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Selectivity of Antibody–Chelate Conjugates for Binding Copper in the Presence of Competing Metals

David L. Kukis, Min Li, and Claude F. Meares*

Department of Chemistry, University of California, Davis, California 95616-0935

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Labeling monoclonal antibodies with radionuclides such as ^{67}Cu or ^{90}Y gives products with cytotoxic properties, which are now being clinically tested. It is essential that metal ions are not lost from the antibody conjugate at significant rates. Conjugates of polyazamacrocyclic bifunctional chelating agents previously developed in our laboratory, the copper complex of 6-(R-benzyl)-1,4,8,11-tetraazacyclotetradecane-*N,N',N'',N'''*-tetraacetic acid (TETA)[1] and the yttrium complex of 2-(R-benzyl)-1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid (DOTA),[2] where R denotes an antibody, linker, or other substituent, have shown remarkable kinetic inertness *in vitro* in human serum and *in vivo*.[3,4]

To avoid radiolytic decomposition, it is necessary to rapidly radiolabel an antibody–macrocycle conjugate with ^{67}Cu. In practice, this requires that the macrocycle selectively bind copper, to the exclusion of other metals present in the labeling mixture. Although solutions of ^{67}Cu are radionuclidically pure,[5] they contain only tiny quantities of this radionuclide relative to stable copper and other metal ions. Four recent lots assayed by the supplier contained on average 0.43 μM ^{67}Cu, 250 μM total copper, and 88 μM zinc. Appreciable amounts of iron and lead were also detected in two lots.

Unsubstituted TETA and related tetraazacyclotetradecanes have shown marked selectivity for binding copper at equilibrium.[6-10] However, calculations based on equilibrium constants are poor predictors of the behavior of copper complexes under physiological conditions.[11] Here we begin to explore how well the equilibrium binding of copper and other metals to the unsubstituted ligands TETA, DOTA, 1,4,7-triazacyclononane-*N,N',N''*-triacetic acid (NOTA), and ethylenediaminetetraacetic acid (EDTA) is correlated with the kinetic properties of those ligands when modified with side chains and attached to large proteins (monoclonal antibodies).

General laboratory techniques were as described previously.[12-14] The syntheses of 2-[*p*-(bromoacetamido)benzyl]-NOTA,[15] 2-[*p*-(bromoacetamido)benzyl]-DOTA,[13,16] 6-[*p*-(bromoacetamido)-benzyl]-TETA,[1] and 1-[*p*-(isothiocyanato)benzyl]-EDTA[12] have been described. Each was conjugated to monoclonal antibody Lym-1[17] by our usual methods and assayed at 1.1–2.7 available ligands per antibody.[12,13,18] The conjugates were transferred to 0.5 M ammonium citrate, pH 5.0.[12] This buffer was chosen because it provides the best results for labeling the antibody–TETA conjugate with ^{67}Cu for clinical use.[19] Zinc, from which ^{67}Cu is produced, was chosen as a competitor because appreciable concentrations of zinc are routinely detected in ^{67}Cu solution. Calcium and magnesium were chosen because, as common contaminants derived from containers and sources of airborne contamination,[20] they are likely to be present in biological buffers and other solutions. Standard solutions of Ca(II), Mg(II), Zn(II), and Cu(II) in 0.5 M ammonium citrate, pH 5.0, were prepared gravimetrically from their chlorides. Copper was trace radiolabeled with ^{67}Cu(II) (Brookhaven National Laboratory).

The antibody–TETA conjugate, copper ion, and metal ion competitors were combined so that the concentration of available TETA groups was 6.5 μM, [Cu(II)] was 10 μM, and metal ion competitor was 0, 100, 1000, 10 000, and 100 000 μM (except that 100 000 μM calcium solutions were not stable in this buffer). For the antibody–EDTA conjugate (12 μM available EDTA),

(1) Moi, M. K.; Meares, C. F.; McCall, M. J.; Cole, W. C.; DeNardo, S. J. *Anal. Biochem.* **1985**, *148*, 249.
(2) Moi, M. K.; Meares, C. F.; DeNardo, S. J. *J. Am. Chem. Soc.* **1988**, *110*, 6266.
(3) Deshpande, S. V.; DeNardo, S. J.; Meares, C. F.; McCall, M. J.; Adams, G. P.; Moi, M. K.; DeNardo, G. L. *J. Nucl. Med.* **1988**, *29*, 217.
(4) Deshpande, S. V.; DeNardo, S. J.; Kukis, D. L.; McCall, M. J.; DeNardo, G. L.; Meares, C. F. *J. Nucl. Med.* **1990**, *31*, 473.
(5) Dasgupta, A. K.; Mausner, L. F.; Srivastiva, S. C. *Appl. Radiat. Isot.* **1991**, *42*, 371.
(6) Hancock, R. D.; Martell, A. E. *Chem. Rev.* **1989**, *89*, 1875.
(7) Thõm, V. J.; Fox, C. C.; Boeyens, J. C. A.; Hancock, R. D. *J. Am. Chem. Soc.* **1984**, *106*, 5947.
(8) Stetter, H.; Frank, W. *Angew. Chem., Int. Ed. Engl.* **1976**, *15*, 686.
(9) Delgado, R.; Fraũsto de Silva, J. J. R. *Talanta* **1982**, *29*, 815.
(10) Cacheris, W. P.; Nickle, S. K.; Sherry, A. D. *Inorg. Chem.* **1987**, *26*, 958.
(11) (a) Meares, C. F. In *Radiolabeled Monoclonal Antibodies for Imaging and Therapy*; NATO Advanced Study Institute on Antibodies (Italy, 7/86), Plenum Press: New York, **1988**; p 229. (b) Moi, M. K.; DeNardo, S. J.; Meares, C. F. *Cancer Res. (Suppl.)* **1989**, *50*, 789s-793s.
(12) Meares, C. F.; McCall, M. J.; Reardan, D. T.; Goodwin, D. A.; Diamanti, C. I.; McTigue, M. *Anal. Biochem.* **1984**, *142*, 68.
(13) McCall, M. J.; Diril, H.; Meares, C. F. *Bioconjugate Chem.* **1990**, *1*, 222.
(14) Studer, M.; Kroger, L. A.; DeNardo, S. J.; Kukis, D. L.; Meares, C. F. *Bioconjugate Chem.* **1992**, *3*, 424.
(15) Studer, M.; Meares, C. F. *Bioconjugate Chem.* **1992**, *3*, 337.
(16) Renn, O. R.; Meares, C. F. *Bioconjugate Chem.* **1992**, *3*, 563.
(17) Epstein, A. L.; Zimmer, A. M.; Spies, S. M. In *Malignant Lymphomas and Hodgkin's Disease: Experimental and Therapeutic Advances*, Cavalli, F., Bonadonna, C., Rozencweig, M., Eds.; Martinus Ninjhoff Publishing Co.: Boston, MA, 1985; p 569.
(18) Rana, T. M.; Meares, C. F. *Bioconjugate Chem.* **1990**, *1*, 357.
(19) Kukis, D. L.; Diril, H.; Greiner, D. P.; DeNardo, S. J.; DeNardo, G. L.; Salako, Q. A.; Meares, C. F. *Cancer*, in press.
(20) Thiers, R. A. *Methods Biochem. Anal.* **1957**, *5*, 273.

0020-1669/93/1332-3981$04.00/0

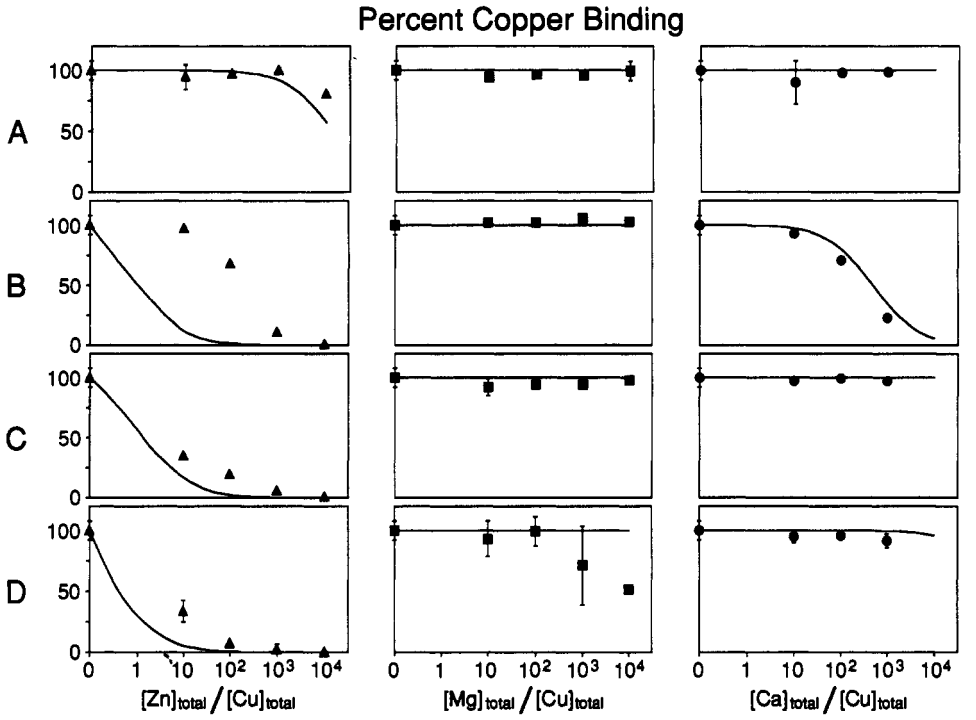

Figure 1. Measured efficiency of copper complexation by the antibody–TETA conjugate (row A), the antibody–DOTA conjugate (B), the antibody–NOTA conjugate (C), or the antibody–EDTA conjugate (D) in the presence of competitors zinc (▲), magnesium (■), or calcium (●). Error bars represent the range of values for duplicate experiments; where no error bars are visible, the range was smaller than the symbol. Curves are the results of equilibrium calculations.

the antibody–NOTA conjugate (17 μM available NOTA), and the antibody–DOTA conjugate (14 μM available DOTA), the metal concentrations were double the values above. The samples were vortex mixed, incubated for 2 h at room temperature, and then purified and transferred to 0.2 M ammonium citrate, pH 6.5, by centrifuged column gel filtration. To remove any nonspecifically bound metal ions, Na_2EDTA (Fisher certified, 0.10 M) was added to the effluents to a final concentration of 14 mM. The solutions were vortex mixed, incubated for 1 h at room temperature, and purified by centrifuged column gel filtration. The EDTA challenge and gel filtration were repeated, and the number of copper ions bound per conjugate was assayed.[12,13]

As shown in Figure 1, only the antibody–TETA conjugate exhibited high selectivity for copper in the presence of each of the three competitors surveyed. For concentrations of calcium, magnesium, or zinc that were respectively 10^3, 10^4, or 10^4 times that of copper, the number of copper ions bound per conjugate were 1.44, 1.44, and 1.17 (compared to 1.45 in the absence of competitor).

Using published equilibrium constants for binding protons and metal ions by citrate and unsubstituted chelators,[21] curves were calculated for the equilibrium complexation of copper relative to the other metal ions. The presence of protein, which did not bind copper under these experimental conditions, was not considered in the calculations. The calculated difference in TETA conditional stability constants for copper and zinc under these conditions is approximately 10^4 and is paralleled by experiment. The zinc/copper competition (panels in the left column of Figure 1) generally indicates a small bias in favor of copper relative to what is predicted by equilibrium calculations. In the case of DOTA (row B), the observed difference is dramatic; copper binding exceeds the theoretical predictions by about 2 orders of magnitude. DOTA generally forms the most kinetically inert complexes, and its apparent rate constant for binding copper exceeds that for zinc by a similar factor ($\approx$50).[22] The observed result would be expected from competing, irreversible binding reactions.

For magnesium and calcium, experiment and theory agree well for all ligands studied, except that magnesium binding to EDTA is stronger than predicted (center panel, row D of Figure 1). The ability of calcium to compete with copper for binding to DOTA is notable (right panel, row B).

To be useful for therapy, a radioimmunopharmaceutical must be kinetically inert to decomposition but readily radiolabeled. Previous studies have shown that copper chelates of the macrocycles NOTA, DOTA, and TETA, conjugated to monoclonal antibodies, all exhibit high kinetic stability in human serum under physiological conditions.[19] Figure 1 shows that for antibody conjugates of these three macrocycles and the acyclic chelator EDTA under practical conditions, the TETA analog has the highest selectivity for binding copper in the presence of calcium, magnesium, or zinc.

The significance of this is demonstrated by the selectivity of TETA for copper relative to other metals present in solutions of purified ^{67}Cu. Measured amounts of EDTA and 6-(*p*-nitrobenzyl)-TETA were added to different lots of ^{67}Cu in 0.1 M ammonium acetate, pH 5.0. After 30 min at room temperature, the solutions were examined by TLC. The amount of EDTA required for complete uptake of copper exceeded the amount of total copper present by a factor of 2–120. The varying excess of EDTA required is due to the variable nature and extent of trace metal contamination. In contrast, no excess of TETA was required for complete uptake of copper, demonstrating its selectivity for copper relative to all metal ions present in ^{67}Cu solutions.

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(21) Martell, A. E.; Smith, R. M. *Critical Stability Constants*; Plenum Press: New York, 1974–1989, Vols. 1, 3, 5, 6.

(22) Kasprzyk, S. P.; Wilkins, R. G. *Inorg. Chem.* **1982**, *21*, 3349.