Enzyme-mediated Conjugation in Molecular Imaging

Subjects: Cell Biology

Contributor: Cristina Bolzati, Barbara Spolaore

Molecular imaging is one of the most fast-developing areas of research. It aims to visualize, characterize, and quantify, in a non-invasive way, processes on molecular or cellular levels in living systems, giving clinicians important information both in the diagnosis and for monitoring the treatment of diseases. Enzymes are powerful tools that efficiently allow the conjugation of proteins under physiological conditions, thus preserving their native structure and activity. Chemoselectivity and site-specificity are also important characteristics of the enzyme-mediated protein conjugation, that allow derivatization of only one type of functional group and to modify a biomolecule at a single defined position (or, in some cases, in a small number of defined positions), respectively. Since nuclear molecular imaging can benefit greatly from the production of homogenous derivatives, enzymatic-based methodologies can be used for the production of site-specific labeled immunoconjugates.

Keywords: radioimmunoconjugates ; transglutaminase ; sortase ; lipoic acid ligase ; galactosyltransferase ; PET ; SPECT ; mAb ; nanobody ; affibody

1. Introduction

Nuclear molecular imaging (MI) by single-photon emission computed tomography (SPECT) and, especially, by positron emission tomography (PET) provides unique advantages over classical diagnostic procedures, which mainly offer visualization of nonspecific changes related to morphology, enabling high detection sensibility, high-resolution images, and quantitative analysis of the tracer. Since the availability of a plethora of radionuclides with nuclear features suited for medical diagnosis and cancer therapy as well as for theranostic purposes, nuclear MI is characterized by remarkable flexibility in the design of radiolabeled probes and has shown the potential to speed up the diagnosis of diseases and the personalization of medical care.

Currently, molecular targeting is one of the most promising approaches to visualize and treat disseminated cancer. It is interesting to note that although the imaging tracers used in biomedical research and in clinical practice are generally based on small molecules and peptides, the research interest has shifted toward the development of radiolabeled naturally occurring or naturally inspired biomolecules, such as antibodies, especially IgG and their truncated counterparts ($F(ab')_2$, Fab, scFv) as well as engineered mAb fragments (miniboby, diaboby, nanobody), and small protein scaffolds (affibody, etc.) [1][2][3][4]. The utility of these biomolecules to treat cancer is due to their ability to bind tumor-associated antigens (e.g., HER2, EGFR, CD20, etc.) overexpressed on the surface of neoplastic cells and their paring with the unique advantages of radionuclides.

To date, looking at the radiopharmaceutical market, all radioimmunoconjugates (RICs) approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) are murine antibodies (Table 1). Moreover, most of them are diagnostic agents radiolabeled with gamma emitter radionuclides, ^{111}In and ^{99m}Tc, and the most recent approval occurred a decade ago [2]. In spite of this, the application of these agents in early phase clinical trials has increased dramatically in recent years [5][6], thanks to the continuous scientific and technological advances in the development of therapeutic mAb for the treatment of disease (as an example, 44 new mAbs gained approval by the FDA over the last five years, among which 10 were for cancer treatment) [7] and to the spread of mAb-based drug conjugates as new paradigm for the selective target delivery of drugs to disease tissues (mainly tumors and disseminate tumor cells) thus combining chemotherapy and immunotherapy [8][9].

Table 1. Radioimmunoconjugates that are approved by FDA and EMA.

Trade Name	Generic Name	Company	Approval Year		Antibody	Target	Cell Line	Radionuclide	I
			EMA	FDA					
OncoScint	Satumomab pendetide	Cytogen	NA	1992	B72.3, mouse IgG1	TAG-72	Hybridoma	^{111}In	
CEA-Scan	Arcitumomab	Immunomedis	1996 (withdrawn in 2005)	1996	IMMU-4, mouse IgG Fab'	CEA	Hybridoma	^{99m}Tc	

Trade Name	Generic Name	Company	Approval Year		Antibody	Target	Cell Line	Radionuclide	I
			EMA	FDA					
Myoscint	Imciromab pentetate	Centocor	NA	1996 (discontinued)	R11D10, mouse IgG2a Fab'	Human cardiac myosin	Murine ascites	^{111}In	
Verluma	Nofetumomab merpentan	Boehringer Ingelheim, NeoRx	NA	1996	NR-LU-10, mouse IgG2b Fab	carcinoma-associated antigen	Hybridoma	^{99m}Tc	
ProstaScint	Capromab pendetide	Cytogen	NA	1996	7E11-C5.3, mouse IgG1	PSMA,	Hybridoma	^{111}In	
Zevalin	Ibritumomab tiuxetan	Spectrum Pharms/Biogen	2004	2002	2B8, mouse IgG1	CD20	CHO	^{90}Y	
Bexxar	Tositumomab	Corixa and GSK	NA	2002 (discontinued in 2014)	B1, mouse IgG2a	CD20	Hybridoma	^{131}I	
NeutroSpec (LeuTech)	Fanolesomab	Palatin Technologies	NA	2004	RB5, mouse IgM	CD15	Hybridoma	^{99m}Tc	
Lymphoscan	Bectumomab	Immunomedics		NA	LL2, mouse IgG2a Fab'	CD22		^{99m}Tc	
HumaSPECT	Votumumab	KS Biomedix Ltd./Organon Teknika	1998 (withdrawn in 2003)	NA	88BV59, human IgG3	Cytokeratin tumor associated antigen	Human lymphoblastoid cell line transformed with EBV	^{99m}Tc	
Indimacis-125	Igovomab	CIS Bio International	1996 (discontinued)	NA	OC125, mouse IgG1 F(ab')2	CA-125		^{111}In	
LeukoScan	Sulesomab	Immunomedics	1997	NA	IMMU MN3, mouse IgG Fab'	NCA-90 NS0		^{99m}Tc	
Scintimun	Besilesomab	CIS Bio	2010	NA	Murine IgG1	NCA-95	Hybridoma	^{99m}Tc	I

Antibody drug conjugates (ADC) and related can quickly be adapted for nuclear imaging through the conjugation of a pertinent radionuclide. This occurrence has driven the idea that radioimmuno-imaging can support in the understanding of therapeutic drugs both during preclinical studies and early phase clinical trials, providing unique information of the mechanism of action and failure of immunotherapy and guiding the rational for new drug development. However, beyond this, imaging probes are important tools in nuclear medicine oncology allowing one to: (a) evaluate the biodistribution of the therapeutic agent; (b) enable better patient selection and stratification to improve trial design; (c) confirm target expression and accessibility before the start of therapy, therefore patients who overexpress target receptors in disease tissues can be identified and a proper therapy defined. This permits to minimize the number of patients who might fail to benefit from therapy and to monitor the effectiveness of the treatment, providing real-time data on early clinical response; (d) assess the organs potentially at risk (i.e., side effects or off-target distribution); (e) determine the efficacy of treatment by measuring the accumulation of therapeutic drug into the tumor. As a result, one of the primary purposes in the creation of antibody-based radiotracers is not preparing stand-alone diagnostic probes but rather generating companion imaging agents that can guide the development and application of therapeutics [7].

2. Enzymatic Methods for the Site-Specific Radiolabeling of Targeting Proteins

The use of enzymes as highly efficient and site-specific alternatives to the classical random chemical approaches has been gaining increasing interest in biotechnology and it is also finding its role in radiopharmaceutical applications for developing site-specific radiolabeling and highly homogeneous RICs [10]. A precise control of the number and location of payloads on the protein should really result in better-defined and more efficient immunoconjugates and more easily reproducible procedures and may facilitate the approval process. In general, the enzymatic derivatization of proteins

offers important advantages such as the use of aqueous mild reaction conditions that are required to preserve the integrity of the biomolecules as well as of BiFunctional Chelating Agents (BFCA) and prosthetic groups. They can be efficiently used to selectively modify proteins and derivatives through one step protocols or stepwise procedures combining mild enzymatic methods with high-yielding bioorthogonal click reactions. The inclusion of groups suitable for bioorthogonal reactions enables a modular approach with the potential for adding multiple functional moieties without any apparent important effect on the mAb function.

From the studies conducted so far on different enzymatic approaches, it is not obvious to define the best methodology for the design of homogeneous RICs, also because they are “biomolecule and radionuclide dependent” and the optimization of the procedure is in some cases necessary. The following four enzymes all proved useful for the radiolabeling of mAb and their truncated derivatives: microbial transglutaminase (mTG), sortase (SortA), galactosidase and galactosyltransferase (GalT) mutant Y289L, and lipoic acid ligase (LplA). However, each enzymatic approach possesses intrinsic pros and cons that are summarized in Table 2.

Table 2. Pros and cons of different enzyme-based approaches.

Enzyme	Substrates	Biomolecules on MI	Pros	Cons
mTG	*Acyl donor*: Gln residues of the protein; Q-tag inserted at any site *Acyl acceptor*: Lys residues of the protein; K-tag inserted at any site; primary ammine	Wild type molecules ranging from whole mAbs to peptides	Homogeneous adducts with a precise control of the number and the location of the payloads Formation of catabolically stable isopeptide bonds Simple 5-aminopentyl groups can be used as lysine surrogates No special antibody engineering is required mTG immobilization increases its reactivity and permits a straightforward purification of IC Minimal off-target reactivity One-step reaction with the cargos Low-cost enzyme	Cross-linked oligomers can be generated in the presence of reactive Gln and Lys residues in the same protein substrate Multiples sites of derivatization are possible Long reaction time for heavy proteins can be required
SortA	*Acyl donor:* LPXTG sequence inserted at the C-terminus or internal sites *Acyl acceptor:* $(Gly)_n$ inserted at the N-terminus	Full-length mAbs and their fragments	Site-specific with no risk of impairing mAb reactivity One-step reaction with the cargos	Need of mAb engineering reversible reaction which requires a high molar excess of SrtA and the nucleophile over the LPXTG-substrate Expensive

Enzyme	Substrates	Biomolecules on MI	Pros	Cons
Galactosidase and GalT(Y289L)	Biantennary complex-type oligosaccharide of mAbs Galactose modified with a functional group for click chemistry	Full-length mAbs with pendant sugar chains	Site-specific with no risk of impairing mAb reactivity No antibody engineering is required Glycans can be manipulated without altering the polypeptide chain The bi-antennary nature of the two oligosaccharide chains allows for at least two and as many as four conjugation events per mAb The labeling sites are easily and rapidly characterized Bioorthogonal click ligation Minimal off-target reactivity	Hardworking (four-step) and time-consuming approach: various buffer exchanges by microspin columns are needed and long incubation times Inability to vary the conjugation site beyond glicans Usefulness limited to the whole mAb with pendant sugar chain and glycosylated proteins
LplA	*Acyl donor*: lipoic acid and its analogues *Acyl acceptor*: LAP-peptide inserted at any site	Full-length mAbs and their fragments	Site-selective with no risk of impairing protein activity Fast one step labeling High catalytic efficiency Formation of catabolically stable isopeptide bonds Bioorthogonal click ligation Minimal off-target reactivity	Need of engineered proteins and lipoate analogues

Site-specific conjugations are also expected to improve the stability and pharmacological profiles of RICs in respect to biomolecules modified using traditional chemical approaches. Indeed, heterogeneous conjugation may result in a high cargo loading that can be responsible for their impaired immunoreactivity, low stability, and suboptimal pharmacokinetic profiles. Nevertheless, if on the one hand, animal studies have shown that site-specifically labeled RICs may feature superior in vivo behavior compared to their randomly constructed counterparts, on the other there is no irrefutable evidence of the added value of site-specific conjugation in comparison to random labeling. In most of the studies comparing both approaches, the site-specifically modified biomolecules seem to not outperform the conjugates prepared by conventional routes. This behavior may be due to the specific biomolecules and/or mouse model used, thus additional studies are urgent needed to support, confirm, or refute that site-specific modification in general will improve nuclear MI with mAb and related.

The examples described in litterature demonstrate the versatility and utility of site-specific enzyme-mediated bioconjugation to improve the efficiency and potential of protein-based radiolabeled products. Most of them are already tested in preclinical studies. However, further efforts concerning scale-up and cost minimization are necessary to realize the entire potential of site-specific protein modification in the clinic. The precise control provided by these bioconjugation methods can be used to identify criteria for selecting modification sites on mAbs and relates as well as determining generalizable design principles to maximize stability and circulation properties, biological activity, and targeted cellular uptake. Future developments in this area will also depend on the identification of new protein scaffolds that have appropriate characteristics in terms of stability, high target affinity, selectivity, rapid clearance, and ease of labeling, etc., as cancer targeting imaging agents. Moreover, by considering the current increased attention to the imaging of inflammatory responses by the direct targeting of T-cells, it is also expected that RICs and relates will be used to specifically target antigens of pathogenic bacteria and viruses, and thus permit the development of new pathogen-specific tracers to discriminate between infectious and sterile sites of inflammation.

References

1. Fu, R.; Carroll, L.; Yahioglu, G.; Aboagye, E.O.; Miller, P.W. Antibody Fragment and Affibody ImmunoPET Imaging Agents: Radiolabelling Strategies and Applications. ChemMedChem 2018, 13, 2466–2478.
2. Salsano, M.; Treglia, G. PET Imaging Using Radiolabelled Antibodies: Future Direction in Tumor Diagnosis and Correlate Applications. Res. Rep. Nucl. Med. 2013, 3, 9–17.
3. Garousi, J.; Orlova, A.; Frejd, F.Y.; Tolmachev, V. Imaging Using Radiolabelled Targeted Proteins: Radioimmunodetection and Beyond. EJNMMI Radiopharm. Chem. 2020, 5, 1–26.
4. Altunay, B.; Morgenroth, A.; Beheshti, M.; Vogg, A.; Wong, N.C.L.; Ting, H.H.; Biersack, H.-J.; Stickeler, E.; Mottaghy, F.M. HER2-Directed Antibodies, Affibodies and Nanobodies as Drug-Delivery Vehicles in Breast Cancer with a Specific Focus on Radioimmunotherapy and Radioimmunoimaging. Eur. J. Nucl. Med. Mol. Imaging 2021, 48, 1371–1389.
5. Mayer, A.T.; Gambhir, S.S. The Immunoimaging Toolbox. J. Nucl. Med. 2018, 59, 1174–1182.
6. Wei, W.; Rosenkrans, Z.T.; Liu, J.; Huang, G.; Luo, Q.-Y.; Cai, W. ImmunoPET: Concept, Design, and Applications. Chem. Rev. 2020, 120, 3787–3851.
7. Lu, R.M.; Hwang, Y.C.; Liu, I.J.; Lee, C.C.; Tsai, H.Z.; Li, H.J.; Wu, H.C. Development of Therapeutic Antibodies for the Treatment of Diseases. J. Biomed. Sci. 2020, 27, 1–30.
8. Khongorzul, P.; Ling, C.J.; Khan, F.U.; Ihsan, A.U.; Zhang, J. Antibody–Drug Conjugates: A Comprehensive Review. Mol. Cancer Res. 2020, 18, 3–19.
9. Chau, C.H.; Steeg, P.S.; Figg, W.D. Antibody–Drug Conjugates for Cancer. Lancet 2019, 394, 793–804.
10. Zhang, Y.; Park, K.Y.; Suazo, K.F.; Distefano, M.D.; Recent progress in enzymatic protein labelling techniques and their applications. *Chemical Society Reviews* **2018**, *47*, 9106-9136, 10.1039/c8cs00537k.