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Facile Metabolic Glycan Labeling Strategy for Exosome Tracking

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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 (Ac₄ManNAz) 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 an 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.

Graphic Abstract

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Disclosure

The authors declare no competing financial interests.

Supporting Information

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Keywords

exosome; click chemistry; metabolic glycan labeling; cancer; distribution

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 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 azidibenzylcyclooctyne (ADIBO) containing fluorescent dyes (Scheme 1). Their application in studying exosome biodistribution within cells and in animals was also demonstrated.

MATERIALS AND METHODS

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 ug/mL streptomycin (Gibco) at 37 °C in humidified 5% CO₂ 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% CO₂ 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% CO₂ incubator.

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 (Ac₄ManNAz; FutureChem, Seoul, Republic of Korea), tetra-acetylated N-azidoacetyl-D-galactosamine (Ac₄GalNAz; Jena Bioscience GmbH, Jena, Germany), or tetra-acetylated N-azidoacetyl-D-glucosamine (Ac₄GlcNAz; Jena Bioscience GmbH, Jena, Germany), for 3 days. After incubation, cell viability was assessed by CellTiter 96[®] AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI) following the manufacturer's protocol.

Exosome isolation

MDA-MB-231 and MCF7 cells ($1 \sim 1.2 \times 10^6$) were seeded in a 100 mm dish with complete medium overnight. The cells were treated with 0 (Control, 0.1% DMSO) or 50 μ M of azido sugars (Ac₄ManNAz, Ac₄GalNAz, and Ac₄GlcNAz) 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 $\times 3,000 g$ for 15 minutes 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 $\times 1,500 g$ for 30 minutes, 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, Ac₄ManNAz, Ac₄GalNAz, and Ac₄GlcNAz were named as DMSO-exo, Man-exo, Gal-exo, and Glc-exo, respectively.

Protein analysis and Western blot analysis

To compare the exosomal protein expression pattern after the azido sugar treatment, the isolated BC derived DMSO-exo, Man-exo, Gal-exo, and Glc-exo (10 μ g) were lysed with cell lysis buffer (Cell Signaling, Danvers, MA), and electrophoretized in 4–12% gradient PAGE gel and stained by silver stain plus kit (Bio-Rad Inc, Hercules, CA).

For CD63 analysis, BC derived DMSO-exo, Man-exo, Gal-exo, and Glc-exo (20 μ g) were lysed with cell lysis buffer, electrophoretized in 5–12% gradient PAGE gel. Standard Western blot procedures were performed. Primary antibody was rabbit anti-human CD63 antibody and secondary antibody was goat anti-rabbit HRP conjugated Ab (System Bioscience).

To check the integrin alpha 6 (ITGA6) expression, MCF7 and MDA-MB-231 cell lysates and MCF7 and MDA-MB-231 derived exosomes (20 μ g) were electrophoretized in 8% PAGE gel and performed by standard western blot procedures. Primary antibodies were rabbit anti-human ITGA6 antibody and rabbit anti-human beat-actin antibody (Cell signaling). Secondary antibody was goat anti-rabbit HRP conjugated Ab (Santa Cruz Biotechnology).

Characterization of exosomes

For zeta potential and size measurement, Cy3 or Cy5.5 labeled DMSO-exo, Man-exo, Gal-exo, and Glc-exo from MCF7 and MDA-MB-231 cells were diluted to 20 µg/mL with Tris buffer solution. The zeta potential of various exosomes was analyzed by ZetaPALS particle analyzer (Brookhaven Instruments Co., Holtsville, NY). The size of various exosomes was analyzed using dynamic light scattering (Zetasizer Nano-ZS, Malvern Instruments Inc., Malvern, UK).

Additionally, the size and morphology of exosomes were obtained by transmission electron microscopy (TEM). The native and Cy3 or Cy5.5 labeled BC derived exosomes (~60 µg/ml based on total proteins) were diluted with Tris-HCl buffer solution. Then 5 µL of diluted exosomes was dropped on the grid and left dry at room temperature. The grid was further washed with one drop of 1.5% Uranyl acetate solution for 4 times to remove the salts from TBS. The samples were stained by one drop of 1.5 % Uranyl acetate for 1 min and the staining droplet was wicked away using a filter paper. The negatively stained samples were observed using a JEOL 1400 transmission electron microscope (JOEL, Tokyo, Japan)

Exosome labeling by ADIBO fluorescence dyes

DMSO-exo, Man-exo, Gal-exo, and Glc-exo (10 µg) derived from MDA-MB-231 and MCF7 were reacted with 1 nmole of ADIBO-Cy3 or ADIBO-Cy5.5 fluorescent dye for 1 h at 37 °C. Labeled exosomes were purified by gel filtration method using PD SpinTrap G-25 (GE Healthcare, Buckinghamshire, UK) following the manufacturer's protocol. Protein concentration of purified exosomes was determined by BCA assay. In most purification processes, recovery rate of exosomes (based on protein concentration) was 52–53%. Labeling yield (%) of exosome was determined by addition of 10% SDS (final concentration to 2%) with Infinite M1000 Pro Fluorometer (TECAN, Männedorf, Switzerland) and calculated as the percentage of (exosome bound fluorescent intensity/total applied fluorescence intensity).

Flow cytometry analysis

To choose the best azido sugar for metabolic cell labelling, MCF7 and MDA-MB-231 cells (1×10^5 cells) were seeded in a 6 well plate with complete medium without phenol red overnight, and then incubated with 0 (control, 0.1% DMSO), 0.5, 5 and 50 µM of azido sugars, Ac₄ManNAz, Ac₄GalNAz, and Ac₄GlcNAz for 3 days. All azido sugars were dissolved in DMSO, and the final amounts of DMSO was 0.1% of total medium. Cells were reacted with the clickable fluorescent dyes, 10 µM of ADIBO-sulfo-Cy3 (FutureChem) for 1 h at 37 °C in CO₂ incubator. The final amounts of DMSO was also 0.1% v/v. After triple washing with PBS, cells detached and analyzed by flow cytometer (Gallios, Beckman Coulter, or FACSCalibur, BD Bioscience). Data represented as the mean fluorescent intensity (MFI) values using Kaluza software (Beckman Coulter) or Cell-Quest software (BD Bioscience). To evaluate the reactivity of cell surface azido groups, MDA-MB-231 and MCF7 cells were treated with the azido sugars, Ac₄ManNAz, Ac₄GalNAz, or Ac₄GlcNAz (50 µM), for 3 days. The cells were then reacted with the clickable fluorescent dyes, 0, 1, 5 and 10µM of ADIBO-sulfo-Cy3 (ADIBO-Cy3) for 1 h at 37 °C in CO₂ incubator.

Cellular uptake of exosomes

To evaluate the uptake patterns of the exosomes, MDA-MB-231 derived exosomes were labeled with ADIBO-Cy3. The MDA-MB-231 cells (5×10^5) were seeded into 6 well plates overnight and then incubated with ADIBO-Cy3 labeled MDA-MB-231 derived exosomes for 4 h in a CO₂ incubator. In the exosome dose-dependency experiment, various doses (0, 5, 10, 20, 50 and 100 µg) of Cy3 labeled exosomes were incubated with cells. In the temperature dependency experiment, Cy3 labeled exosomes (40 µg) were incubated with cells at 4°C or 37°C. In the blocking experiment, various doses (0, 1, 5 and 10 µg/mL) of exogenous heparin (Sigma-Aldrich), a heparan sulfate mimetic, was added together with Cy3 labeled exosomes (40 µg). The cellular uptake of the Cy3 exosomes in MDA-MB-231 was represented by Cy3 fluorescence intensity (arbitrary unit, a.u.).

To evaluate the impact of surface modifications by azido metabolic incorporation and ADIBO-Cy3 labeling, MDA-MB-231 derived exosomes (DMSO-exosomes, Man-exosomes and Cy3-labeled Man-exosomes) were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, System Biosciences), per the manufacturer's recommended protocol. The labeled exosomes were then incubated with MDA-MB-231 cells for 4 h prior to FACS analysis.

To evaluate the uptake of the exosomes in various cells, MDA-MB-231 and MCF7 derived exosomes were labeled with ADIBO-Cy3. MCF7, MDA-MB-231, MDA-MB-468, BT-549 and NIH-3T3 cells (5×10^5) were seeded into 6 well plate overnight. The cells were incubated with ADIBO-Cy3 labeled MDA-MB-231 and MCF7 derived exosomes (2 µg) for 24 h at 37 °C in CO₂ incubator. The uptake of Cy3 exosomes in various cells was analyzed and represented as Cy3 fluorescence intensity (gated %).

Fluorescence microscopy

To choose the best azido sugar for metabolic cell labeling, MCF7 and MDA-MB-231 (2,000 cells) were seeded in a 96 well plate with black wall and clear bottom (Corning Costar) with complete medium without phenol red and then incubated with 0 (control, 0.1% DMSO) and 50 µM of azido sugars, Ac₄ManNAz, Ac₄GalNAz, or Ac₄GacNAz for 3 days. All azido sugars were dissolved in DMSO, and the final amounts of DMSO was 0.1% of total medium. The cells were reacted with 10 µM of ADIBO-Cy3 for 1 h at 37°C in CO₂ incubator. After triple washing with DMEM without phenol red and FBS, fluorescence images were obtained by EVOS FL Auto imaging system (ThermoFisher Scientific) using a RFP LED light cube (Ex/Em=531 ± 40 nm/593 ± 40 nm). Bright-field and fluorescence images were acquired at ×200 magnification and imaging data were represented as overlaid images.

To evaluate the uptake patterns of various exosome doses, MDA-MB-231 cells were incubated with Cy3 labeled MDA-MB-231 derived exosomes for 4 h in an CO₂ incubator, as aforementioned. The fluorescence images were obtained by a confocal laser scanning equipment, LSM 880 (Zeiss), with a plan-apochromat 20× objective using DAPI and RFP filters and Zen 2.3 software.

To evaluate the time-dependent uptake of the exosome into various cells, MDA-MB-231 derived exosomes were labeled with ADIBO-Cy3. MCF7, MDA-MB-231 and NIH-3T3 cells (5×10^5) seeded in 96 well plates were incubated with ADIBO-Cy3 labeled MDA-MB-231 derived exosomes (2 $\mu\text{g}/\text{well}$) for 4 h and then 24 h in CO_2 incubator, after which the cells were analyzed with an EVOS FL Auto imaging system.

To evaluate the uptake of various exosomes in cells, MDA-MB-231 and MCF7 derived exosomes were labeled with ADIBO-Cy3. MDA-MB-231, MCF7, MDA-MB-468, BT-549 and NIH-3T3 (2×10^3 cells) were seeded into a 96 well plate overnight. The cells were incubated with ADIBO-Cy3 labeled MDA-MB-231 and MCF7 derived exosomes (2 $\mu\text{g}/\text{well}$) for 24 h at 37° in CO_2 incubator. The uptake of ADIBO-Cy3 labeled exosomes in various cells were analyzed with EVOS FL Auto imaging system.

Biodistribution of exosome in mice

All animal studies were conducted in accordance with guidelines of the Institutional Animal Care and Use committee (IACUC) at the Korea Institute of Radiological and Medical Sciences (KIRAMS, IACUC approval No. KIRAMS 2016-0081). MDA-MB-231 or MCF7 cells (5×10^6 cells in 0.1 mL PBS) were subcutaneously injected into athymic female mice (NaraBiotech, Suwon, Republic of Korea). For MCF7 tumor model, a 17β -estradiol pellet (Innovative Research of America, Sarasota, FL) was subcutaneously implanted into mice one week before cell injection. When tumor diameter was approximately 1 cm, Cy5.5 labeled exosomes (10 $\mu\text{g}/0.1$ mL in TBS) from MDA-MB-231 or MCF7 cells were intravenously injected into the tail vein of their own tumor bearing mice ($n=3$). Mice were sacrificed 1 day later. Excised organs and tissues were collected and imaged.

To evaluate the distribution of exosomes at different time points, the *ex vivo* biodistribution of Cy5.5 labeled MDA-MB-231 derived exosomes (10 $\mu\text{g}/0.1$ mL in TBS) was performed at 4 h and 24 h post-injection.

To determine the modulator's effect on pharmacokinetics, Cy5.5 labeled MDA-MB-231 derived exosomes (10 $\mu\text{g}/0.1$ mL in TBS) were pre-treated with a fibronectin binding blocker, HYD-1 peptide (kikmvviswkg) (1 nmole/10 μg of exosomes, Peptron, Seoul, Korea), or an anti-integrin $\alpha 6$ neutralizing rat antibody (1 $\mu\text{g}/10$ μg of exosomes, NKI-GoH3 (Millipore, Burlington, MA) at 37°C for 1 h. The untreated or modulator pre-incubated Cy5.5 labeled MDA-MB-231 derived exosomes were then IV injected into a MDA-MB-231 tumor model ($n=3$ per group). The animals were sacrificed at 24 h. Excised organs and tissues were collected and imaged. Fluorescence images were acquired with IVIS-200 fluorescence system (PerkinElmer, Waltham, MA) with Cy5.5 filter set (Ex: 615~665 nm, Em: 695~770 nm) (exposure time: 2 sec). The fluorescence intensity of each tissue or organ was analyzed by Living Image 2.50 software. The biodistribution data were represented as relative fluorescent unit (RFU) of photon/sec/ $\text{cm}^2/\text{steradian}$ (sr).

Statistical analysis

Statistical analyses were executed using Student *t*-test, one-way and two-way Anova with Prism 7 (GraphPad Software, Inc.). Values of *P* less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The preparation of the exosomes chosen began with the selection of the most suitable sugar anchors. Ideally, the anchors must be nontoxic and could provide a reactive azido group on cell surface glycans. Three acetylated sugars were selected for testing, due to their cell-permeability. Ac₄ManNAz can be incorporated into cell surface glycans as azido sialic acid (SiaNAz) residues, Ac₄GalNAz can be incorporated into mucin-type O-linked glycoproteins, and Ac₄GlcNAz can be placed onto cytosolic and nuclear proteins at sites normally occupied by *O*-GlcNAc.[25] The acetylated unnatural azido sugar molecules, tetra-acetylated N-azidoacetyl-D-mannosamine (Ac₄ManNAz), tetra-acetylated N-azidoacetyl-D-galactosamine (Ac₄GalNAz), and tetra-acetylated N-azidoacetyl-D-glucosamine (Ac₄GlcNAz), were tested with two breast cancer (BC) cell lines, MDA-MB-231 and MCF-7 (Figure S1). It was found that all azido-sugars showed no cytotoxic effects on BC cells, below 50 μ M, after 3 days of incubation. Interestingly, the addition of an azido-sugar, except Ac₄GlcNAz, slightly increases cell viability (Figure S1). It is not clear how does this occur, but a similar trend has been observed in literatures.[26, 30, 31]

To validate the accessibility and reactivity of the incorporated azide moieties, BC cells were treated with three azido-sugars, Ac₄ManNAz, Ac₄GalNAz, and Ac₄GlcNAz (50 μ M) for 3 days, and then a clickable fluorescent dye, ADIBO-sulfo-Cy3 was added (Figure S2). Since fluorescent dyes can be lipophilic, sulfonated fluorescent dyes were chosen to minimize non-specific binding to cell membranes and exosomes. Based on flow cytometry analysis, Ac₄ManNAz treated MCF7 and MDA-MB-231 cells showed significantly higher mean fluorescent intensity among three azido-sugars (Figure 1 and Figure S3). The Cy3 fluorescent intensity in BC cells increased in a azido-sugar dose-dependent manner (Figure 1b and Figure S3b). The fluorescent intensity of 50 μ M Ac₄ManNAz-treated BC cells was more than 60-fold higher than that of control cancer cells without Ac₄ManNAz treatment. The 5 μ M Ac₄ManNAz-treated BC cells showed about 20-fold increased fluorescent intensity over the control, while the enhancement was negligible in the case of 0.5 μ M of Ac₄ManNAz. Compared to Ac₄GalNAz and Ac₄GlcNAz treatments, the Ac₄ManNAz treated BC cells showed significant enhancement of fluorescent intensity following the increases of Ac₄ManNAz. When cells were treated with a fixed amount of azido sugar (50 μ M) but various concentrations of clickable ADIBO-Cy3 dye, all cells showed increasing fluorescent intensity as a dye dose-dependent manner (Figure 1c and Figure S3c). The fluorescence images of Ac₄ManNAz treated BC cells displayed the highest fluorescent intensity when compared to the others (Figure 1d and Figure S3d). While the no azido-sugar treated cell controls showed only minimal fluorescence. This data suggested that the ADIBO-sulfo-Cy3 dye doesn't penetrate nor adhere to the outer plasma membrane of cells non-specifically. Ac₄ManNAz was reported as a good precursor for metabolic glycan labeling. It could be easily incorporated into natural sialic acids on the tumor cell's surface. [25] In our hands, Ac₄ManNAz was indeed the most effective anchor in cell surface glycan modification under the testing condition.

To confirm that the best azido-sugar for cell membrane glycan labeling is also the best analog for exosome labeling, MCF7 and MDA-MB-231 cells were incubated with azido sugars (50 μ M) or DMSO control (dimethyl sulfoxide, final concentration 0.1%) for 3 days.

The exosomes were isolated using ExoQuick reagent. To prevent bovine exosome contaminants from FBS, the cells were cultured in a serum deprived DMEM medium for 2 days prior to exosome collection. In our hands, no significant cytotoxicity was observed during these two days. However, if serum starvation compromised exosome production is a concern, exosome-deprived FBS or growth factors supplemented culture media could be considered.

The collected exosomes were then reacted with ADIBO-Cy3 or -Cy5.5 dyes, and purified by gel filtration spin column. The recovery rate of labeled exosome based on protein concentration was ~ 53 %. Western blot analysis was performed to validate the integrity of the labeled exosomes. After treatment with various azido-sugars, all isolated BC-derived exosomes contained the CD63 exosomal marker (Figure 2a and Figure S4a). Additionally, SDS-PAGE electrophoresis and silver staining of isolated BC-derived exosomes was done to identify whether the azido-sugar treatments would affect protein expression patterns in their exosomes. All azido-sugars treated BC-derived exosomes showed similar exosomal protein expression patterns, compared to DMSO treated BC-derived exosomes (Figure S5). This data suggested that the azido-sugar treatment (50 μ M) does not affect exosomal protein contents.

To verify that the azido moieties were highly presented on the surface of the exosomes, which were derived from the azido-sugar treated cancer cells, clickable fluorescent dyes were reacted with the isolated exosomes for 1 h at 37°C. After purification, the fluorescence intensities of the fluorescent dye labeled BC-derived exosomes were measured (Figure 2b and Figure S4b). Reacting with ADIBO-Cy3, the Ac₄ManNAz treated BC cell-derived exosomes showed 4.0- and 2.8-fold higher labeling yield than the DMSO treated BC cell-derived exosomes (14.8% vs. 3.7% in MDA-MB-231 exosome; 17.3% vs. 6.3% in MCF7 exosome). The fluorescence intensity of the Ac₄GalNAz and Ac₄GlcNAz modified exosomes was much lower. The parallel experiment showed that ADIBO-Cy5.5 offered a similar labeling pattern with these sugar derivatives (Figure 2b and Figure S4b). This exosome labeling efficiency indicated that Ac₄ManNAz is an excellent metabolic precursor in glycan modification for bioorthogonal click chemistry and also the best azide moiety for exosome label and modification.

The prepared Cy3 or Cy5.5 labeled exosomes were further characterized by measuring their zeta potential and size in an aqueous condition using dynamic light scattering (DLS) analysis and TEM. As expected, the zeta potential and size of the fluorescent labeled BC-derived exosomes was no different than what was displayed by the other azido-sugars treated exosomes (Figure 2 and Figure S4). The size of the exosomes in the TEM images was slightly smaller, compared with that was seen in the DLS analysis (Fig 2d, Fig S4d). However, there was no statistically significant difference in the size and zeta potentials between all preparations.

For the exosome labeling step, ADIBO dyes were dissolved in DMSO. Therefore, DMSO controls (0.1% final concentration) were included in all evaluations. The low extent of non-specific dye loadings was observed in the DMSO treated exosomes (Figure 2b and Figure S4b). In fluorescence microscope images and flow cytometry analysis, scanty amount of

fluorescence from DMSO treated BC derived exosomes was detected in a few cells (Figure 4 and Figure S7). Though this result indicated that tiny amount of clickable ADIBO-fluorescent dyes were non-specifically bound to DMSO-treated exosomes without an azide moiety, it had little effect on the uptake study because this non-specific label was only at the background level.

To study the uptake of exosomes, MDA-MB-231 cells were treated with Cy3 labeled MDA-MB-231 derived exosomes. Exosome dose-dependent uptake was observed (Figure 3a) through flow cytometry analysis. The confocal microscopic images supported the exosome dose dependency on exosome uptake (Figure 3b). Flow cytometry and confocal microscopic analyses also supported the temperature dependent uptake, which was efficiently inhibited at 4°C (Figure 3c and 3d). Cy3 labeled exosomes mostly bound to the cell's surface at 4°C (Figure 3d, upper detailed image), but the exosomes were internalized by the cells at 37°C (Figure 3d, lower detailed image).

Heparan sulfate proteoglycans in recipient cells have been identified as controllers of the exosome's internalization.[32] To confirm that this metabolic glycan labelling strategy did not affect the uptake process, Cy3 labeled exosomes and various doses of heparin, which act as a heparan sulfate mimetic, were co-incubated with MDA-MB-231 cells. A dose-dependent inhibition was observed, and, at 10 µg/mL of heparin, exosome uptake was reduced by approximately 62% compared to untreated cells (Figure 3e). Our data indicates that the labelling strategy did not disrupt the interaction of the exosome with its receptor, heparan sulfate proteoglycan. Several recent studies have suggested that exosomal tetraspanin family proteins function as a “zip code” for selective targeting towards specific tissue and cell ligands.[33–36] For example, tetraspanin 8 expressing exosomes preferentially bind cells expressing Mac1 (CD11b) and/or CD54. Our exosome labeling strategy using bioorthogonal click chemistry could be a handy tool to decipher the code between exosomal proteins and specific cells and tissues by using specific exosomal protein knockdowns.

To elucidate the potential stereo impact on uptake, which was caused by the added azido moiety and clicked fluorescent dye, MDA-MB-231 derived DMSO-exosomes, Man-exosomes and Cy3-Man-exosomes were labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE). CFSE is a membrane permeable dye widely used to label intracellular and intra-exosomal proteins.[37] The uptake study showed that all CFSE modified exosomes in MDA-MB-231 cells were picked up at similar degrees. The fluorescence intensities of the CFSE labeled Man-exosomes and Cy3-Man-exosomes were very close to that of CFSE labeled DMSO-exosomes (Figure 3f). This data suggests that the optimized modification, including the metabolically incorporated azido sugar and chemically appended fluorescent dyes, on the surface of exosome has minimal impact on cellular uptake.

To evaluate the time-dependency of exosome uptake, MDA-MB-231 derived Cy3 labeled exosomes were incubated with MCF7, MDA-MB-231 and NIH-3T3 cells at 4 h and 24 h (Figure S6). The exosome uptake was increased with incubation time at all cell lines (Figure S6). To study the preferential uptake of exosomes, azido functionalized exosomes collected from MDA-MB-231 and MCF7 were labeled with ADIBO-Cy3, and then fed to

MCF7, MDA-MB-231, MDA-MB-468, BT-549 and NIH-3T3 cells for 24 h (Figure 3 and Figure S7). MDA-MB-231 exosomes were picked up efficiently by MDA-MB-231 cells, compared to other cells. Interestingly, MCF7 exosomes were also quickly loaded by MDA-MB-231 cells. MDA-MB-231 cells accumulated similar amounts of exosomes regardless of their sources. MCF7 and NIH-3T3 cells picked up similar amounts of MDA-MB-231 and MCF7 derived exosomes, while MDA-MB-468 and BT-549 cells picked up more MCF-7 derived exosomes than MDA-MB-231 derived exosomes (Figure 4 and Figure S7). This observation supported a recent report that suggested the constituents of MCF7 derived exosomes may be responsible for their greater adherence and internalization in various cell types.[38] Furthermore, our results support that recipient cells have their own selection mechanisms and that exosomes from different sources are different.

The *in vivo* distribution of exosomes is a critical factor in the current development of exosome technologies, especially in exosomes that have been suggested as messengers, drug carriers and tumor metastasis primers.[39] Recent reports have indicated that exosomes from highly metastatic melanomas increased the metastatic behavior of primary tumors by educating bone marrow progenitor cells. Pancreatic ductal adenocarcinomas-derived exosomes were observed to have induced liver pre-metastatic niche formations via the exosomal macrophage migration inhibitory factor (MIF), and exosomes derived from cancer cells bearing specific metastatic organotropisms were taken up by organ-specific cells to prepare the pre-metastatic niche.[9, 35, 40]

An initial demonstration of our exosome labeling strategy in exosome tracking *in vivo* was performed with Cy5.5 labeled exosomes, originated from MCF7 and MDA-MB-231 cells. The exosomes were intravenously administered into their own tumor bearing mice, and the organs and tissues were collected a day later. *Ex vivo* fluorescence imaging was acquired using an optical imaging system (IVIS-200). Both MCF7 and MDA-MB-231-derived exosomes homed to tumors. The accumulated fluorescence signals were about two times higher in tumors than in the blood and muscle. As expected, most fluorescent exosomes accumulated in the liver and intestines. There was no statistical difference between the biodistribution of MCF7 and MDA-MB-231 derived exosomes in almost all organs and tissues. However, it was found that MDA-MB-231 exosomes gave about a 2-fold higher fluorescence signal in the lungs compared to the MCF7-derived exosomes ($p < 0.05$). This data suggests that highly metastatic MDA-MB-231 exosomes are more selectively localized in their premetastatic niches than other organs and tissues.

Surface integrin proteins are known to correlate with organotropic metastasis [40]. For example, the integrin $\alpha_6\beta_1$ and $\alpha_6\beta_4$ has been linked to lung metastasis and integrin $\alpha_v\beta_5$ is associated with liver metastasis. Integrins have been suggested to also dictate the specific distribution of extracellular vesicles.[40] Integrin alpha 6 (ITGA6), a transmembrane glycoprotein adhesion receptor protein, is upregulated in breast cancer cells and promotes the migration and invasion of breast cancer cells. ITGA6 is known as a metastasis promoter in human MDA-MB-231 breast cancer cells [41–43], and to also participate in cell adhesion as well as cell surface mediated signalling.[43] The western blot analysis confirmed a high level of ITGA6 in highly metastatic MDA-MB-231 cells and their exosomes; while low levels of ITGA6 was seen in MCF7 and their exosomes (Figure S8).

To further validate the pivotal role of ITGA6 in the biodistribution of exosomes *in vivo*, two blocking strategies were tested, one with HYD-1 peptide, an extracellular matrix (ECM) protein binding blocker, and the other with an anti-ITGA6 neutralizing antibody. HYD-1 is known to inhibit tumor cell adherence to ECM proteins, such as laminin I and II, fibronectin, and collagen IV, in tumor progression and metastasis processes.[44] The anti-ITGA6 neutralizing antibody is expected to block surface ITGA6. The exosomes from MDA-MB-231 were pre-treated with HYD-1 or anti-ITGA6 antibody, and then injected into MDA-MB-231 tumor bearing mice. One day later, the *ex vivo* biodistribution analysis showed that the uptake of Cy5.5 labeled exosomes in the liver markedly decreased with the pre-incubation of HYD-1 peptide ($p < 0.05$) compared to the exosome only (Figure 6). In the case of the anti-ITGA6 antibody, the liver uptake of the Cy5.5 labeled exosomes was significantly lower compared with the one pre-incubated with HYD-1 ($P < 0.05$). This data suggests that the organ uptake of exosomes relies on the exosome's binding to ECM proteins.

CONCLUSION

In summary, our results successfully demonstrated the potential of an effective exosome labeling strategy with the introduction of an azido moiety on the surface of exosomes through metabolic glycan synthesis and bioorthogonal SPAAC chemistry by ADIBO containing fluorescent dyes. Ac₄ManNAz, which is an azide containing unnatural monosaccharide, is the most compatible building block to the tested cells. The azido groups were introduced onto the membrane of cells and their exosomes. The bioorthogonal SPAAC reaction specifically labels azide-sialic acid containing exosomes. The use of these fluorescently labeled exosomes was demonstrated in tracking their preferential uptake *in vitro* and biodistribution *in vivo*. This exosome labeling strategy can be highly useful in studying the interaction and communication between exosome and target cells. It could also be applied to screen therapeutic candidates that inhibit the interaction both *in vitro* and *in vivo*. Furthermore, based on this flexible SPAAC chemistry, many other ligands, in addition to the fluorescent dyes, could be anchored onto exosomes. For instance, radio chelators could be added for whole body imaging and quantitation, or targeting ligands could improve the delivery efficiency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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.

Highlight

- A facile and effective exosome labeling strategy
- Long lasting exosome label
- A promising tool for exosome tracking study

Figure 1.

Flow cytometry analysis and fluorescence microscopic images of MDA-MB-231 cells after azido-sugars treatment and click reaction with ADIBO-Cy3 fluorescent dye. (a) MDA-MB-231 cells treated with 50 μM of Ac₄ManNAz, Ac₄GalNAz, or Ac₄GlcNAz for 3 days and reacted with ADIBO-Cy3 (10 μM) for 1 h. (b) MDA-MB-231 cells treated with various concentration of azido-sugars and reacted with ADIBO-Cy3 (10 μM) for 1 h. Data is presented as mean ± SD (n = 3). (c) MDA-MB-231 cells treated with 50 μM azido-sugars and reacted with various concentrations of ADIBO-Cy3 for 1 h. Data is presented as mean ± SD (n = 3). (d) Fluorescence images of MDA-MB-231 cells treated with 50 μM concentrations of Ac₄ManNAz, Ac₄GalNAz, or Ac₄GlcNAz for 3 days and reacted with ADIBO-Cy3 (10 μM) for 1 h. MFI: mean fluorescent intensity.

Figure 2.

The characterizations and fluorescence labeling efficiency of MDA-MB-231 derived exosomes. (a) Western blot of CD63 exosomal marker in various azido-sugars treated MDA-MB-231 cells derived exosomes. (b) Fluorescence labeling yields and (c) transmission electron microscopic (TEM) images of MDA-MB-231 cells derived exosomes. Scale bar is 100 nm. The exosomes isolated from MDA-MB-231 cells treated with 50 μ M of Ac₄ManNAz, Ac₄GalNAz, and Ac₄GlcNAz for 3 days and then reacted with ADIBO-Cy3 or Cy5.5 for 1 h. Exosome labeling yields were calculated as the percentage of (exosome bound fluorescent dye/total applied fluorescent dye). Data is presented as mean $\pm$ SD (n = 3). After treated with various azido-sugars and reacted with clickable fluorescent dyes, (d) the size of MDA-MB-231 cells derived exosomes was measured by Zetasizer Nano-ZS (Malvern Instruments) and (e) the zeta potential of MDA-MB-231 cells derived exosomes was determined by ZetaPALS potential analyzer (Brookhaven Instruments).

Figure 3. Effects of exosomes dose, temperature, heparin and labeling on exosome uptake
 (a) MDA-MB-231 cells were incubated with various doses (0, 5, 10, 20, 50, and 100 μg/mL) of MDA-MB-231 derived Cy3-labeled exosomes for 4 h and then analyzed for exosome uptake by flow cytometry. (b) Representative confocal microscopic images of cells incubated with exosomes without or with 10 and 50 μg/mL of exosomes. (c) MDA-MB-231 cells were incubated with 40 μg/mL of MDA-MB-231 derived Cy3-labeled exosomes at 4°C and 37°C for 4 h. (d) Representative confocal microscopic images of cells incubated with exosomes at 4°C and 37°C. (e) MDA-MB-231 cells were simultaneously co-incubated with 40 μg/mL of MDA-MB-231 derived Cy3-labeled exosomes and heparin (0, 1, 5 and 10 μg/mL) for 4 h. (f) The CFSE fluorescent intensities in MDA-MB-231 cells after incubating with CFSE labelled DMSO-Exo, Man-Exo and Cy3-Man-Exo for 4 h. The data presented are exosome uptake (arbitrary unit, a.u.) and mean ±S.D (n=3).

Figure 4.

Uptake of ADIBO-Cy3 labeled exosomes by cells. (a, c) Fluorescence microscopic images of MCF-7 and MDA-MB-231 derived exosomes to MCF-7, MDA-MB-231 and NIH-3T3 cells (Magnification: $\times 200$). (b, d) Flow cytometry analysis of Cy3 labeled exosome from MDA-MB-231 and MCF-7 cells to MCF-7, MDA-MB-231, MDA-MB-468, BT-549 and NIH-3T3 cells for 24 h. FI: fluorescence intensity. Data is presented as mean $\pm$ SD (n = 3).

Figure 5.

Ex vivo fluorescence images and biodistribution of ADIBO-Cy5.5 labeled MCF-7 and MDA-MB-231 exosomes in MCF-7 and MDA-MB-231 tumor models. (a) Cy5.5 labeled MCF-7 exosomes (10 μg) intravenously injected into MCF-7 tumor bearing mice ($n=3$). (b) Cy5.5 labeled MDA-MB-231 exosomes (10 μg) intravenously injected into MDA-MB-231 tumor bearing mice ($n=3$). The fluorescence images were acquired at 24 h after injection of Cy5.5 labeled exosomes using IVIS-200 for 2s. (c) *Ex vivo* biodistribution data were analyzed and quantified by using Living Image 2.50 software (Perkin Elmer, USA). Data is presented as mean $\pm$ SD ($n = 3$). * $P < 0.05$.

Figure 6.

Ex vivo biodistribution of Cy5.5 labeled MDA-MB-231 exosomes in MDA-MB-231 tumor model. (a) Biodistribution of Cy5.5 labeled MDA-MB-231 exosome in each time point. Cy5.5 labeled MDA-MB-231 exosome (10 ug) intravenously injected into MDA-MB-231 tumor bearing mice (n=3). The fluorescence images were acquired at 4h and 24 h treatment of Cy5.5 labeled exosomes using IVIS-200 for 2 s. Data were analyzed and quantified by using Living Image 2.50 software (Perkin Elmer, USA). (b) Biodistribution of Cy5.5 labeled MDA-MB-231 exosome without and with HYD-1 peptide and integrin $\alpha 6$ antibody. Cy5.5 labeled MDA-MB-231 exosome pre-incubated with HYD-1 peptide and Integrin $\alpha 6$ antibody and then intravenously injected into MDA-MB-231 tumor bearing mice (n=3). The fluorescent intensities were obtained at 24 h with IVIS-200 for 2 s. * P = 0.03, ** P = 0.01.

Scheme 1.

Schematic illustration of metabolic exosome labeling with strain-promoted azide-alkyne click (SPAAC) chemistry. Tetra-acetylated N-azidoacetyl-D-mannosamine (Ac₄ManNAz) 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.