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Facile metabolic glycan labeling strategy for exosome tracking

Tae Sup Lee[a,b,1], Young Kim[a,1,2], Weiqi Zhang[a], In Ho Song[b], Ching-Hsuan Tung[a,*]

[a] Molecular Imaging Innovations Institute, Department of Radiology, Weill Cornell Medicine, New York, NY 10021, USA
[b] Division of RI-convergence Research, Korea Institute of Radiology and Medical Sciences, Seoul 01812, Republic of Korea

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ABSTRACT

Background: Exosomes are nano-sized vesicles derived from the fusion of multivesicular bodies with the surrounding plasma membrane. Exosomes have various diagnostic and therapeutic potentials in cancer and other diseases, thus tracking exosomes is an important issue.

Methods: Here, we report a facile exosome labeling strategy using a natural metabolic incorporation of an azido-sugar into the glycan, and a strain-promoted azide-alkyne click reaction. In culture, tetra-acetylated *N*-azidoacetyl-D-mannosamine (Ac_4ManNAz) was spontaneously incorporated into glycans within the cells and later redistributed onto their exosomes. These azido-containing exosomes were then labeled with azadibenzylcyclooctyne (ADIBO)-fluorescent dyes by a bioorthogonal click reaction.

Results: Cellular uptake and the *in vivo* tracking of fluorescent labeled exosomes were evaluated in various cells and tumor bearing mice. Highly metastatic cancer-derived exosomes showed an increased self-homing *in vitro* and selective organ distribution *in vivo*.

Conclusion: Our metabolic exosome labeling strategy could be a promising tool in studying the biology and distribution of exosomes, and optimizing exosome based therapeutic approaches.

General significant: A facile and effective exosome labeling strategy was introduced by presenting azido moiety on the surface of exosome through metabolic glycan synthesis, and then conjugating a strain-promoted fluorescent dye.

1. Introduction

Exosomes are extracellular vesicles of endocytic origin with a size of 30 to 150 nm and can be released by most cells including tumor cells, macrophages, dendritic cells, fibroblasts, epithelial cells, B cells and T cells [1–3]. Although exosomes from different cells are similar in size, their contents and functions may vary. Exosomes carry various messages, such as enzymes, structural proteins, adhesion molecules, lipid raft, DNA, messenger RNAs and microRNAs [4–7]. These contents are believed to make exosomes unique and multifunctional. However, an exosome's function exceeds that of mere a messenger. They protect their contents from degradation, transmit information to distant cells, and functionally change recipient cell states in specific transformations. Tumor-derived exosomes, which transfer oncogenes and onco-miRNAs, are able to alter the tumor microenvironment, stimulate angiogenesis, promote carcinogenesis and tumor growth, modulate immune responses, and temper therapeutic responses [8,9]. Therefore, exosomes have been proposed as both diagnostic markers and therapeutic targets. Furthermore, dendritic cells are able to place tumor antigens onto the surface of their exosomes. Thus, these nano-sized vehicles have been represented as cell-free cancer vaccines to trigger immune responses [10,11]. Exosome cancer vaccines derived from dendritic cells are currently being tested in several phase I and 2 clinical trials [10,12,13]. Although its efficacy still needs to be improved, the potential found in a vaccine strategy is encouraging. Exosomes derived from different cells have various target-specific homing efficiencies [14,15]. To increase its targeting specificity and therapeutic efficacy, cell-derived exosomes have been engineered to carry specific surface proteins or peptides, and loaded with chemotherapeutics and/or magnetic nanoparticles [16–18]. Despite great attention to their therapeutic potential, the lack in knowledge of their *in vivo* behavior is a major drawback. Exosome biology and its application is a new field that shows great potential in cancer diagnosis and treatment. Nevertheless, the current understanding of these endogenous nano-sized vesicles is inadequate, and should be improved.

To extend the biomedical application of exosomes, it is essential

* Corresponding author at: Molecular Imaging Innovations Institute, Department of Radiology, Weill Cornell Medicine, 413 East 69th Street, New York, NY 10021, USA.
E-mail address: cht2018@med.cornell.edu (C.-H. Tung).
[1] These authors contributed equally to this work.
[2] Current affiliation: Department of Oral Pathology, School of Dentistry, Chonnam National University, Gwangju 61186, Republic of Korea.

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that their distribution in cells and *in vivo* is well studied. Currently, only a few labeling technologies have been developed for exosome studies. CD63, an exosomal marker that has been tagged with a green fluorescent protein (GFP), is a popular method in cellular studies [19]. A similar protein engineering method was developed by fusing *Gaussian luciferase* to the transmembrane domain of a platelet-derived growth factor receptor (PDGFR), for bioluminescence imaging and to monitor systemically administered exosomes *in vivo* [20]. Genetic engineering strategies are useful for the evaluation of exosomal protein transfers, however, the transfection methods are not accessible by some laboratory. In contrast, direct non-covalent membrane associations using lipophilic dyes have widely been used [15,21,22]. Although simple, the retention of the dye is a concern [23]. Thus a copper catalyzed click strategy, which is not fully biocompatible, was recently introduced by conjugating alkyne groups to the exosome using a carbodiimide coupling reaction [24].

A copper-free and strain-promoted azide-alkyne click (SPAAC) chemistry, which reacts between an azide and a strained alkyne, cyclooctyne to form a triazole linkage, is a non-toxic conjugation method [25]. Bioorthogonal metabolic labeling strategies using biocompatible SPAAC chemistry have been applied to image cells in animals [26]. Through the natural biosynthetic pathway of glycosylation, unnatural metabolic precursors, azide containing sugars, can be incorporated into the glycoproteins [27]. Exosomes are intrinsically a type of plasma membrane. It is therefore expected, that bioorthogonal click chemistry *via* metabolic glycan engineering can be effectively applied to functionalize exosomes. While we are working on this exosome labelling strategy, Wang et al. briefly reported the same strategy for exosome label [28]. Herein, we presented a systematic optimization of this functionalization methodology. Through a SPAAC chemistry, the azide decorated exosomes were labeled with azadibenzylcyclooctyne (ADIBO) containing fluorescent dyes (Scheme 1). Their application in studying exosome biodistribution within cells and in animals was also demonstrated.

2. Materials and methods

2.1. Cell culture

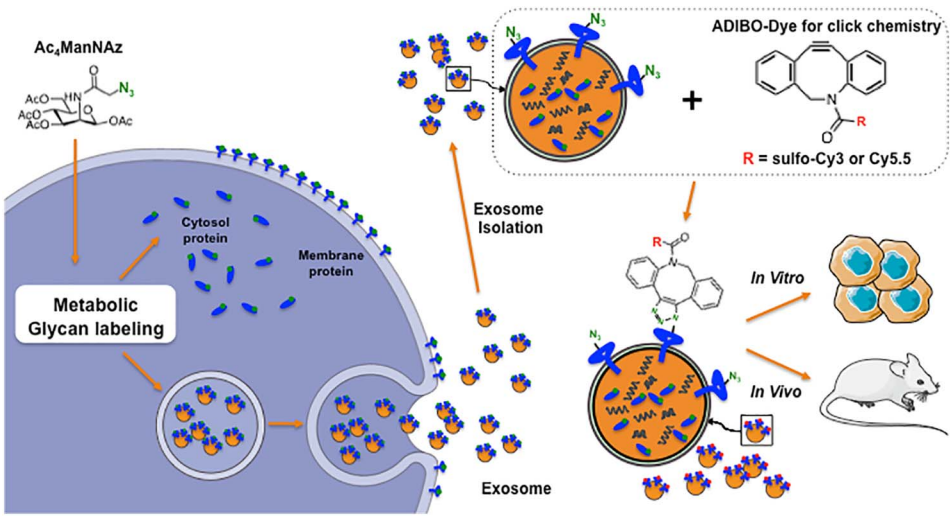
Human breast cancer cell lines, MDA-MB-231, MCF7, BT-549, MDA-MB-468, and mouse fibroblast NIH-3T3 were purchased from American Type Culture Collection (ATCC, Manassas, VA). MDA-MB-231, MCF-7, NIH-3T3 cells were maintained in DMEM (Cellgro, Manassas, VA), supplemented with 10% fetal bovine serum (FBS; Gibco, Life Technologies, Carlsbad, CA), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco) at 37 °C in humidified 5% CO_2 incubator. BT-549 cells were maintained in RPMI-1640 (Cellgro, Manassas, VA) with 0.023 IU/mL insulin, 10% FBS and 1% Penicillin/streptomycin at 37 °C in humidified 5% CO_2 incubator. MDA-MB-468 cells were cultured in L-15 (Cellgro, Manassas, VA) with 10% FBS, 1% of penicillin/streptomycin at 37 °C in humidified 0% CO_2 incubator.

2.2. Cytotoxicity of azido sugars

MDA-MB-231 and MCF7 cells (2×10^3 cells/well) were seeded in a 96 well plate (Corning Costar, Corning life sciences, Pittston, PA) a day before treatment. The cells were incubated with various concentrations of azido sugars, tetra-acetylated *N*-azidoacetyl-D-mannosamine (Ac_4ManNAz; FutureChem, Seoul, Republic of Korea), tetra-acetylated *N*-azidoacetyl-D-galactosamine (Ac_4GalNAz; Jena Bioscience GmbH, Jena, Germany), or tetra-acetylated *N*-azidoacetyl-D-glucosamine (Ac_4GlcNAz; Jena Bioscience GmbH, Jena, Germany), for 3 days. After incubation, cell viability was assessed by CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay Kit (Promega, Madison, WI) following the manufacturer's protocol.

2.3. Exosome isolation

MDA-MB-231 and MCF7 cells ($1–1.2 \times 10^6$) were seeded in a 100 mm dish with complete medium overnight. The cells were treated with 0 (Control, 0.1% DMSO) or 50 μM of azido sugars (Ac_4ManNAz, Ac_4GalNAz, and Ac_4GlcNAz) for 3 days. The cells were washed and incubated with phenol red free DMEM medium without FBS for 2 more days. Exosomes were isolated by Exoquick-TC reagent (System Bioscience, Palo Alto, CA) following the manufacturer's procedures. Cell culture supernatants were centrifuged at 3000 ×*g* for 15 min to remove cell debris, and then the supernatants were mixed with Exoquick-TC Exosome isolation reagent as 5:1 ratio and refrigerated overnight. The solutions were centrifuged at 1500 ×*g* for 30 min, the exosomes were isolated as a precipitated form, and then resuspended in a Tris buffered solution (pH 7.4). It should be aware that this isolation method cannot guarantee exosome-specific capture, thus other extracellular vesicles such as microvesicles or lipoproteins may be co-precipitated [29]. Exosome protein concentration was measured by BCA assay (Pierce, Rockford, IL). The isolated exosomes treated with DMSO, Ac_4ManNAz, Ac_4GalNAz, and Ac_4GlcNAz were named as DMSO-exo, Man-exo, Gal-exo, and Glc-exo, respectively.



Scheme 1. Schematic illustration of metabolic exosome labeling with strain-promoted azide-alkyne click (SPAAC) chemistry. Tetra-acetylated *N*-azidoacetyl-D-mannosamine (Ac_4ManNAz) metabolically incorporated into the sialic acid of cells and exosome glycoconjugates. Azide-containing exosomes were labeled with azadibenzylcyclooctyne (ADIBO)-sulfo-Cy3 or Cy5.5 fluorescent dyes by bioorthogonal click chemistry. Fluorescent dye labeled exosomes were evaluated in *in vitro* cellular uptake experiments and *in vivo* tracking studies. The helical structures within exosomes represent DNA as a double helix and RNA as a single helix.