DOI: 10.21776/ub.jpacr.2023.012.03.659 *J. Pure App. Chem. Res.*, **2023**, 12 (3), 148-156
28 December 2023

# Computational Analysis on The Development of New Technetium-99m-labeled Pentapeptide for Cancer Molecular Imaging Targeting Integrin $\alpha_5\beta_1$

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Received 15 July 2022; Accepted 28 December 2023

## ABSTRACT

Cancer continues to be a major leading cause of death despite huge efforts dedicated to developing anticancer drugs. Radiopeptide is currently used for targeted therapy and diagnosis of cancer. The structure selection of new radiopeptide should be determined to minimize the decrease in binding affinity because of metal radioisotope and its chelator. In this research, pentapeptide Pro-His-Ser-Cys-Asn (PHSCN) and its technetium-based radiopeptides were analyzed computationally to provide information of their interactions to RGD binding pocket of integrin $\alpha_5\beta_1$ and synergy pocket of integrin by docking simulation using Autodock Vina and Autodock 4.2. PHSCN had two possible conformations to interact with integrin $\alpha_5$, thus provided two possible positions for conjugation with bifunctional chelator – technetium. Even though the results showed that the binding value of radiolabeled compounds was lower than PHSCN's, some radiolabeled compounds in this simulation might have biological activity. The use of HYNIC-EDDA as a chelator produces better value than DTPA and MAS3. Radiolabeling procedures in the N-terminal position of the peptide are preferable and have higher affinity than C-terminal modification. Further laboratory experiments are required to confirm the activity of EDDA-Tc-HYNIC-PHSCN-$NH_2$ on the integrin $\alpha_5\beta_1$ receptor.

Keywords: radiopeptide, technetium, integrin $\alpha_5\beta_1$, computational simulations

## INTRODUCTION

Cancer incidence and mortality are still rapidly growing worldwide [1]. Cancer treatment can be performed using one or a combination of surgery, chemotherapy, and radiation therapy. However, ineffectively cancer treatment commonly because the therapeutic drugs and radiopharmaceuticals have not target cancer tissues specifically which also causing side effects to normal tissues. Recently, specific radiolabeled peptides for diagnostic imaging and/or therapy of cancers have been developed. Peptides play an important role in the growth and cellular functions of normal and cancer cells [2]. The overexpression of peptide receptors on cancer patients offers advantages on anticancer peptides development over small molecules, proteins, and antibodies [3]. High binding affinity and selectivity of peptides provide fewer side effects on cancer treatment. Peptides are also easy to synthesize and convenient to conjugated with chelator and linker for radiopharmaceuticals preparation.

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**p-ISSN : 2302 – 4690 | e-ISSN : 2541 – 0733**

In the recent decade, integrin $\alpha_5\beta_1$ (fibronectin receptor) has attracted interest as cancer therapeutic targets mainly colon, breast, ovarian, lung, brain tumors, and anti-angiogenesis therapy [4]. Overexpression of this type of integrin is correlated with a poor state for cancer patients. Specific integrin $\alpha_5\beta_1$ antagonists based on small peptides as anti-angiogenic agents have been developed. Acetylated amidated pentapeptide Pro-His-Ser-Cys-Asn (PHSCN) inhibited the sequence Pro-His-Ser-Arg-Asn (PHSRN) in the 9th type III repeat of fibronectin that is required to allow full adhesion of integrin $\alpha_5\beta_1$ to fibronectin which is called synergy site [4]. This drug blocks several cancers including prostate, colorectal, lung, and breast cancer without side effects reported. In combination with radiotherapy, this drug was also reported to be triggered apoptosis of breast cancer.

Nuclear medicine provides non-invasive evaluations that utilize radioactive materials/ radiopharmaceuticals in the diagnosis and treatment of cancers [5]. The radiopharmaceutical consists of a carrier/ active molecule and radioisotope. The carrier plays an important role to deliver radionuclide to a specific biological target [6]. The higher the specific activity of the carrier, the better the imaging and the fewer the side effects in therapeutic procedures. For this reason, a new radiopharmaceutical based on pentapeptide PHSCN in combination with diagnosis and/ or therapeutic radioisotope that accumulates in integrin $\alpha_5\beta_1$ needs to be developed. It visualizes information about the phenotypic functional changes associated with the development of cancer by modern imaging technologies such as Positron Emission Tomography (PET) or Single-photon emission computed tomography (SPECT). In this project, we used a molecular docking simulation to be screening the best radiolabeling method of radiopharmaceuticals based on pentapeptides and technetium radioisotope by determining the binding affinity of each compound in synergy pocket and RGD pocket of integrin $\alpha_5\beta_1$. The result of these simulations was tracers that are chemically, and geometry fit into integrin $\alpha_5\beta_1$ binding pocket without massive changes in inhibition activity of PHSCN.

## EXPERIMENT

### Procedure of computational simulations

The initial crystal structure of integrin $\alpha_5\beta_1$ is extracted from RCSB Protein Data Bank (PDB entry: 3vi4 [7]). Integrin $\alpha_5\beta_1$ antagonist Ac-PHSCN-$NH_2$ was blindly docked into integrin $\alpha_5\beta_1$ using Autodock Vina. Then, the position of ligands was checked by investigated pocket positions using online software FTMAP and from references to acquired grid box positions of RGD pocket of integrin $\alpha_5\beta_1$ and synergy pocket of integrin $\alpha_5$. Docking preparation and the determination of binding pocket location were achieved using Autodocktools-1.5.6. Further simulations were conducted on the center value of x, y, z is -8, 4, and 8 respectively (synergy pocket), and -14, -14, and -21 (RGD pocket). Ac-PHSCN-$NH_2$, Ac-PHSRN-$NH_2$, amino acid modified of PHSCN on N terminal and C terminal were the ligands used for the simulations using Autodock Vina. Technetium-based radiolabeled of pentapeptides were created and simulated using Autodock 4.2. Diethylenetriaminepentaacetic acid (DTPA), S-acetylmercaptoacetyltriserine (MAS3), and HYNIC-EDDA on radiolabeled compounds structures were bifunctional chelating agents. Interaction of radiolabeled compounds and integrin $\alpha_5\beta_1$ were analyzed on the grid box with the center value of x, y, z is -8, 9, and 8 respectively (synergy pocket), -14, -14, and -21 (RGD pocket) and with wider box size than pentapeptide's box. Docking results were analyzed and visualized using Autodocktools-1.5.6, Biovia Discovery Studio 2021, VMD 1.9.4, and UCSF Chimera 1.16.



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## RESULT AND DISCUSSION

Autodock Vina [8] and Autodock 4 [9] is an open-source program for molecular docking to calculate the binding affinity of compounds (peptides and radiopeptides) to a molecular target (integrin $\alpha_5\beta_1$) rapidly and accurately. Molecular docking is one of computational chemistry used to minimize failure rate, cost, and efficiency of drug discovery and development. Computational chemistry (molecular modeling) has been growing fast through past decades as one of the most effective strategies to speed up the drug discovery from 10-16 years to only 6-8 years and cut down the development cost by almost a third [10]. AutoDock Vina has better accuracy, faster and easier to use compared to AutoDock 4 AutoDock Vina tends to be faster than Autodock 4, Autodock Vina uses the hybrid scoring function of knowledge-based and empirical scoring function [11] and Autodock 4 uses scoring function based on the AMBER force field.

Among the integrin family, several subunits were essential for vasculo- and angiogenesis during cancer growth i.e., $\alpha_4$, $\alpha_5$, $\alpha_v$ or $\beta_1$ [4]. Integrin $\alpha_5\beta_1$ interacts with high affinity and specificity to fibronectin. The α subunit is responsible for the high specificity of integrin $\alpha_5\beta_1$ for fibronectin by PHSRN interaction in the synergy site. The primary interaction is RGD sequence occurs at RGD site of integrin $\alpha_5\beta_1$ [3]. Thus, blocking integrin $\alpha_5$ with a non-RGD peptide (pentapeptide Ac-PHSCN-$NH_2$/ ATN-161) showed anti-angiogenic and reduced tumor growth. Experiment results also demonstrated that PHSCN also binds competitively to the RGD pocket of integrin $\alpha_5\beta_1$ [12]. For this reason, the binding affinity of radiolabeled compounds of PHSCN with technetium radioisotope on synergy and RGD pocket of integrin $\alpha_5\beta_1$ was compared to initial pentapeptides to determine the effect of geometry changes after the radiolabeling process.

### Pentapeptides and Integrin $\alpha_5\beta_1$ Interactions

The first step was determining the location of synergy and RGD pocket by blind docking of Ac-PHSCN-$NH_2$ into fibronectin receptor. Two locations were observed which are confirmed by the references and computational mapping server FTMAP. FTMAP identifies binding spots by distributes small organic probe molecules of varying size, shape, and polarity on a macromolecule surface, finds the most promising positions for each probe type, then clusters the probes and ranks the clusters based on their average energy [13]. The first location of the binding spot is at $\alpha_5$ subunit (purple) which indicated a synergy pocket and the second is between $\alpha_5$ and $\beta_1$ subunit which indicated RGD pocket (Figure 1).

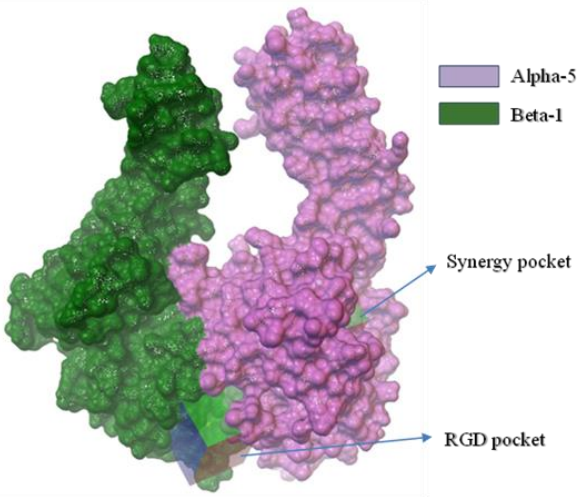


**Figure 1.** Position of synergy pocket of $\alpha_5$ and RGD pocket of $\alpha_5\beta_1$.



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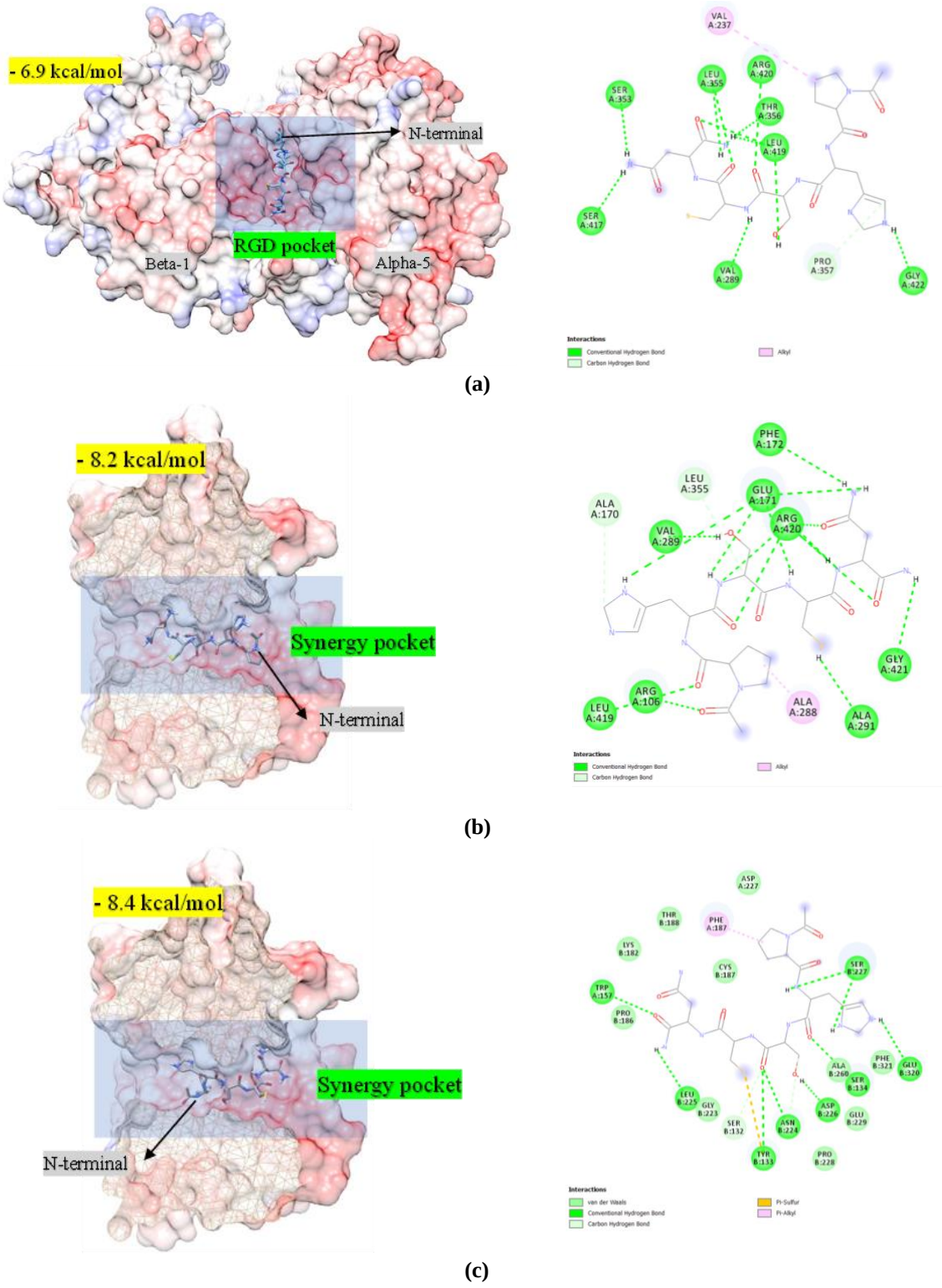


**Figure 2.** Position and interaction of Ac-PHSCN-$NH_2$, (a) RGD pocket, (b) synergy pocket (conformation 1), (c) synergy pocket (conformation 2).



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The primary interaction between Ac-PHSCN-$NH_2$ and integrin $\alpha_5\beta_1$ is conventional hydrogen bonds. Ac-PHSCN-$NH_2$ block PHSRN sequence of fibronectin by occupying synergy pocket of integrin $\alpha_5$ by two possible conformations (Figure 2b and 2c) with quite similar binding affinity value and might block RGD sequence of fibronectin (Figure 2a) with lower binding affinity. N-terminal of PHSCN on conformation 1 located on the outside of the binding pocket, opposite to conformation 2. On conf 1, strong hydrogen bonds occur within $C_{PHSCN}$-Val289 (2.07 Å), $N_{PHSCN}$-Ser353 (2.22 Å), $N_{PHSCN}$-Ser417(2.38 Å), $S_{PHSCN}$-Leu419(2.13 Å), and medium to weak hydrogen bonds to Leu355, Thr356, Arg420, and Gly422. On conf 2, strong interactions appeared between $N_{PHSCN}$-Glu171(2.16 Å), $N_{PHSCN}$-Val289 (2.33 Å), and weaker interaction to Arg106, Phe172, Ala291, Leu419, Arg420, and Gly421. On the RGD pocket, the main interactions were $N_{PHSCN}$-Leu225 (2.21 Å), $S_{PHSCN}$-Asp226 (1.79 Å), $H_{PHSCN}$-Ser227 (2.09 Å), and other bonds with longer distances to Tyr133, Ser134, Trp157, Asn224, and Glu320.

The visualizations above showed the possibility to modify PHSCN by substitute amino acid residue and/or add technetium radioisotope supported by a bifunctional agent at proline residue or an asparagine residue. N-contained amino acid residues (histidine/H, lysine/K, asparagine/N, glutamine/Q, and arginine/R) were used to facilitated conjugation with a bifunctional agent. However, this modification might influence specificity, penetrating capability, stability, and efficacy. Some amino also could as a spacer between peptide and radioisotope and minimize the steric effect of radioisotope and its chelator at binding pocket. The value of binding affinities and position resulted from molecular docking of pentapeptides are shown in Table 1 and Figure 3.

**Table 1**. Docking result of pentapeptides targeting integrin $\alpha_5\beta_1$ binding pocket

| Pentapeptides | Binding affinity (kcal/ mol) | | | Remark/ Key residues |
|---|---|---|---|---|
| | RGD pocket ($\alpha_5\beta_1$) | Synergy pocket ($\alpha_5$) | | |
| | | Conf 1 | Conf 2 | |
| Ac-PHSRN-$NH_2$ | -7.2 | -8.6 | -8.6 | **Ac-PHSCN-$NH_2$** *RGD pocket* Tyr133, Ser134, Trp157, Asn224, Leu225, Asp226, Ser227, Glu320 (conventional hydrogen bond) *Synergy pocket (conf 1)* Val289, Ser353, Leu355, Thr356, Ser417, Leu419, Arg420, Gly422 (conventional hydrogen bond) *Synergy pocket (conf 2)* Arg106, Glu171, Phe172, Val289, Ala291, Leu419, Arg420, Gly421 (conventional hydrogen bond) |
| Ac-PHSCN-$NH_2$ | -6.9 | -8.2 | -8.4 | |
| PHSCN-$NH_2$ | -7.5* | -8.4* | -8.7 | |
| HHSCN-$NH_2$ | -7.4 | -8.2 | -9.0 | |
| KHSCN-$NH_2$ | -7.2 | -8.1 | -8.6 | |
| NHSCN-$NH_2$ | -7.8 | - | -8.6 | |
| QHSCN-$NH_2$ | -7.5 | - | -8.8 | |
| RHSCN-$NH_2$ | -6.0 | -8.4 | -8.8 | |
| Ac-PHSCN | -7.1* | -8.3 | -8.4* | |
| Ac-PHSCH | -7.0 | - | -8.7 | |
| Ac-PHSCK | -6.1 | -8.2 | -8.0 | |
| Ac-PHSCQ | -7.1 | -8.5 | -8.1 | |
| Ac-PHSCR | -7.3 | -8.7 | -8.6 | |

* Used as comparison value for selection of pentapeptides.

Some pentapeptides produce only one conformation in interaction with integrin $\alpha_5$ which are C-terminal located outside the binding pocket. Substitution of proline residue by histidine, lysine, and arginine has no significant movement of location, interaction, and binding affinity, where the other two do not generate similar conformations with conformation 1.



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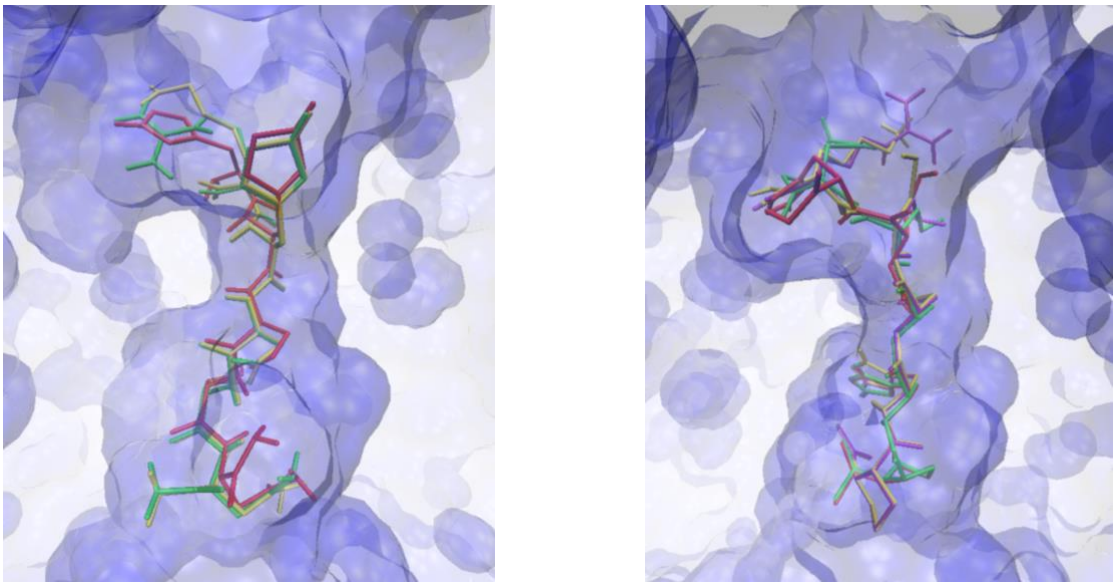
Asparagine replacement by histidine and arginine produces higher affinities than PHSCN at second conformation. P to H and N to R replacement also seems no major effect on peptide interaction at RGD pocket. Thus, these pentapeptides also could simulate further to develop a radiolabeled compound with a higher affinity to regulate metal radioisotope position relative to the binding pocket.



(a) (b)

**Figure 3.** Position of pentapeptides on synergy pocket, (a) AA-HSCN-NH2 (red: AA=H; yellow: AA=K; green: AA=R), (b) Ac-PHSC-AA (red: AA=H; yellow: AA=K; green: AA=Q; purple: AA=R).

**Technetium-based radiolabeled compounds and Integrin $\alpha_5\beta_1$ Interaction**

As mentioned before, PHSCN can be labeled with radionuclides that accumulate in the macromolecule target. Besides anti-angiogenic and reduced tumor growth, radiolabeled compounds of PHSCN can be utilized for cancer detection or diagnosis using modern imaging methods such as single-photon emission computed tomography (SPECT) and positron emission tomography (PET). For diagnosis purposes, gamma or positron emitters are used while beta, alpha, or Auger electron emitters are applied for cancer therapy. The radiohalogens ($^{18}$F, $^{124}$I, $^{131}$I, etc) or radiometals ($^{99m}$Tc, $^{177}$Lu, etc) are commonly used to formulate peptide-based radiopharmaceuticals. The most frequently used SPECT radioisotopes are technetium-99m/ $^{99m}$Tc which has a half-life of 6 hours and emits low energy gamma-ray (141 keV, 89%). Technetium has multi-oxidation states which allow the formation of a variety of complexes with specific characteristics. However, the chemical structure becomes important to develop new technetium-based radiopharmaceuticals aimed at a specific target, [14]. Radiopharmaceutical for imaging must have high-target selectivity, binding affinity, stability, and slow metabolism in vivo [15].

The design of these $^{99m}$Tc radiopharmaceuticals for molecular imaging consisting of the bifunctional chelator conjugated peptide and technetium radioisotope. Peptides are separated by a linker or spacer chain to avoid interference of the metal complex in the interaction with the target. Many bifunctional chelators have been used for technetium radioisotope. HYNIC, MAS3, and DTPA have been used in this computational simulation. $^{99m}$Tc is eluted from the



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generator by the saline solution to get pertechnetate in aqueous media $^{99m}TcO_4^-$ (oxidation state of +7). By the presence of reducing agents such as $Na_2S_2O_4$, $SnCl_2$, phosphines, or zinc, the oxidation state of $^{99m}Tc$ varies to +5, +4, or +3. The most technetium-based radiopharmaceuticals are in the oxidation state of +5. The labeling process is performed by adding conjugated peptides into the solution.

**Table 2**. Docking result of radiopeptides with chelator variation and conjugation position

| Radiopeptides | Binding affinity (kcal/ mol) | | |
|---|---|---|---|
| | RGD pocket ($\alpha_5\beta_1$) | Synergy pocket ($\alpha_5$) | |
| | | Conf 1 | Conf 2 |
| Ac-PHSCN-$NH_2$ | -7.29 | -7.46 | 7.86 |
| Tc-DTPA-PHSCN-$NH_2$ | -7.26 | -4.03 | - |
| Ac-PHSCN(Tc-DTPA)-$NH_2$ | -7.37 | - | -2.18 |
| EDDA-Tc-HYNIC-PHSCN-$NH_2$ | -6.52 | -4.65 | - |
| Ac-PHSCN(HYNIC-Tc-EDDA)-$NH_2$ | -5.37 | - | -2.65 |
| Tc-MAS3-PHSCN-$NH_2$ | -3.09 | -1.86 | - |
| Ac-PHSCN(Tc-MAS3)-$NH_2$ | -2.27 | - | -2.19 |

The docking result showed a decrease in the binding affinity of the radiolabeled compound compared to Ac-PHSCN-$NH_2$. Binding affinities at synergy pocket were fall as MAS3 was used as a bifunctional chelator and as conjugation was simulated at asparagine residue. While at the RGD pocket, a significant drop of binding affinity only occur when MAS3 was used and no or slight changes on DTPA and HYNIC contained radiolabeled compounds. The highest binding affinity was obtained from EDDA-Tc-HYNIC-PHSCN-$NH_2$. HYNIC acts as a mono or bidentate ligand and required co-ligands such as EDDA, tricine, or nicotinic acid to complete the coordination of the Tc-HYNIC core [2]. Tc-HYNIC core has been widely used in radiopharmaceuticals. EDDA-Tc-HYNIC and Tc-DTPA radiolabeled compounds of PHSCN and N-terminal modified pentapeptides are recommended to be synthesized and tested at the laboratory.

**Table 3**. Docking result of radiopeptides with amino acid modification

| Radiopeptides | Binding affinity (kcal/ mol) | | |
|---|---|---|---|
| | RGD pocket ($\alpha_5\beta_1$) | Synergy pocket ($\alpha_5$) | |
| | | Conf 1 | Conf 2 |
| Ac-H(Tc-DTPA)HSCN-NH2 | -6.78 | -4.25 | - |
| Ac-H(Tc-HYNIC-EDDA)HSCN-$NH_2$ | -5.71 | -4.88 | - |
| Ac-PHSCR(Tc-DTPA)-NH2 | -7.60 | - | -3.42 |
| Ac-PHSCR(Tc-HYNIC-EDDA)-$NH_2$ | -6.68 | - | -3.50 |

By replacement of residue at the terminal position with longer sidechain residue, the binding affinity seems to be increasing. It indicated that the possible reason for the low binding value of the radiolabeled compound is because the space between peptide and metal radioisotope was insufficient. The big structure of technetium and its chelator cause the molecule could not to penetrate to the deep inside of the synergy pocket as shown in Figures 4 and 5. Based on this simulation result, the radiolabeled compounds may require a linker to



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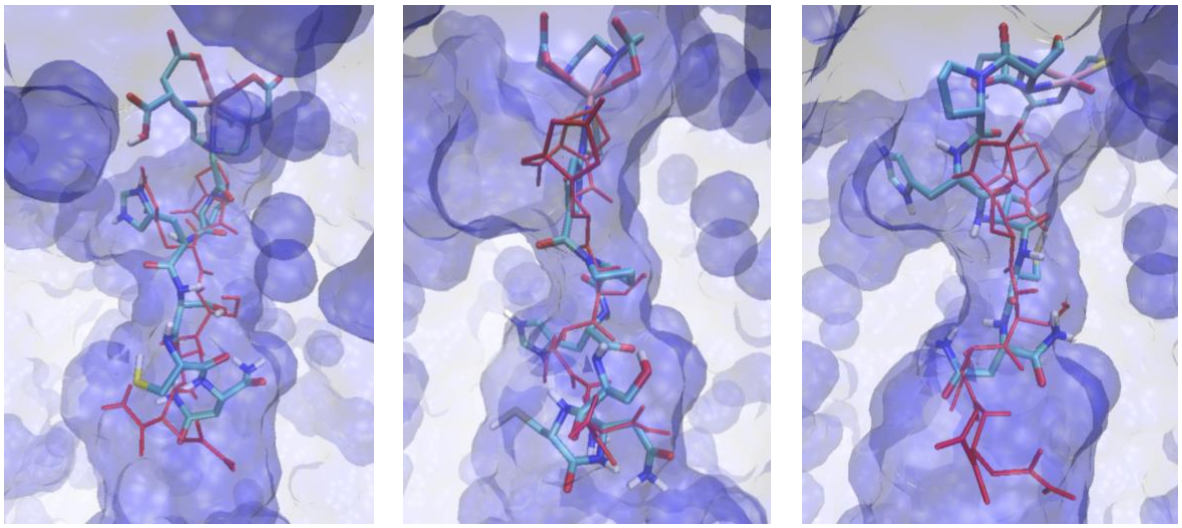
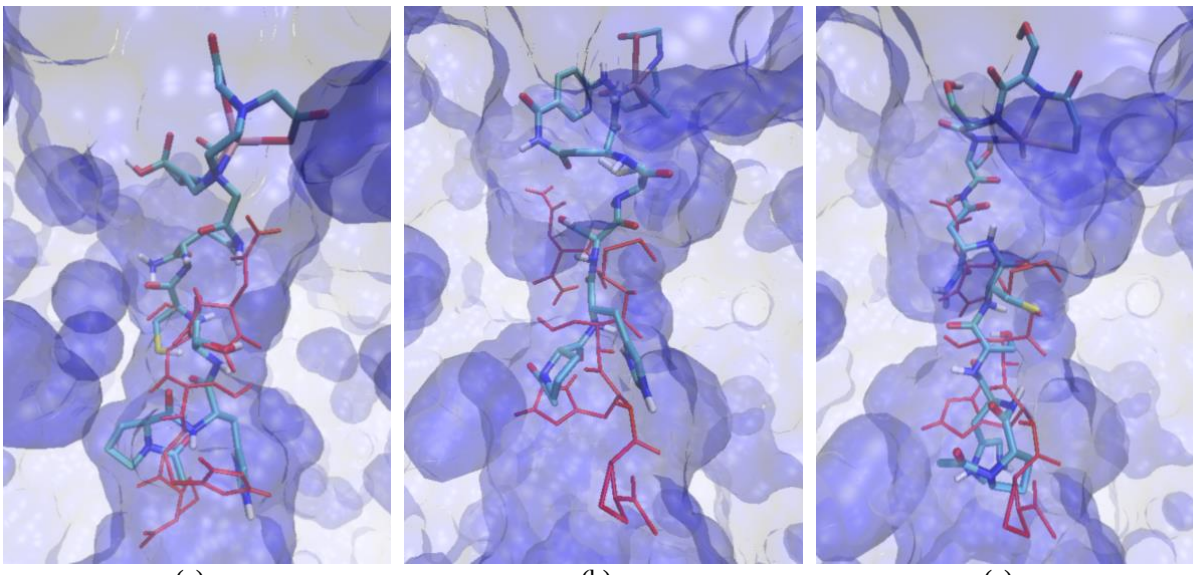
minimize the steric effect of added group hence improve binding affinity and inhibition activity at integrin $\alpha_5\beta_1$. Further computational simulation on the variation of linker used is also required to minimize failure rate and experimental effort in the laboratory.



(a) (b) (c)

**Figure 4.** Radiolabeled PHSCN in comparison with conformation 1 of Ac-PHSCN-$NH_2$ (red), (a) Ac-PHSCN(Tc-DTPA)-$NH_2$, (b) EDDA-Tc-HYNIC-PHSCN-$NH_2$, (c) Tc-MAS3-PHSCN-$NH_2$.



(a) (b) (c)

**Figure 5.** Radiolabeled PHSCN in comparison with conformation 2 of Ac-PHSCN-$NH_2$ (red), (a) Tc-DTPA-PHSCN-$NH_2$, (b) Ac-PHSCN(HYNIC-Tc-EDDA)-$NH_2$, (c) Ac-PHSCN(Tc-MAS3)-$NH_2$.



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## CONCLUSION

Based on the simulation result, radiolabeling procedures of pentapeptide Pro-His-Ser-Cys-Asn (PHSCN) using technetium metal radioisotope reduce the binding affinity of peptides on both synergy and RGD pocket of integrin $\alpha_5\beta_1$. However, the inhibitory activity for the labeled compound may still occur. EDDA-Tc-HYNIC-PHSCN-$NH_2$ and Tc-DTPA-PHSCN-$NH_2$ radiolabeled compounds of PHSCN and N-terminal modified pentapeptides are recommended to be synthesized and tested for physicochemical properties, stability, and bioactivity at the laboratory in the further research for the development of cancer detection and therapy by targeting integrin $\alpha_5\beta_1$.

## ACKNOWLEDGMENT

The authors would like to thank the Center for Applied Nuclear Science and Technology, National Nuclear Energy Agency for facility support.

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