ABSTRACT: The widespread and successful use of radiopharmaceuticals in diagnosis, treatment, and therapeutic monitoring of cancer and other ailments has spawned significant literature. The transition from untargeted to targeted radiopharmaceuticals reflects the various stages of design and development. Targeted radiopharmaceuticals bind to specific biomarkers, get fixed, and highlight the disease site. A new subset of radioprobes, the bioresponsive radiopharmaceuticals, has been developed in recent years. These probes generally benefit from signal enhancement after undergoing molecular changes due to the fluctuations in the environment (pH, redox, or enzymatic activity) at the site of interest. This review presents a comprehensive overview of bioresponsive radioimaging probes covering the basis, application, and scope of development.

INTRODUCTION
The prevalent application of noninvasive molecular imaging methods for diagnosis and subsequent therapeutic interventions of diseases has spawned significant literature. Compared to conventional imaging, molecular imaging techniques [positron emission tomography (PET), single photon emission computed tomography (SPECT), magnetic resonance imaging (MRI), and optical imaging] rely on alterations at the molecular level rather than anatomical anomalies.1 Such probes are also referred as stimuli-responsive or bioresponsive probes,1c consisting of a signaling unit, a biological unit that helps reach a specific site, and a stimuli-responsive unit that gets activated due to changes in the microenvironment. Depending on the application, the biological and the stimuli response may be the same or different. A plethora of examples of bioresponsive probes in molecular imaging have been reported and reviewed. Recent reports include a review by (1) Cho et al. covering stimuli-responsive $^{19}$F molecular probes, (2) Pinto et al. contribution highlighting responsive metal (gadolinium, europium, and manganese) complexes for MRI, and, (3) van Duijnhoven et al.1a summarization on bioresponsive probe for various molecular imaging modalities. This mini-review focuses on bioresponsive probes specifically for nuclear imaging (PET/SPECT) and highlights the advances through specific examples.

BIORESPONSIVE PROBES
As is true for the development of any radiopharmaceuticals, the development of a bioresponsive probe is also an iterative process focusing on selectivity and sensitivity with the capability to yield qualitative and quantitative information. Some salient features include the following:1b,3
1. It should be easy to synthesize and well-characterized.
2. It should be capable of being radiolabeled with high specificity.
3. It should be retained specifically in cells with the altered microenvironment.
4. The cellular retention and internalization mechanism should be well-established.
5. The lipophilicity parameters should be optimized for cellular penetration across biological membranes but without nonspecific binding.
6. The pharmacokinetic behavior should be well-established with fast systemic clearance.
7. The pharmacokinetic parameters should have no or minimal dependence on parameters covarying with the microenvironment.

Received: July 28, 2020
Accepted: September 15, 2020
Published: October 5, 2020
8. The probe should have high stability against metabolism except the expected changes induced by the microenvironment of interest.

9. The probe should conform to imaging requirements.

The difference between MRI/optical and PET–SPECT bioresponsive radioprobes is that, the efficiency of the MRI/optical probe itself changes due to the microenvironment, thereby switching to an on–off state, whereas the PET/SPECT probes rely on selective localization due to changes in the biological moiety.\(^1a\)

**pH-Responsive Probes.** Metabolic alteration is an inherent characteristic of many diseases, including cancer and neurodegenerative diseases. The altered metabolic cycles activate the hypoxia-inducible factor (HIF), resulting in enhanced glycolysis and accumulation of acidic metabolites like lactic acid. At the tissue level, the change manifests as a decrease in pH. Thus, pH becomes an essential biomarker for monitoring a disease, the therapy response, and as a stimuli for drug release.\(^2\) MRI imaging, primarily using various variants of Gd(III)-DOTA chelates, has been at the forefront for ascertaining the pH status. The mechanism relies on the change in gadolinium hydration state with the change in pH, which can be translated into quantitative information.\(^2b\)

For PET/SPECT probes, pH-dependent localization is the basis for contrast imaging rather than changes in the physicochemical state of the radiometal. A prominent example of pH sensing in nuclear imaging has been the utilization of 36 residues long pH low insertion peptide (pHLIP)\(^4\) derived from the bacteriorhodopsin C-helix. When the intracellular pH is acidic, the peptide having a pKa of 6.0 assumes an \(\alpha\)-helix structure and gets embedded into the cellular membrane.

Due to the difficulties involved in the \(^{18}\)F radiolabeling of long peptides, especially the involvement of harsh radiolabeling conditions adversely affecting peptide integrity, the pragmatic approach has been to utilize the pHLIP using the metal complexes. Vävere et al.\(^4b\) labeled the pHLIP peptide with \(^{64}\)Cu using DOTA as the chelator (1). The assessment of the probe to establish pH-dependent (extracellular pH, pHe) uptake was reported using cell lines (1) LLC and PC-3 tumor cells grouped as less acidic or less aggressive with nonmodulated PC-3 volume averaged pH 7.23 ± 0.10, and (2) LnCaP as a more acidic or more aggressive model cell line having nonmodulated volume average pH 6.78 ± 0.29 and modulated volume averaged pH 6.94 ± 0.56. Bicarbonate treatment of the cell lines PC-3 and LnCaP leads to decreased uptake of \(^{64}\)Cu-DOTA-pHLIP, indicating pH-dependent uptake. Preclinical data also supported the pH-LIP uptake in correlation with pH. The biodistribution data showed that the uptake of \(^{64}\)Cu-DOTA-pHLIP in the nonmodulated LnCaP tumors was 3-fold greater than the uptake in the bicarbonate water-modulated tumors. As the probe depends on pH-induced structural modifications, the method is semiquantitative and not quantitative due to the lack of a linear response between pH and the embedded peptide. Further studies concentrated on varying the sequence of pHLIP and employing truncated sequences to deeply understand the structure–activity relationship.\(^4c,d\)

The selection among the various pHLIP variants in the pH range of 6–7.4 was based on favorable binding at acidic pH and lower uptake at neutral pH. The study\(^5\) concentrated on the effect of radiometal (\(^{68}\)Ga vs \(^{64}\)Cu) and chelator (DOTA vs NOTA). The author’s findings reflected the importance of time pairing between radiometal half-life and residence time of the peptide favoring \(^{64}\)Cu-based radioprobes, better chelation with NOTA and peptide sequence with \(\varepsilon\)-amino acids to discourage peptide degradation. The authors also noted the complexity involved in consideration of volume averaged pHe, spatial gradient of pH in tumors, and the effect of pKa of the peptides. In continuation with the work, Demoin et al.\(^4d\) appended pHLIP peptide and its truncated modified variants with the macrocycle NOTA (2) and NOTA derived NO2A (3). The complexes were radiolabeled as a PET imaging agent with \(^{18}\)F using the Al–F radiolabeling metal-chelate method and \(^{64}\)Cu. The combined studies\(^4c,d\) led to two leads among the 12 variants tested. The peptide has also been labeled with \(^{99m}\)Tc as a SPECT tracer using the tetraamine chelator at the N-terminus of the peptide (4, \(^{99m}\)Tc–AH114567).\(^4e\) In order to negate the effect of the chelate and metal on insertion of the peptide, the chelate is appended at the noninserting N-terminus. The results showed a positive correlation between the tumor uptake and extracellular pH.

The \(^{18}\)F labeling, though not simple, was carried out using click chemistry without compromising the integrity of the peptide using mild conditions (5).\(^4f\) Rather than labeling the peptide, the radiolabeled 6-\(^{18}\)F-fluoro-2-ethynlypyridine procthetic group was synthesized initially with good radiochemical purity of \(\geq 98\%\) followed by click conjugation with an azide bearing pHLIP peptide.

Other than pHLIP, acidic sensing was also tried using fluorodeoxyglucose (FDG) analogues. Extending the application of FDG, Flavell et al.\(^4g\) reported \(^{18}\)F-FDG amines (6–\(e\)) that are acid-labile prodrugs. Analogues with different glycosylamine appended at the 1-position of FDG were synthesized. The acid-catalyzed hydrolysis of the \(^{18}\)F-FDG amine leads to the formation of \(^{18}\)F-FDG, which was taken up and selectively accumulated in cancerous cells by the usual entrapment mechanism. Five \(\beta\)-glycosides were synthesized and compared. The analogues showed pH-dependent uptake in PC-3 cells. Following alkalization with sodium bicarbonate, or carbonic anhydrase overexpression, the \(^{18}\)F-FDG amine’s uptake is reduced. The analogue was evaluated for pharmacological profiling in the PC-3 xenograft model.

The clinical validation for the above pH-responsive probes (Figure 1) is yet to be realized.

Few PET-MR dual probes (Figure 2) have been reported for sensing of pH, though the role of sensing is accomplished primarily using the MR moiety. These probes aid in ascertaining whether the change in MR signal is the result of either pH response or the concentration. The confirmation can be done with a simultaneous yet independent determination of the concentration of the contrast agent. The contrast agent has two units: a pH-responsive Gd(III) unit and PET/SPECT radioisotope bearing another group, which estimates the concentration. Notable PET-MR dual probes include a heterodimeric ligand of Gd(III)–Ga(III) diatomic complex \(^7\)\(^a\) and a click conjugate of Gd(III) with fluoroethylazide \(^8\)\(^a\) as a potential pH-sensitive MRI/PET probe.

**ROS-Responsive Probes.** The reactive oxygen species (ROS)-like superoxide radical anion (\(\mathrm{O}_2^{*-}\)), hydrogen peroxide (\(\mathrm{H}_2\mathrm{O}_2\)), hydroxyl radical (\(\mathrm{OH}^-\)), and hypochlorous acid (\(\mathrm{HOCl}\)) are byproducts in oxidative metabolism. ROS have a role in normal cellular processes, particularly in
signaling and are tightly regulated by the enzymes superoxide dismutase, catalase, and NADH peroxidase. However, in diseased states, the alteration in the ROS level is observed, leading to a pro-oxidative state or the oxidative stress and protein, lipid, and DNA damage. Aging, cardiovascular, neurodegenerative diseases, cancer, and chronic inflammation are associated with dysregulation and ROS imbalance. Monitoring of ROS levels has been mainly carried using in vitro cell-based methods and rely on near-IR optical, bioluminescence, and chemiluminescence probes. The advantage of imaging ROS imbalance using PET/SPECT is sensitivity, good spatial resolution, and low toxicity.\(^6\) The reaction-based approach in which radiotracers accumulate in cells following cleavage or oxidation of a ROS-sensitive moiety is employed. Figure 3 summarizes some ROS-responsive radioprobes described below.

![Figure 3. Summarization of ROS-responsive probes.](https://dx.doi.org/10.1021/acsomega.0c03601)

Initially, fluorescent molecules were tagged with PET radioisotopes to form ROS-sensing probes such as the \(^{18}\)F-labeled analogue of dihydroethidium (DHE; 9a).\(^{6a}\) The uptake mechanism remains the same as that of neutral unlabeled DHE. Once it crosses the membrane inside the cell, DHE is oxidized and forms a planar charged compound entrapped inside the cell. The study was claimed to be first-of-its-kind to demonstrate the in vivo PET imaging of ROS. Later, a variant of DHE was reported for imaging neuroinflammation. The variation was suited for permeation through the blood–brain barrier, and the tracer \(^{18}\)F-ROStrace was evaluated in the LPS-treated murine model for neuroinflammation (9b).\(^{6b}\) For sensing superoxides and hydroxyl radicals, near-IR cyanine dye was \(^{18}\)F-radiolabeled using standard nucleophilic fluorination in the presence of methanolic borohydride (10).\(^{6c}\)

Response against oxidative stress includes the over-expression of certain receptors and transporters. Few radiotracers have been reported to employ this as a strategy for cellular internalization and add further selectivity of the probe. A radiotracer boronate-caged \(^{18}\)F-FLT (FLT = fluoro-labeled thymidine, 11) probe was developed on lines similar to clinical PET agent–FLT entrapment following phosphor-
have been developed, which lead to preferential entrapment from this work, Carroll et al. reported the synthesis and evaluation of $^{11}$C-VitC. The salient observations were ROS-dependent $^{11}$C-VitC accumulation in U-87 cells and the tracer’s ability to detect endogenous ROS in activated neutrophil lineage cells. The probe is speculated to have clinical utility in imaging of inflammation and/or monitoring immunotherapy. Cystine transporter substrate $^{18}$F-5-fluoro-aminosuberic acid ($^{18}$F-FASu (12)), having potential as a ROS diagnostic tracer via indirect measure of xC activity, was evaluated using a transduced HEK::xC CT cell line and in xenograft mice using the breast cancer cell line.

**Hypoxia-Responsive Probes.** In the event of inadequate oxygen supply or hypoxia, tissues display a response due to the activation of hypoxia-induced factor (HIF). The tissues that can be affected are deep solid tumors due to poor vasculature and ischemic tissues due to reduced blood flow, resulting in an inadequate supply of oxygen and nutrients. To the best of our knowledge, no probe can directly image the HIF activity. Indirect methods based on reductase activity or $pO_2$ have been developed, which lead to preferential localization of the probe. Nitroimidazole and derivatives, a serendipitous discovery, were used for therapy and imaging for a long time. The nitroimidazole is an electro-affinic compound capable of undergoing enzyme-mediated one-electron reduction. In hypoxic cells, the reduced nitroimidazole gets trapped due to its interaction with macromolecules. This preferential entrapment lays the basis for applications in therapy and imaging (Figure 4).

Radio-halogen-labeled nitroimidazole compounds have been widely applied in imaging hypoxia and have dedicated reviews covering their cellular-trapping mechanism and applications. At present, $^{18}$F-MISO is the radioprobe of choice and a gold standard for hypoxia imaging using PET. The unfavorable points for $^{18}$F-MISO are low S/N due to poor tissue uptake limit the image quality and slow cellular release delaying the imaging. This led to further research and some other nitroimidazole-containing radioprobes. The difference is in the mechanism of membrane permeability and pharmacokinetic parameters. Once inside the cell, the mechanism of nitroimidazole undergoing one-electron reduction and cellular retention remains the same as depicted in Figure 4. Nevertheless, $^{18}$F-MISO and $^{18}$F-FAZA are clinically relevant radiotracers for hypoxia imaging (Figure 5). Readers are also referred to reviews for the detailed discussion.

In order to monitor $pO_2$, chimeric fusion protein (POS), having oxygen-dependent degradation domains (ODD), and protein transduction unit (PTD) were explored as hypoxia imaging probes. Having streptavidin–biotin for labeling with $^{125}$I (for SPECT imaging), the probe showed a retention in hypoxic cells 2-fold greater than that of normal cells where the probe was degraded. PET imaging of the peptide has also been reported using the $^{18}$F-analogue (POS and (4-$^{18}$F-fluorobenzoyl)norbiotinamide ($^{18}$F-FBB)) along with preclinical validation in mice models.

Parallel, researchers continued to search for metal complexes of nitroimidazole and other bioreductive compounds (aromatic and aliphatic N-oxides, quinones, and heterocycles) as alternatives. A few prominent examples of metallic radiopharmaceuticals are described below (Figure 6).

An extensive literature on the history and genesis of $^{99m}$Tc-nitroimidazole compounds with chelates based on propylene amine oxime (PnAO), butyleneamine oxime (BnAO), boronic acid adducts, cyclam, hydroximinoamide, iminodiacetic acids (IDA), diethylenetriamines (DETAA), dithiolate and cysteine-based, and triazoles has been presented in the review.

As a summarization, three $^{99m}$Tc-nitroimidazole analogues (14a–c) have been illustrated: BMS 181321, the first $^{99m}$Tc-labeled 2-nitroimidazole (14a) to be widely studied for hypoxia imaging, and $^{99m}$Tc-HL-91 or Prognox
(14b), a non-nitroaromatic compound for hypoxic cell detection that was designed to counter the high hepatic uptake of 14a, and BRU59-21 (previously known as BMS194796) 14c, a second-generation radioligand successfully designed to lower the hepatic radioactivity dose with superior imaging capability particularly for myocardial hypoxia imaging.7g-i 99mTcO-peptidic complexes containing the 2-nitroimidazole group and having an N3S chelating system were also reported.7j However, the utility of these 99mTc-ligands is yet to be established clinically for hypoxia imaging.

Hoigebazar et al.7k reported the formation of Al–F complexes of NODA-nitroimidazole (15). The driving reason was to have an alternative to tedious 18F. Two complexes were synthesized with high labeling efficiency (84–88%). The researchers selected NODA as the chelator as it does not bear the third −COOH group present in NOTA, assumed to interfere and destabilize Al binding. In proof-of-concept studies based on preclinical PET validation, the complexes retained preferential accumulation in hypoxic cells and even displayed better tumor to nontumor (T/N) ratios than 18F-FMISO and 18F-FAZA. However, clinical applications are yet to be tested.

Multivalent interactions are well-recognized to increase localization and affinity because of enhanced feasibility of ligand–receptor interactions. A series of bivalent and multivalent nitroimidazole derivatives have been reported (16a–d).8a–d Loading of additional nitroimidazole moieties generally increases the tumor specificity but has varied compound-specific effects on lipophilicity, reduction potentials, and pharmacokinetic parameters. 68Ga-labeled nitroimidazole derivatives (17)8e having the hexadentate H2-DEDPA (1,2-[[6-carboxypyrind-2-yl]methylamino]ethane) chelator were synthesized and tested for hypoxia imaging. Of the nine derivatives, four of the analogues exhibited good hypoxic–normoxic differentiating ability. The effectiveness was governed by the probe’s ability to penetrate inside the cells rather than the substituents on nitroimidazole.
In nuclear imaging and among the metallic radiopharmaceuticals, the copper-complexed bisthiosemicarbazone compound, $^{64}$Cu-diacyl-bis(N4-methylthiosemicarbazone) ($^{64}$Cu-ATSM) has been the most exploited clinically for imaging hypoxia. The complex was reported subsequent to Cu-PTSM$^{9a}$ (PTSM = pyruvaldehyde bis(N4-methylthiosemicarbazone), a blood perfusion and hypoxia imaging agent. Due to the high reduction potential of Cu-PTSM, the molecule had lower hypoxia selectivity. Structural modifications led to Cu-ATSM,$^{9b}$ which showed better cellular membrane permeation due to its lipophilicity and better hypoxia imaging due to its 100 mV lower reduction potential. Once inside the cell through a combination of passive diffusion and carrier-mediated penetration, the ligand undergoes one electron change from Cu(II) to Cu(I) due to the reductive environment. Detailed studies on the mechanism of Cu-ATSM indicate that the tracer is an indicative of an over-reductive environment generated by an increase of NADH and NADPH levels and, consequently, an indirect marker of reduced intracellular state. 

Mechanism of Cu-ATSM to sense hypoxia and intracellular trapping.

Figure 7. Mechanism of Cu-ATSM to sense hypoxia and intracellular trapping.

[Cu-ATSM], although excellent for oncology applications, it may not be suitable for delineating non-oncological hypoxia-like cardiovascular or neurological hypoxia. Design considerations like the variation in the alkylation pattern of diimine backbone and sulfur–selenium swapping can significantly influence the properties of the complexes. An additional single methyl group can lower the Cu(II/I) redox potential by approximately 80 mV. This was reflected in Cu-PTSM and Cu-ATSM, with Cu-ATSM being a better hypoxic imaging agent. Cu-ATSM and Cu-ATS are hypoxia-selective at the cellular level, but only Cu-ATSM is blood–brain barrier permeable (Figure 8 for chelate scaffolds). It has been suggested that the marked effects of backbone alkylation are mediated by frontier orbitals that differ little in energy but considerably in spatial properties. Even subtle energy shifts have profound chemical and pharmacokinetic consequences because they alter the order, and hence the occupancy, of frontier orbitals. The first copper bis(selenosemicarbazone) complexes were reported by Castle et al.$^{9c}$ in 2003. The researchers observed that swapping of S with Se had less effects on the oxidation potentials with a mere shift of approximately 10 mV. In contrast, the backbone alkylation had profound effects on the reduction potentials. The conclusions supported the involvement of spatially different orbitals. Although the stability of bis(selenosemicarbazone) complexes increased upon the addition of methyl groups to the diimine backbone, the fully alkylated species, $^{64}$Cu-ASSM, demonstrated no hypoxia selectivity. However, the additional alkylation present in $^{64}$Cu-ATSE modifies the hypoxia selectivity and in vivo properties when compared with $^{64}$Cu-ATSM.

Enzyme-Responsive Probes. The cellular functions are affected by enzymes, and the signaling pathways influence the activity of the enzyme. Enzymes can be imaged either for their expression using an inhibitor-based molecular probe or activity using a bioresponsive probe. An inhibitor-based probe is used to assess the localization of the enzyme in a diseased state. However, strategies which only detect the presence of an enzyme may lead to erroneous results. Quite often, the inactive form of the enzyme is expressed in a cell that is activated as per the requirement of the cell. Inhibitor-based probes present another challenge, especially if intended for therapy subsequent to imaging. Such probes may themselves inhibit the protease activity, thereby excluding the possibility for protease produg therapy. Usually designed as a one-to-one complex, the targeted molecular probes may not reflect the enzymatic activity. The inhibitor-based probes are also prone to yield incorrect results if subjects undergoing inhibitor therapy. A responsive probe will be the substrate of the enzyme and, when catalyzed, undergo a chemical transformation. The chemically transformed probe then generates the signal through selective entrapment. The selective entrapment will be proportional to the enzymatic activity and reflected in signal amplification.$^{1a,10}$ This strategy has been widely explored for MRI agents, wherein after the chemical transformation, change in the hydration state of gadolinium results in signaling.

Few examples of enzyme responsive PET/SPECT probes (Figure 9) include probes for matrix metalloproteinase (MMP). MMP peptide substrate (GPLGVR) was conjugated to polyethylene glycol (PEG$_{5000}$). This conjugate was linked with a tetramethylrhodamine group (TMR) and radiolabeled with $^{18}$F. Upon protease cleavage inside the cell, the released hydrophobic $^{18}$F-TMR selectively accumulated at the sites of MMP activity and detected using PET.$^{10b}$

Another category of intracellular enzymes that have been assessed for activity using PET/SPECT probe is thymidine kinases, used in reporter gene imaging approaches.
Thymidine kinase (TK) phosphorylates pyrimidine nucleoside and acycloguanosine derivatives. Compared to mammalian TK, the herpes simplex virus-1 TK (HSV1-TK) phosphorylates a broader category of substrates, leading to preferential trapping of molecules in cells expressing HSV1-TK. The most extensively validated probe is FHBG (18F-labeled 9-[4-fluoro-3-(hydroxymethyl)butyl]guanine, 18) studied for clinical applications with detailed pharmacokinetics.10c

Caspases, indicative of apoptosis, are another class of enzymes for which substrate-based probes are being explored. The probes are peptide-based substrates having the DEVDG sequence. Initial work was based on radiolabeling with 131I (D-3-131I-iodotyrosine) for SPECT imaging of a series of variants and ascertaining the effects of substituents and uptake in apoptotic cells.10d The variants comprise substrate-specific sequences (DEVDG and NQVNG) and cell-penetrating peptide Tat fragments that are trapped inside the cell. The experimental results indicate favorable uptake by apoptotic cells.

Among the PET tracers, 18F-CP18 (19)10e was among the first in series followed by 18F-C-SNAT and recently investigated 18F-DEVD-Cys(StBu)-PPG(CBT)-AmBF3.10f 18F-CP18 is a tetrapeptide DEVD probe specific for caspase 3 and caspase 7. Further, the peptide is conjugated with a polyethylene glycol (PEG) chain and galactose moiety for cellular penetration and pharmacokinetics. After entering the cell, the caspase cleaves PEG, resulting in entrapment of the tracer. The probe was 18F-radiolabeled using click chemistry. Early clinical trials in healthy volunteers indicate its safety and feasibility for use in humans.10g 18F-C-SNAT10h (20) and 18F-DEVD-Cys(StBu)-PPG(CBT)-AmBF310f are caspase-sensitive nanoaggregation probes developed with an intent of longer retention and signal amplification due to high density of 18F.

### SUMMARY AND FUTURE PERSPECTIVES

This review has comprehensively covered the bioresponsive PET/SPECT radioprobes stimulated due to the microenvironment of cells. The bioresponsive probes will have a pivotal role in disease diagnosis and exploring information at the molecular level of disease processes. As is true for any radiopharmaceuticals, the emphasis should be to develop viable probes that are chemically easy to synthesize and economical. 18F and 11C probes dominate the radiopharmaceuticals, but the development of metal radiopharmaceuticals can be beneficial for society. However, the design of metal radiopharmaceuticals can be tedious because of the influence of the metal and the chelate on the biological vector. The tailored chelator design provides control over the metal complex properties such as labeling efficiency, stability, overall charge, lipophilicity, and size, thereby serving as a valuable tool for radiotracer optimization. The critical role that the chemical nature of the metal chelate has on the biological performance of targeted radiopharmaceuticals demands continued development and optimization of new chelates.

Despite great advances and a surfeit of radiotracers reported, only a handful of radiotracers are successful clinically. Chemistry considerations and lessons learned from the biological evaluation of reported molecules will help design future radiotracer modifications. Transformation and fusion of small molecule radiotracers as nanoprobes and microrobots is expected to pave the way for smart and biocompatible radiotracers.

### AUTHOR INFORMATION

**Corresponding Author**

Anil K. Mishra – Division of Cyclotron and Radiopharmaceutical Sciences (DCRS), Institute of Nuclear Medicine and Allied Sciences (INMAS), Timarpur, Delhi 110054, India; orcid.org/0000-0003-2523-9045; Email: akmishra@inmas.drdo.in

**Authors**

Shubhra Chaturvedi – Division of Cyclotron and Radiopharmaceutical Sciences (DCRS), Institute of Nuclear Medicine and Allied Sciences (INMAS), Timarpur, Delhi 110054, India; orcid.org/0000-0003-0285-2111

Puja Panwar Hazari – Division of Cyclotron and Radiopharmaceutical Sciences (DCRS), Institute of Nuclear Medicine and Allied Sciences (INMAS), Timarpur, Delhi 110054, India; orcid.org/0000-0003-0285-2111

---

**Figure 9.** Examples of enzyme-responsive PET/SPECT probes.
Medicine and Allied Sciences (INMAS), Timarpur, Delhi 110054, India

Ankur Kaul – Division of Cyclotron and Radiopharmaceutical Sciences (DCRS), Institute of Nuclear Medicine and Allied Sciences (INMAS), Timarpur, Delhi 110054, India

Anju – Division of Cyclotron and Radiopharmaceutical Sciences (DCRS), Institute of Nuclear Medicine and Allied Sciences (INMAS), Timarpur, Delhi 110054, India

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c03601

Notes
The authors declare no competing financial interest.

Biographies

Dr. S. Chaturvedi is presently working as Senior Scientist at INMAS, Delhi. Dr. Chaturvedi obtained a Bachelor’s degree (Chemistry) from St. Stephen’s College, Delhi, a Master’s degree (Chemistry) from Indian Institute of Technology, and Ph.D. in Chemistry from Banaras Hindu University, Varanasi. For the past 15 years she has been working in development of radiopharmaceuticals and radiation sciences. Her research focuses on the design strategies for targeted radiopharmaceuticals. Her research contributions have been part of several research projects, with application in onco-imaging and neuro-imaging. She is also pursuing research for drug delivery systems based on biocompatible scaffolds.

Dr. Puja P. Hazari is currently working as a Biomedical Scientist at INMAS. After her Masters and Ph.D. in Biomedical Sciences from Delhi University, she joined INMAS. She is an enthusiastic scientist with more than 70 publications in reputed journals. Her research interest is centered on radiotracers for SPECT and PET imaging and has validated different approaches in design and radiolabeling for developing radiotracers. Her expertise for new PET radiolabeling methodology of small molecules with carbon-11 and fluorine-18 is well-accomplished. She has experience in radiolabeling a gamut of sensitive biomolecules and has been able to successfully translate basic research into clinical application.

Dr. A. Kaul has a Bachelor’s degree in Pharmacy from Mumbai University. She joined INMAS as a Technical officer and completed her Ph.D. (Life Sciences) from Bharathiar University, Coimbatore. She has immense experience in radiolabeling and radiopharmacy. Her research focuses on radiochemistry and preclinical evaluation of radiopharmaceuticals.

Ms. Anju is a research scholar at INMAS, who is actively involved in synthesis and evaluation of metallo-molecular probes for MRI and nuclear imaging. She did her graduation and post-graduation from Punjab University.

Dr. Anil K. Mishra is presently affiliated with INMAS as Chief Scientist. After acquiring a M.S. (Chemistry) from Gorakhpur University and Ph.D. (Chemistry) from Banaras Hindu University, Varanasi, India, Dr. Mishra gained experience in Université de Bourgogne, France, where he developed macrocycle chemistry with...
Prof Guilard. This was followed by a brief stint with Prof. Meares at the University of California, Davis, USA. He also served as a Scientist at INSEMr, Nantes, France, on the team of Prof. Chatal. With more than 200 publications to his credit, his varied and vast experience includes development of targeted radiopharmaceuticals using different classes of molecules and applying novel chemistries and approaches.

**ACKNOWLEDGMENTS**

The authors thank the Director INMAS for providing the necessary facilities. The authors also acknowledge the grants received under the collaborative project of INMAS-SAMEER funded by Ministry of Electronics and Information Technology (Meity), India.

**REFERENCES**

(1) (a) van Duijnhoven, S. M.; Robillard, M. S.; Langereis, S.; Grüll, H. Bioreponsive probes for molecular imaging: concepts and in vivo applications. *Contrast Media Mol. Imaging* 2015, 10 (4), 282–308. (b) Chaturvedi, S.; Kaul, A.; Hazari, P. P.; Mishra, A. K. Mapping small molecule receptors with metal-labeled radiopharmaceuticals. *MedChemComm* 2017, 8 (5), 855–870. (c) Chaturvedi, S.; Mishra, A. K. Small molecule radiopharmaceuticals—a review of current approaches. *Front. Med.* 2016, 3, 5.

(2) (a) Cho, M. H.; Shin, S. H.; Park, S. H.; Kadayakkara, D. K.; Kim, D.; Choi, Y..Targeted, Stimuli-Responsive, and Theranostic Nanocteated probes for molecular imaging: concepts and in vivo applications. *Contrast Media Mol. Imaging* 2015, 10 (4), 282–308. (b) Chaturvedi, S.; Kaul, A.; Hazari, P. P.; Mishra, A. K. Mapping small molecule receptors with metal-labeled radiopharmaceuticals. *MedChemComm* 2017, 8 (5), 855–870. (c) Chaturvedi, S.; Mishra, A. K. Small molecule radiopharmaceuticals—a review of current approaches. *Front. Med.* 2016, 3, 5.

(3) (a) Hunt, J. F.; Rath, P.; Rothschild, K. J.; Engelman, D. M. [11 C] Ascorbic and [11 C] dehydroascorbic acid, an oxygen-dependent degradable streptavidin and a novel 18 F-labeled probe. *Org. Biomol. Chem.* 2013, 11 (10), 1683–1690. (b) Frullano, P.; Catana, C.; Benner, T.; Sherry, A. D.; Caravan, P. Bimodal MR–PET agent for quantitative pH imaging. *Angew. Chem., Int. Ed.* 2010, 49 (13), 2382–2384.

(4) (a) Chu, W.; Chepetan, A.; Zhou, D.; Shoghi, K. I.; Xu, J.; Dungan, L. L.; Gropler, R. J.; Mintun, M. A.; Mach, R. H. Development of a PET radiotracer for non-invasive imaging of the reactive oxygen species, superoxide, in vivo. *Org. Biomol. Chem.* 2014, 12 (25), 4421–4431. (b) Hou, C.; Hsieh, C.-J.; Li, S.; Lee, H.; Graham, T. J.; Xu, K.; Weng, C.-C.; Doot, R. K.; Chu, W.; Chakraborty, S. K.; Dungan, L. L.; Mintun, M. A.; Mach, R. H. Development of a positron emission tomography radiotracer for imaging elevated levels of superoxide in neuroinflammation. *ACS Chem. Neurosci.* 2018, 9 (3), 578–586. (c) Al-Karmi, S.; Albu, S. A.; Vito, A.; Janzen, N.; Czorny, S.; Barvenicis, L.; Nanao, M.; Zubieta, J.; Capretta, A.; Valliant, J. F. Preparation of an 18F-labeled hydroxycinnine as a multimodal probe for reactive oxygen species. *Chem. – Eur. J.* 2017, 23 (2), 254–258. (d) Carroll, V.; Michel, B. W.; Blecha, J.; VanBrocklin, H.; Keshari, K.; Wilson, D.; Chang, C. J. A boronate-caged [18F] FLT probe for hydrogen peroxide detection using positron emission tomography. *J. Am. Chem. Soc.* 2014, 136 (42), 14742–14745. (e) Yamamoto, F.; Shibata, S.; Watanabe, S.; Masuda, K.; Maeda, M. Positron-labeled antioxidant 6-deoxy-6-[18F] fluoro-l-ascorbic acid: increased uptake in transient global ischemic rat brain. *Nucl. Med. Biol.* 1996, 23 (4), 479–486. (f) Carroll, V. N.; Truillet, C.; Shen, B.; Flavell, R. J.; Shao, X.; Evans, M. J.; VanBrocklin, H. F.; Scott, P. J. H.; Chin, F. T.; Wilson, D. M. [11 C] Ascorbic and [11 C] dehydroascorbic acid, an endogenous redox pair for sensing reactive oxygen species using positron emission tomography. *Chem. Commun.* 2016, 52 (27), 4888–4890. (g) Yang, H.; Jenni, S.; Colovic, M.; Merkens, H.; Poleschuk, C.; Rodrigo, I.; Miao, Q.; Johnson, B. F.; Rishel, M. J.; Sossi, V.; Webster, J. M.; Benard, F.; Schaffer, P. 18F-5-Fluorooxalosuberic acid as a potential tracer to gauge oxidative stress in breast cancer models. *J. Nucl. Med.* 2017, 58 (3), 367–373. (h) (f) Fleming, I. N.; Manavaki, R.; Blower, P. J.; West, C.; Williams, K. J.; Harris, A. L.; Domarkas, J.; Lord, S.; Baldry, C.; Gilbert, F. J. Imaging tumour hypoxia with positron emission tomography. *Br. J. Cancer* 2015, 112 (2), 238–250 and references therein. (b) Kudo, T.; Ueda, M.; Kuge, Y.; Mukai, T.; Tanaka, S.; Masutani, M.; Kiyono, Y.; Kizaka-Kondoh, S.; Hiraoka, M.; Saji, H. Imaging of HIP-1-active tumor hypoxia using a protein effectively delivered to and specifically stabilized in HIP-1-active tumor cells. *J. Nucl. Med.* 2009, 50 (6), 942–949. (c) Kudo, T.; Ueda, M.; Konishi, H.; Kawashima, H.; Kuge, Y.; Mukai, T.; Miyano, A.; Tanaka, S.; Kizaka-Kondoh, S.; Hiraoka, M.; Saji, H. PET imaging of hypoxia-inducible factor-1-active tumor cells with pretargeted oxygen-dependent degradable streptavidin and a novel 18 F-labeled biotin derivative. * Mol. Imaging Biol.* 2011, 13 (5), 1003–1010. (d) Reichert, D. E.; Lewis, J. S.; Anderson, C. J. Metal complexes as diagnostic tools. *Coord. Chem. Rev.* 1999, 184 (1), 3–66 and references therein. (e) Giglio, J.; Rey, A. 99mTc Labeling Strategies for the Development of Potential Nitroimidazole Hypoxia Imaging Agents. *Inorg. Chim. Acta* 2019, 451, 23–39. (f) Ricardo, C. L.; Kumar, P.; Wiebe, L. I. Bifunctional metal-nitroimidazole complexes for hypoxia theranosis in cancer. *J. Diagn. Imaging Ther.* 2015, 2, 103–158. (g) (l) Linder, K. E.; Chan, Y. W.; Curr, J. E.; Malley, M. J.; Nowotnik, D. P.; Nunn, A. D. TecO (PnAO-1-(2-nitroimidazole)) [BMS-181321], a new techtetium-containing nitroimidazole complex for imaging hypoxia: synthesis, characterization, and xanthine oxidase-catalyzed reduction. *J. Med. Chem.* 1994, 37 (1), 9–17. (h) Cook, G. J. R.; Barrington, S.; Houston, S.; Maisey, M. N.; Fogelman, I. HL91, a new Tc-99m-labelled agent with potential for identifying tumour hypoxia: correlation with FDG PET. *J. Nucl. Med.* 1996, 37, 877–881. (j) Melo, T.; Duncan, J.; Ballinger, J. R.; Rauth, A. M. BRUS9-21, a second-generation 99mTc-labeled 2-nitroimidazole for imaging hypoxia in tumors. *J. Nucl. Med.* 2000, 41 (1), 169–176. (j) Su, Z. F.; Zhang, X.; Ballinger, J. R.; Rauth, A. M.; Pollak, A.; Thornback, J. R. Synthesis and evaluation of two technetium-99m-
labeled peptidic 2-nitroimidazoles for imaging hypoxia. Bioconjugate Chem. 1999, 10 (5), 897–904. (k) Hoigebazar, L.; Jeong, J. M.; Lee, J. Y.; Shetty, D.; Yang, B. Y.; Lee, Y. S.; Lee, D. S.; Chung, J. K.; Lee, M. C. Syntheses of 2-nitroimidazole derivatives conjugated with 1, 4, 7-triazacyclononane-N, N′-diacetic acid labeled with F-18 using an aluminum complex method for hypoxia imaging. J. Med. Chem. 2012, 55 (7), 3155–3162. (8) Huang, H.; Zhou, H.; Li, Z.; Wang, X.; Chu, T. Effect of a second nitroimidazole redox centre on the accumulation of a hypoxia marker: synthesis and in vitro evaluation of 99mTc-labeled bisnitroimidazole propylene amine oxime complexes. Bioorg. Med. Chem. Lett. 2012, 22 (1), 172–177. (b) Mei, L.; Wang, Y.; Chu, T. 99mTc/Re complexes bearing bisnitroimidazole or mononitroimidazole as potential bioreductive markers for tumor: Synthesis, physicochemical characterization and biological evaluation. Eur. J. Med. Chem. 2012, 58, 50–63. (c) Mukai, T.; Suwada, J.; Sano, K.; Okada, M.; Yamamoto, F.; Maeda, M. Design of Ga-DOTA-based bifunctional radiopharmaceuticals: Two functional moieties can be conjugated to radiogallium–DOTA without reducing the complex stability. Bioorg. Med. Chem. 2009, 17 (13), 4285–4289. (d) Seelam, S. R.; Lee, J. Y.; Lee, Y. S.; Hong, M. K.; Kim, Y. J.; Banka, V. K.; Lee, D. S.; Chung, J. K.; Jeong, J. M. Development of 68Ga-labeled multivalent nitroimidazole derivatives for hypoxia imaging. Bioorg. Med. Chem. 2015, 23 (24), 7743–7750. (e) Ramogida, C. F.; Pan, J.; Ferreira, C. L.; Patrick, B. O.; Rebollar, K.; Yapp, D. T.; Lin, K. S.; Adam, M. J.; Orvig, C. Nitroimidazole-Containing H2dedpa and H2 CHX dedpa Derivatives as Potential PET Imaging Agents of Hypoxia with 68Ga. Inorg. Chem. 2015, 54 (10), 4953–4965. (9) (a) Mathias, C. J.; Welch, M. J.; Perry, J. D.; McGuire, A. H.; Zhu, X.; Connett, J. M.; Green, M. A. Investigation of copper-PTSM as a PET tracer for tumor blood flow. International journal of radiation applications and instrumentation. Part B. Nucl. Med. Biol. 1991, 18 (7), 807–811. (b) Fujibayashi, Y.; Tanuchi, H.; Yonekura, Y.; Ohtani, H. Copper-62-ATSM: a new hypoxia imaging agent with high membrane permeability and low redox potential. J. Nucl. Med. 1997, 38 (7), 1155–1160. (c) Colombié, M.; Gouard, S.; Frindel, M.; Vidal, A.; Chérel, M.; Kraeber-Bodéré, F.; Rousseau, C.; Bourgeois, M. Focus on the controversial aspects of 64Cu-ATSM in tumoral hypoxia mapping by PET imaging. Front. Med. 2015, 2, 58. (d) Castle, T. C.; Maurer, R. I.; Sowrey, F. E.; Went, M. J.; Reynolds, C. A.; McInnes, E. J.; Blower, P. J. Hypoxia-targeting copper bis (selenoemicarbazone) complexes: comparison with their sulfur analogues. J. Am. Chem. Soc. 2003, 125 (33), 10040–10049. (e) McQuade, P.; Martin, K. E.; Castle, T. C.; Went, M. J.; Blower, P. J.; Welch, M. J.; Lewis, J. S. Investigation into 64Cu-labeled Bis (selenoemicarbazone) and Bis (thiosemicarbazone) complexes as hypoxia imaging agents. Nucl. Med. Biol. 2005, 32 (2), 147–156. (10) (a) Holland, J. P.; Cumming, P.; Vasdev, N. PET radiopharmaceuticals for probing enzymes in the brain. Am. J. Nucl. Med. Mol. imaging 2013, 3 (3), 194–216. (b) Chuang, C.-H.; Chuang, K.-H.; Wang, H.-E.; Rolfler, S. R.; Shiea, J.-t.; Tsou, S.-C.; Cheng, T.-C.; Kao, C.-H.; Wu, S.-Y.; Tseng, W.-L.; Cheng, C.-M.; Hou, M.-F.; Wang, J.-M.; Cheng, T.-L. In vivo positron emission tomography imaging of protease activity by generation of a hydrophobic product from a noninhibitory protease substrate. Clin. Cancer Res. 2012, 18 (1), 238–247. (c) Yaghoubi, S. S.; Gambhir, S. S. PET imaging of herpes simplex virus type 1 thymidine kinase (HSV1-tk) or mutant HSV1-sr39tk reporter gene expression in mice and humans using [18 F]FHBG. Nat. Protoc. 2006, 1 (6), 3069–3074. (d) Bauer, C.; Bauder-Wuest, U.; Mier, W.; Haberkorn, U.; Eisenhut, M. 131I-labeled peptides as caspase substrates for apoptosis imaging. J. Nucl. Med. 2005, 46 (6), 1066–1074. (e) Su, H.; Chen, G.; Gangadhrarmath, U.; Gomes, L. F.; Liang, Q.; Xu, F.; Mochlara, V. P.; Sardenings, A. K.; Walsh, J. C.; Xia, C.; Yu, C.; Kolb, H. C. Evaluation of [18 F]-CP18 as a PET imaging tracer for apoptosis. Mol. Imaging Biol. 2013, 15 (6), 739–747. (f) Qiu, L.; Wang, W.; Li, K.; Peng, Y.; Lv, G.; Liu, Q.; Gao, F.; Seimbille, Y.; Xie, M.; Lin, J. Rational design of caspase-responsive smart molecular probe for positron emission tomography imaging of drug-induced apoptosis. Theranostics 2019, 9 (23), 6962–6975. (g) Doss, M.; Kolb, H. C.; Walsh, J. C.; Mochlara, V.; Fan, H.; Chaudhary, A.; Zhu, Z.; Alpaugh, R. K.; Lango, M. N.; Yu, J. Q. Biodistribution and radiation dosimetry of 18F-CP18, a potential apoptosis imaging agent, as determined from PET/CT scans in healthy volunteers. J. Nucl. Med. 2013, 54 (12), 2087–2092. (h) Shen, B.; Jeon, J.; Palner, M.; Ye, D.; Shuhendler, A.; Chin, F. T.; Rao, J. Positron emission tomography imaging of drug-induced tumor apoptosis with a caspase-triggered nanoaggregation probe. Angew. Chem., Int. Ed. 2013, 52 (40), 10511–10514.