Project Title: A Novel Approach to Prepare ^{99m}Tc-Based Multivalent RGD Peptides

1. OBJECTIVE: This project presents a novel approach to prepare the ^{99m}Tc-bridged multivalent RGD (arginine-glycine-aspartate) peptides. This project will focus on fundamentals of ^{99m}Tc radiochemistry. The main objective of this project is to demonstrate the proof-of-principle for the proposed radiotracers. Once a kit formulation is developed for preparation of the ^{99m}Tc-bridged multivalent RGD peptides, various tumor-bearing animal models will be used to evaluate their potential for SPECT (single photon-emission computed tomography) imaging of cancer.

2. APPROACH AND PROGRESS: This project is directed towards fundamental radiochemistry associated with integrin $\alpha_v\beta_3$-targeted ^{99m}Tc radiotracers. The synthetic methodology described in this project applies to the incorporation of ^{99m}Tc into a wide range of small biomolecules with high stability and specific activity. Over the last 15 months, we have successfully prepared two isonitrile-RGD peptide conjugates, $CNCH_2CH_2CO$-G_3–c(RGDfK) (G_3 = glycine-glycine-glycine) and $CNCH_2CH_2CO$-PEG_4–c(RGDfK) (PEG_4 = 15-amino-4,7,10,13-tetraoxapentadecanoic acid). We found that $CNCH_2CH_2CO$-G_3–c(RGDfK) and $CNCH_2CH_2CO$-PEG_4–c(RGDfK) are highly stable in aqueous solution under basic conditions (pH >7.5). We were also able to prepare their ^{99m}Tc(I)-tricarbonyl complexes: $[^{99m}Tc(L)_3(CO)_3]^+$ (L = $CNCH_2CH_2CO$-G_3–c(RGDfK) and $CNCH_2CH_2CO$-PEG_4–c(RGDfK)). We found that ^{99m}Tc(I)-tricarbonyl complexes show multiple peaks (Figure 1), caused by the presence of multiple species with various number of isonitrile-conjugated RGD peptides. However, we were not able to prepare complexes $[^{99m}Tc(L)_6]^+$ (L = $CNCH_2CH_2CO$-G_3–c(RGDfK) and $CNCH_2CH_2CO$-PEG_4–c(RGDfK)) in spite of intensive efforts. Therefore, we turned our direction to a fundamental question: are multimers really multivalent?

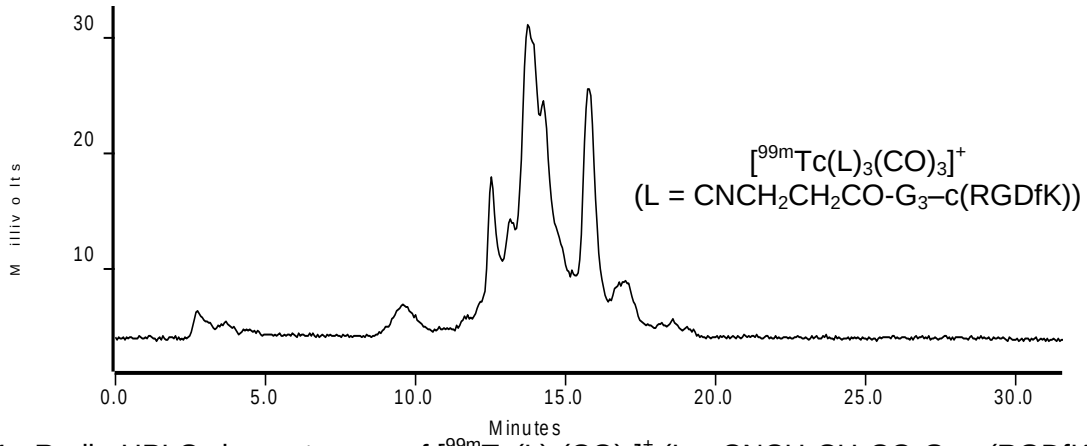

Figure 1. Radio-HPLC chromatogram of $[^{99m}Tc(L)_3(CO)_3]^+$ (L = $CNCH_2CH_2CO$-G_3–c(RGDfK)).

3. MULTIMERS NOT MULTIVALENT: On the basis of integrin $\alpha_v\beta_3$ binding assays and ex-vivo biodistribution data, we know that cyclic RGD dimers (3P-RGD_2 and 3G-RGD_2) and the tetramer (RGD_4) are bivalent in binding to integrin $\alpha_v\beta_3$. However, it remains unclear if RGD_4 will become tetravalent if the G_3 and PEG_4 linkers are incorporated between its four cyclic RGD motifs. To answer this fundamental question, two novel DOTA-conjugated cyclic RGD tetramers (Figure 2: 6P-RGD_4 and 6G-RGD_4) have been prepared. We also compared tumor uptake of ^{111}In-labeled cyclic RGD dimers (3P-RGD_2 and 3G-RGD_2) and tetramers in the athymic nude mice bearing U87MG glioma xenografts. We found that ^{111}In(DOTA-3P-RGD_2) and ^{111}In(DOTA-6P-RGD_4) shared a similar tumor uptake over the 24 h p.i., suggesting that 6P-RGD_4 and 6G-RGD_4 are not tetravalent. In contrast, ^{111}In(DOTA-RGD_4), ^{111}In(DOTA-6P-RGD_4) and ^{111}In(DOTA-6G-RGD_4) all had the longer tumor retention times than ^{111}In(DOTA-3P-RGD_2) and ^{111}In(DOTA-3G-RGD_2) (Figure 3), probably due to the presence of two extra RGD motifs. This finding is particularly important for the future development of ^{90}Y and ^{177}Lu radiotracers with great potential for radiotherapy of integrin $\alpha_v\beta_3$-positive solid tumors.

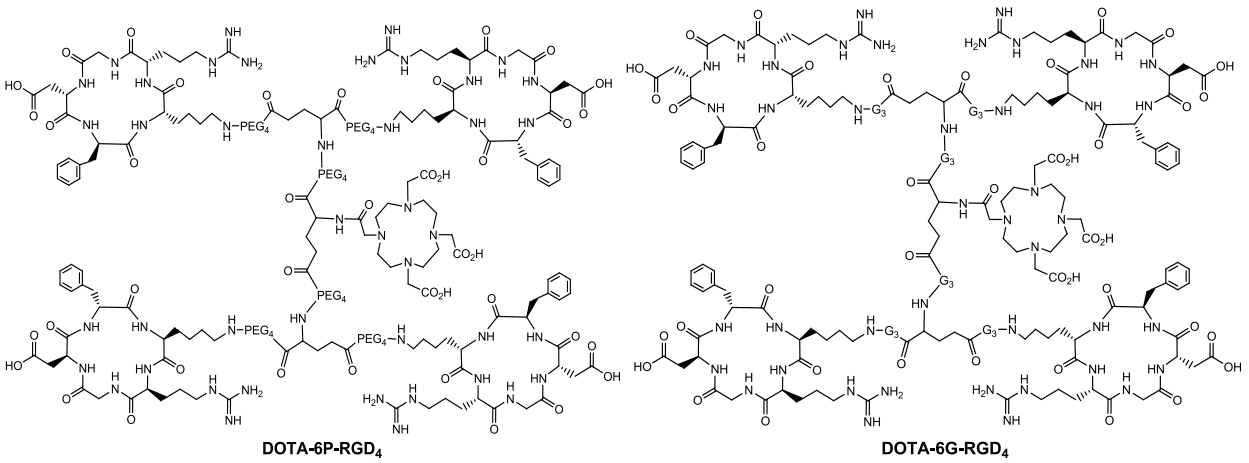

Figure 2. DOTA-conjugated cyclic RGD tetramers: DOTA-6P-RGD_4 and DOTA-6G-RGD_4.

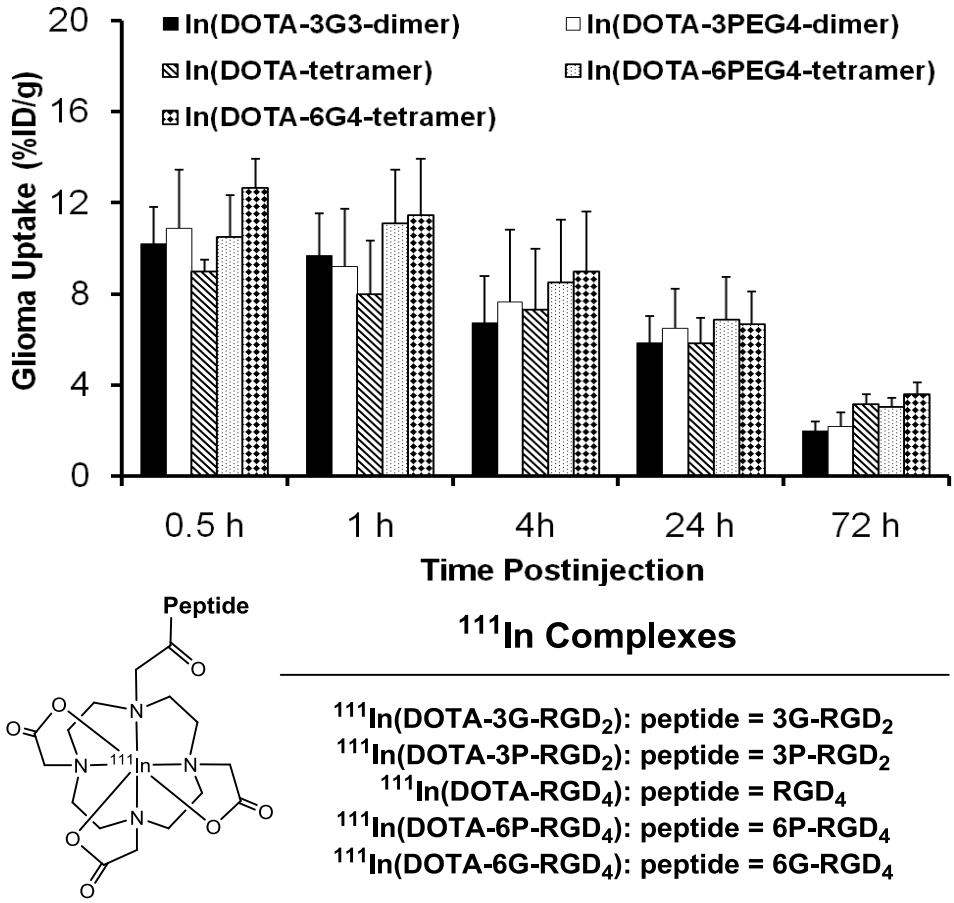

Figure 3. Direct comparison of the tumor uptake of the ^{111}In-labeled RGD dimers (3P-RGD_2 and 3G-RGD_2) and tetramers (RGD_4, 6P-RGD_4 and 6G-RGD_4) in athymic nude mice bearing U87MG glioma xenografts.

As discussed previously, two factors (Figure 4: bivalency and the enhanced local RGD concentration) contribute to the high integrin $\alpha_v\beta_3$ binding affinity of multimeric cyclic RGD peptides. The concentration factor exists in all multimeric cyclic RGD peptides regardless of spacers or linkers. The key for bivalency is the distance between two RGD motifs. For example, this distance in 3P-RGD_2 (38 bonds) and 3G-RGD_2 (26 bonds) is long enough for them to achieve bivalency, which leads to the higher integrin $\alpha_v\beta_3$ binding affinity of 3P-RGD_2 and 3G-

RGD_2 than that of RGD_2, and much higher tumor uptake of ^{111}In(DOTA-3P-RGD_2) and ^{111}In(DOTA-3G-RGD_2) than that of ^{111}In(DOTA-P-RGD_2). In contrast, the concentration factor might be responsible for the longer tumor retention times (Figure 4**)** of ^{111}In(DOTA-RGD_4), ^{111}In(DOTA-6P-RGD_4) and ^{111}In(DOTA-6G-RGD_4) as compared to that of ^{111}In(DOTA-3P-RGD_2) and ^{111}In(DOTA-3G-RGD_2). Even though 6P-RGD_4 and 6G-RGD_4 are not tetravalent, the presence of two extra RGD motifs definitely helps to improve the radiotracer tumor retention time, which might become important for ^{90}Y and ^{177}Lu radiotracers with great potential for radiotherapy of integrin $\alpha_v\beta_3$-positive solid tumors. It must be noted that the ability of a multimeric RGD peptide to achieve bivalency also depends on the tumor integrin $\alpha_v\beta_3$ density. If the tumor integrin $\alpha_v\beta_3$ density is very high, the distance between two neighboring integrin $\alpha_v\beta_3$ sites will be short, which makes it easier for the multimeric RGD peptide to achieve bivalency. If the integrin $\alpha_v\beta_3$ density is very low, the distance between two neighboring integrin $\alpha_v\beta_3$ sites will be long, and it might be more difficult for the same multimeric RGD peptide to achieve simultaneous integrin $\alpha_v\beta_3$ binding.

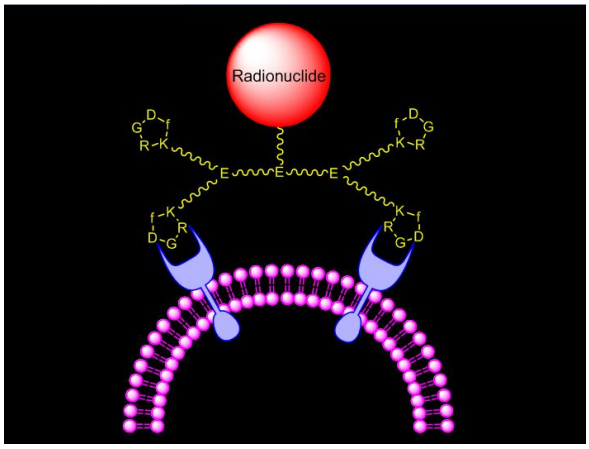

Figure 4. Schematic illustration of interactions between a cyclic RGD multimer and integrin $\alpha_v\beta_3$. The targeting moiety is c(RGDfK). The spacer is glutamic acid (E). If this distance is long enough, the cyclic RGD multimer will bind to integrin $\alpha_v\beta_3$ in a bivalent fashion. If this distance is not long enough for simultaneous integrin $\alpha_v\beta_3$ binding, the RGD concentration is still "locally enriched" in the vicinity of neighboring integrin $\alpha_v\beta_3$ sites once the first cyclic RGD motif is bound. The concentration factor exists in all RGD multimer regardless of spacer or linker. The combination of "bivalency" and "enriched RGD concentration" will result in higher integrin $\alpha_v\beta_3$ binding affinity for cyclic RGD multimers and better tumor uptake for their radiotracers.

4. INTEGRIN $\alpha_v\beta_3$ and RGD SPECIFICITY. integrin $\alpha_v\beta_3$ specificity of ^{111}In(DOTA-6P-RGD_4) was demonstrated using excess of RGD_2 (14 mg/kg or ~350 μg/mouse) as the blocking agent. Figure 5**A** compares the 60-min uptake of ^{111}In(DOTA-6P-RGD_4) in the absence/presence of RGD_2. Co-injection of RGD_2 almost completely blocked tumor uptake of ^{111}In(DOTA-6P-RGD_4). The normal organ uptake of ^{111}In(DOTA-6P-RGD_4) was also blocked by RGD_2. For example, the uptake of ^{111}In(DOTA-6P-RGD_4) in the heart, intestine, liver, lungs, and spleen was 1.43 ± 0.25, 0.78 ± 0.24, 11.53 ± 6.94, 4.01 ± 0.41, 3.69 ± 0.92 and 2.12 ± 0.20 %ID/g, respectively, without RGD_2, while its uptake in the same organs was only 0.13 ± 0.02, 0.38 ± 0.03, 0.47 ± 0.08, 0.66 ± 0.04, 1.15 ± 0.30, and 0.47 ± 0.06 %ID/g, respectively, with RGD_2. Figure 5**B** compares the 60-min uptake of ^{111}In(DOTA-6P-RGD_4) and ^{111}In(DOTA-6P-RGK_4) (non-sense peptide conjugate, c(RGKfD) instead of c(RGDfK)) in the tumor and normal organs. As expected, the non-sense compound had much lower ($p < 0.01$) tumor uptake (0.78 ± 0.06 %ID/g) than the original radiolabeled peptide (11.08 ± 2.08 %ID/g). ^{111}In(DOTA-6P-RGD_4) also had considerably higher ($p < 0.01$) uptake in normal organs such as intestine, kidneys, liver, lungs and spleen than ^{111}In(DOTA-6P-RGK_4) (Figure 5**B**).

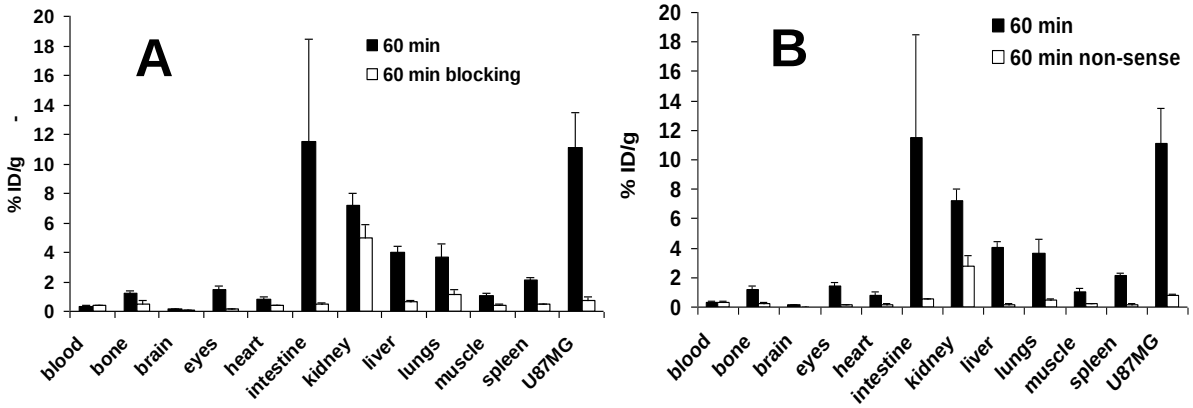

Figure 5. **A**: Comparison of the 60-min biodistribution data of ^{111}In(DOTA-6P-RGD$_4$) in athymic nude mice bearing U87MG glioma xenografts in the absence/presence of excess RGD$_2$ to demonstrate its integrin $\alpha_v\beta_3$–specificity; **B**: comparison of the 60-min biodistribution of ^{111}In(DOTA-6P-RGD$_4$) and ^{111}In(DOTA-6P-RGK$_4$) in athymic nude mice bearing U87MG glioma xenografts to demonstrate the RGD-specificity.

4. ACCOMPLISHMENTS. The following summarizes our accomplishments:

1). We have demonstrated that (1) multimerization of cyclic RGD peptides enhances the integrin $\alpha_v\beta_3$ bonding affinity and radiotracer tumor uptake; (2) addition of G$_3$ or PEG$_4$ linkers makes it possible for two RGD motifs in 3P-RGD$_2$ and 3G-RGD$_2$ to achieve simultaneous integrin $\alpha_v\beta_3$ binding; and (3) multimers are actually bivalent (not multivalent), the presence of extra RGD motifs can enhance the tumor retention time of the radiotracer.

2). One Ph.D. student graduated early 2009, two postdoctoral fellows trained, 6 papers published on refereed journals.

1. Zhou, Y.; Shao, G.; Wang, F.; and Liu, S. Imaging breast cancer lung metastasis by u-SPECT-II/CT with an integrin $\alpha_v\beta_3$-targeted radiotracer ^{99m}Tc-3P-RGD$_2$. *Theranostics* **2012**, 2:577-587.
2. Zhou, Y.; Kim, Y. S.; Lu, X.; and Liu, S. Evaluation of ^{99m}Tc-labeled cyclic RGD dimers: impact of cyclic RGD peptides and ^{99m}Tc chelates on biological properties*. Bioconj. Chem.* **2012**, 23, 586−595. (**Impact factor: 5.001**)
3. Zhou, Y.; Kim, Y. S.; Chakraborty, S.; Shi, J.; Gao, H.; and Liu, S. ^{99m}Tc-Labeled cyclic RGD peptides for noninvasive monitoring of tumor integrin $\alpha_v\beta_3$ expression. *Mol. Imaging* **2011**, 10: 386-97. (**Impact factor: 3.329**)
4. Shi, J.; Zhou, Y.; Chakraborty, S.; Kim, Y. S.; Jia, B.; Wang, F.; and Liu, S. Evaluation of ^{111}In-labeled cyclic RGD peptides: effects of peptide and PEG$_4$ multiplicity on their tumor uptake, excretion kinetics and metabolic stability. *Theranostics,* **2011**; *1,* 322-340.
5. Shi, J.; Jia, B.; Kim, Y. S.; Chakraborty, S.; Zhou, Y.; Wang, F.; and Liu, S. Impact of bifunctional chelators on biological properties of ^{111}In-labeled cyclic peptide RGD dimers. *Amino Acids* **2011**, 41, 1059-1070. (**Impact factor: 4.106**)
6. Chakraborty, S.; Liu, S.; Kim, Y. S.; Shi, J.; Zhou, Y.; and Wang, F. Evaluation of ^{111}In-labeled cyclic RGD peptides: tetrameric not tetravalent. *Bioconj. Chem.* **2010**, *21*, 969-978. (**Impact factor: 5.001**)