^{133}Ba Cryptate Labeled Immunoglobulin G

William A. Pettit* and Barbara K. Swailes

Nuclear Medicine Service, V.A. Medical Center
Iowa City, Iowa 52240, U.S.A.

Summary

A method for the attachment of cryptates containing radioactive metal ions to proteins has been developed. The aminocryptand, 4,7,13,16,21,24-hexaoxa-5,6-(4'-aminobenzo)1,10-diazabicyclo[8.8.8]-hexacosane, was complexed with divalent ^{133}Ba and subsequently converted to the isothiocyanatocryptate. The bioconjugate with immunoglobulin G was formed in better than 40% radiochemical yield in a three step procedure. Alternatively, the radiolabeled bioconjugate could be formed by addition of ^{133}Ba to immunoglobulin G that had been reacted with the isothiocyanate derivative of the cryptand. Stability studies indicated the radiolabeled bioconjugate has a half-life of 6 h and 12 h in phosphate buffers of pH=7.4 and pH=9.2, respectively. Attempts to label the immunoglobulin with the corresponding ^{142}Pr cryptate resulted in considerable radioactive colloid formation with no detectable bioconjugate produced.

Key Words: barium-133, cryptand, cryptate, immunoglobulin G

Introduction

Effective utilization of radionuclide labeled antibodies to tumor associated antigens has been achieved in the diagnosis and treatment of cancer (1). Although radioiodine has been used extensively in this regard, some radiometals offer a greater variety of isotopic half-lives and higher linear energy transfer radiations (2). Although bifunctional chelating agents based on DTPA or EDTA have been successfully used with isotopes of indium, gallium, and technetium, they do not form stable chelates *in vivo* with some metal ions considered for therapy, e.g., ^{90}Y (3) or ^{212}Bi (4). Preparation of radiometal chelates and evaluation of their stabilities remain topics of investigational interest (5). In order to diversify the types of complexing agents available for attaching metal ions to proteins, we have prepared and used a cryptand derivatized for this purpose. In addition, we have set forth some methods that are useful in monitoring the formation and stability of the resulting bioconjugate.

Cryptands are known to form inclusion complexes or cryptates with alkali, alkaline earth, and other metal ions (6). Although Gansow first reported attachment of a labeled cryptate to a protein (7) using a diazonium salt reaction, the preparation of ^{133}Ba cryptate and its attachment to an immunoglobulin through a thiourea linkage should provide bioconjugates with greater retention of native biological function due to the milder reaction conditions employed (8).

0362–4803/93/040305–07$08.50

Received 10 October, 1992
Revised 7 December, 1992

Experimental Procedures

The cryptand, 4,7,13,16,21,24-hexaoxa-5,6-(4'-aminobenzo)-1,10-diazabicyclo[8.8.8]hexacosane, $2BNH_2$:2:2, was prepared from 1,2-bis-(oxyacetylchloride)-4-nitrobenzene and 1,4,10,13-tetraoxa-7,16-diazacyclooctadecane following procedures analogous to those used by Gansow (9). The details of this synthesis are reported elsewhere (10). ^{133}Barium chloride was obtained from New England Nuclear (Billerica, MA) with a specific activity of 3.04 mCi/mg. ^{142}Pr trichloride was obtained from the University of Missouri Reactor Facility (Columbia, MO) with an initial specific activity of 3.66 mCi/mg. Aqueous solutions were prepared from water processed through in-line Millipore (Bedford, MA) Milli-RO-15 and Super-Q water systems. Sephadex G-25 disposable PD-10 columns were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Silica gel TLC strips (Gelman Instruments, Ann Arbor, MI) were used as supplied, i.e., not heat activated. Procedures were conducted at ambient temp unless specified.

Preparation of radionuclide immunoglobulin bioconjugates. An aliquot of ^{133}Ba chloride (20 μCi, $3.3x10^{-8}$ mol) in HCl solution was taken to dryness in an oven at 105° C. To the dry salt was added 5 mg of $2BNH_2$:2:2 ($1.1x10^{-5}$ mol) in dry $CHCl_3$. The $CHCl_3$ was evaporated at reduced pressure and the resulting residue stirred with 600 μL of dry MeOH for 20 min. Cryptate formation was monitored by Gelman strip TLC in methanol:1% acetic acid, 7:1. Following chromatogram development, the strips were placed in an iodine chamber for detection of organic components and subsequently cut into 0.5 cm sections and counted. The MeOH was evaporated *in vacuo* and to the flask was added 500 μL $CHCl_3$, 200 μL of H_2O, 10 μL of thiophosgene ($1.3x10^{-5}$ mol), and 350 μL of saturated aqueous lithium carbonate. The resulting mixture was stirred for 2 h. The $CHCl_3$ layer was removed by aspiration and the aqueous layer was washed with 1 ml of $CHCl_3$. Following removal of the $CHCl_3$ wash, the aqueous phase was partially evaporated at water aspirator pressure for 5 min to remove residual volatile material. The aqueous phase was then added dropwise to an agitated 1 mL solution of IgG (10 mg/mL, $6.2x10^{-8}$ mol) in 0.1M phosphate buffer, pH 9.2, cooled in ice. The resulting solution was stirred and the reaction was followed by scintillation counting of the fractions collected from PD-10 gel filtration of aliquots of the reaction mixture as described below. A similar procedure was followed in attempts to prepare the ^{142}Pr labeled bioconjugate except that the procedure of Gansow (12) was used to prepare the ^{142}Pr labeled cryptate prior to protein attachment. HPLC gel filtration with in-line UV (254 nm) and NaI scintillation detectors for monitoring the column eluate was used to ascertain association of protein absorbance and radioactivity.

Monitoring of bioconjugate formation. The ^{133}Ba labeled bioconjugate was prepared in the manner described. At timed intervals, aliquots of the reaction mixture were removed and placed over PD-10 columns and eluted with 0.1M phosphate buffer, pH 9.2. Fractions of 0.53 mL (9 drops) were collected and counted. Counting standards from the initial reaction and radioactivity associated with the protein peaks from the column runs were used to determine the % radioactivity incorporated.

Alternative labeling procedure. A sample of $2BNH_2$:2:2 was converted to the isothiocyanate derivative as described above. The aqueous solution of the isothiocyanate was added dropwise to a cooled stirred solution of the IgG in pH 9.2 phosphate buffer. After 20 h the mixture was chromatographed over a PD-10 column with pH 7.4 phosphate buffer and aliquots of the fractions assayed for protein content using the Pierce BCA reagent. The protein fractions were pooled and added to 20 μCi of dry ^{133}Ba chloride. The resulting solution was stirred for 24 h. During the reaction, specifically at 6 and 24 h, aliquots of the reaction mixture were separated on PD-10 columns and the fractions counted.

Control experiments for the labeling procedure.
A. A 20 μCi aliquot of ^{133}Ba chloride was dried as described, mixed with 1 mL of 0.1M phosphate buffer, pH 9.2, and allowed to stand at room temp for 47 h. This solution was chromatographed over a PD-10 column in the manner analogous to that of the protein assay and the fractions collected and counted. An aliquot of the ^{142}Pr solution was treated in a similar manner.

B. Samples of dried ^{133}Ba and ^{142}Pr chlorides were mixed with 1 mL of 10 mg/mL of IgG in pH 9.2 phosphate buffer and allowed to stand for 47 h. Aliquots of these solutions were placed over PD-10 columns and the fractions obtained counted.

Stability studies. Duplicate 0.5 mL samples of the ^{133}Ba bioconjugate recovered from the void volume of PD-10 gel filtration were placed in a well-type dialysis apparatus and dialyzed against 0.1M phosphate buffer, pH 9.2, for 24 h. Buffer changes of 90 mL were made at 3, 6, and 12 h. Aliquots of the original samples and duplicate aliquots from the dialysis wells were removed and counted at timed intervals. An analogous study was conducted at pH 7.4.

Aliquots of a solution ^{133}Ba labeled bioconjugate prepared and isolated by PD-10 gel filtration in the described manner were chromatographed over PD-10 columns with phosphate buffers of differing pH.. Eluting phosphate buffers of pH=5.01, 7.45, 8.35, 8.68, and 9.29 were used. In each case the recovered protein fractions were pooled and stored at 5°C for 20 h. Equal portions of these solutions were then rechromatographed over PD-10 columns and the fractions obtained counted.

Results

Preparation of radionuclide labeled immunoglobulin bioconjugates. A diffinitive assay for the formation of the ^{133}Ba and ^{142}Pr complexes with 2BNH$_2$:2:2 was developed using unactivated Gelman silica gel strips. The uncomplexed metal ions and the free amine had R_f values of 0-0.2 and 1.0, respectively. For the Ba^{2+} and Pr^{3+} cryptates the R_f values in this chromatographic system were 0.4-0.5 and 1.0, respectively. Unfortunately, the HPLC system developed for the analysis of 4,7,13,16,21,24-hexaoxa-5,6-(benzo)-1,10-diazabicyclo[8.8.8]hexacosane, 2B:2:2, Ba^{2+}, and the Ba^{2+} complex of 2B:2:2 reported previously (11) was not satisfactory for the amine derivative of the cryptand due to the long retention times of components in reversed-phase chromatography. The ^{133}Ba cryptate of 2BNH$_2$:2:2 was produced typically in better than 90% yield by the methanol procedure described. The ^{142}Pr cryptate of 2BNH$_2$:2:2 could be obtained by Gansow's procedure (12) using refluxing anhydrous acetonitrile and triethylorthoformate in 34% yield.

Formation of the isothiocyanate derivative of the cryptate was essentially complete after 2 h as indicated by disappearance of the amine with fluorescamine (13). Attachment of the derivatized complex to IgG was monitored as indicated in Figure 1. This study was repeated for a 70 h period and equilibrium appeared to be established at about 20 h as indicated by a constant amount of bioconjugate over the interval of 20-70 h. During the first 20 h the production of bioconjugate was defined by: % bioconjugate = 14.6 $t_{(h)}^{0.39}$. Radiochemical yields were 44-47%. Gel filtration HPLC showed the coincidence of the UV absorbance and radioactivity with the same retention time as IgG albeit with considerable tailing of the radioactivity peak. No radioactivity was eluted before protein.

Although the Pr^{3+} cryptate formation was indicated by TLC on Gelman strips, considerable Pr colloid formation occurred during the subsequent attempts to attach it to the protein. Only a few percent of the total Pr radioactivity passed into the void volume of the PD-10 column and this was almost quantitatively retained (> 99.5%) retained on a 0.45 μm filter when the fraction was prepared for HPLC analysis.

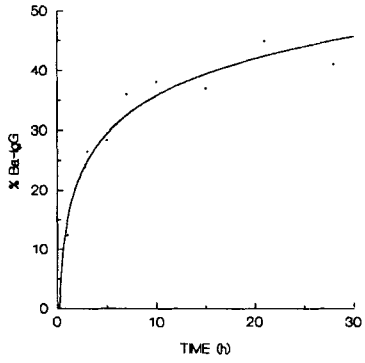

Figure 1. Logarithmic curve fit of the production of Ba-133 cryptate labeled IgG from the reaction of ^{133}Ba isothiocyanatocryptate and IgG in 0.1 M phosphate buffer, pH 9.2.

Alternative labeling procedure. IgG which had been modified by previous attachment of the cryptand could be labeled with ^{133}Ba in pH 7.4 phosphate buffer in 30% radiochemical yield at 6 h. In addition the amount of radioactivity associated with the protein remained constant at 24 h.

Control experiments for the labeling procedure.

A. At pH 9.2 under the conditions used for labeling the protein with the isothiocyanatocryptate, ^{133}Ba produced no detectable colloid in the void volume of PD-10 (Figure 2). Column recovery of the Ba radioactivity was quantitative. In the identical experiment with ^{142}Pr, only 7% column recovery was obtained and 72% of that was associated with the void volume fraction. In this case most of the radioactivity was retained at the top of the column.

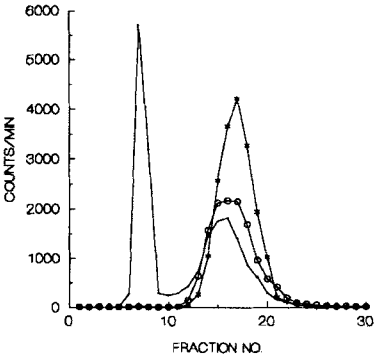

Figure 2. Superimposed plots of the PD-10 gel filtration results of Ba-133 isothiocyanatocryptate + IgG (points), Ba-133 chloride + IgG (stars), and Ba-133 chloride alone (circles) in 0.1 M phosphate buffer, pH 9.2. Labeled protein (fraction 7) is produced only by the reaction of the cryptate and IgG. No radioactive Ba^{2+} colloid (also fraction 7) or non-specific association of Ba^{2+} and protein occurs under the labeling conditions. Fractions 12-21 contain low molecular weight radioactive species, i.e., Ba^{+2} and/or Ba^{+2} cryptate.

B. Under the conditions of labeling there was no significant non-specific association of ^{133}Ba and IgG. The PD-10 column recovery of radioactivity was quantitative with less than 0.3% of total radioactivity associated with the void volume fraction. In the study to detect non-specific interaction of ^{142}Pr with IgG under the conditions of the labeling process, 90% of the radioactivity applied to the column was retained by the column. Of the 10% radioactivity recovered from the column, 78% was obtained in the void volume.When the void volume fraction was filtered through a 0.45 µm filter in preparation for HPLC analysis; however, greater than 99.5% of the radioactivity was retained on the filter. These results were comparable to those obtained from the ^{142}Pr protein labeling experiments.

Stability studies. The dialysis data of the ^{133}Ba bioconjugate indicate a logrithmic relationship and apparent first order kinetics for loss of ^{133}Ba from the bioconjugate. The half-life of the bioconjugate at pH 7.4 and pH 9.2 is about 6 h and 12 h, respectively (Figure 3).

The results of the stability of the bioconjugate as a function of pH are provided in Figure 4.

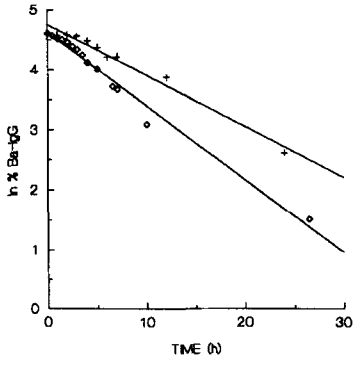

Figure 3. Linear fit of the loss of Ba-133 radioactivity from the Ba-133 cryptate labeled IgG with time during dialysis against pH 7.4 (diamonds) and pH 9.2 (pluses) phosphate buffers.

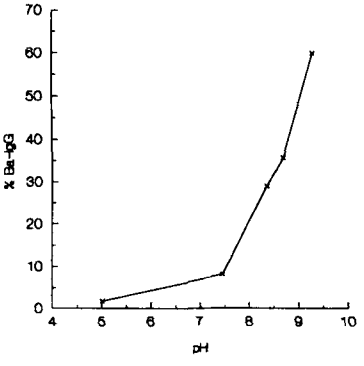

Figure 4. Relative amount of Ba-133 cryptate labeled IgG remaining after 20 h at 5°C in phosphate buffers of different pH.

Discussion

Bioconjugate formation initially demonstrated a logarithmic relationship with time. In view of our observations indicating the existance of an equilibrium between labeled bioconjugate and free Ba^{2+} ions, this experiment was repeated for a longer period of time to determine at what time the maximum yield was obtained. Under the conditions used, this occurs at about 20 h. This labeling method consistantly provided better than 40% radiochemical yield of the ^{133}Ba bioconjugate. The alternative labeling method was not investigated extensively, but served to demonstrate that Ba^{2+} could be attached to the protein provided that the cryptand had previously been attached to the protein. The results also suggest that the establishment of the cryptand/cryptate equilibrium is rapid compared to the rate of attachment of the derivatized cryptate to the protein. This method may be more useful in applications with relative short-lived radionuclides. The control experiments described were designed to determine if and to what extent metal colloid formation occurred in the labeling procedure. Sephadex G-25 gel filtration has been used as a means of fractionating chemical species primarily on molecular weight differences (14). Large components such as proteins or colloidal materials are eluted first. Colloid can be confused with labeled protein if the assay method does not distinguish it as demonstrated here with Pr^{3+}. Our previous work noted the difficulty in assaying metal labeled proteins due to colloid formation (15). The expected pH dependency of bioconjugate stability was demonstrated by the dialysis data of the ^{133}Ba bioconjugate (Figure 3) and the pH stability study (Figure 4). Protonation of the bridgehead nitrogens destabilizies cryptates by reducing the interaction of the free electron pairs of the nitrogens with the metal ion. This mode of decomposition of cryptates appears to be the one that most significantly affects the stability of the labeled bioconjugate. Since Ba^{2+} ion will become associated to IgG which has been previously treated with the isothiocyanate derivative of the cryptand, it is reasonable to assume that an equilibrium is established between free and complexed Ba^{2+} ions and that the equilibrium is pH dependent.

X-ray crystallographic data (16) of the Ba^{2+} cryptate of 2B:2:2 indicates that Ba^{2+} deforms the cryptand such that the ethoxy bridges are not symmetrically arranged around the Ba^{2+} ion. The Pr^{3+} ion is somewhat smaller than Ba^{+2} (17), yet apparently provides a less stable cryptate with $2BNH_2$:2:2. Rare earth metal ions are known to avidly scavenge water and in dilute solution are likely to form colliodal hydroxides. The results reported here strongly suggest that under aqueous equilibrium conditions, Pr^{3+} preferentially forms a colloidal hydroxide instead of complexing with the 2:2:2 cryptand.

A considerable number of studies have been conducted on cryptate stabilities (6,18,19,20), and much of this early work was limited to the alkali and alkaline earth metals. Although the Ba^{2+} cryptate model chosen for our initial work was not expected to provide a very stable bioconjugate, it has been useful for methods development. Indeed, Hg^{+} and Pb^{2+} cryptates of the 2:2:2 cryptand system are known to be more stable complexes (18) and other metal ion cryptates have been described as substitutionally inert (21). Moreover, the application of cryptates to radioimmunotherapy for cancer will depend upon the selection of suitable isotopes that are stabily bound to the protein *in vivo*.

Acknowledgements

This work was supported by the Research and Development Service of the Department of Veterans Affairs.

References

1. Ford C.H.J. and Casson A.G.- Cancer Chemother. Pharmacol. 17: 197 (1986)

2. Gansow O.A.- Nucl. Med. Biol. 18: 369 (1991)

3. Roselli M., Schlom J., Gansow O.A., Raubitschek A., Mirzadeh S., Brechiel M.W., and Colcher D.- J. Nucl. Med. 30: 672(1988)

4. Macklis, R.M., Kinsey, B.M., Kassis, A.I., Farrara J.L.M., Atcher R.W., Hines J.J., Coleman C.N., Adelstein S.J., and Burakoff S.J.- Science 240: 1024 (1988)

5. Deshpande S.V., DeNardo S.J., Kukis D.L., Moi M.K., McCall M.J., DeNardo G.L.,and Meares C.F.- J. Nucl. Med. 31: 473 (1990)

6. Lehn J.M.- Struct. Bonding 16: 1 (1973)

7. Gansow O.A. and Kausar A.R.- Inorg. Chim. Acta 91: 213 (1984)

8. Ward H.A. and Frothergill J.E.- Fluorescent Protein Tracing, 4th ed., R.C. Nairn, Ed., pp. 14-31, Longmans Green, New York (1976)

9. Gansow O.A., Kausar A.R., and Triplett K.B.- J. Heterocyclic Chem. 18: 297 (1981)

10. Pettit W.A., Iwai Y., Barfknecht C.F., and Swenson D.C.- J. Heterocyclic Chem. 29: 877 (1992)

11. Pettit W.A., Swailes B.K., and Peterson R.E.- J. Chromatogr. 469: 440 (1989)

12. Gansow O.A. and Kausar A.R.- Inorg. Chim. Acta 72: 39 (1983)

13. Udenfriend S., Stein S., Bohlen P., Dairman W., Leimgruber W., and Weigele M.- Science 178: 871 (1972)

14. Sephadex Gel Filtration in Theory and Practice. pp. 1-63, Pharmacia Fine Chemicals, Uppsala, Sweden (1975)

15. Pettit W.A., DeLand F.H., and Bennett S.J.- Int. J. Appl. Radiat. Isotopes 29: 344 (1978)

16. Pettit W.A., and Baenziger N.C.- Acta Crystallogr. (in press)

17. Handbook of Chemistry and Physics, 1st Student ed.,Weast R.C., Ed., p. F 105, CRC Press, Boca Raton, FL. (1987)

18. Yee E.L., Gansow O.A., and Weaver M.J.- J. Amer. Chem. Soc. 107(7): 2278 (1980)

19. Lehn J.M. and Sauvage J.P.- J. Amer. Chem. Soc. 97(23): 6700 (1975)

20. Kauffmann E., Dye J.L., Lehn J.M., and Popov A.I.- J. Amer. Chem. Soc. 107(7): 2274 (1980)

21. Gansow O.A., Kausar A.R., Triplett K.M., Weaver M.J., and Yee E.L.- J. Am. Chem. Soc. 99(21): 7087 (1977)