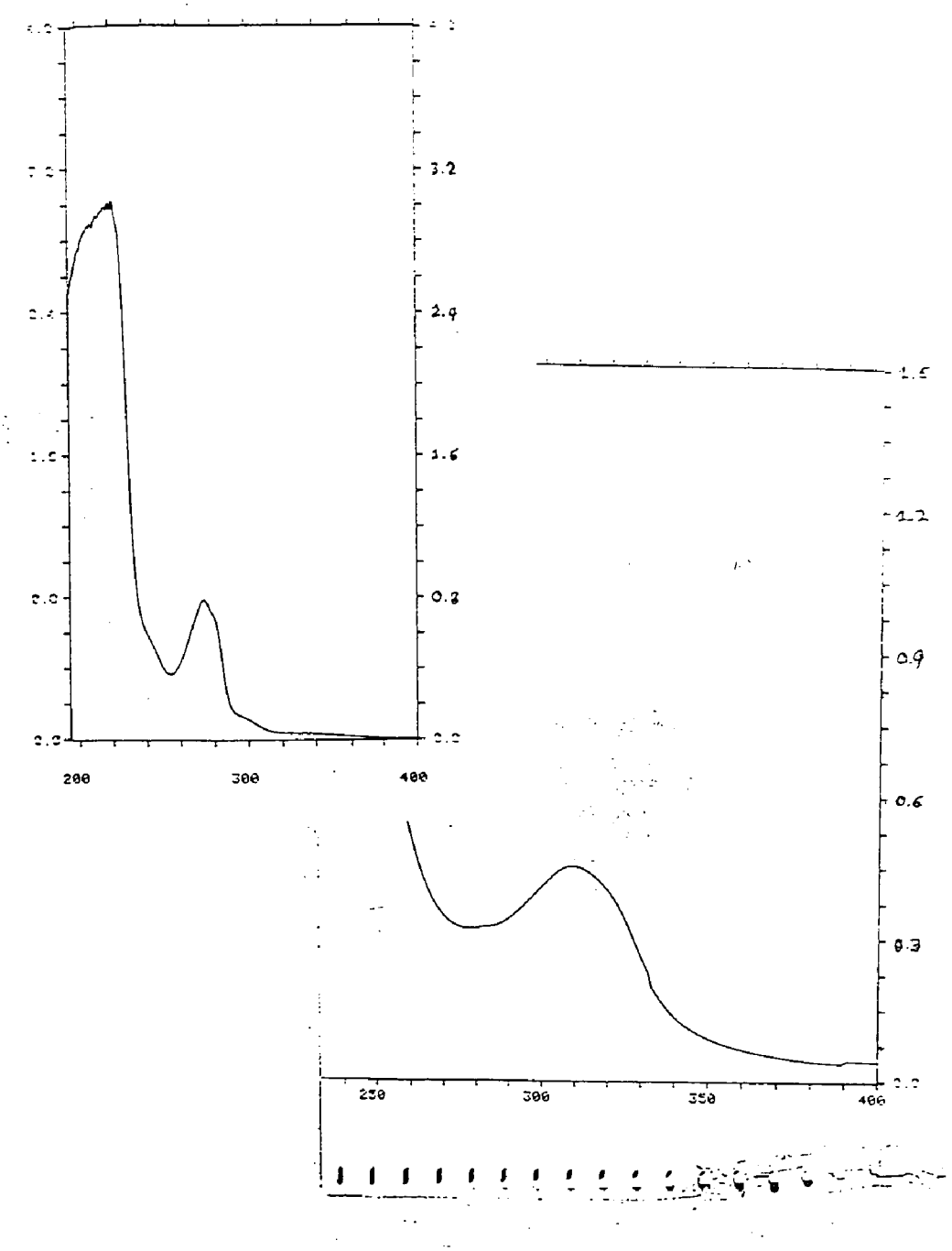


Figure B.4 Top; UV spectra of ii.1 Bottom; UV spectra of ii.1 after cold iodination.

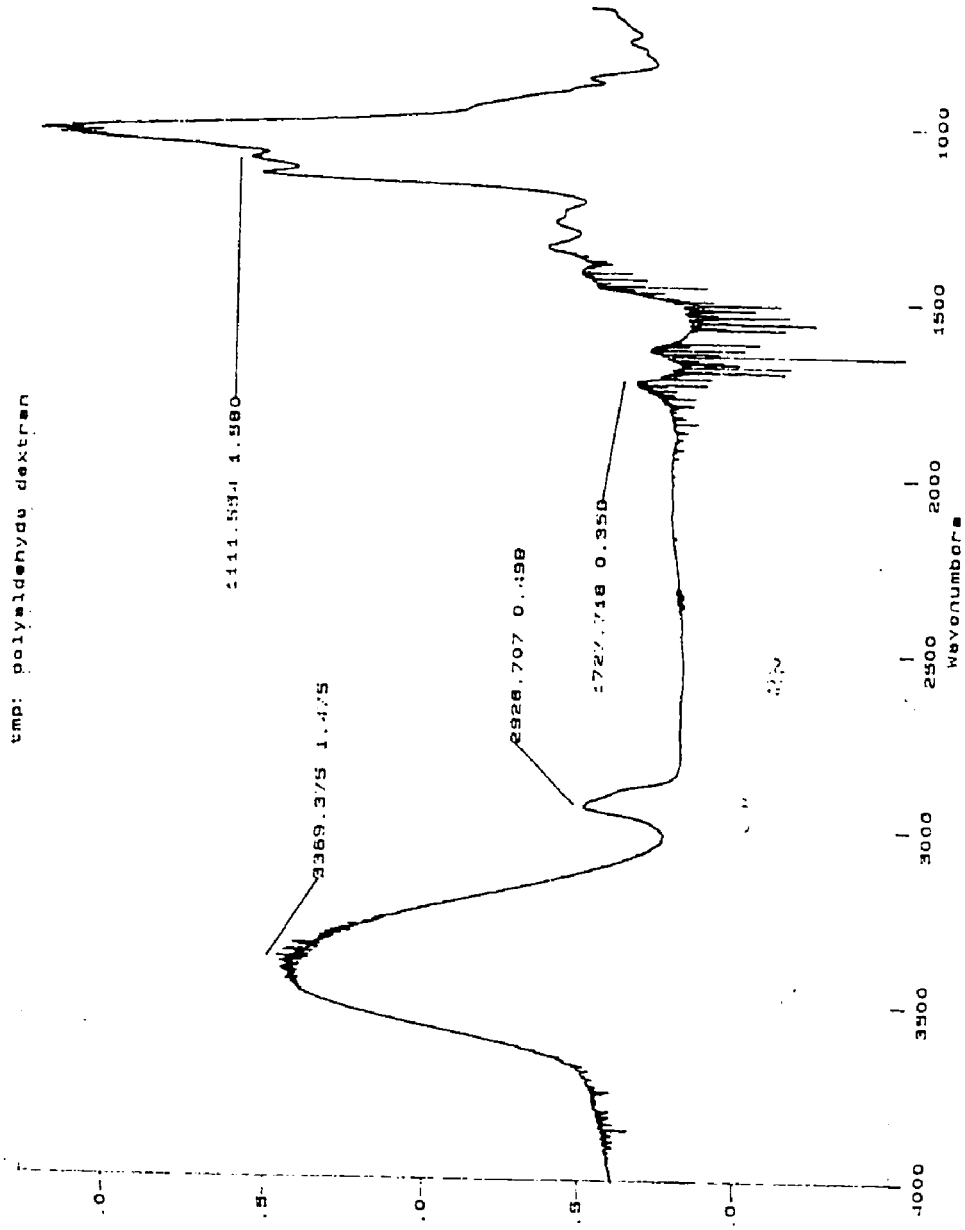


Figure B.5 IR of Polyaldehyde Dextran iii.1 (1% mixture in KBr)

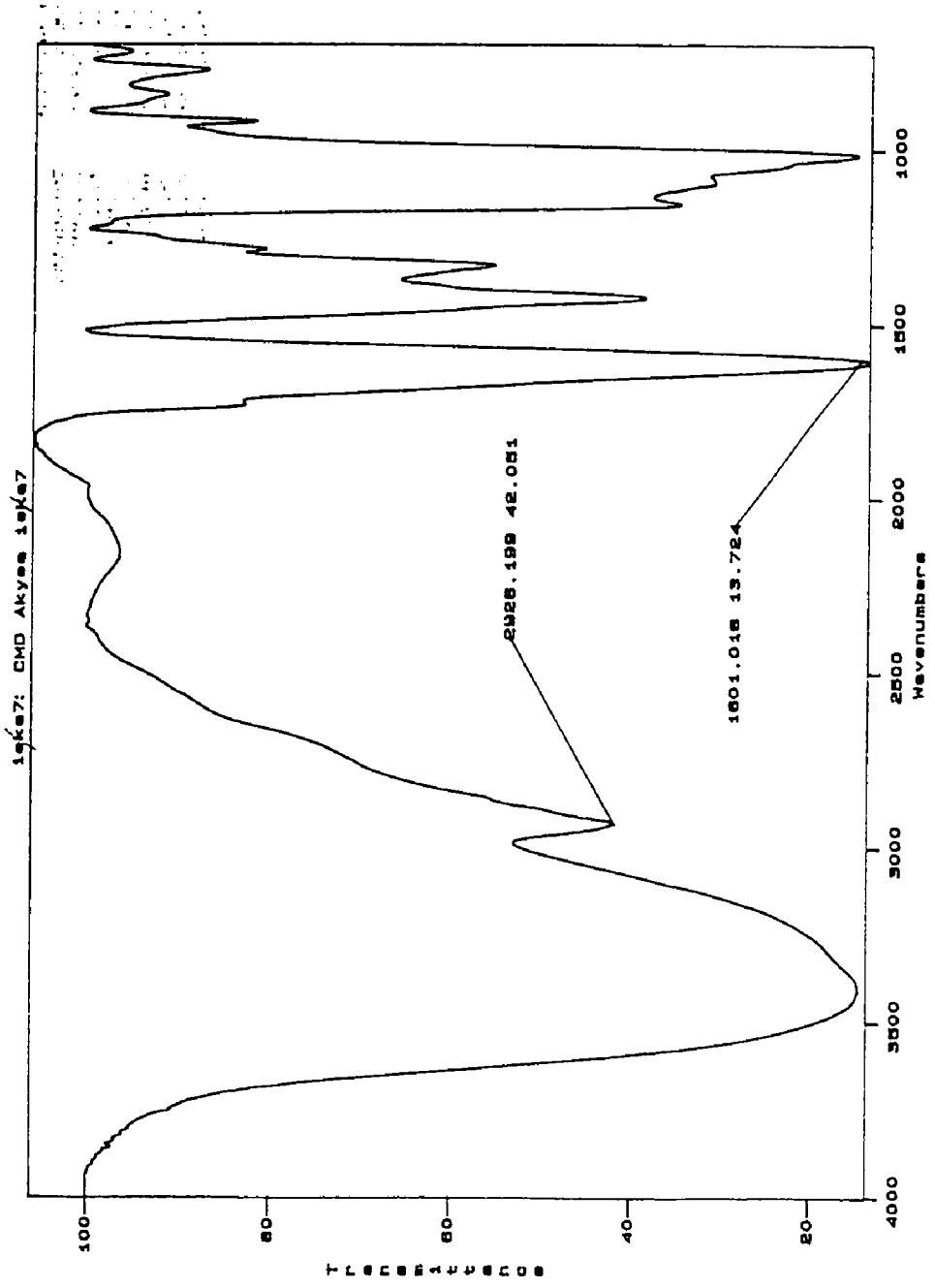


Figure B.6 IR of Carboxymethyl dextran Sodium Salt

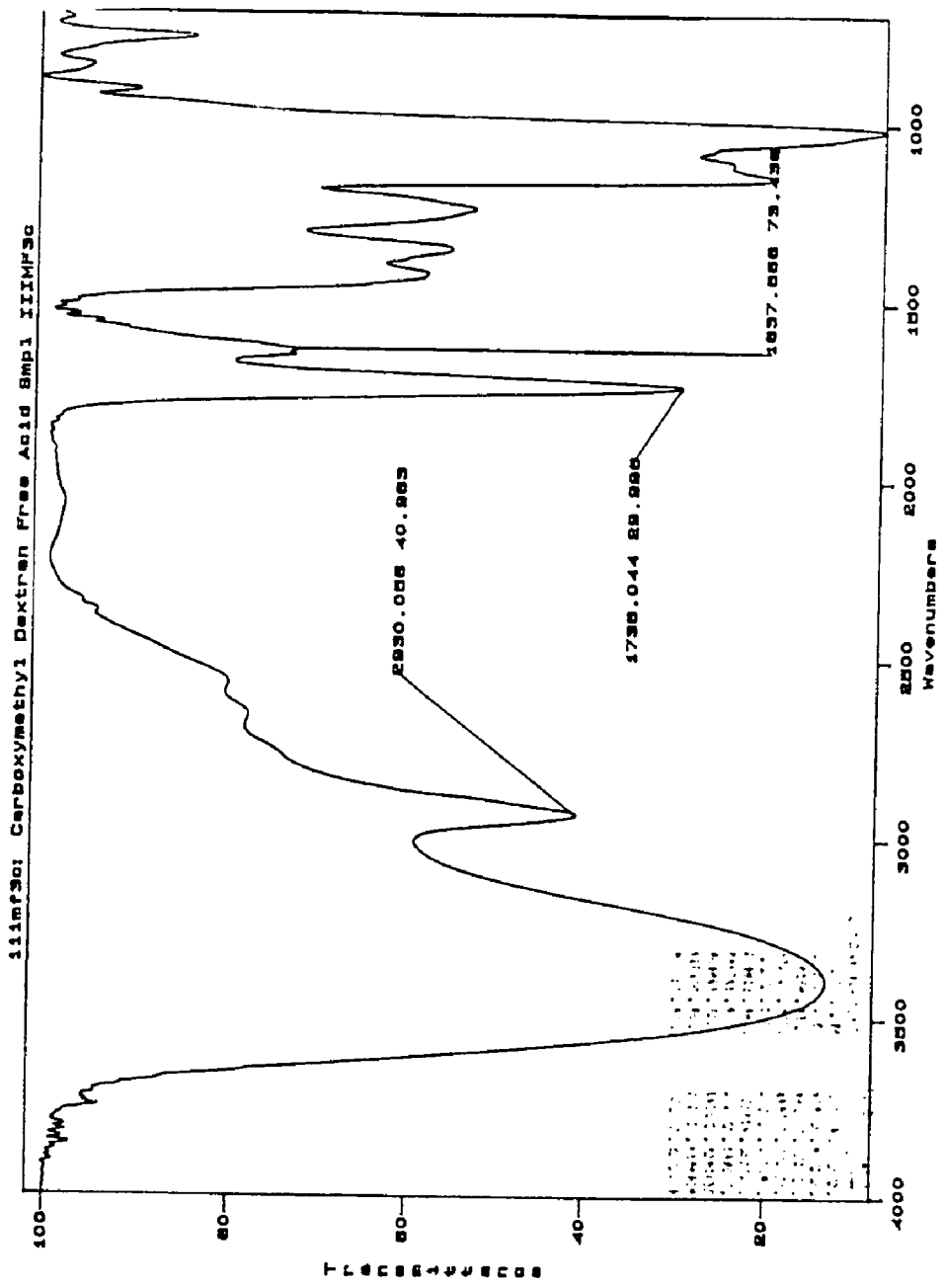


Figure B.7 IR of Carboxymethyldextran Free Acid iii.3

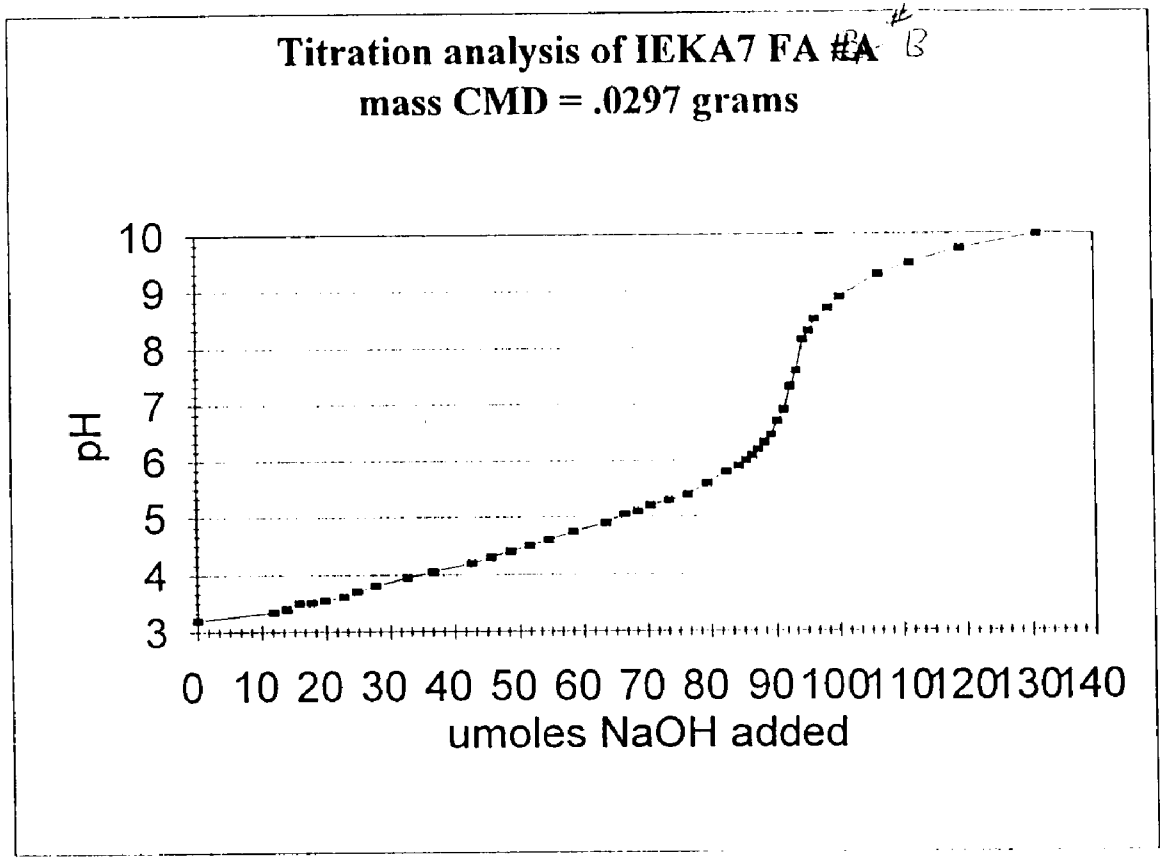


Figure B.8 Titration analysis of iii.3

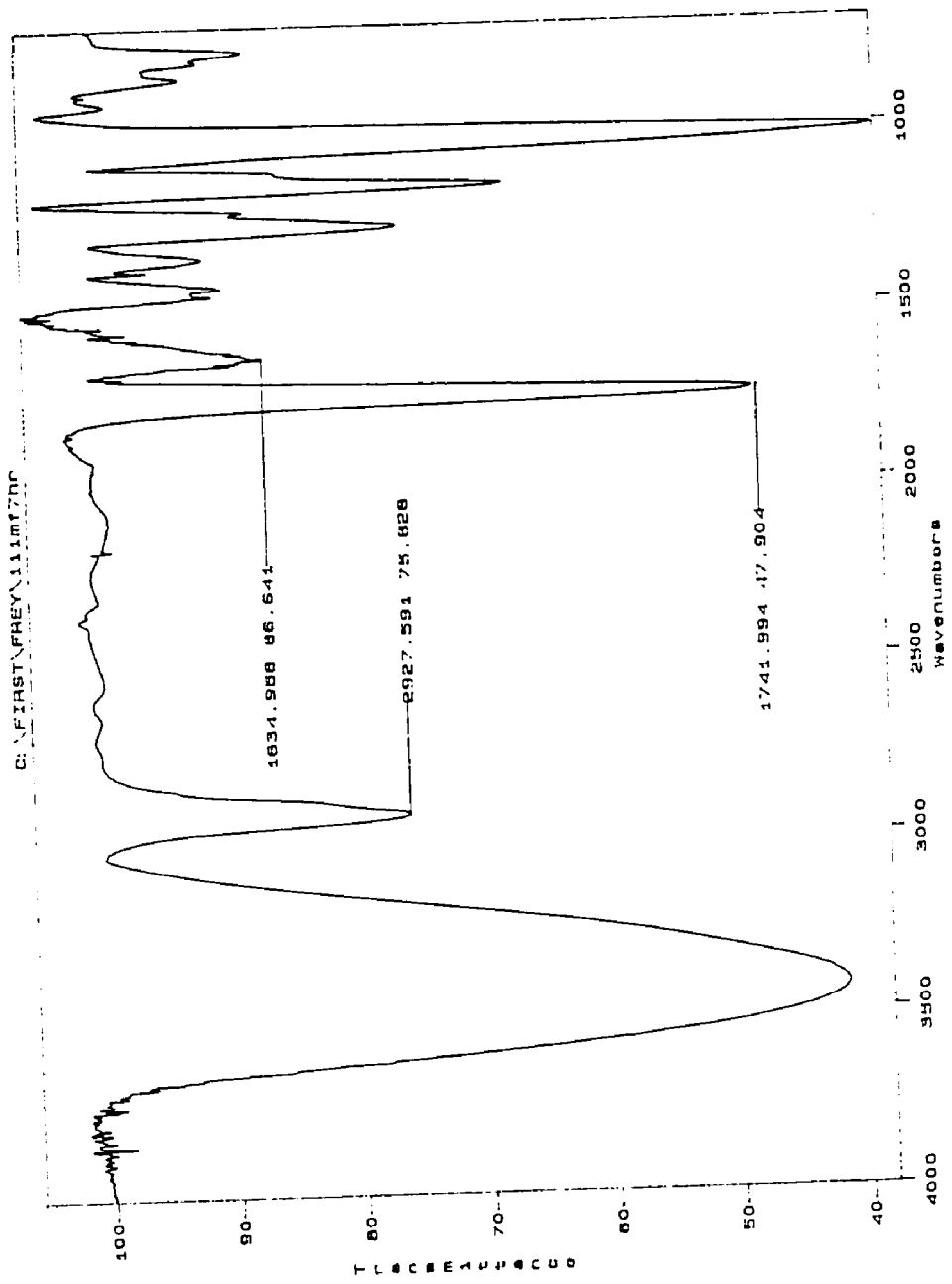


Figure B.9 IR spectra of Carboxymethyldextran lactone iii.4

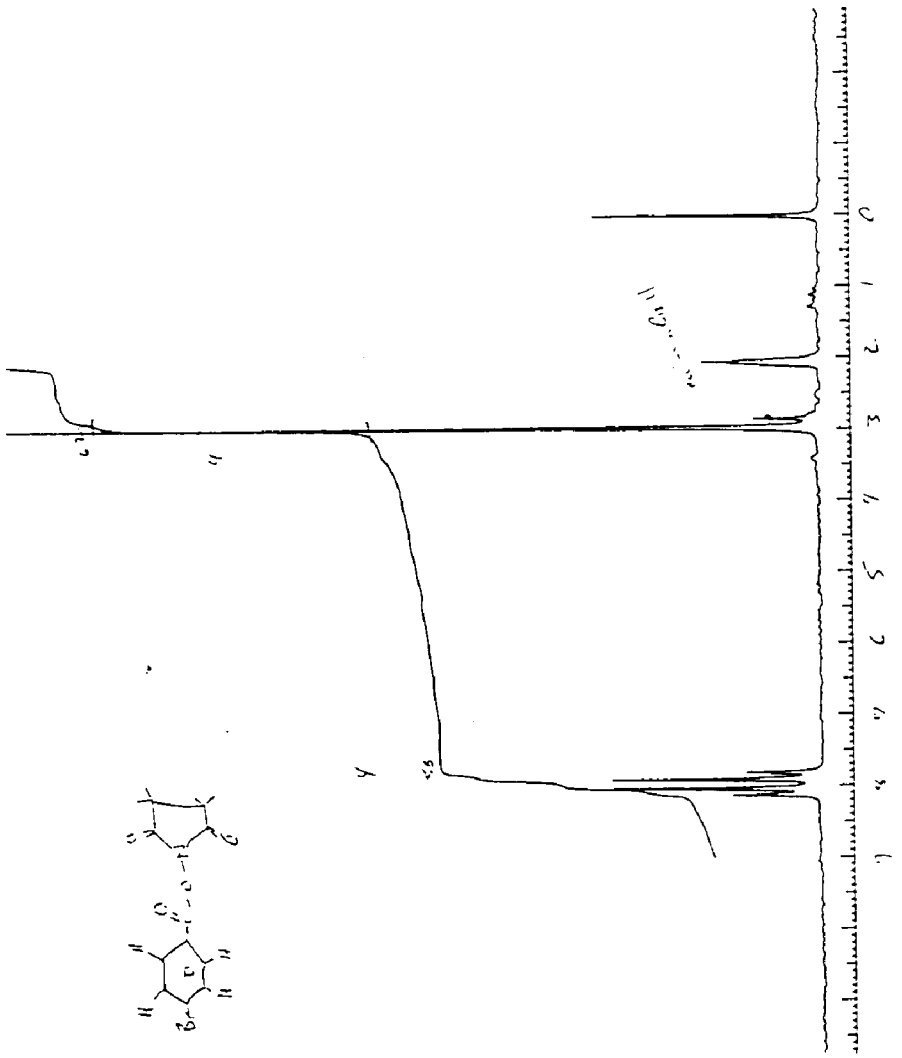


Figure B.10 ^{1}H NMR of N-succinimidyl p-bromobenzoate in $DMSO_{d6}$

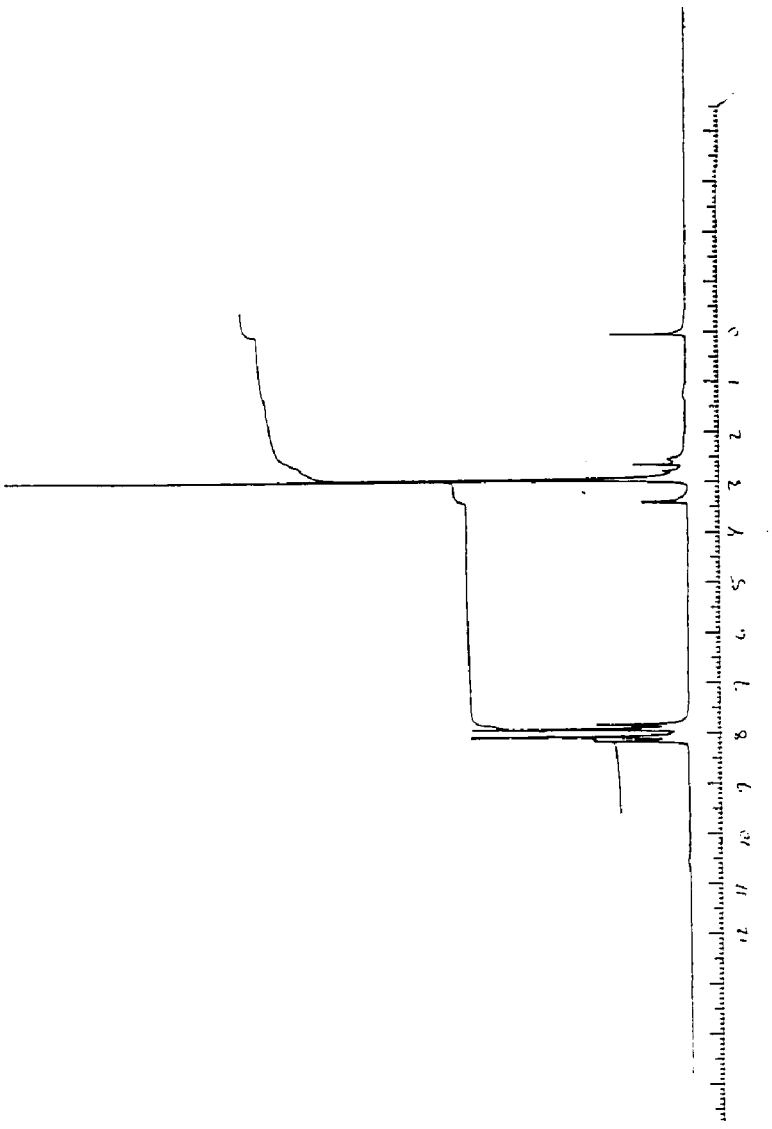


Figure B.11 ^{1}H NMR of N-succinimidyl p-iodobenzoate in $DMSO_{d6}$

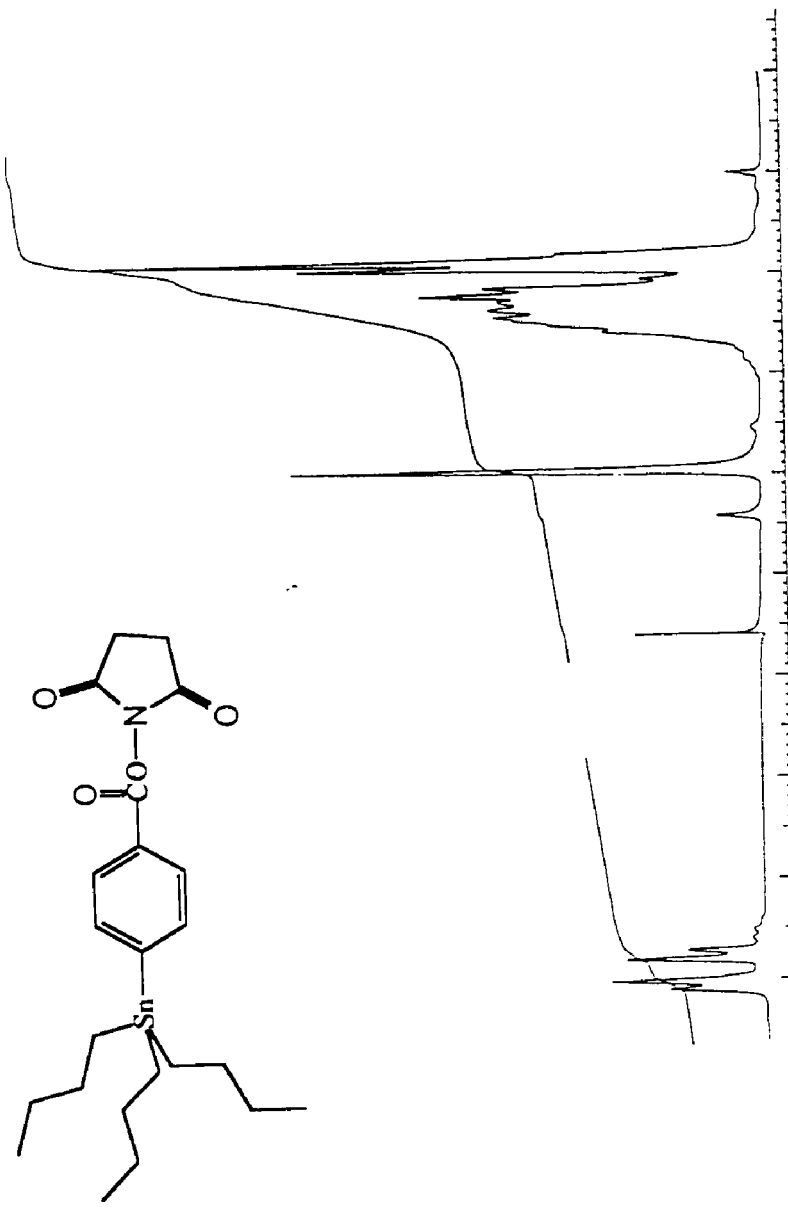


Figure B.12 N-succinimidyl-p-tri-Nbutyl-stannyl benzoate (iv.1) ^{1}H NMR

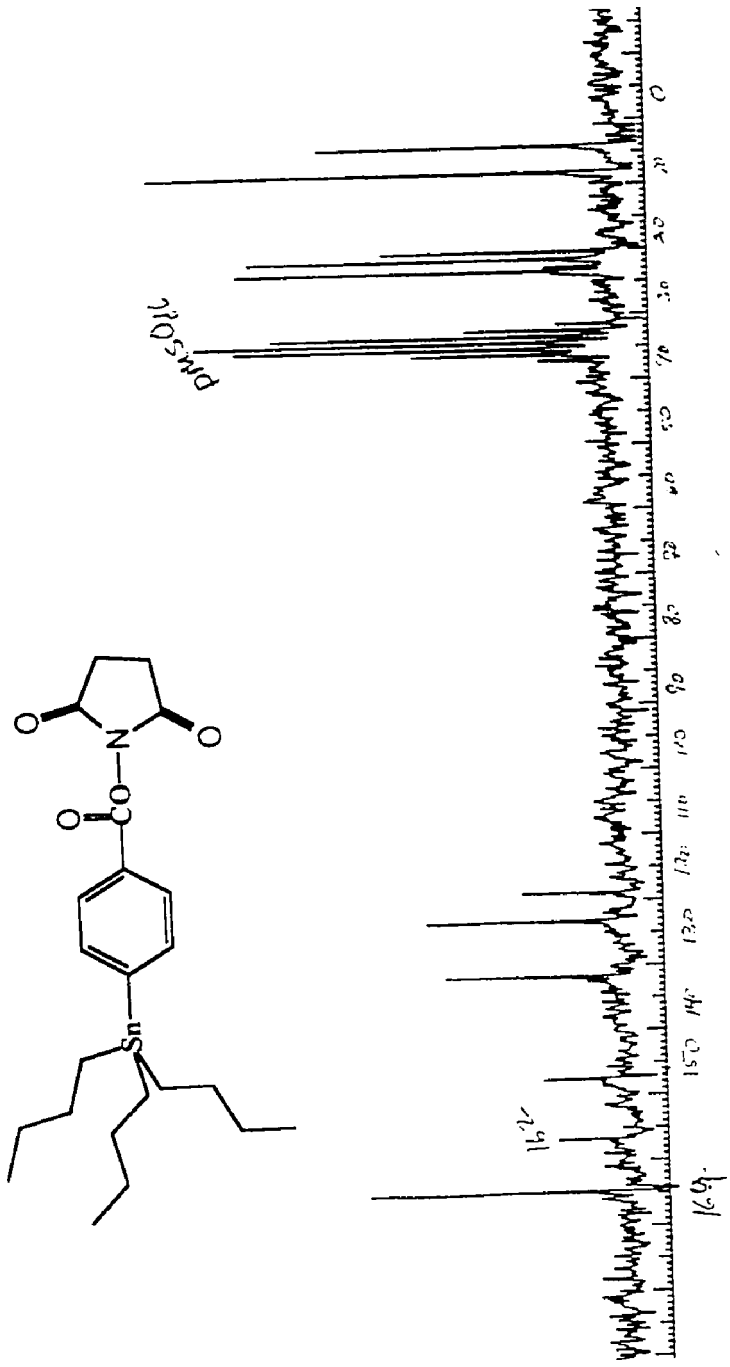
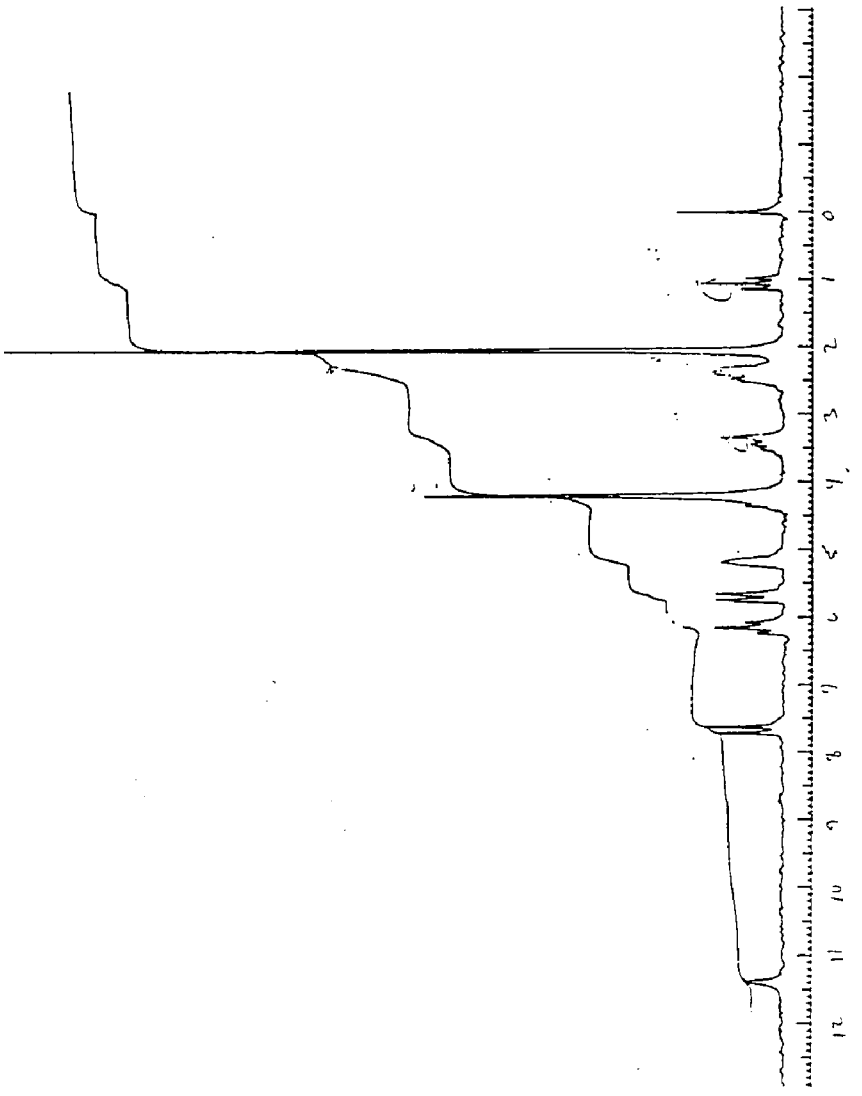


Figure B.13 N-succinimidyl-p-tri-Nbutyl-stannyl benzoate (iv.1) ^{13}C NMR



B.14 3'5'diacetyl 2'deoxyuridine (v.1) ^{1}H NMR.

VITA

Michael Frederick Frey was born to Frederick Phillip Frey and Christine Joyce Giuliano Frey on October 7, 1966 in Fountain Hill, Pennsylvania. Following a very short stay in Spring Hill, PA the family moved to Rochester, NY where the author spent most of his young adult life.

The author attended McQuaid Jesuit High School in Brighton, NY from 1980-1984. In 1984 he began his studies in Chemistry at Loyola University of New Orleans. He participated in the student affiliates program of the American Chemical Society at Loyola as well as Chairing the Dean of Arts and Sciences Student Advisory Council. After completing the accredited Bachelor of Science degree program in Chemistry, Mr. Frey enrolled in the Graduate School of Lehigh University where he completed the Master of Science degree in Chemistry (Biochemistry). The author continued his studies at Lehigh University. In the Fall of 1993 Mr. Frey was employed as an Adjunct Instructor at Lafayette College where he taught Introductory Biochemistry and is currently teaching at Northampton Community College in Bethlehem, PA.