Final Technique Report of SMIRTP

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Abstract
Positron emission tomography (PET) imaging agents play significantly roles in detection and management of diseases. There are urgent needs to increase output and translate probes more rapidly for clinical use. Moreover, there is a crucial lack of trained radiochemists and nuclear medicine physician scientists to meet the increasing demand of developing novel PET imaging probes for clinical use. With strong support and commitment from Office of Science, Department of Energy, coupled with an expanding interest in the benefits of molecular and biomedical imaging, Stanford Molecular Imaging Research and Training Program (SMIRT) has advanced generalizable strategies to develop PET agents and multimodality radiotracers that can target most cell surface receptors. These radioactive imaging agents have been based on novel protein scaffolds such as knottins and Affibodies, and newly developed gold nanoparticles and organic nanoparticles. The projects yielded several new classes of knottin-based imaging probes that target $\alpha_v\beta_6$, $\alpha_v\beta_3$, EGFR, melanin, etc. as single or multimodal imaging agents. More importantly, the projects firmly established the development of knottins based PET probes as a generalizable approach for many tumor targets. Three novel PET probes have been successfully developed from research lab and translated into pilot clinical patient imaging studies during the course of the funding period. We also increased the number of trained radiochemists/nuclear medicine physician-scientists and provided them with comprehensive training regarding probe development and the clinical translational process. The research opportunities of this SMIRT engaged trainees in cutting-edge projects with experienced mentors and improve cross-training between radiochemists and nuclear medicine physicians. The training enabled an overall understanding of the complete probe development and clinical translation process, and encouraged trainees to pursue rewarding careers in radionuclide based imaging.

Key words: PET; Molecular imaging; Multimodality imaging; Molecular probe; Scaffold protein

Final progress made on each aims

Specific aim 1: To develop a generalizable process to accelerate PET probe discovery for clinical use.

In this aim, our goal is to establish the use of protein scaffold based approach as a generalizable method for PET probe development and future clinical translation.

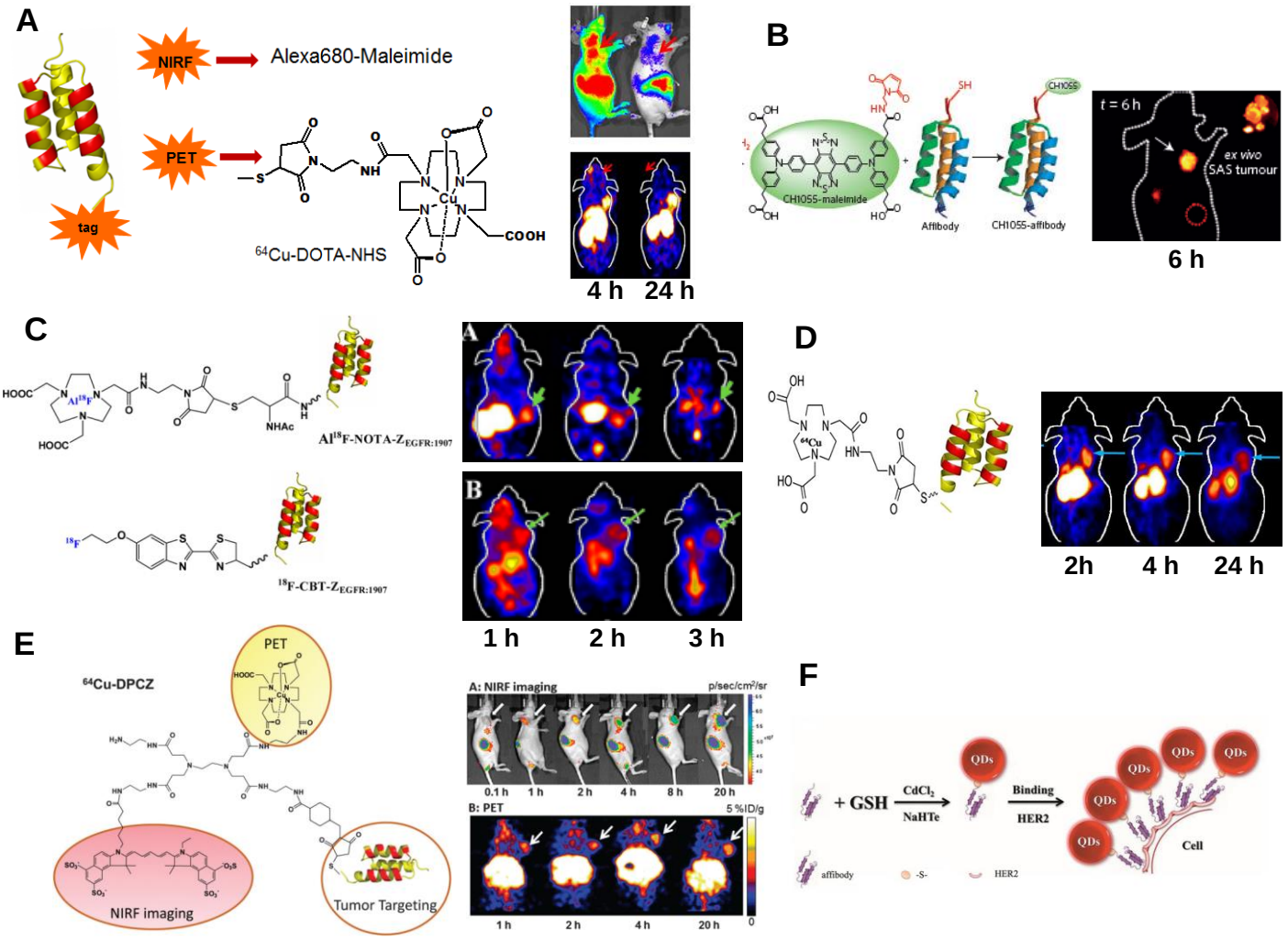

Figure 1. Affibody based probes for cancer imaging. (A). Anti-EGFR Affibody labeled with NIR-I dye (Alex680) or PET radionuclide (^{64}Cu) for liver cancer imaging. **(B)** Anti-EGFR Affibody labeled with NIR-II dye (CH1055) for brain cancer imaging. **(C)** Anti-EGFR Affibody labeled with PET radionuclide (^{18}F) for imaging of A431 tumor. **(D)** Anti-IGF1R Affibody labeled with ^{64}Cu for imaging of U87MG tumor. **(E)** Anti-HER2 Affibody labeled with both NIR-I dye (Cy5.5) and PET radionuclide (^{64}Cu) for dual modality imaging of SKOV3 tumor. **(F)** Anti-HER2 Affibody labeled with QDs for dual modality imaging of SKOV3 tumor.

Two protein scaffolds have been investigated in our program: Affibody and Knottin.

Affibody molecules are based on a 58 amino acid residue protein domain, derived from one of the IgG-binding domains of staphylococcal protein A, and they have been engineered to be chemically stable and to bind target proteins with high affinity. Because of their small size (~7 kDa) and high affinity, Affibody molecules generally show fast and good tumor tissue penetration and accumulation, and rapid clearance from the blood, resulting in high imaging contrast within a short period (for example, 0.5−1 h) after injection. Anti-human epidermal growth factor receptor 2 (HER2), the epidermal growth factor receptor (EGFR), and the insulin-like growth factor 1 receptor (IGF-1R) affibody molecules (Z_{HER2}, Z_{EGFR}, Z_{IGF1R}) have been developed. In our study, we successfully radiolabeled these small proteins with different radionuclides (^{18}F, ^{64}Cu) and

optical imaging dyes for in vivo tumor imaging (**Figure 1**). First, Z_{EGFR} was labeled with ^{64}Cu and near-infrared window I (NIR-I, 650-900 nm) dye Alex680 and used for imaging of hepatocellular carcinoma (HCC) (***1***). Second, for the first time, we developed a small molecule near-infrared window II (NIR-II, 1000-1700 nm) dye CH1055 and its derivatives, which greatly facilitates the clinical translation of NIR-II imaging technology (***2, 3***). This dye was further used to conjugate with Z_{EGFR}, and the resulting optical probe showed outstanding tumor imaging quality, with much higher imaging contrast and deeper tissue imaging capability than those of the Cy5.5 labeled Z_{EGFR} (***2***). Our work on NIR-II imaging represents the major advancement in the field of optical imaging. Third, two different radiofluorination labeling strategies were explored to label Z_{EGFR}. The ^{18}F-labeled Z_{EGFR} probes showed the high promise for clinical imaging (***4***). Forth, besides the development of Z_{EGFR} based probe, Z_{IGF1R} was also labeled with ^{64}Cu and tested in animal models. Our work indicates that ^{64}Cu-Z_{IGF1R} can be used for tumor targeted PET imaging (***5***). Fifth, a Z_{HER2} based dual imaging probe (PET and optical imaging) was successfully developed. Dendrimer PAMAM G0 was used as a platform to assemble an NIRF dye, a metal chelator, and Affibody for dual modality imaging of ovarian cancer. Excellent tumor imaging quality was achieved in both modalities in the living tumor mice models (***6***). Lastly, complex chemical functionalization, surface modification, and bioconjugation chemistry are generally required to tag biomolecules such as Affibody to fluorescent nanoparticles including quantum dots (QDs) for imaging of different biomarkers. To address this issue, we developed a simple method for production of QDs stabilized by the small protein, Affibody (AF-QDs) for fluorescent imaging of the human HER2 in human A549 lung cancer cells. This one-pot synthesis of AFQDs avoids complex chemical conjugation procedures and demonstrates a promising approach for the preparation of fluorescent nanoprobes for imaging of cancer targets (***7***).

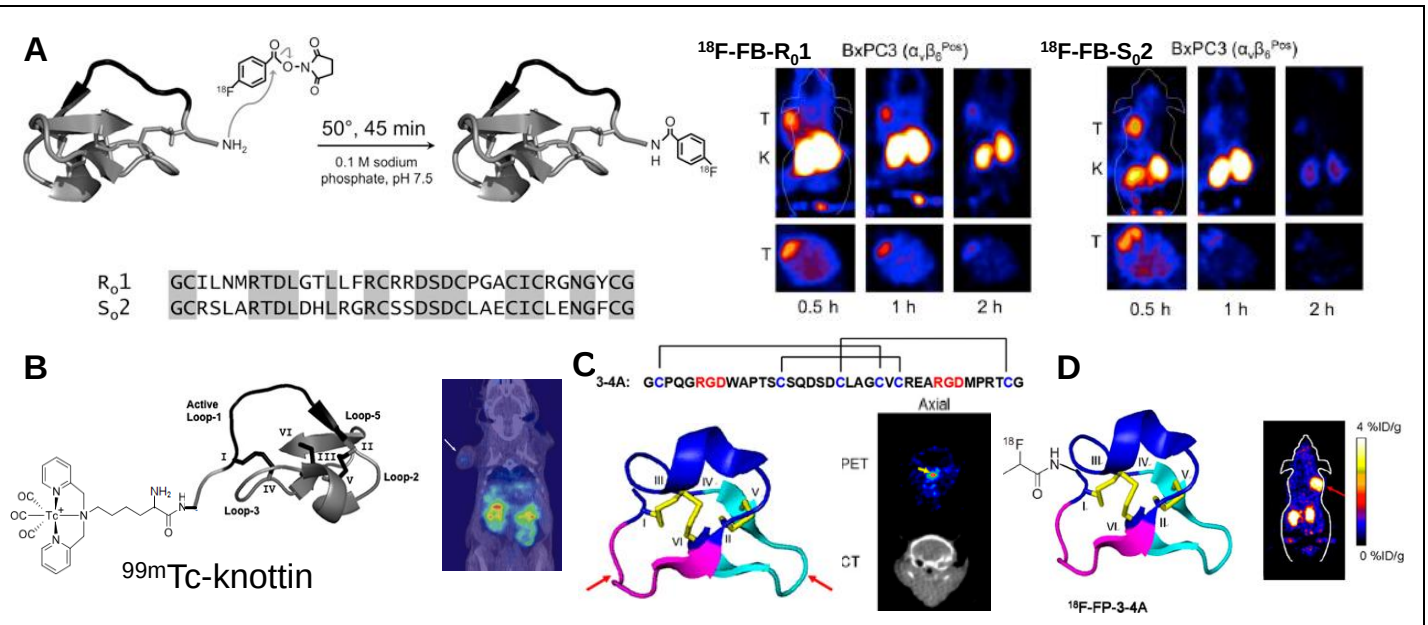

Figure 2. Knottin based probes for cancer imaging. (A) Cystine knot peptides $R_0$1 and $S_0$2 developed that can bind to integrin $\alpha_v\beta_6$ and labeled with ^{18}F-fluorobenzoate (FB) for PET imaging of pancreatic cancer. **(B)** The SAAC conjugated $S_0$2 labeled with ^{99m}Tc for SPECT imaging of human lung cancer. **(C)** Knottin 3-4A that can bind with $\alpha_v\beta_3$ labeled with ^{64}Cu for imaging of carotid atherosclerotic plaque. **(D)** 3-4A labeled with 4-nitrophenyl 2-^{18}F-fluoropropinate (^{18}F-NFP) for imaging of $\alpha_v\beta_3$ positive tumor.

Cystine knot peptides (also known as knottins) are small polypeptides that are characterized by a stable core motif formed by 3 disulfide bonds that are interwoven into a knotted conformation. As a result, knottins have high thermal and proteolytic stability. In addition, the relatively small size (30−50 amino acids) makes them accessible by standard solid-phase peptide synthesis techniques. More importantly, knottins demonstrate fast blood clearance, high and specific tumor targeting ability, and biocompatibility, highlighting their potential use for patient imaging. In our studies, we engineered cystine knot peptides $R_0$1 and $S_0$2 that can bind to integrin $\alpha_v\beta_6$ with a 3–6 nM affinity. These small proteins were radiolabeled with ^{18}F-fluorobenzoate (FB), and the

resulting probes ^{18}F-fluorobenzoate-$R_0$1 (^{18}F-FB-$R_0$1) and ^{18}F-fluorobenzoate-$S_0$2 (^{18}F-FB-$S_0$2) showed excellent tumor imaging quality, demonstrating their high translational promise for molecular imaging of integrin $\alpha_v\beta_6$ overexpression in pancreatic and other cancers (***8***). The knottin $S_0$2 was also conjugated with a single amino acid chelate (SAAC) and labeled with $[^{99m}Tc(H_2O)_3(CO)_3]^+$. The resulting probe, ^{99m}Tc-SAAC-$S_0$2 demonstrated promise for use as a single photon emission computed tomography (SPECT) agent to image integrin $\alpha_v\beta_6$ expression in living systems (***9***). The $\alpha_v\beta_6$ knottin was also labeled with optical dye to achieve image guided surgery of pancreatic cancer (***10***). Furthermore, a divalent knottin containing two separate integrin binding epitopes (RGD) in the adjacent loops, 3-4A, was recently developed by us. We conjugated knottin 3-4A with 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) and radiolabeled it with ^{64}Cu. The resulting PET probe, ^{64}Cu-NOTA-3-4A displayed specific accumulation in carotid atherosclerotic plaques in which macrophage infiltration and angiogenesis are responsible for elevated integrin $\alpha_v\beta_3$ levels. It demonstrated promise for atherosclerosis imaging or for the evaluation of therapies used to treat atherosclerosis (***11***). In another study, integrin $\alpha_v\beta_3$ binding knottin 3-4A was radiofluorinated with a 4-nitrophenyl 2-^{18}F-fluoropropinate (^{18}F-NFP) group. The resulting divalent PET probe, ^{18}F-FP−3-4A was characterized by rapid and high tumor uptake and excellent tumor-to-normal tissue ratios. ^{18}F-FP−3-4A is a highly promising knottin based PET probe for translating into clinical imaging of tumor angiogenesis (***12***) (**Figure 2**).

Besides the research on using scaffold proteins for imaging probe development, we also explored the use of other molecule platforms including peptides (***13-21***), small molecules (***22,23***), simple radiometal cations (***24-26***) for development of molecular probes, in order to understand the advantages and limitations of different molecular platforms. More specifically, we used integrin $\alpha_v\beta_3$ targeted RGD peptide, GRPR targeted bombesin peptides, uPAR targeted AE105 peptide, alpha-MSH peptide, and follicle stimulating hormone for developing peptide based PET, SPECT, optical and multimodality probes (***13-21***). We developed benzamide molecule based PET probe, N-(2-(diethylamino)-ethyl)-^{18}F-5-fluoropicolinamide (^{18}F-P3BZA), and TPP-F16 as mitochondria targeted optical probe (***22,23***). Lastly, radiocopper cations ($^{64}Cu^{2+}$, $^{64}Cu^{+}$) were used directly to image and treat tumor and image inflammation as well (***24-26***).

Overall, our studies reveal that both Affibody and Knottin are versatile, robust and excellent protein scaffolds. They generally have predicable in vivo behavior with high targeting efficacy and in vivo stability. They can be easily used to quickly develop a variety of imaging probes against different targets for different imaging modalities, thus greatly facilitating the imaging probe development. Several Affibody and Knottin based lead probes show high potential for clinical translation have been identified, and they thus moved forward into clinical evaluation in our Aim 2. In contrast, peptides, small molecules, and simple radiometal cations can be used for imaging probe development. But their biologically activity, in vivo pharmacokinetics and targeting ability can be highly depended on labeling approaches, linker, and other modification applied. Significant efforts need to be spent for probe optimization using these molecular platforms.

Our papers published on the research topic of aim 1:

Publications on Affibody based probes

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2. Antaris AL, Chen H, Cheng K, Sun Y, Hong G, Qu C, Diao S, Deng Z, Hu X, Zhang B, Zhang X, Yaghi OK, Alamparambil ZR, Hong X*, Cheng Z*, Dai H*. A small-molecule dye for NIR-II imaging. ***Nature Materials.*** 2016;15(2):235-42. PMID: 26595119. [This work has received significant media coverage including Medical Daily, Nanotec, Imaging&Microscopy, etc.]. doi: 10.1038/nmat4476.
3. Antaris AL, Chen H, Diao S, Ma Z, Zhang Z, Zhu S, Wang J, Lozano AX, Fan Q, Chew L, Zhu M, Cheng K, Hong X*, Dai H*, Cheng Z*. A high quantum yield molecule-protein complex fluorophore for near-infrared II imaging. ***Nature Communications.*** 2017;8:15269. doi: 10.1038/ncomms15269. PMID:28524850.

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Publications on Knottin based probes

8. Hackel BJ, Kimura RH, Miao Z, Liu H, Sathirachinda A, Chin FT, Cheng Z, Gambhir SS. ^{18}F-Labeled Cystine Knot Peptides for PET Imaging of Integrin $\alpha_v\beta_6$. ***Journal of Nuclear Medicine*.** 2013; 54(7):1101-5. PMID:23670900. doi: 10.2967/jnumed.112.110759
9. Zhu X, Li J, Hong Y, Kimura RH, Liu H, Qin C, Hu X, Hayes TR, Benny P, Gambhir SS, Cheng Z*. ^{99m}Tc-Labeled Cystine Knot Peptide Targeting Integrin $\alpha_v\beta_6$ for Tumor SPECT Imaging. ***Molecular Pharmaceutics***. 2014; 11(4): 1208-1217. PMID: 24524409. doi: 10.1021/mp400683q
10. Development and Preclinical Validation of a Cysteine Knottin Peptide Targeting Integrin αvβ6 for Near-infrared Fluorescent-guided Surgery in Pancreatic Cancer. Tummers WS, Kimura RH, Abou-Elkacem L, Beinat C, Vahrmeijer AL, Swijnenburg RJ, Willmann JK, Gambhir SS. ***Clin Cancer Res.*** 2018 Apr 1;24(7):1667-1676. doi: 10.1158/1078-0432.CCR-17-2491. Epub 2018 Jan 3.PMID: 29298796
11. Jiang L, Kimura RH, Ma X, Tu Y, Miao Z, Shen B, Chin FT, Shi H, Cheng Z.* A Radiofluorinated Divalent Cystine Knot Peptide for Tumor PET Imaging. ***Molecular Pharmaceutics***. 2014, 11(11):3885-92. PMID: 24717098. doi: 10.1021/mp500018s
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Publications on peptide based probes

13. Sun Y, Ma X, Cheng K, Wu B, Duan J, Chen H, Bu L, Zhang R, Hu X, Deng Z, Xing L, Hong X*, Cheng Z.* Strained Cyclooctyne as a Novel Molecular Platform for Construction of Multimodal Imaging Probes. ***Angewandte Chemie Int Ed***. 2015, 54(20):5981-4. PMID: 25800807 (Hot paper). doi: 10.1002/anie.201500941
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17. Sun Y, Qu C, Chen H, He M, Tang C, Kang S, Yang M, Jiang Y, Ding B, Hong X*, Cheng Z* Novel benzo-bis(1,2,5-thiadiazole) fluorophores for *in vivo* NIR-II optical imaging. ***Chemical Science***. 2016, 7: 6203-6207. DOI: 10.1039/C6SC01561A. (Edge article).
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19. Loft MD, Sun Y, Liu C, Christensen C, Huang D, Kjær A*, Cheng Z*. Synthesis and In Vivo Evaluation of PEG Modified ^{68}Ga-labelled urokinase Plasminogen Activator Receptor (uPAR) Targeting Peptides For PET Imaging of Glioblastoma. ***Amino Acids***. 2017;49(6):1089-1100. PMID: 28316028. doi: 10.1007/s00726-017-2407-4
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Publications on small molecule based probes

22. Bu L, Li R, Liu H, Feng W, Xiong X, Zhao H, Vollrath D, Shen B, Cheng Z.* In Vivo Longitudinal Molecular Imaging of Intrastriatal Transplanted RPE Cells by ^{18}F-P3BZA PET/CT. ***Radiology***. 2014;272(1):174-83. PMID: 24758555. [Commented by Radiology]. doi: 10.1148/radiol.14132042
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Publications on radiometal cation based probes

24. Qin C, Liu H, Chen K, Hu X, Lan X, Zhang Y, Cheng Z.* Theranostic of Malignant Melanoma with $^{64}CuCl_2$. ***Journal of Nuclear Medicine***. 2014; 55: 812-817. PMID: 24627435. doi: 10.2967/jnumed.113.133850
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Specific aim 2: To translate an anti-$\alpha_v\beta_6$ ^{18}F-knottin for clinical imaging of pancreatic cancer.

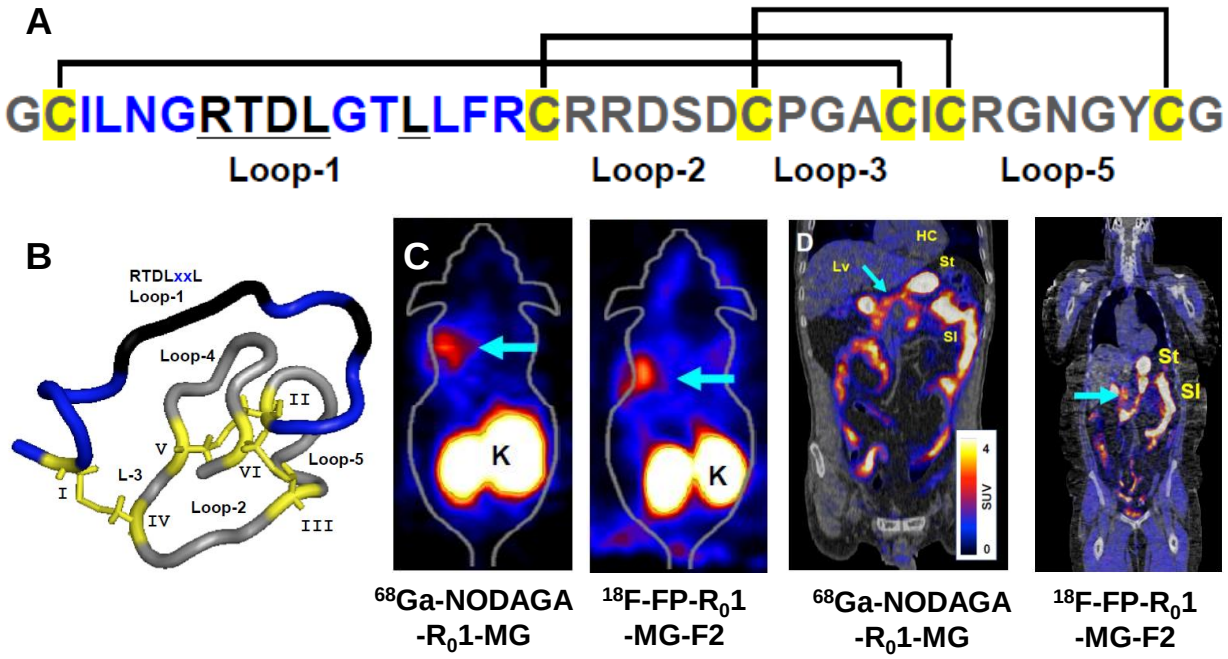

Figure 3. $\alpha_v\beta_6$ knottin PET probes for animal and pancreatic cancer imaging. (A) Primary structure of lead candidate $R_0$1-MG. **(B)** Three dimensional ^{1}H NMR of $R_0$1-MG. **(C)** PET imaging at 1 hr post injection of the ^{68}Ga-NODAGA-$R_0$1-MG (left) and ^{18}F-FP-$R_0$1-MG-F2 (right) in integrin $\alpha_v\beta_6$ positive tumor models. **(D)** Coronal PET/CT images acquired from a patient diagnosed with pancreatic cancer after ~1 h injection of ^{68}Ga-NODAGA-$R_0$1-MG (left), and another patient at ~1 h injection of ^{18}F-FP-$R_0$1-MG-F2 (right).

Through the research of Aim 1, we identified the lead PET probes for clinical translation. In the Aim 2, we continued to optimize the anti-integrin $\alpha_v\beta_6$ knottin PET probes and tested two of them in clinic studies. Specifically, knottin $R_0$1 labeled with different radiolabels such as ^{64}Cu-DOTA, ^{68}Ga-NODAGA and ^{18}F-FP were evaluated in the mice models. The lead probes ^{18}F-FP-$R_0$1-MG-F2 and ^{68}Ga-NODAGA-$R_0$1-MG were further evaluated including the toxicological testing of the lead compound, determining optimal dosimetry for human use, obtaining FDA approval for clinical trials, evaluating lead PET tracer's safety, biodistribution and pharmacokinetics in 5 healthy human volunteers, and evaluating the tracer's ability to detect multiple cancers (pancreatic, cervical and lung) in 7 patients at two study locations (**Figure 3**). Overall, our results indicated that the newly developed cystine knot PET tracer has potential utility in multiple cancers that are associated with overexpression of integrin $\alpha_v\beta_6$. A paper was prepared and submitted for consideration of publication (***27***).

Our small molecule based PET probe development also resulted in several promising clinical translatable imaging probes. ^{18}F-P3BZA is one of them and a novel radiotracer that demonstrates high binding selectivity and affinity to melanoma. We thus tested the toxicity of the ^{19}F-P3BZA, evaluated the biodistribution and clinical radiation dosimetry of ^{18}F-P3BZA in healthy volunteers, and performed a preliminary clinical application for melanoma PET imaging in 5 patients (**Figure 4**). Our study suggests that the biodistribution and effective radiation dose of ^{18}F-P3BZA are safe and compatible for clinical use. We also investigated the first-in-human clinical application of melanoma which showed favorable delineated tumors in patients, demonstrating the potential of ^{18}F-P3BZA for diagnostic PET imaging of melanoma (***28***).

Overall, our research efforts in Aim 2 successfully led to two knottin based PET probes (^{18}F-FP-$R_0$1-MG-F2 and ^{68}Ga-NODAGA-$R_0$1-MG) and a small molecule based PET probe (^{18}F-P3BZA) into clinical imaging study. These three probes show high potential for broad clinical applications.

Our manuscripts prepared on the research topic of aim 2:

27. Kimura RH, Wang L, Shen B, Huo L, Tummers W, Filipp FV, Abou-Elkacem L, Baratto L, Habte F, Devulapally R, Witney TH, Cheng Y, Haywood T, Tikole S, Chakraborti S, Nix J, Bonagura CA, Hatami N, Visser BC, Poultsides GA, Norton J, Natarajan A, Ilovich O, Srinivas S, Srinivasan A, Paulmurugan R, Willmann J, Chin FT, Cheng Z, Iagaru A, Li F, Gambhir SS. A First-in-Human Study of Integrin $\alpha_v\beta_6$ Cystine Knot Positron Emission Tomography Tracers. ***Nature.*** Submitted in March, 2018.
28. Ma X, Wang S, Wang S, Liu D, Zhao X, Chen H, Kang F, Li G, Yang W, Wang J*, Cheng Z*. Biodistribution and Radiation Dosimetry of ^{18}F-P3BZA for Human Melanoma PET Imaging and First Application in Patients. ***Journal of Nuclear Medicine.*** 2018, April, revised manuscript under for consideration of publication.

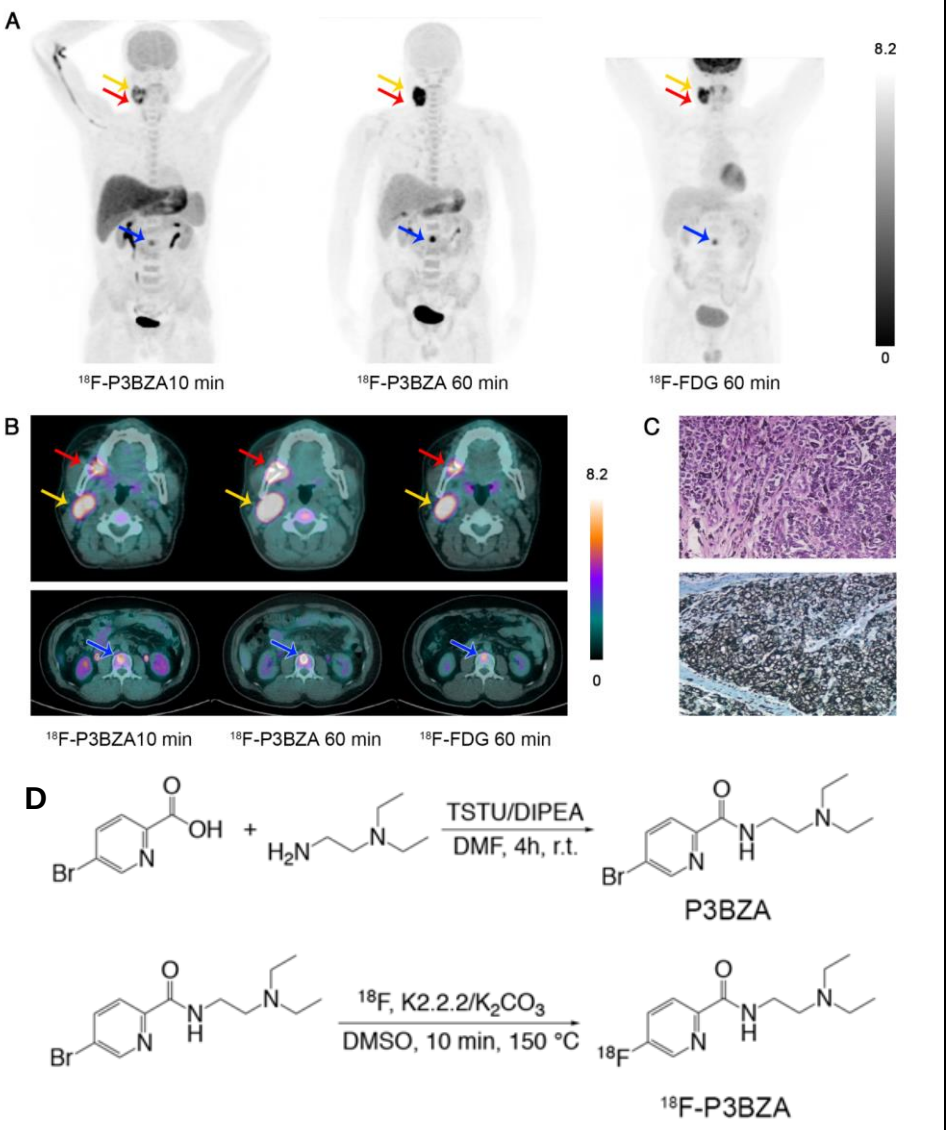

Figure 4. Sample images of a melanoma patient. (A) PET images at 10 and 60 min after ^{18}F-P3BZA injection and at 60 min after ^{18}F-FDG injection. **(B)** Transverse images of primary melanoma (Red arrow), lymph node metastasis (Yellow arrow), and bone metastasis (Blue arrow). **(C)** H&E (upper part) and IHC (lower part) images of primary melanoma. **(D)** The synthetic scheme for ^{18}F-P3BZA.

Specific Aim 3: To develop novel scaffold protein modified gold nanoparticles for tetra-modality imaging (MRI/PET/PAI/Raman) of tumors in living subjects.

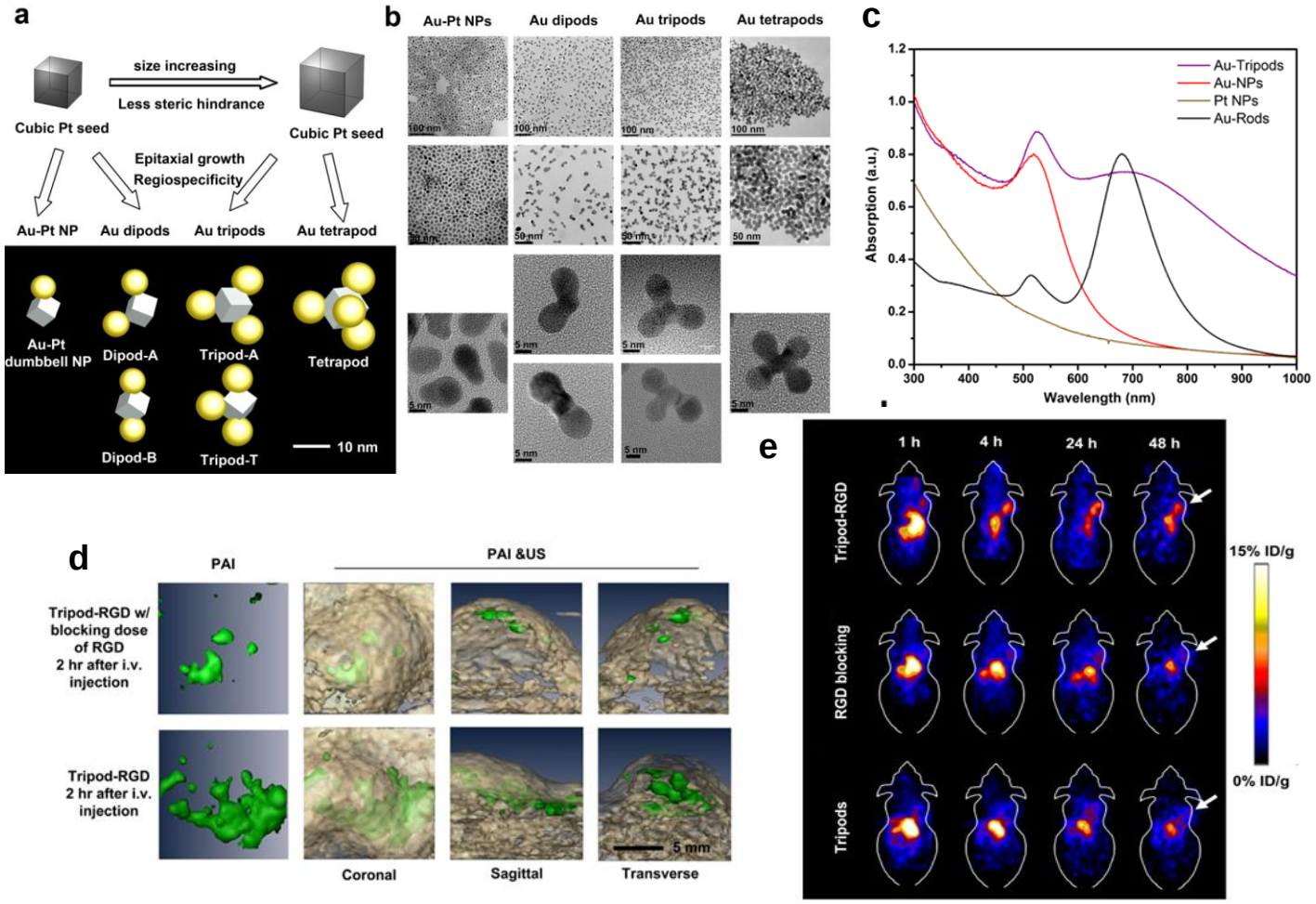

Figure 5. Novel Au-tripod NPs for PAI and PET. (a) Construction of gold multipods (including Au-Pt dumbbell NPs, Au-dipods, Au-tripods and Au-tetrapods). **(b)** TEM images of the resultant gold multipods at different magnifications. HRTEM of representative gold multipods were shown in the bottom-right panel. **(c)** UV-vis extinction curves of various gold multipods (including Au-Pt dumbbell NPs, Au-dipods, Au-tripods, and Au-tetrapods) at the same sample weight (based on ICP-MS), gold nanospheres, cubic platinum NPs and gold nanorods. (**d**) The perspective views of 3D volume rendering of photoacoustic images (green) and ultrasound images (brown). (**e**) Decay corrected representative coronal microPET images of nude mice bearing U87MG tumor at 1, 4 and 24 h after tail vein injection of ^{64}Cu-DOTA labeled Au-tripods modified with (top)/without (bottom) RGD peptide. Arrows indicate the location of tumors.

In our preliminary research, we prepared gold (Au)-tripod nanoparticles (NPs), which showed promising properties for photoacoustic imaging (PAI) of tumors. In this aim, we further expanded the nanoplatform for multimodality imaging. During the funding period, we synthesized branched gold nano-architectures with small particle size (<20 nm) including god dipods, tripods and tetrapods (**Figure 5a and b**) (***29***). The measurement of optical properties of these nanostructures revealed that Au-tripods have a much stronger extinction peak in the NIR region, compared to the other Au-multipods. After deconvolution of the broad extinction peak from 400 to 1000 nm, two plasmon resonances at 540 nm and 700 nm can be distinguished (**Figure 5c**). The correlation between the morphology and optical spectra of Au-tripods is critical to design desired contrast enhancers for PAI and suggests that stringently controlled shapes could result in strong, discrete optical absorption peaks in visible-NIR regions. The photoacoustic signal produced by Au-tripods was observed to be linearly dependent on the concentrations. We then developed a strategy based on the ligand exchange to anchor bifunctional polyethylene glycol (PEG) to the Au surface via a thiol-gold interaction. The dithiol ligand, lipoic acid, was first converted to a succinimide ester which is capable of conjugating with one of primary amine groups of PEG-

3400. Lipoic acid terminated PEG-3400 enables the phase transfer of the Au-tripods from organic solvents to aqueous solution and provides a steric barrier to prevent NPs agglomeration. Moreover, the PEG-3400-NH_2 could facilitate subsequent immobilization of various biological molecules via bioconjugation chemistry. Thus the c(RGDfC) can site-specifically conjugate with the maleimide-terminated NPs in an oriented and homogeneous fashion. In order to non-invasively track the RGD-Au-tripods *in vivo* by PET, the radiometal chelator, 2-(4-isothio cyanatobenzyl)-1,4,7-triazacyclo nonane-1,4,7-triacetic acid (p-SCN-Bn-NOTA) was also conjugated with the amine group presented on the surface of RGD-Au-tripods in a well-defined manner for ^{64}Cu radiolabeling. The PAI imaging ability of RGD-Au-tripods to $\alpha_v\beta_3$ integrin positive tumor was evaluated in the U87MG bearing tumor mice. Before the injection, the photoacoustic and ultrasound images of the mice were taken. Photoacoustic images with lateral step size of 0.25 mm were acquired at 670, 700, and 725 nm wavelength. Following the photoacoustic scan, an ultrasound image of the entire tumor area was acquired. Mice injected with RGD-Au-tripods showed significantly higher photoacoustic signal in the tumor compared with the blocking group co-injected with RGD after 2 h post-injection (**Figure 5d**). Similarly, PET imaging revealed that the ^{64}Cu labeled DOTA-tripods or RGD-DOTA-tripods showed good tumor uptake and good tumor to background contrast in subcutaneous U87MG mouse models. The tumor uptake of ^{64}Cu-RGD-DOTA-tripods was much higher than that of ^{64}Cu-DOTA-tripods (**Figure 5e**), suggesting the targeting specificity of the nanoprobe and the use of Au-tripod as a platform for multimodality imaging. In summary, our study suggested that Au-tripod nanoparticles can be reliably synthesized through stringently controlled chemical synthesis, and the highly selective and sensitive detection of cancer cells in a living subject is possible using molecular specific Au-tripods based multimodality imaging nanoprobes. This is a significant advancement in the field of using novel nanomaterials for cancer molecular imaging.

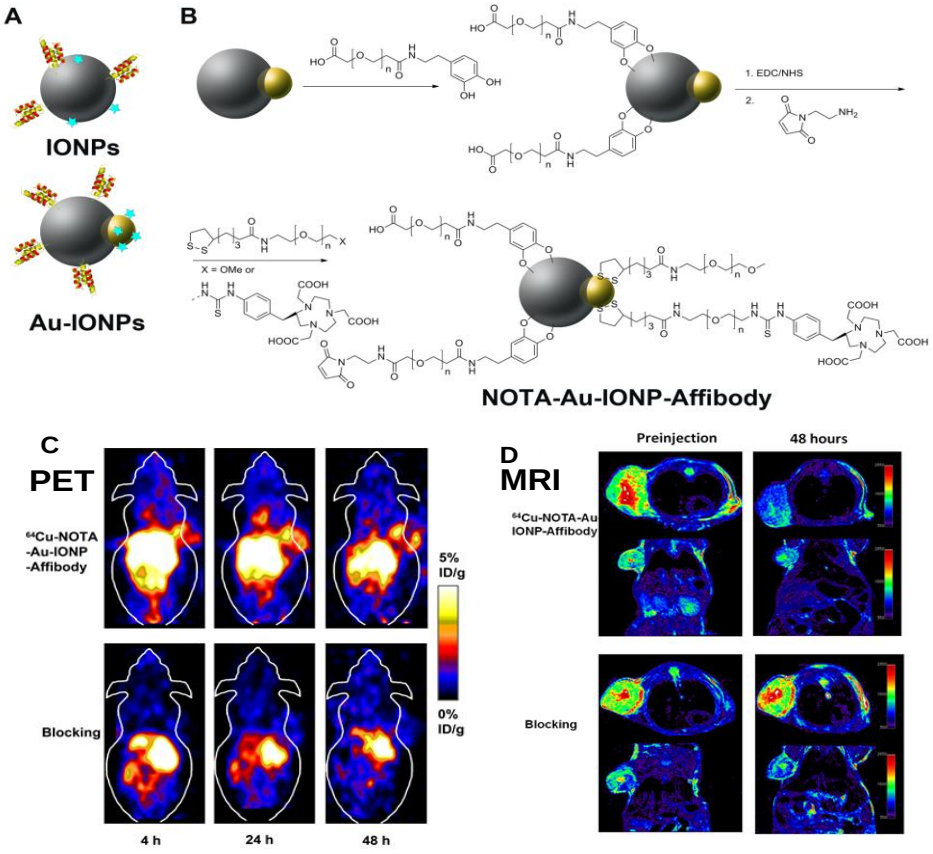

Figure 6. Dumbbell nanoparticles (Au-IONPs) for PET/MRI imaging of EGFR positive tumor. (A): Schematic three dimensional illustration of the Affibody binding domain with conventional single-component IONP (top) and dumbbell Au-IONP (bottom). (**B):** Schematic illustration of Au-IONPs surface functionalization and conjugation with Affibody. **(C)**: *In vivo* decay-corrected whole body coronal PET images of A431 tumor bearing mice acquired 4h, 24h and 48h after injection of ^{64}Cu-NOTA-Au-IONP-Affibody and the blocking dose of Affibody (n = 3/group). ^{64}Cu-NOTA-Au-IONP-Affibody shows excellent and specific tumor imaging ability (n = 3/group). **(D):** *In vivo* T_2-weighted MR images of A431 tumor bearing mice acquired before and at 48h after injection of ^{64}Cu-NOTA-Au-IONP-Affibody and blocking dose of Affibody.

Moreover, in this Aim, a highly monodispersed hetero-nanostructure with two different functional nanomaterials (gold, Au) and iron oxide (Fe_3O_4, IO) within one structure was also successfully developed by us as Affibody based trimodality nanoprobe (PET; optical imaging; and MRI) for imaging of EGFR positive tumors (**Figure 6A and B**) (***30***). Unlike other regular nanostructures with a single component, the Au-IO hetero-nanostructures (Au-IONPs) with unique chemical and physical properties have capability to combine several imaging modalities together to provide complementary information. The IO component within hetero-nanostructures serve as a T_2 reporter for MRI; and gold component serve as both optical and PET reporters. Moreover, such hetero-nanoprobes could provide a robust nano-platform for surface-specific modification with both targeting molecules (anti-EGFR Affibody protein) and PET reporters (radiometal ^{64}Cu chelators) in highly efficient and reliable manner. In vitro and in vivo study showed that the resultant nanoprobe provided high specificity, sensitivity, and excellent tumor contrast for both PET and MRI in the human EGFR-expressing cells

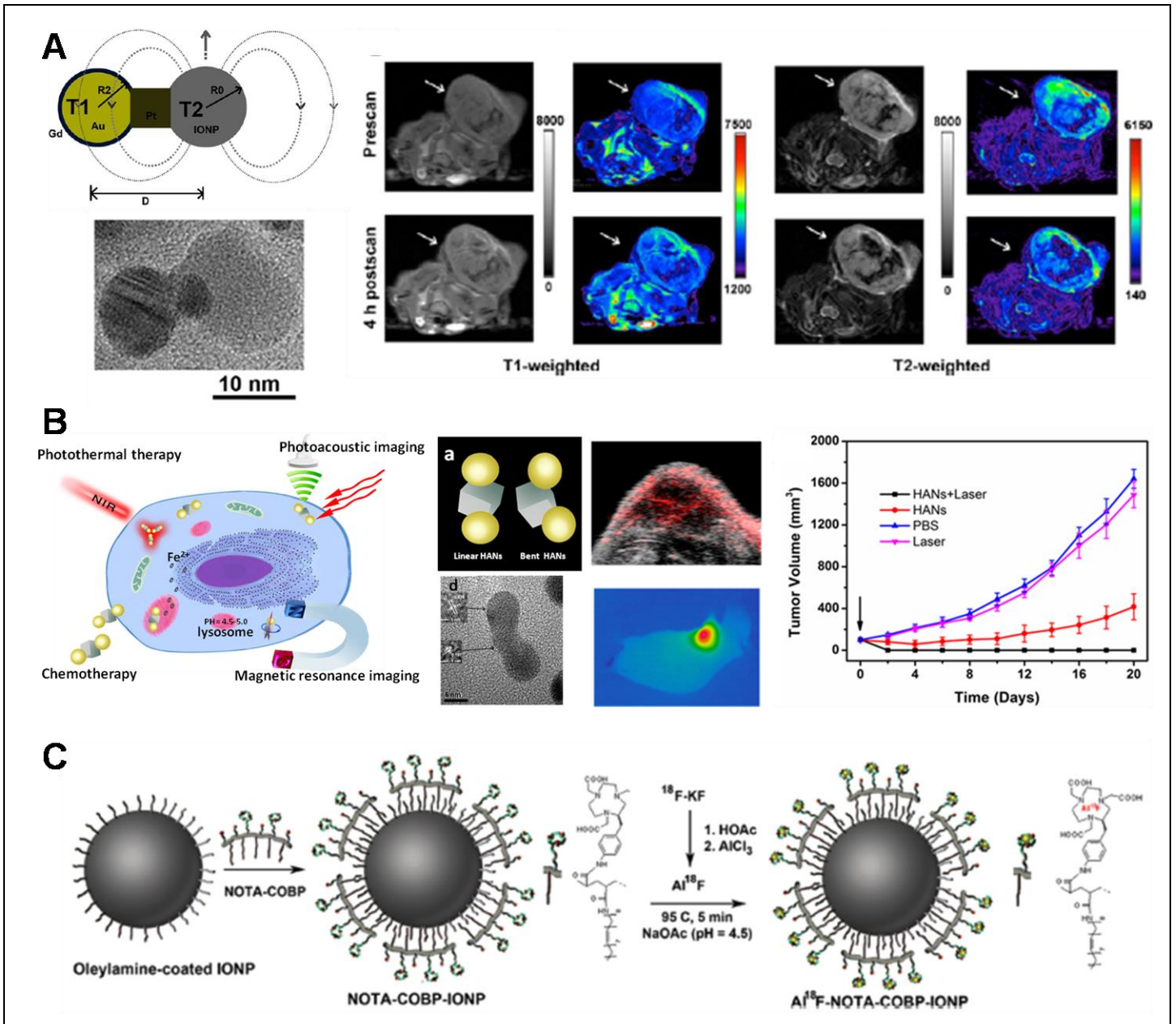

Figure 7. Au and IONP based nanoparticles for multimodality imaging. (A): Hybrid nanotrimers based on Au-Pt-IONPs for dual T1 and T2-weighted MRI. **(B):** Design of Au-FePt-Au hybrid anisotropic nanostructures for dual modal cancer imaging and chemo-thermo therapies. **(C)**: Preparation of water-soluble NOTA–COBP-IONPs (or NOTA-IONPs) and radiolabeling of NOTA-IONPs for PET/MRI imaging.

and tumors (**Figure 6C and D**). Our study highlights the EGFR targeting efficiency of hetero-nanoparticles and the feasibility for their further theranostic applications.

We continued to study the hetero-nanostructure for imaging and treatment. Hybrid nanotrimers based on Au-Pt-IONP was developed for dual T1 and T2-weighted MRI, which could allow us to image and diagnose the tumors or other abnormalities in an exceptionally accurate and reliable manner (***31***). In our study, by fusing distinct nanocrystals via solid-state interfaces, we built hybrid heteronanostructures to combine both T1 and T2-weighted contrast agents together for MRI with high accuracy and reliability. The resultant hybrid heterotrimers showed high stability in physiological conditions and could induce both simultaneous positive and negative contrast enhancements in MR images. Small animal PET imaging study revealed that the hybrid heterostructures displayed favorable biodistribution and were suitable for in vivo imaging. Their potential as dual contrast agents for T1 and T2-weighted MRI was further demonstrated by in vitro and in vivo imaging and relaxivity measurements (**Figure 7A**). In another study, we developed an efficient and novel strategy to synthesize hybrid anisotropic nanoparticles (HANs) with intrinsic multimodal theranostic capability (chemotherapy, photothermal therapy, MRI, and PAI) (***32***). For the first time, under the guidance of MRI and PAI, the chemotherapy and thermotherapy induced by administration of multifunctional hybrid nanoprobes were applied simultaneously to the treatment of colon cancer-bearing mice in vivo (**Figure 7B**). Meanwhile, we also developed a polymer coating procedure for providing colloidal stability to the nanoparticles and, more importantly, for applying a fast, facile ^{18}F labeling of iron oxide nanoparticles (IONPs) for PET/MR dual-modality imaging (***Figure 7C, 33***). The structure of the amphiphilic polymer was based on a backbone of polyacrylic acid, conjugated with multiple oleylamines to form a comb-like branched structure. The dense polymer shell provided high colloidal stability to the IONPs against harsh conditions such as high temperature, low pH value, and high ion strength. By incorporating a NOTA chelator to the comb-like amphiphilic polymer for the chelation of aluminum fluoride ions, we applied a one-step radiolabeling approach for a fast, facile radiofluorination of magnetic nanoparticles. The new strategy can significantly reduce the procedure time and radiation exposure. The PET/MR dual modality imaging was successfully achieved in living subjects by using ^{18}F labeled magnetic nanoparticles.

Besides the research on using Au and IONP based hetero-nanostructures for nanoprobe development and tumor imaging and treatment, we have also explored the use of IONPs and QDs to label macrophages as a potential tumor-microenvironment target for noninvasive imaging of early response to anticancer therapy (***34***), use zwitterionic manganese and gadolinium metal-organic frameworks (MOFs) as contrast agents for MRI (***35***). Lastly, we have also developed organic nanoplatforms such as melanin dots for theranostic and chemical applications (***36-40***), amphiphilic phospholipids encapsulated with NIR-II dyes for optical imaging and PAI (***41,42***), smart self-assembled organic nanoprobe for protein-specific detection (***43***), and perylene-diimide-based nanoparticles as highly efficient photoacoustic agents for brain cancer imaging (***44***). Overall, our work on nanoprobe and nanomedicine development has significant impact to the field and provides a variety of novel nanoplatforms for research and potential clinical translation.

Our papers published on the research topic of aim 3:

Publications on Affibody based probes

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Lastly, supported by DOE funding, we also published review articles to summarize the recent progress in field of molecular imaging and nanomedicine, and to provide insight to scientists (***45-54***).

Our papers published as review articles:

45. Bu L, Shen B*, Cheng Z.* Fluorescent imaging of cancerous tissues for targeted surgery. ***Advanced Drug Delivery Review.*** 2014; 76:21-38. PMID: 25064553. doi: 10.1016/j.addr.2014.07.008
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We also aimed to provide a superb and extensive hands-on training program to postdoctoral fellows through a diverse group of basic scientists (radiochemistry, chemistry) and clinician scientists in nuclear medicine. Our training efforts were seamlessly integrated with research efforts to develop novel clinically translatable PET probes and to foster the next generation of radiochemists and physicians. Specific training aims include the following: ***Training Aim 1: To learn about fundamental and novel radiosynthetic procedures to label a generalizable biomolecule platform to accelerate PET probe discovery using knottins (corresponding to SA1); Training Aim 2: To learn how to make clinical-grade radiolabeled imaging probes while maintaining regulatory compliance (corresponding to SA2)*****; *****Training Aim 3: To learn how to radiolabel gold-based nanoparticles for the development of novel tetramodal imaging probes (corresponding to SA3)*****.***

The overall training goal was to train individuals to be fully capable of understanding and developing a PET probe from its measurement phase (i.e., compounding, labeling, and analyses) in the Radiochemistry Facility to dose formulation/QC and application to patients in our nuclear medicine clinic. We provided training for 19 new trainees (10 Ph.D. and 9 MD fellows) during our grant funding cycle: 13 candidates were funded through this grant and leveraged funding from the Department of Radiology for 6 additional trainees. Research opportunities were given to engage trainees in our cutting-edge projects with mentors and improve radiochemistry and nuclear medicine cross-training. This enabled the trainees to gain an overall understanding of the complete probe development and clinical translation process, and encourage new trainees to pursue future rewarding careers in radionuclide-based imaging. Continued and reinforced involvement in molecular imaging research training empowered us to proactively tackle the short supply problem of trained scientists/clinicians in the molecular imaging/Nuclear Medicine community. Our Department, with its state-of-the-art resources in facilities/equipment and expertise, trained these new personnel in radiopharmaceutical discovery, production, and clinical use. Fellows took advantage of attending/auditing Stanford courses and the opportunity to discuss with their mentor their plans for any coursework, lectures, and seminar series that might be helpful for them to fill any deficits they may have.

Lucia Baratto MD (DOE-funded)
Jessica Klockow PhD (DOE-funded)
Kenneth Hettie PhD (DOE-funded)
Walid Alsharif PhD (DOE-funded)
Khun Visith Keu MD (DOE-funded)
Camila Mosci, MD (DOE-funded)
Su Hyun Hong PhD (DOE-funded)
Hongguang Liu MD PhD (DOE-funded)
Kai Cheng PhD (DOE-funded)
Xiang Hu MD, PhD (DOE-funded)
Xiaowei Ma, MD (DOE-funded)
Lihong Bu, MD PhD (DOE-funded)
Hao Chen, PhD (DOE-funded)
Thomas Haywood PhD (Stanford-funded)
Negin Hatami MD (Stanford-funded)
Ophir Vermesh MD, PhD (Stanford-funded)
Stephan Rogalia MD (Stanford-funded)
Chirag Patel MD (Stanford-funded)
Henry Guo, MD (Stanford-funded)

Importantly, Dr. Hongguang Liu and Xiaowei Ma learnt the fundamental and novel radiosynthetic procedures for labeling a generalizable biomolecule platform to accelerate PET probe discovery using knottins (**TA 1**). Drs. Cheng, Liu, Hu, Ma, Hong, Chen, and Bu were trained on radiolabeling nanoparticle scaffold for multimodality imaging (**TA3**). The radiolabeling technique these trainees learnt include: ^{18}F-SFB, ^{18}F-NFP, ^{18}F-AlF-NOTA and ^{64}Cu, ^{68}Ga-DOTA or NOTA labeling and quality control studies. Moreover, scientists from Drs. Sam Gambhir, Andrei Iagaru and Frederick Chin's lab at MIPS were trained. **Radiochemistry trainees:** Bin Shen, PhD was trained with respect to the clinical-grade production of several PET radiotracers. He set-up the ^{18}F and ^{68}Ga radiochemistry for making clinical-grade ^{18}F-FP-R01-MG-F2 and ^{68}Ga-R01-MG. The final clinical radiochemistry training for making ^{18}F-FP-R01 was provided to Jessica Lee Klockow, Kenneth Scott Hettie, Walid Alsharif and Xiaowei Ma, MD. **Clinical trainees:** We trained Camila Mosci, MD, Khun Visith Keu, Henry Guo, Lucia Baratto with respect to develop clinical-grade materials for human use with fluorine-18 radiochemistry and using the ^{18}F-labeled clinical research tracers in the clinic (**TA2**). In addition, trainees also learnt details of mandatory regulatory approvals and documentation that is necessary for human PET studies (**TA2**). Overall, through the research and training, we successfully accomplished our training aims and proposed pilot human studies.