

Design and Applications of Bispecific Heterodimers: Molecular Imaging and beyond

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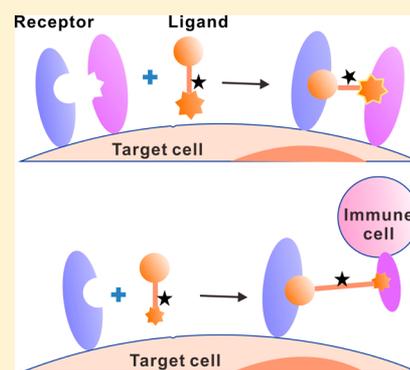
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ABSTRACT: Ligand-based molecular imaging probes have been designed with high affinity and specificity for monitoring biological process and responses. Single-target recognition by traditional probes can limit their applicability for disease detection and therapy because synergistic action between disease mediators and different receptors is often involved in disease progression. Consequently, probes that can recognize multiple targets should demonstrate higher targeting efficacy and specificity than their monospecific peers. This concept has been validated by multiple bispecific heterodimer-based imaging probes that have demonstrated promising results in several animal models. This review summarizes the design strategies for bispecific peptide- and antibody-based heterodimers and their applications in molecular targeting and imaging. The design and application of bispecific heterodimer-conjugated nanomaterials are also discussed.

KEYWORDS: *molecular imaging, heterobivalent ligands, bispecific antibodies, dual targeting, cancer, positron emission tomography (PET)*



INTRODUCTION

Over the last 2 decades, various molecular imaging technologies, including positron emission tomography (PET), computed tomography (CT), single photon emission computed tomography (SPECT), magnetic resonance imaging (MRI), ultrasound, and fluorescence reflectance imaging, have revolutionized the way that we investigate complex biochemical phenomena.^{1,2} Along with the rapid advances in molecular and cell biology, molecular imaging can greatly enhance the ability for researchers and clinicians to identify novel molecular targets and biomarkers, especially those involved in disease (particularly cancer) initiation, progression, and treatment response. Detection of such biomarkers can lead to faster diagnosis and treatment, better prognosis and staging, and improved management.

Molecular imaging is defined as “the noninvasive visualization, characterization, and measurement of biological processes at the cellular and molecular level in humans and other living systems”.³ Because molecular imaging provides both anatomical and physiological information, it has become an essential tool in bench-side research, clinical trials, and medical practice. One of the central challenges for molecular imaging is the development of specific imaging probes that have a high target-to-background ratio and improved contrast in vivo.⁴ The ideal imaging probe should possess high affinity and specificity for target, adequate retention in the target, low nonspecific uptake, and efficient capillary permeability. To date, many ligand-mediated targeting probes have been explored, and some of them have been approved for clinical use. A few

examples are the cyclic octapeptide octreotide, a peptide that targets the somatostatin receptor,^{5,6} trastuzumab (Herceptin), an antibody that binds to the antiepidermal growth factor receptor 2 (ErbB2, HER2) receptor,⁷ and bevacizumab (Avastin), an antibody that binds to the extracellular vascular endothelial growth factor A (VEGF-A).^{8,9}

Many diseases, especially inflammatory disorders and cancer, result from complex interactions between disease-mediated ligands and growth-promoting receptors. The crosstalk with other signaling pathways complicates the use of ligand-based probes for molecular imaging. Thus, accurate knowledge of the receptor's role in the interaction between cells and their microenvironment is important. Solid tumors, for instance, are usually composed of an assemblage of distinct cell types (e.g., endothelial cells, pericytes, immune inflammatory cells, cancer-associated fibroblasts, cancer cells, cancer stem cells, etc.) that interact through the reciprocal heterotypic signaling pathway to maintain and orchestrate the tumor microenvironment.¹⁰ For example, epidermal growth factor (EGF), VEGF, other proangiogenic factors (e.g., fibroblast growth factor 2 (FGF2), chemokines, and cytokines) can amplify the inflammatory state and serve as effectors of tumor progression.¹¹ Additionally, tumor heterogeneity^{12,13} and binding site barriers between ligand and receptor¹⁴ can limit the

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Table 1. Representative Examples of Ligand-Based Bispecific Heterodimers

	target 1	target 2	heterodimers	disease model	ref
peptide heterodimers	MCIR	CCK-2R	MSH × CCK	cancer	26
	GRPR	$\alpha_v\beta_3$	BBN × RGD	cancer	19, 20, 24, 75, 79, 116
	c-Met	$\alpha_v\beta_3$	cMBP/cRGDk	cancer	28
	GRPR	Y1	t-BBN/BVD15-DO3A	in vitro	117
	hMC4R	CCK-2R	MSH-7/CCK-6	cancer	25, 26, 118
antibody heterodimers	CD90	MLC1	anti-CD90/anti-MLC1	stem cells	37
	EGFR	HER2	EGF/Trastuzumab Fab	cancer	91
	HER2	HER3	Trastuzumab Fab/HRG	cancer	89
	CD3	TAC	anti-CD3/anti-TAC F(ab'-zipper) ₂	in vitro	44
	Met	VEGFR-2	Met-Fc/VEGFR-2-Fc	cancer	66
	HER2	HER3	anti-HER2/anti-HER3 scFv		46
	CD3	CD4	anti-CD3/CD4-IgG		65
	c-Mpl	HER3	anti-Mpl/anti-p-HER3	cancer	63
	EGFR	CD2	anti-EGFR/anti-CD2	cancer	57
	HER2	CD3	anti-p185 ^{HER2} /anti-CD3	cancer	58
	CD3	CD19	CD3 × CD19 diabody	B cell leukemia	100
	HER2	CD3	F(ab') ₂ HER2 × CD3	cancer	98

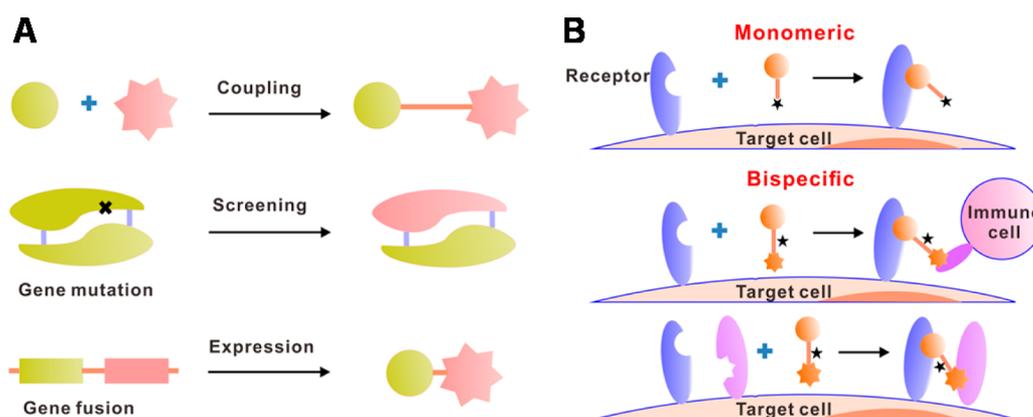


Figure 1. Schematic illustration of the synthesis strategies and receptor interaction of bispecific heterodimers. (A) Synthesis strategies of bispecific heterodimer based on chemical coupling, mutation/screening, and gene fusion. (B) Interactions between bispecific heterodimer/monospecific ligand and cellular receptors during the molecular imaging process. Stars stand for the imaging labels for the ligands.

targeting and therapeutic efficiency of ligands because they are typically monospecific. In fact, mounting evidence has demonstrated that acquired resistance to antibody therapy can occur if the antibody is against a single receptor, and this resistance is often related to pathway switching between receptors.^{15,16} Consequently, multiple targeting, or the ability to bind multiple targets simultaneously, has become a more advantageous approach for the development of ligand-based imaging probes and therapeutics.

Over the past few decades, dual targeting with bispecific peptides or antibodies has been explored in clinical trials as an alternative combination therapy for cancer patients (with over 50 ongoing or completed trials listed at clinicaltrials.gov). Heterodimers ligands are composed of two covalently linked targeting subunits and are a simple, beneficial model for the investigation of dual targeting. Recently, many bispecific heterodimers, summarized in Table 1, have been developed. Compared with monoreceptor targeting compounds, bispecific heterodimers have several advantages including increased affinity, avidity, and efficacy,^{17,18} which establishes them as strong applicants for use in molecular imaging. In this review, we will discuss the design of bispecific peptide and antibody heterodimers and their applications in molecular targeting and imaging, with special emphasis on antibody heterodimers. We

will also briefly discuss the design and application of bispecific heterodimer-conjugated nanomaterials.

■ DESIGN OF BISPECIFIC HETERODIMERS

Two major strategies for the design of heterodimers exist. In the first strategy, the heterodimer is formed by cross-linking two ligands that target two receptors from different cells at a given location (Figure 1A).^{19,20} This strategy is commonly used in the design of peptide heterodimers. In the second strategy, the heterodimer is usually generated by methods of genetic engineering or manipulation.¹⁷ This strategy is more applicable in the design of protein-based heterodimers in which the structural integrity is of great concern during their development. Clear advantages of this strategy include greater flexibility, higher production yield, and lower binding affinity loss. Three primary approaches are readily adopted in the production of protein heterodimer: the first approach involves gene fusion and expression in *Escherichia coli* to produce protein heterodimers in a tandem manner (Figure 1A).²¹ The second approach uses somatic hybridization by two protein-secreting cells (e.g., hybridomas) along with affinity chromatography purification and is employed in the production of bispecific antibodies.²² The third approach is comparatively rare; it introduces mutations into monospecific proteins and

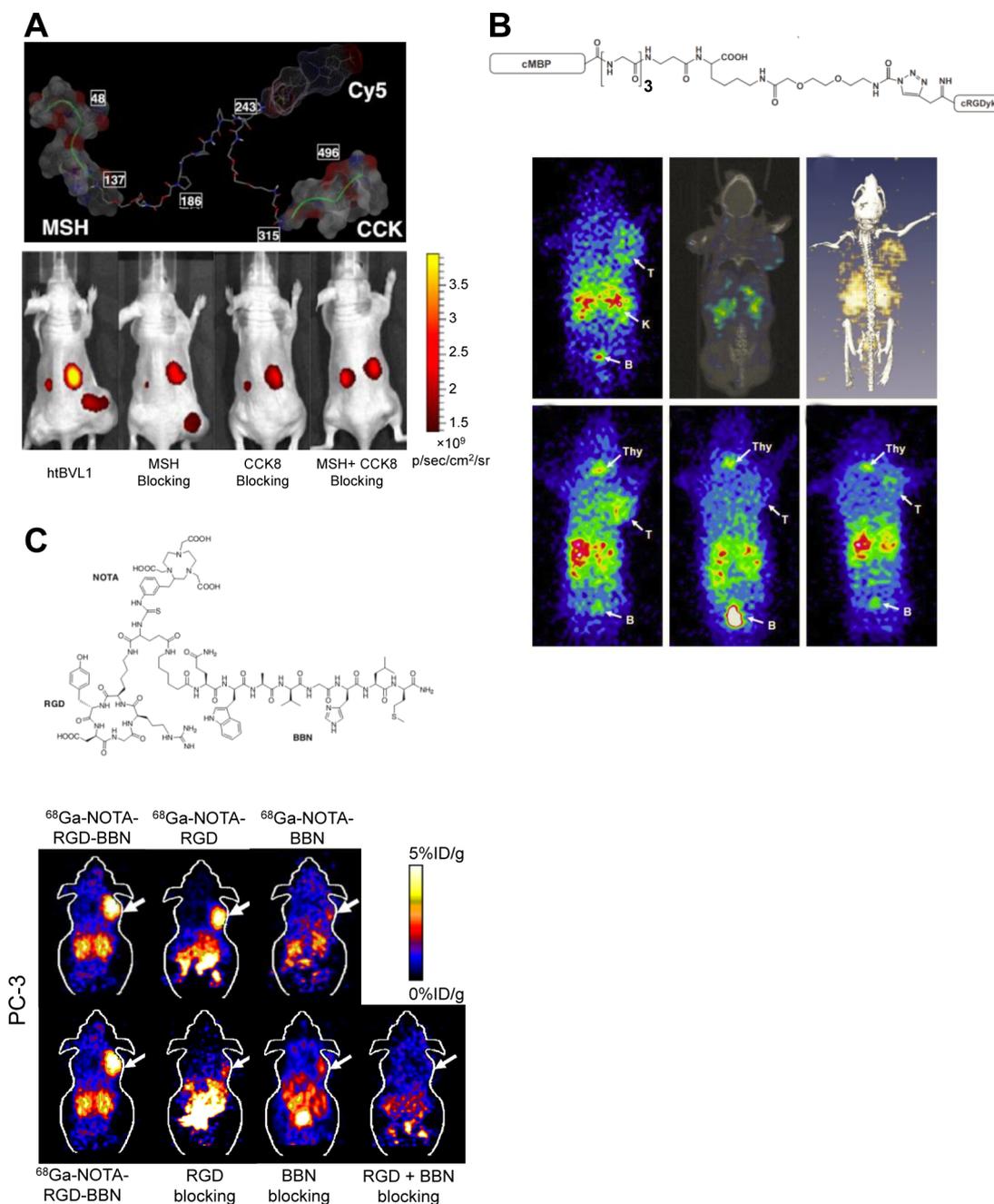


Figure 2. Representative peptide heterodimers used for molecular imaging. (A) Molecular structure of Cy5-labeled heterobivalent ligand 1 (htBVL1) and representative in vivo fluorescence images showing its specific uptake in target tumor (right flank, target tumor with MC1R and CCK-2R expression; left flank, control tumor with only MC1R expression). Adapted with permission from ref 26. Copyright 2012 National Academy of Sciences. (B) Structure and SPECT/CT images of ¹²⁵I-cMBP-click-c(RGDyK) heterodimer in U87MG tumor (c-MET and integrin $\alpha_v\beta_3$ positive) at 1 (upper panel) and 4 h p.i. (lower left image). Blocking with cRGDyK (lower middle image) or cMBP (lower right image) was carried out at 4 h p.i. T, tumor; B, bladder; Thy, thyroid; and K, kidney. Adapted with permission from ref 28. Copyright 2011 Japanese Cancer Association. (C) PET images of ⁶⁸Ga-NOTA-RGD-BBN, ⁶⁸Ga-NOTA-BBN, and ⁶⁸Ga-NOTA-RGD at 1 h p.i. in PC-3 tumor-bearing mice. Adapted with permission from ref 79. Copyright 2009 Springer-Verlag.

screens bispecific candidates out from the mutant library (Figure 1A). One representative example was carried out by Papo et al. in which they introduced an integrin $\alpha_v\beta_3$ binding capacity into the single-chain VEGF (scVEGF) by a yeast-displayed mutant library to generate a dual-specific scVEGF mutant with high affinity to both VEGFR2 and integrin $\alpha_v\beta_3$.²³ Compared with monospecific mutants that bind only to VEGFR2 or integrin $\alpha_v\beta_3$, the dual-specific scVEGF proteins demonstrated more effective inhibition of VEGF-mediated

receptor phosphorylation, endothelial cell proliferation, and blood vessel formation both in vitro and in vivo. In the following text, we will categorize the heterodimers based on these design strategies.

Peptide-Based Heterodimers. Chemical conjugation is practically the only design concept for peptide heterodimers. After the cross-linking of two peptides, evaluation of binding affinity and specificity is essential for their imaging applications. Generally speaking, there are two primary approaches to

evaluate these parameters: the first approach can be carried out in two different cell types in which one cell type overexpresses a single receptor and the other cell type overexpresses both target receptors.²⁴ In the second approach, binding affinity/specificity is examined in one cell type with high expression of both receptors (Figure 1B). Ligands with strong affinity for each individual receptor compete with the heterodimer during its interaction with the target cells.²⁰ In both of these approaches, the key point is to confirm that the peptide heterodimer has satisfactory a binding affinity and high specificity for each of its target receptors.

On the basis of these strategies, heterobivalent ligands (htBVLs) were developed that contain both melanocyte-stimulating hormone (MSH) and cholecystokinin (CCK) peptide ligands tethered with linkers of different rigidity and length.²⁵ These heterodimers could simultaneously bind melanocortin-4 receptor (MC4R) and CCK-2 receptor (CCK-2R), which are overexpressed in multiple cancer types including pancreatic cancer.²⁵ The monovalent binding capacity of these ligands was evaluated in HEK293 cells transfected with either MC4R, CCK-2R, or both. The binding affinity of the optimized heterodimer to cells expressing both MC4R and CCK-2R was over 20-fold higher than for cells expressing only MC4R. More recently, the same research group assessed the *in vivo* targeting efficacy of one heterodimer compound (named htBVL1) composed of similar peptide ligands and optimized the linker between the two ligands.²⁶ Flow cytometry analysis indicated that cells expressing both receptors had higher cellular uptake of heterodimer than those expressing either receptor at a concentration of 50 nM. After systemic injection of Cy5-labeled htBVL1 in tumor-bearing mice, higher uptake and longer retention were observed in tumors that overexpressed both receptors compared with single-receptor-positive tumors. Blocking with MSH, CCK, or both reduced the uptake of each target tumor significantly (Figure 2A). These studies provide valuable insights into the design of heterobivalent ligands with high avidity: the length and conformation of the linker can be very crucial during the design of peptide heterodimers. Because the binding of one pharmacophore to its corresponding site at the target brings the second pharmacophore in close proximity to that target, the enhanced tumor affinity from heterodimers mainly arises from increases in local ligand concentration.²⁷ However, when the pharmacophores of peptide heterodimers overlap, simultaneous binding of two peptide ligands to two different receptors is impossible.²⁷

Another important factor for peptide heterodimer design is the careful evaluation of the receptors of interest. The selection of targets is important for the development of bispecific heterodimers with improved tumor uptake. For example, a mesenchymal–epithelial transition factor (c-Met) binding peptide (cMBP) was conjugated with cyclic RGD (c(RGDyK)) through a click reaction to form a cMBP-click-c(RGDyK) peptide heterodimer.²⁸ This heterodimer was designed to possess the ability to recognize both c-Met and integrin $\alpha_v\beta_3$ receptors simultaneously. However, biodistribution studies and SPECT/CT imaging showed that despite the uptake of ¹²⁵I-cMBP-click-c(RGDyK) in a U87MG tumor (positive for both c-MET and integrin $\alpha_v\beta_3$) that could be blocked partially by cMBP or c(RGDyK) (Figure 2B), it did not demonstrate any improvement to that of a cMBP-scrambled peptide.

Antibody-Based Heterodimers. Monoclonal antibodies are well-established workhorses for therapeutic and diagnostic purposes, particularly in oncology.²⁹ Because of their excep-

tional ability to recognize specific antigens, monoclonal antibodies play a central role in targeted therapeutics. However, targeting only one antigen is usually insufficient in oncology, where tumors can progress after a latency period during antibody treatment. Compared with classic monospecific antibodies, bispecific antibodies can further improve the specificity for particular antigens and serve as more powerful tools for studying the molecular mechanisms of disease and developing more potent therapeutics. By artificial manipulation of antibody genes, bispecific antibody heterodimers are being developed to enable targeting of different epitopes on the same cell surface receptors, targeting two different receptors simultaneously, and enhancing cell–cell interactions.³⁰

Bispecific antibodies can be produced by three main methods: chemical conjugation, hybridoma cell line fusion, and protein engineering involving recombinant DNA.³¹ Chemical cross-linking of two different fragment-antigen-binding (F(ab')) fragments was the first strategy introduced in 1980s to generate bispecific antibodies.³² In this method, two different F(ab') arms are bridged through disulfide or thioether bond coupling.³³ A number of bispecific F(ab')₂ fragments heterodimers has been produced in this manner, including anti-CEA/anti-indium-DTPA,³⁴ anti-Id/anti-HSG,³⁵ anti-CEA/anti-DTPA-In,³⁶ and anti-MLC1/Anti-CD90.³⁷ However, chemical modification may cause the inactivation of antibody binding sites or dysfunction of the effector agents.³⁸ Furthermore, chemical cross-linking requires extra purification compared to homodimer formation and often results in poor dimer stability. Bispecific antibodies can also be generated by the fusion of two different hybridomas. The resulting hybrid hybridoma secretes a heterogeneous population of antibodies, including bispecific antibodies.³⁹ However, this technology also requires extensive purification procedures, and the production efficiency of bispecific antibodies is comparatively low. By far, recombinant DNA technology is the most frequently used and trustworthy method for producing bispecific antibodies. This method can produce bispecific antibodies in large quantities, does not involve chemical linkage, and requires minimum purification process. A variety of antibody-based heterodimers, such as knobs-into-holes structure, bispecific F(ab)₂, heterodimeric scFv, and heterodimeric Fab, have been produced using recombinant DNA technology.⁴⁰ Detailed examples of bispecific antibody production will be discussed in the following paragraphs.

Linear fusion of genes encoding different antibody single-chain variable (scFv) fragments was the initially used technology to produce bispecific antibodies.⁴¹ A recombinant linear CD3/EGP-2-directed bispecific monoclonal antibody, BIS-1 F(ab')₂, was produced by this method.⁴² To improve the production yield of heterodimers, leucine zipper sequences were introduced into the C terminus of two different scFv fragments.⁴³ Bispecific anti-CD3/anti-Tac F(ab'-zipper)₂ heterodimers were produced by this method and demonstrated high efficacy for cytotoxic T cell recruitment.⁴⁴ A variety of other linkers have also been used to produce heterodimers, such as the CD3/17-1A bispecific antibody⁴⁵ and anti-HER3/anti-HER2 bispecific scFv.⁴⁶ By fusing with a helical dimerization domain (e.g., cysteine-containing peptide^{47,48} helix–loop–helix motif,⁴⁹ and barnse–dibarnase domain^{50,51}), the affinity and specificity of scFv heterodimers can be tuned. The length and 3D structure of the linker between scFv's are closely associated with the expression abundance and immunogenicity of the final heterodimers. At the same time,

this gene fusion strategy can also be applicable in the formation of bispecific antibody fragments such as diabody (Db), a dimeric antibody fragment composed of the variable region of IgG heavy and light chains (V_H and V_L) connected with a peptide linker.²⁹ Bispecific diabody can be produced in the format of $V_{HA}-V_{LB}$ and $V_{HB}-V_{LA}$ or $V_{LA}-V_{HB}$ and $V_{LB}-V_{HA}$.⁵² Coexpression of V_H-V_L fusions in the periplasm of *E. coli* enables the stable production of bispecific diabodies. However, the comparatively short linker between V_H and V_L inside diabodies may restrict their ability to simultaneously access two antigens on two different cells.^{53,54}

Complementary fragments from antibodies can also be used as heterodimerization scaffolds for the production of recombinant Fab-scFv fusion proteins.⁵⁴⁻⁵⁶ For example, spontaneous interactions between heavy chain constant domains 1 (C_{H1}) and light chain constant domains (C_L) can result in heterodimerization that forms a covalently linked heterodimer named a $C_H C_L$ miniantibody. One humanized immunoglobulin (IgG) C_{H1} was connected with the C_L domains of another antibody in this manner to form a bispecific anti-EGFR/anti-CD2 heterodimer that has high avidity for both EGFR and CD2 as well as low immunogenicity.⁵⁷ When both domains were coexpressed in *E. coli*, 63% of the total proteins formed were bispecific.^{57,58} Another option is the interaction between F_d and L chains of Fab and the C terminus of a scFv molecule; in this way, bispecific Fab-scFv and trispecific Fab-(scFv)₂ can be generated with up to a 90% production yield.^{54,55}

The engineering of an antibody's fragment crystallizable (Fc) region is another means to produce bispecific antibody heterodimers. In antibodies, Fab arms are connected via a flexible hinge region to the homodimeric Fc fragment. The Fc region of IgG mediates antibody effector functions through interactions with Fc receptors⁵⁹ and serves as an important factor for an antibody's long serum half-life in vivo through interactions with neonatal Fc receptors.⁶⁰ The Fc is often used to generate antibody-like fusion proteins because of its inherent dimeric nature⁶¹ and can be used to create complex heterogeneous antibody mixtures.⁶² Methods based on Fc engineering, such as knobs-into-holes (KiH), have been frequently adopted to generate heterodimeric antibody assemblies.^{59,63,64} In the KiH method, amino acids are mutated within the CH₃ domains of antibody heavy chains, forcing complementary heterodimeric assembly between two different heavy chains.⁶⁵ A bispecific antibody heterodimer targeting both c-MET and VEGFR-2 was created by this method and exhibited potent antitumor efficacy in gastric cancer.⁶⁶ Mutations induced via the KiH method change the charge complementarity at the CH₃ domain interface, promoting Fc heterodimer formation and suppressing the formation of knob-knob or hole-hole homodimers.⁵⁹ Unfortunately, the KiH method sometimes produces bispecific antibodies with unnatural domain junctions and a loss of natural antibody architecture. Correct association of the light chains and their cognate heavy chains can be achieved by exchange of heavy-chain and light-chain domains within the Fab of one-half of the bispecific antibody.⁶⁷ Another strategy is to utilize an IgG4 antibody, which readily engages in Fab-arm exchange with other IgG4 antibodies.⁶⁸ Novel bispecific IgG4-CD20/IgG4-EGFR and IgG4-CD20/gemtuzumab antibodies were produced by this manner. The sequences essential for arm exchange are present only in the CH₃ and core hinge regions of the IgG4 isotype. To facilitate arm exchange in antibodies of these

isotypes, minimal point mutations were introduced into the CH₃ and core hinge sequences of IgG1 and IgG2 antibodies.⁶⁹ The generated IgG1 bispecific antibodies had faster clearance than the parental IgG1 antibodies in rats, but the impact of these modifications on immunogenicity was not investigated.

More recently, a modified KiH method that relied on coculture of two bacterial strains (one expressing the knob and the other expressing the hole half of the antibody) was developed for the generation of nonimmunogenic, stable bispecific antibodies.⁷⁰ After inoculating with an appropriate ratio of bacteria expressing anti-EGFR and anti-MET, respectively, the purified bispecific antibodies demonstrated similar monomeric stability and heterodimer purity as the bispecific antibody produced by the half antibody redox method. The resulting bispecific antibody against MET and EGFR could bind both targets monovalently, inhibit their signaling, and suppress MET and EGFR-driven cell and tumor growth.

■ MOLECULAR IMAGING WITH BISPECIFIC HETERODIMERS

Peptide-Based Heterodimers. Molecular imaging of cancer with peptide ligands has attracted widespread research attention because they have relatively high affinity and excellent tissue penetration. A number of different peptide receptors are massively overexpressed in numerous cancers; examples include, but are not limited to, somatostatin receptor, gastrin-releasing peptide receptor (GRPR), CCK2/CCK-B, glucagon-like peptide-1 receptor (GLP-1), and integrin $\alpha_v\beta_3$.^{71,72} Measuring receptor expression is crucial for accurate diagnosis as well as for monitoring the response to therapy. Several peptide-based radiotracers have shown promising results in animal studies, and some of them have been investigated in clinical trials.^{73,74} Chemically linked peptide heterodimers, which bind to two different receptors, can increase the functional affinity and binding specificity of the probe.

Recently, an integrin $\alpha_v\beta_3$ and GRPR dual-targeted peptide (RGD-BBN heterodimer) was developed and utilized in PET imaging of cancer.⁷⁵ Arginine-glycine-aspartate (RGD) peptides can specifically target integrin $\alpha_v\beta_3$, a molecular marker of angiogenesis, and have been successfully used for imaging by PET and SPECT techniques.^{76,77} Bombesin (BBN) is an amphibian homologue of mammalian GRP, which can specifically bind to GRPR, and has been extensively investigated for the diagnosis and treatment of GRPR-positive tumors.⁷⁸ Li et al. used a glutamate linker to bridge cyclic RGD and BBN and radiolabeled the fused peptide with ¹⁸F ($t_{1/2}$: 110 min, β^+ , 100%) to investigate its dual-receptor-targeting ability in PC-3 prostate tumor xenografts (integrin $\alpha_v\beta_3$ positive; GRPR positive).⁷⁵ They found that tumor uptake of ¹⁸F-FB-RGD-BBN was significantly higher than that of ¹⁸F-FB-BBN and ¹⁸F-FB-RGD, respectively. Compared with ¹⁸F-FB-BBN and ¹⁸F-FB-RGD, ¹⁸F-FB-RGD-BBN also showed substantially lower liver and renal uptake. One limitation of this study is that the heterodimer was merged by a short glutamate linker, which compromised its simultaneous binding capacity to GRPR and integrin $\alpha_v\beta_3$. To overcome this limitation, the same group further modified the structure of RGD-BBN by using orthogonally protected Fmoc-Glu-Oal.²⁴ The optimized RGD-BBN heterodimer was radiolabeled with ⁶⁸Ga ($t_{1/2}$: 68 min; β^+ , 89%) for PET imaging.⁷⁹ Biodistribution studies showed that PC-3 tumor uptake of ⁶⁸Ga-NOTA-RGD-BBN was significantly higher than that of ¹⁸F-FB-RGD-BBN. The

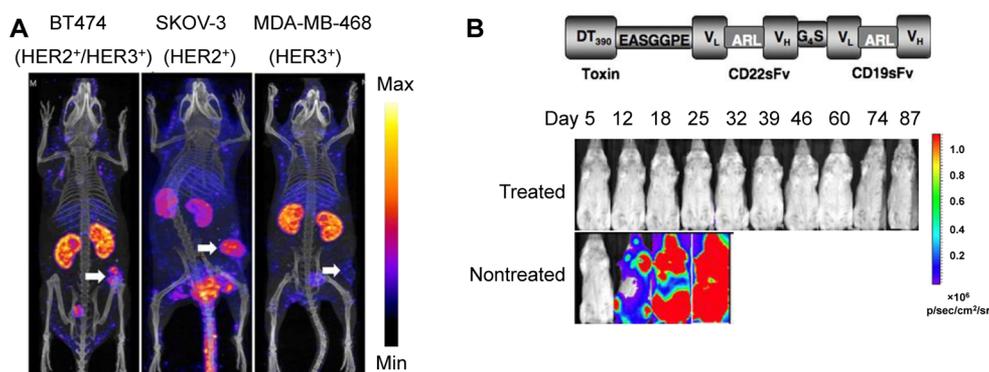


Figure 3. Representative antibody heterodimers used for molecular imaging. (A) Whole-body SPECT/CT images of ^{111}In -Fab-PEG₂₄-HRG in CD1 athymic mice bearing BT-474 (HER2⁺/HER3⁺), SKOV-3 (HER2⁺/HER3⁻), or MDA-MB-468 (HER2⁻/HER3⁺) tumors (arrows) at 48 h. Adapted with permission from ref 89. Copyright 2012 Society of Nuclear Medicine and Molecular Imaging. (B) Structure of diphtheria toxin (DT₃₉₀)/anti-CD19 and anti-CD22 scFv conjugates (DT2219ARL) and serial bioluminescence images of mice bearing Raji-luc Burkitt's lymphomas treated with or without DT2219ARL. Adapted with permission from ref 115. Copyright 2009 Elsevier.

tumor uptake of ^{68}Ga -NOTA-RGD-BBN was also evaluated in two different cell types: PC-3 and MDA-MB-435 (GRPR negative, integrin $\alpha_v\beta_3$ positive). In the PC-3 tumor model, the tumor uptake of ^{68}Ga -NOTA-RGD-BBN was slightly higher than that of ^{68}Ga -NOTA-BBN and significantly higher than that of ^{68}Ga -NOTA-RGD at 1 h p.i. (Figure 2C). MDA-MB-435 tumors had significantly lower tumor uptake of the heterodimer compared with PC-3 tumors. In blocking studies with RGD or BBN alone (Figure 2C), a partial decrease in tumor uptake was observed. When blocking with both RGD and BBN, tumor uptake of the tracer was reduced to background levels. These results indicated that the dual-targeting tracer could still bind to one available receptor while the other receptor was blocked.

A number of peptide heterodimers have been developed for molecular imaging of inflammation.^{80,81} The migration and activation of leukocytes is one of the hallmarks of inflammation.⁸² Several groups have used the radiolabeled chemotactic peptide cFLFLFK and its analogues to detect neutrophils as they target the formyl peptide receptor (FPR) on leukocytes.^{83,84} However, poor pharmacokinetic parameters and low detection sensitivity have limited their utility. To address these issues, a heterobivalent peptide was designed using cFLFLF and TKPPR connected with a PEG linker. The resulting cFLFLF-(PEG)₁₂-TKPPR-^{99m}Tc was able to target the FPR and tuftsin receptor simultaneously.⁸⁵ High expression of these two receptors activates polymorphonuclear leukocytes (PMNs), and sites of inflammation can be monitored by imaging of PMNs with cFLFLF-(PEG)₁₂-TKPPR-^{99m}Tc. cFLFLF-(PEG)₁₂-TKPPR-^{99m}Tc demonstrated high stability in serum and favorable pharmacological properties. SPECT/CT imaging showed that the accumulation of cFLFLF-(PEG)₁₂-TKPPR-^{99m}Tc in the inflamed tissue was 3.15-fold higher than in the control tissue.

Antibody-Based Heterodimers. Bispecific antibody or antibody fragments-based heterodimers have shown potential for the molecular imaging of cancer. One favorable target set is the HER (ErbB) family. The heterodimerization of HER2 with HER3 results in aberrant proliferation of tumor cells.⁸⁶ The anti-HER2 monoclonal antibodies trastuzumab and pertuzumab can inhibit the proliferation of breast cancer by preventing receptor dimerization.⁸⁷ In one study, the tumor-targeting efficacy of anti-c-HER2 741F8-1 (sFv')₂ homodimers was compared with that of 741F8/26-10 (sFv')₂ heterodimers,

which has specificity for digoxin and related cardiac glycosides.⁸⁸ SKOV-3 tumor accumulation of ^{125}I -741F8(sFv')₂ was significantly higher than that of ^{125}I -741F8/26-10 (sFv')₂ at 24 h p.i. The difference in tumor retention between 741F8-1 (sFv')₂ and 741F8/26-10 (sFv')₂ might have been caused by the greater effective affinity for 741F8-1 (sFv')₂ compared with 741F8/26-10 (sFv')₂. Later, another bispecific anti-HER3/HER2 A5-linker-ML3.9 bs-scFv (ALM) was engineered with similar selective binding capacity to both target antigens in tumor cells.⁴⁶ The accumulation of ^{125}I -ALM in SKOV-3 tumor xenografts (HER2 positive/HER3 positive) was significantly higher than that in either the MVM2 tumors (HER2 positive/HER3 negative) or MDA-MB-468 tumors (HER2 negative/HER3 positive). At the same time, SKOV-3 tumor uptake of ^{125}I -ALM was statistically higher than either that of A5 scFv or ML3.9 scFv. Together, these results indicate that the similar binding affinity and expression levels of both targeting tumor-associated antigens are important for increasing the overall tumor retention of bispecific antibodies.

Antibody fragments fused with other nonantibody proteins is another important category of heterodimers for molecular targeting and imaging. Recently, a novel bispecific radio-immunoconjugate (bsRIC) consisting of trastuzumab Fab fragments and human heregulin- β 1 (HRG) was developed with the goal of imaging HER2/HER3 heterodimers selectively.⁸⁹ HRG is the targeting ligand of HER3 that promotes the recruitment of HER2 to the complex.⁹⁰ Razumienko et al. found that tumor uptake of the bsRIC ^{111}In -DTPA-Fab-PEG₂₄-HRG in BT-474 human breast cancer xenografts (HER2 positive/HER3 positive) was higher than that of ^{111}In -DTPA-HRG in MDA-MB-468 xenografts (HER2 negative/HER3 positive) (Figure 3A).⁸⁹ Excessive HRG or trastuzumab Fab blocking decreased the uptake of ^{111}In -bsRICs in MDA-MB-468 tumors, which demonstrated the specificity of ^{111}In -bsRICs for HER2 and HER3 in vivo. Using a similar strategy, trastuzumab Fab fragments were chemically cross-linked with human EGF to synthesize bsRICs that recognize HER2 and EGFR.⁹¹ However, the different binding affinity of Fab and EGF to 231-H2N (HER2 positive/EGFR positive) human breast cancer xenografts and SKOV-3 (HER2 negative/EGFR positive) human ovarian cancer xenografts resulted in similar tumor uptake of ^{111}In -bsRICs in both tumor models. Future studies with bispecific heterodimers need to focus on

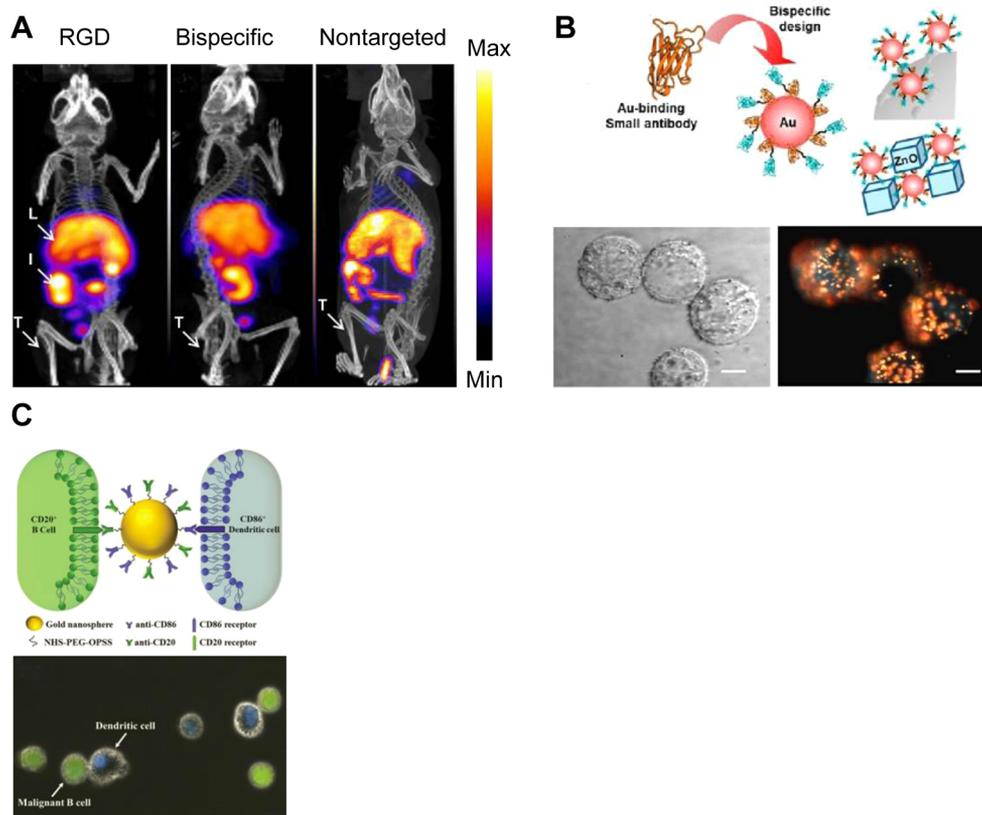


Figure 4. Bispecific nanomaterials for molecular targeting and imaging. (A) SPECT/CT images of U87MG tumor-bearing mice 4 h p.i. of ^{111}In -labeled RGD-liposome, RGD/substance P-liposome (bispecific), and nontargeted liposome. Adapted with permission from ref 108. Copyright 2013 Dove Medical Press. (B) Diagram depicting the bottom-up assembly of the ZnO-binding E32 VHH dimer and surface plasmon resonance (SPR) images of A431 cells treated with the gold-binding E32 VHH fragment. Adapted from ref 109. Copyright 2012 American Chemical Society. (C) Schematic illustration of nanoparticle-mediated coupling between a malignant B cell and a DC and fluorescence image of B220 cells (green cytoplasm) attached to DCs (blue nuclei). Scale bar represents 10 μm . Adapted with permission from ref 111. Copyright 2013 Wiley-VCH Verlag GmbH.

choosing the correct flexible linker, ligands with similar binding affinities, and receptors that have appropriate expression levels.

Bispecific antibodies can also cross-link different target antigens on two different cells and have been used to retarget immune effector cells to tumor cells. Multiple successful studies have demonstrated the capacity of bispecific antibodies to enhance the interactions between malignant cells and cytotoxic T cells (CTLs),⁹² macrophages,⁹³ or natural killer (NK) cells.⁹⁴ If one binding site specifically recognizes the tumor-associated antigens and the other binding site is oriented against a marker for effector cells of the immune system (e.g., CD3 on T cells and CD16 on NK cells⁹⁵), then immune effector cell retargeting can be achieved. For instance, CD3-directed bispecific antibodies have proven to be beneficial for redirected tumor therapies. De Jonge et al. developed an anti-CD3/anti-idiotype (Id, a tumor-specific antigen) bispecific scFv that could retarget CTLs toward BCL1 lymphoma cells and exhibited antitumor activity toward BCL1.⁹⁶ Although bispecific antibodies can trigger direct killing of tumor cells, Id variants are not always tumor exclusive and can lead to destructive immune response.⁹⁷ Therefore, a humanized bispecific $\text{F}(\text{ab}')_2\text{-HER2} \times \text{CD3}$ was further developed to retarget cytotoxic CD8^+ NKT cells for the immunotherapy of HER2-expressing tumors.⁹⁸ $\text{F}(\text{ab}')_2\text{-HER2} \times \text{CD3}$ was found to substantially enhance cytotoxic activity of CD8^+ NKT cells. To directly assess the specific cytotoxic activity of CD8^+ NKT cells in vivo, genetically modified SKOV-3 tumor cells expressing luciferase were used

for monitoring tumor growth and the response to therapy. They found that the bioluminescence from tumors treated with CD8^+ NKT cells redirected with $\text{F}(\text{ab}')_2\text{-HER2} \times \text{CD3}$ was significantly weaker than that of tumors treated with CD8^+ NKT cells alone or $\text{F}(\text{ab}')_2\text{-HER2} \times \text{CD3}$. Additionally, animals treated with CD8^+ NKT cells redirected with $\text{F}(\text{ab}')_2\text{-HER2} \times \text{CD3}$ had the highest survival rate at week 21.

The CD19 antigen is expressed in virtually all B-cell malignancies.⁹⁹ To treat leukemia and malignant lymphomas, a bispecific heterodimeric diabody, $\text{CD3} \times \text{CD19}$, specific for the ϵ -chain of the $\text{CD3}/\text{TCR}$ complex and CD19 on B cells, was constructed.¹⁰⁰ The $\text{CD3} \times \text{CD19}$ diabody could specifically interact with both CD3-positive and CD19-positive cells and inhibited the growth of B lymphoma xenografts in immunodeficient mice before preactivated human peripheral blood lymphocytes could. To enhance the selective killing efficiency of tumor cells, antibodies can be also coupled with immunotoxins. Anti-CD19 immunotoxins have reported anticancer effects,¹⁰¹ and anti-CD22 immunotoxins have been successfully used to treat rare hairy cell leukemia.¹⁰² However, toxin-related side effects limited their clinical application. To address these issues, Vallera et al. fused diphtheria toxin (DT_{390}) with anti-CD19 and anti-CD22 scFv to generate a novel bispecific fusion protein DT2219, which had broader reactivity in recognizing and inhibiting B-cell malignancies.¹⁰³ To increase the targeting ability of DT2219, reverse-oriented $\text{V}_\text{H}\text{-V}_\text{L}$ domains of anti-CD19 and anti-CD22 sFv were

genetically engineered with aggregation-reducing linkers. The resulting variant protein, DT2219ARL, had enhanced affinity and protein yield. Seventy five percent of the DT2219ARL-treated mice were found to be completely tumor free on day 87 after intravenous injection with Raji-luc Burkitt's lymphoma (CD22 positive/CD19 positive). Additionally, luciferase bioluminescent imaging of untreated mice showed tumor present in the lung, bone marrow, and spinal cord on day 21 (Figure 3B). These data indicate that DT2219ARL can prevent and kill malignant B cells in vivo. By retargeting immune effector cells or targeted delivery of immunotoxins to tumor cells, bispecific antibody heterodimers can be used in tumor therapy, and this therapy can be monitored using molecular imaging.

Although a number of successful studies have been carried out with bispecific antibodies and antibody fragments, molecular targeting/imaging with bispecific antibody heterodimers is still in its infancy. The careful choice of targets, optimization of protein fusion technology, and improved binding capacity for both of the targets will always be needed during the development of bispecific antibody-based molecular targeting/imaging agents. To meet these requirements, nanomaterials have been introduced to boost the performance of bispecific heterodimers.

Bispecific Nanoparticles. Nanoparticles offer the ability to deliver a larger therapeutic payload per target recognition event than traditional probes and are able to carry multiple targeting agents for therapy or imaging of tumor cells. Researchers have developed nanomaterials capable of interacting with different molecular targets. These multispecific nanoparticles can be divided into two categories: nanomaterials conjugated with two different ligands that target their individual receptors or nanomaterials conjugated with bispecific ligands. Although dual-ligand modification of nanomaterials is not readily achievable, mounting attempts have already been devoted to produce bispecific nanomaterials for drug delivery,^{104,105} gene delivery,¹⁰⁶ or combination therapy of cancer.¹⁰⁷ However, molecular imaging research with those types of bispecific nanomaterials is extremely limited, and so far, there is only one report using a bispecific liposome to target integrin $\alpha_v\beta_3$ and neurokinin-1 receptor in glioblastoma.¹⁰⁸ Unfortunately, there was no observable tumor-uptake enhancement compared with that of an unconjugated liposome in this study, which was monitored by SPECT/CT (Figure 4A). However, bispecific ligand-modified nanomaterials have also generated various exciting results. For instance, Hattori et al. combined peptide-grafting and phage-display techniques to generate a high-affinity bispecific antibody fragment that can be strongly absorbed onto gold nanoparticles.¹⁰⁹ They designed and constructed multispecific antibodies by joining gold-binding and EGFR-binding antibody fragments; these antibodies were used to enhance the surface plasmon resonance (SPR) scattering signal from gold nanoparticles followed by their use for SPR imaging of cancer cells (Figure 4B). Using a similar strategy, an anti-gold antibody fragment, A14P-b2, was fused with an anti-hen egg white lysozyme antibody fragment, HyHEL10 Fv, to generate a bispecific diabody.¹¹⁰ The resulting diabody enabled the functionalization of gold nanoparticles and allowed for selective protein accumulation on a gold-patterned silicon substrate.

Another application for heterodimer-modified nanoparticles is to redirect the immune cells to recognize and eliminate the tumor cells. Bispecific antibody (anti-CD20/anti-CD86)-conjugated gold nanoparticles were recently designed to selectively attach malignant cells (Burkitt lymphoma B cells;

BJAB) to antigen-presenting cells (human monocyte-derived dendritic cells; DCs) (Figure 4C).¹¹¹ The resulting nanoparticles caused widespread cell fusion and the formation of hybrid cells after femtosecond pulse irradiation. A relatively uniform distribution of the individual gold nanoparticles on the plasma membranes of both cells was observed. After mixing BJAB and DC in a 1:1 ratio, the cells formed pairs or small clusters in the bispecific nanoparticle-treated group at levels that were more than 4-fold higher than the single antibody nanoparticle or nonspecific anti-EGFR-coated nanoparticle-treated groups. Thus, the anti-CD20/anti-CD86-conjugated gold nanoparticles offer a simple and effective method to boost specific fusion/interaction between different cells.

As an interdisciplinary field involving physics, chemistry, engineering, biology, and medicine, nanotechnology has the potential to improve the early detection, accurate diagnosis, and personalized treatment of various diseases, especially cancer. The interaction between heterodimers and nanoparticles is bilateral. On one hand, heterodimers can assist in the functionalization of certain nanoparticles as well as link different nanomaterials,¹⁰⁹ allowing for the generation of new nanomaterials with novel characteristics. On the other hand, nanomaterials provide a versatile platform to enhance the applicability of different types of heterodimers for drug delivery and theranostic purposes.

CONCLUSIONS

Given the wide range of physiological processes involved in disease progression, a number of promising molecular targets exist for the development of molecular imaging probes. Powerful probes with optimal in vivo biodistribution and imaging characteristics are required for such technologies. Bispecific ligand-based heterodimers have potent binding affinity and efficacy compared to traditional probes, making them promising candidates for molecular targeting and imaging applications. On the basis of the quantitative data acquired from different imaging studies, bispecific heterodimers usually display a significantly improved target-to-background ratio (the ultimate "Holy Grail" pursued in molecular imaging) compared with their monospecific peers because of the enhanced specificity brought by the binding of two targets.

Bispecific peptide heterodimers can be easily prepared by chemical conjugation of two peptides that bind different targets via a flexible linker. However, the length and rigidity of the linkers play an important role in the in vitro and in vivo characteristics of peptide heterodimers. Thus, the choice of appropriate linkers is crucial for their design and screening. Apart from this, careful analysis of the receptor expression patterns and selection of appropriate imaging labels are prerequisites for the development of suitable bispecific peptide heterodimers for a certain disease. In many scenarios, the cell signaling pathways of two targeted receptors are interconnected, which provides the foundation for bispecific peptides to trigger additive or synergistic biological effects in vivo. Following these guidelines, a number of bispecific heterodimers have demonstrated excellent tumor-targeting capability.^{19,75}

Compared with bispecific peptide heterodimers, most bispecific antibodies possess higher affinities for both targets and are readily applicable for the treatment of cancer and inflammatory diseases. Although the use of bispecific antibody heterodimers in molecular imaging is still in a very preliminary stage, their enhanced affinity and ability to target different epitopes make them promising imaging probes. Recent studies

have shown that molecules with molecular weights of approximately 60–100 kDa (i.e., diabodies, triabodies, or tetramers) are ideal for tumor targeting because of their increased tumor penetration and fast clearance. These probes have the potential to make “same-day” imaging possible for clinical applications.^{112–114} Exploring suitable truncated forms is necessary for the future development and optimization of bispecific antibodies for a variety of molecular applications.

For the success of bispecific heterodimers in molecular imaging applications, several factors must be addressed during both design and development. Understanding a receptor's expression pattern and its role in the cross-talk between tumor cells and their microenvironment is crucial. Fine tuning the heterodimer's properties (e.g., size, fusion types, specific amino acid mutations, pharmacokinetic adjustment, etc.) can also impact its stability, biodistribution, and tumor-to-background ratio. The affinity between ligand(s) and receptor(s) is dependent on a series of parameters such as charge, polarity, aromaticity, residue volume, surface area, or solvent accessibility. Inside a bispecific heterodimer molecule, a ligand with lower affinity could still serve to further improve the binding capacity of a ligand with higher affinity as long as the relevant physical/chemical properties of the high-affinity ligand can be optimized. Through careful analysis of these parameters, bispecific heterodimers with optimized pharmacokinetic and imaging characteristics can be developed, improving both the management of patient's with various diseases and disease-related bench-side research.

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Notes

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