Final Scientific/Technical Report

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Recipient:	Children's Hospital Boston
Project Title:	In Vitro Assessment of the In Vivo Stability of Cu-64 Radiopharmaceuticals
Principal Investigator:	Alan B. Packard, Ph.D.
Distribution Limitations:	None

DOE/Office of Science Program Office: Office of Biological and Environmental Research (BER)
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Executive Summary

Objective – There were three objectives to this project: First, to develop a method with which to measure the rate of loss of ^{64}Cu from the ligands that are used in the preparation of ^{64}labeled radiopharmaceuticals; second, to validate the method by comparing the measured *in vitro* stability of these ^{64}Cu complexes with the *in vivo* stability of ^{64}Cu-labeled antibodies prepared using the corresponding bifunctional chelators (BFCs); and third, to use the results of studies carried out in the first two objectives to determine the chemical factors that define the *in vivo* stability of ^{64}Cu radiopharmaceuticals.
Hypothesis tested – The proposed method would 1) provide quantitative measures of the *in vitro* stability of ^{64}Cu radiopharmaceuticals and 2) enable the systematic evaluation of the chemical factors that define the *in vivo* stability of ^{64}Cu radiopharmaceuticals, thereby laying the groundwork for the future development of superior, high-specific-activity ^{64}Cu radiopharmaceuticals.
Experimental design – We evaluated two techniques for the measurement of the kinetic stability of ^{64}Cu-labeled radiopharmaceuticals: batch extraction of free ^{64}Cu and free-ion selective radiotracer extraction (FISRE). These methods were validated by measuring the dissociation rate of ^{64}Cu from a known compound - Cu-EDTA. During this validation, we observed that the FISRE (free-ion-selective radiotracer extraction) method that was to be the primary method used in this project was not, in fact, applicable to the more stable complexes used as BFCs. This required that we develop an alternative method with which to effect these measurements.
Results – Although initial studies using FISRE suggested that it could be applied to the target complexes, more in-depth investigations revealed significant problems. The most important problem is that the rate of exchange between the Chelex-100 resin used in the column for the FISRE experiments was slower than the rate of exchange of ^{64}Cu from the complexes that were the subject of this study and was found to be dependent on the flow rate of the experimental solution through the column. Because of this, ^{64}Cu that was lost from the complexes during the experimental period was not completely trapped on the Chelex-100 obviating the use of this method to measure the stability of these complexes. An alternative method was, therefore, developed and used to accomplish these measurements. This method relies on the measurement of the rate of replacement of ^{64}Cu from the complex by non-radioactive copper. Studies carried out using this method revealed that at pH 7.5 the order of stability is ^{64}Cu-NOTA$\approx$^{64}Cu-TETA>^{64}Cu-DOTA and that ^{64}Cu is more readily displaced from the DOTA ligand at pH 7.5 than at pH 6.0 or 5.0. In contrast, neither ^{64}Cu-TETA nor ^{64}Cu-NOTA show a similar pattern of pH dependence. This method is now being applied to other ligands (e.g., CB2a) as well as to the evaluation of physiologically relevant factors other than pH.

Potential Impact (Benefits/Outcomes): Prior to this project, there was no biologically relevant method to measure the kinetic stability of ^{64}Cu radiopharmaceuticals. The innovative method developed in this project has provided the first quantitative data about the kinetic stability of the ^{64}Cu complexes of NOTA, DOTA, and TETA at physiologically relevant pH and NaCl concentrations. This accomplishment will provide several immediate benefits. First, it is now possible to actually measure the kinetic stability of the ^{64}Cu complexes that are used as BFCs whereas this data was previously inferred from differences in biodistribution. Second, this method can be applied to the conjugates of these BFCs with proteins and the rate at which ^{64}Cu is lost from the materials can be directly measured allowing differences in biodistribution to be ascribed to factors other than loss of ^{64}Cu from the complex. Furthermore, differences in the rate of loss of ^{64}Cu from the bioconjugates versus the free ligands can be measured allowing the evaluation of the effect of protein conjugation on chelate stablity. Third, as more data is acquired with a larger variety of ligands, the factors that control the kinetic stability of the complexes can be more precisely evaluated. Fourth, as this method is not specific for ^{64}Cu complexes, it can be applied to other metalloradiopharmaceuticals thus allowing the more precise evaluation of optimal chelation conditions for radiometals such as ^{68}Ga and ^{89}Zr. In the longer-term, improved knowledge of the stability of the complexes should have and appreciable and lasting effect upon the way in which various diseases (especially cancer) are detected, diagnosed, staged, and treated.

Report

The objective of this project was to develop a method with which to measure the bioavailability of ^{64}Cu ("free" ^{64}Cu) in ^{64}Cu radiopharmaceuticals. This method would be used to measure the dissociation rate constants (k_d) of the ^{64}Cu complexes of a series of chelators that are frequently used in the preparation of ^{64}Cu radiopharmaceuticals as well as the k_ds of the radioimmunoconjugates prepared using these chelators. The method would then be applied to the determination of the chemical factors that define the *in vivo* stability of ^{64}Cu radiopharmaceuticals.

The successful development of high specific-activity (HSA) ^{64}Cu radiopharmaceuticals depends upon retention of the ^{64}Cu atom in the radiopharmaceutical. While there have been many efforts to develop new chelators that better retain ^{64}Cu *in vivo*, there have been no corresponding efforts to develop an effective *in vitro* method by which this increased retention may be measured. Instead, the *in vivo* stability of ^{64}Cu radiopharmaceuticals has been assessed indirectly, using parameters such as liver uptake, with decreased liver uptake suggesting higher *in vivo* complex stability. This approach is, however, inadequate in the context of ^{64}Cu radiopharmaceuticals that are expected to accumulate to some degree in the liver even in the complete absence of free ^{64}Cu (e.g., ^{64}Cu-labeled antibodies), and furthermore it is, at best, an indirect measure of complex stability. The development of core technologies such as those developed in this project is, therefore, essential to the successful development of radiopharmaceuticals that use bifunctional chelators to attach radiometals to targeting vectors such as peptides and antibodies. It is also essential to the elucidation of the chemical factors that give rise to increased *in vivo* stability of ^{64}Cu.

Hypothesis – The development of method with which to measure the bioavailability of ^{64}Cu ("free" ^{64}Cu) in ^{64}Cu radiopharmaceuticals will provide an innovative tool that reliably predicts the bioavailability of ^{64}Cu in ^{64}Cu-labeled radioimmunoconjugates and can also be used for the systematic evaluation of the chemical factors that define the *in vivo* stability of ^{64}Cu radiopharmaceuticals, effectively laying the groundwork for the future development of superior ^{64}Cu radiopharmaceuticals.

This project included two Specific Aims:

Aim #1 – Develop and validate a method by which to measure the *in vitro* stability of ^{64}Cu radiopharmaceuticals may be measured Copper(II) complexes are typically extremely labile, and the bioavailability of Cu^{II} is determined not only by the amount of "free" Cu that is present at any given time, but also by the rate at which the Cu^{II} dissociates from the ligand *in vivo*. In this project, we developed and validated an *in vitro* method to measure the rate at which ^{64}Cu is lost from ^{64}Cu complexes of chelators used in the preparation of metalloradiopharmaceuticals.

Aim #2 – Determine the chemical factors that define the *in vivo* stability of ^{64}Cu complexes Copper-64 radiopharmaceuticals are generally based on existing chelating agents(e.g., DOTA), and efforts to develop more stable ^{64}Cu complexes have focused on wrapping the Cu atom more tightly (e.g., cross-bridged cyclam and SarAr) and observing decreased liver uptake. This approach does not, however, take into account other factors that may affect complex stability *in vivo* (e.g., chelate ring size, chelate flexibility, and ring substitution). The second aim of this project was to apply the system developed in Aim #1 to the measurement of bioavailable ^{64}Cu in two series of well-defined ^{64}Cu complexes and use these measurements to evaluate how these chemical factors affect *in vitro* stability. Because of the delays encountered in developing and validating the measurement method (Aim #1), we were not able to complete Aim #2.

The primary method by which Aim #1 was to be accomplished was through the use of FISRE (Free-Ion-Selective Radiotracer Extraction). In this method, a solution of the ^{64}Cu complex of interest is added to a solution of non-radioactive copper in a suitable buffer and passed through a custom-built column containing Chelex-100 resin saturated with non-radioactive copper. For this method to work effectively, any ^{64}Cu that is lost from the complex must be instantly complexed by the Chelex-100 resin while the ^{64}Cu that is lost from the complex is replaced by non-radioactive copper. This requires that the Chelex-100 resin have a higher affinity for copper than the target complex. While this was the case in previous studies of complexes such as Cu-DTPA (ref??), it is not the case for more kinetically stable complexes

such those used as BFCs. With these more kinetically stable complexes, the measured rate of exchange becomes a function of flow rate of the sample through the column rather than complex stability. This result led to the development of an alternative method by which to accomplish this measurement.

The alternative method that was developed overcame this limitation. In summary, the ^{64}Cu complex of the ligand of interest was prepared in a suitable buffer at pH 5.0 (piperazine), 6.0 (MES), or 7.5 (HEPES) in 0.01 M NaCl. The preformed complex was then added to a solution of non-radioactive copper (1, 2, or 5 μM) and the appearance of free ^{64}Cu in the solution was measured as a function of time by thin-layer chromatography. The method was applied to the three ligands that form the cores of the bifunctional chelators that are most commonly used to prepare ^{64}Cu-labeled proteins; DOTA, TETA, and NOTA.

In the first series of experiments, the kinetic stability of ^{64}Cu-DOTA, NOTA, and TETA were compared at pH 7.5 in the presence of 1.0 μM Cu(II). The results of this study are shown in Figure 1.

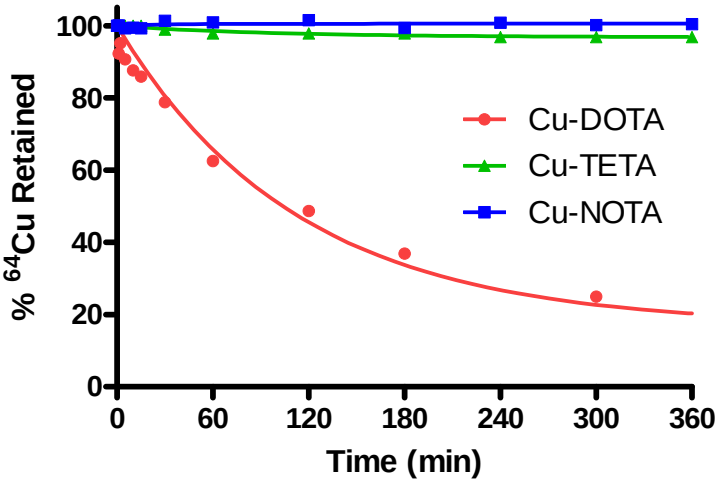

Figure 1. Measurement of the rate of loss of ^{64}Cu at pH 7.5, 1 μM Cu(II).

Under these conditions, more than 50% of the ^{64}Cu is lost from the DOTA ligand within two hours while more than 90% of the ^{64}Cu is retained by both TETA and NOTA. This observation led us to further evaluate the stability of ^{64}Cu-DOTA under a wider range of conditions.

Figure 2 (following page) summarizes the results of the measurement of the effects of pH and Cu(II) concentration on the stability of ^{64}Cu-DOTA. At pH 7.5 (data shown in red), increasing the non-radioactive Cu(II) concentration increases the rate at which ^{64}Cu is lost from the complex. A similar effect is seen at pH 6.0 (blue) and pH 5.0 (green), but the effect is less at lower pHs. One can also compare the effect of pH at a given Cu(II) concentration. For example, at 1 μM Cu(II), ^{64}Cu is lost from the ^{64}Cu-DOTA complex approximately twice as fast at pH 7.5 (<50% remaining at 2h) as it is at pH 6.0 (~90% remaining at 2 h). At pH 5.0, approximately 95% of the ^{64}Cu-DOTA remains at 2 h. This result is significant because it is contrary to the expectation that loss of ^{64}Cu from the Cu-DOTA should be by a disssociative rather than an associative mechanism. If the loss of ^{64}Cu was by a dissociative mechanism, it would be independent of the concentration of non-radioactive Cu(II) in the test solution. Similarly, one might expect that the rate of loss of 64Cu would be higher at lower pH and lower at higher pH because the dissociation of Cu(II) from complexes such as this is typically facilitated by protonation of the coordinated amines, which increases at lower pH.

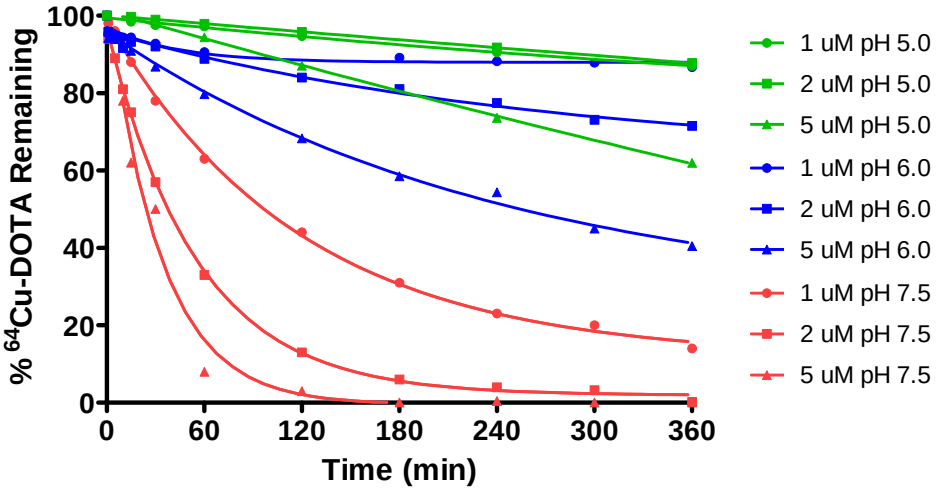

Figure 2. Effect of pH and Cu(II) concentration on loss of ^{64}Cu from ^{64}Cu-DOTA.

One possible explanation for this observation is that the loss of ^{64}Cu from the complex is facilitated by the "extra" carboxylate arms on the DOTA ligand. The structure of Cu-DOTA (F**ig. 3**., *Helv Chim Acta* **69**, 2067 (1986)) shows that the four N atoms and two of the carboxylate arms are coordinated to the Cu and two of the carboxylate arms are free. These free carboxylates can, therefore, coordinate to a non-radioactive Cu(II) atom from the solution facilitating loss of ^{64}Cu from the complex. Furthermore, the ability of these free carboxylates to coordinate Cu(II) will be inversely proportional to pH – At higher pH, the carboxylate will be less protonated and more available for complexation. This hypothesis can be tested by comparing the kinetic stability of ^{64}Cu-DOTA to that of ^{64}Cu-DODA, which only contains two carboxylate arms. If the hypothesis is correct, that stability of ^{64}Cu-DOTA should not decrease with increased Cu(II) concentration and increasing pH. Unfortunately, we were not able to carry out this experiment prior to completion of this project.

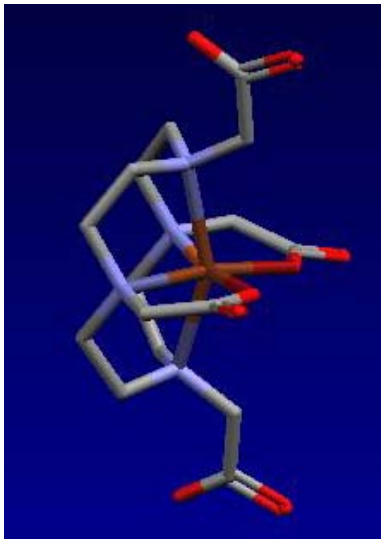

Figure 3. Structure of Cu-DOTA

An additional observation that was made during the evaluation of the stability of ^{64}Cu-DOTA is that the observed stability of the complex is affected by the specific activity of the ^{64}Cu used for the measurements. While this was initially somewhat surprising, it is consistent with the above observation that increasing the concentration of non-radioactive Cu(II) decreases the stability of the ^{64}Cu-DOTA complex.

Similar studies were carried out with NOTA and TETA. These results are summarized in Figures 4 and 5.

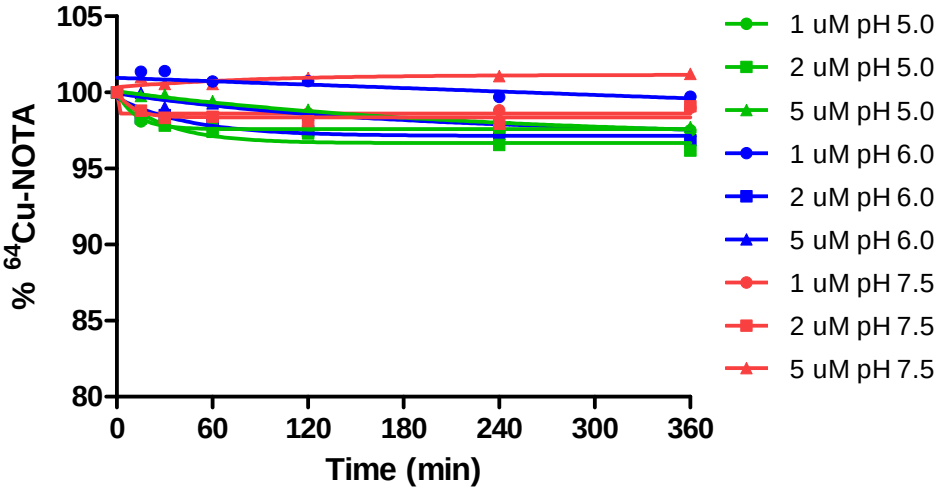

Figure 4. Effect of pH and Cu(II) concentration on stability of ^{64}Cu-NOTA

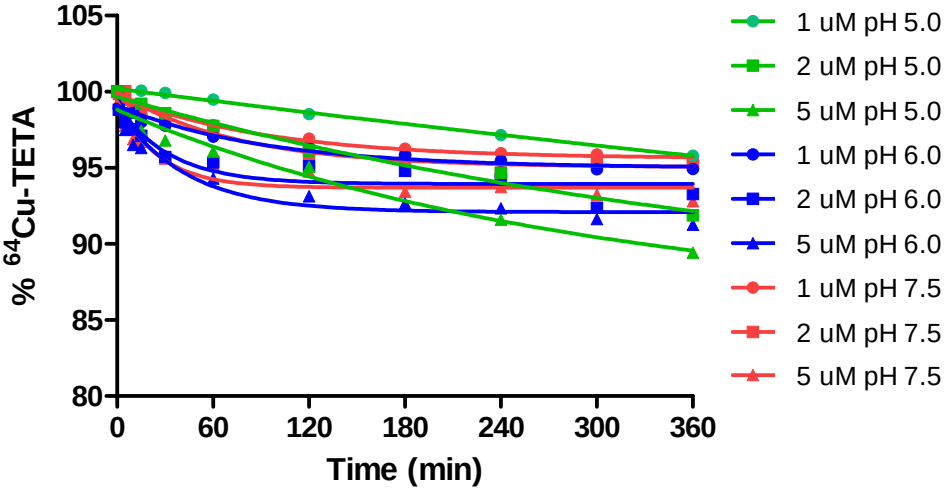

Figure 5. Effect of pH and Cu(II) concentration of the stability of ^{64}Cu-TETA

These data show that both ^{64}Cu-NOTA and ^{64}Cu-TETA are significantly more stable than ^{64}Cu-DOTA with respect to both pH and Cu(II) concentration, although there is perhaps a small effect with ^{64}Cu-TETA. This latter result would be consistent with the hypothesis that the extra carboxylate arms facilitate displacement of ^{64}Cu from these ligands with the key difference being that for ^{64}Cu-TETA, the Cu atom is located in the core of the macrocycle and is thus a great deal more difficult to displace than it is in ^{64}Cu-DOTA where the macrocyclic core is smaller and the Cu(II) atom sits above the plane defined by the four N atoms rather than in the center of the ligand.

In summary, this project has provided that first quantitative data about the stability of ^{64}Cu complexes of the three ligands that are most commonly used as BFCs to attach ^{64}Cu to proteins. These data show that, under these experimental condidtions, ^{64}Cu-NOTA and ^{64}Cu-TETA are both more stable that ^{64}Cu-DOTA. They also show that dissociation of ^{64}Cu from ^{64}Cu-DOTA is facilitated by increased Cu(II) concentration and increased pH. Additional experiments will be necessary to elucidate the reason for this increased lability, and we are currently seeking funding to support these studies.

Products

1) Publications
   a) Direct measurement of the kinetic stability of Cu-64 complexes of bifunctional chelators. Vidhi Maheshwari, Jason Dearling, S. Ted Treves and Alan Packard, *J Nucl Med*. 2010; **51** (Supplement 2):135.
   b) In-vitro stability measurement of Cu-64 complexes of bifunctional chelators. Vidhi Maheshwari, Jason Dearling, Ted S Treves, Alan B Packard, 240th Annual Meeting of the American Chemical Society, Boston, MA, 2010, abstract #NUCL64.
   c) Measurement of copper(II) exchange kinetics for Cu-64 complexes of bifunctional chelators. Jason Dearling, Vidhi Maheshwari, S. Ted Treves, Alan Packard, *J Nucl Med*. 2011; **52** (Supplement 1):408.
   d) *In vitro* evaluation of loss of ^{64}Cu from bifunctional chelators under physiologically relevant conditions. Maheshwari V, Dearling JL, Treves ST, Packard AB, *J Labelled Compd Radiopharm*. 2011; **54** (Supplement 1):S367.
   e) Direct measurement of the rate of loss of ^{64}Cu from bifunctional chelators under physiologically relevant conditions. Maheshwari V, Dearling JL, Treves ST, Packard AB. *Inorg Chim Acta*. 2012 (manuscript in preparation).

2) Networks or collaborations fostered

We were very fortunate in this project to be able to establish collaborations with several investigators who are developing new bifunctional chelating agents for ^{64}Cu in their laboratories. These investigators were kind enough to provide us with samples of their novel ligands for evaluation in our system. Unfortunately, due to the system problems that we encountered at the beginning of the project (described in the Technical Report), we were not able to carry out measurements using these new ligands. We do, however, hope to secure new funding for this project in the future, and if this proves possible, we will measure the kinetic stability of these new ligands.

Ed Wong/Gary Weissman – University of New Hampshire
Provided samples of CB2a and related ligands and valuable discussions

Cara Ferreira – MDS Nordion
Provided samples of CPTA and related ligands and valuable discussions

Elena Ryback-Akimova – Tufts University
Provided samples of novel macrocyclic ligands and valuable discussions

Claude Meares – UC Davis
Provided samples of BAT-6 and related ligands and valuable discussions

3) Technologies/Techniques

This project produced a simple straightforward method for the in vitro measurement of the kinetic stability of ^{64}Cu (and other radiometal) complexes of biological interest under physiologically relevant conditions. This method can be applied to measurement of the stability of other ^{64}Cu complexes and can be used to evaluate the effects of changes in ligand structure on the kinetic stability of the ^{64}Cu complexes. The method can also be used to measure the effect of conjugation of ligands to proteins on the kinetic stability of the ^{64}Cu complexes as well as the effect, if any, of protein size (e.g., peptide vs. antibody) on kinetic stability. Overall, this provides a direct, physiologically relevant measure that was not previously available. These measurements will ultimately therefore allow better decisions to be made about the optimal chelator to use with a particular metal in the development of metal-based radiopharmaceuticals, both for imaging and therapy.