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Understanding Selectivity of Metabolic Labelling and Click-Targeting in Multicellular Environments as a Route to Tissue Selective Drug Delivery

Angel Tan, Qingtao Liu, Dedy Septiadi, Shuiling Chu, Tianqing Liu, Sarah-Jane Richards, Barbara Rothen-Rutishauser, Alke Petri-Fink, Matthew I. Gibson, and Ben J. Boyd

Cancer cells generally exhibit higher metabolic demands relative to that of normal tissue cells. This offers great possibilities to exploit metabolic glycoengineering in combination with bio-orthogonal chemistry reactions to achieve tumour site-targeted therapeutic delivery. This work addresses the selectivity of metabolic glycan labelling in diseased (i.e., cancer) versus normal cells grown in a multicellular environment. Dibenzocyclooctyne (DBCO)-bearing acetylated-D-mannosamine (Ac4ManNDBCO) was synthesised to metabolically label three different types of cell lines originated from the human lung tissues: A549 adenocarcinomic alveolar basal epithelial cells, MeTSA non-cancerous mesothelial cells, and MRC5 non-cancerous fibroblasts. These cell lines were found to display different labelling sensitivity, which trended with their doubling time in the following order: A549 > MeTSA > MRC5. The higher metabolic labelling efficiency inherently led to a higher extent of specific binding and accumulation of the clickable N3-conjugated gold nanoparticles (N3-AuNps, core diameter = 30 nm) in the DBCO-glycan modified A549 and MeTSA cells, but to a less prominent effect in MRC5 cells. These findings demonstrate that relative cell metabolism rates can be exploited by metabolic labelling to recruit nanotherapeutics whilst minimising off-specific targeting of surround tissues.

Introduction

Nanomedicine for oncology has heavily relied on systemic passive targeting of particles through the enhanced permeability and retention (EPR) effect due to vasculature leakiness in the malignant tissues. However, existing preclinical and clinical outcomes have remained sub-optimal with only < 1% of the doses of drug-nanoparticles ultimately reaching the specific targeting of surround tissues. In the last two decades, a chemoselective, active targeting strategy that combines cell metabolic glycoengineering and bio-orthogonal chemistry reactions to achieve tumour site-selective delivery. This work addresses the selectivity of metabolic glycan labelling in diseased (i.e., cancer) versus normal cells grown in a multicellular environment. Dibenzocyclooctyne (DBCO)-bearing acetylated-D-mannosamine (Ac4ManNDBCO) was synthesised to metabolically label three different types of cell lines originated from the human lung tissues: A549 adenocarcinomic alveolar basal epithelial cells, MeTSA non-cancerous mesothelial cells, and MRC5 non-cancerous fibroblasts. These cell lines were found to display different labelling sensitivity, which trended with their doubling time in the following order: A549 > MeTSA > MRC5. The higher metabolic labelling efficiency inherently led to a higher extent of specific binding and accumulation of the clickable N3-conjugated gold nanoparticles (N3-AuNps, core diameter = 30 nm) in the DBCO-glycan modified A549 and MeTSA cells, but to a less prominent effect in MRC5 cells. These findings demonstrate that relative cell metabolism rates can be exploited by metabolic labelling to recruit nanotherapeutics whilst minimising off-specific targeting of surround tissues.

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constant than the SPAAC (i.e., >1000 M⁻¹s⁻¹ versus 0.1 M⁻¹s⁻¹, respectively); nevertheless, the Tz and TCO derivatives are not as favourable in terms of molecular sizes, which pose the risks of perturbation to the original physiological functions.⁹

Among the synthetic monosaccharide analogues developed, sialic acid and N-acetylmannosamine (ManNAc) compounds are the most extensively studied for labelling the sialic acid residues.¹¹,¹² The glycan synthesis method has been well established, and their bio-orthogonal reactions successfully applied in vitro and in vivo especially for tumour pre-targeting prior to click-chemistry guided imaging and nano-therapeutic delivery.⁶,⁷,¹⁰,¹³,¹⁴ However, most studies on synthetic glycans for glyco-engineering have been on monoculture of cancer cells,²,⁴,⁶,⁷,¹⁰,¹¹,¹³,¹⁶,¹⁹,²¹ and to a lesser extent on monoculture of normal cell lines,¹⁰,¹⁷,²²,²³ which limits the wider understanding of application to other diverse cell systems. A majority of in vivo assessments utilising tumour xenograft mouse models have reflected either the need of intratumoural injection,¹⁹ or the use of carrier-/ligand-assisted delivery of the synthetic sugars to achieve cancer-specific labelling intravenously.⁶,⁷,¹⁴,¹⁵ Despite the general assumptions that malignant cells often display higher sugar metabolic demands than their normal neighbouring cells,²⁴ uncertainties remain with respect to their metabolic competition and thus, the selectivity of glycan labelling towards cancer cells when they are grown in a multicellular environment.

This work employed an in vitro multicellular testing platform using the ibidi® μ-Slide co-culture dishes to address two major research questions in the field: (i) is there any relationship between the type of cells (i.e., cancer versus non-malignant) and the specificity of metabolic glycan labelling, and (ii) whether any differential labelling is sufficiently specific to drive click-targeting of the complementary probes to tumours under multicellular settings (Fig. 1, ‘Feed-and-Click’ study). Despite the extensive reports on using azido-sugars for metabolic glycan labelling, in this work a DBCO-tagged, tetra-O-acetylated D-mannosamine (Ac₄ManNDBCO) was used to metabolically label the cells (Fig. 1a). The main reason for using Ac₄ManNDBCO is that conjugating nano-sized carriers with the relatively large and hydrophobic reaction molecules (such as DBCO) is likely to compromise colloidal stability and density of ligation.¹⁹ Previously, our laboratory has also verified that functionalisation of a lipid-based cubosome nanosystem with N₃ enabled reduced polydispersity in the particle size distribution and higher binding efficiency to its reactive DBCO dye partner.⁵ In this basis, this work employs gold nanoparticles (AuNps) bearing the smaller-sized, clickable N₃ moieties as a model nanocarrier to evaluate the competitive binding/uptake in a multicellular culture. To enable fluorescence-based detection of the cell-nanoparticle association, a fluorescence tag, hostosol methacrylate (λₑₓ 488 nm), was conjugated to the N₃-AuNps. Three types of cell lines derived from the human lung tissues were selected for their varying tumorigenic and proliferating characteristics: A549 adenocarcinomic alveolar basal epithelial cells, MeT5A non-cancerous mesothelial cells and MRC5 non-cancerous lung fibroblasts (scale bar = 20 μm).

![Fig. 1](image-url) Overview of the “Feed-and-Click” study using an in vitro multicellular testing platform: (a) molecular structure of N-DBCO, tetra-O-acetylated, D-mannosamine (Ac₄ManNDBCO), a synthetic sugar naturally consumed and processed by the malignant cells to integrate the intact DBCO tag on the cell surface glycans. The abiotic DBCO-tag can be specifically click-targeted by the complementary N₃-bearing nanoparticles (Nps); (b) multicellular culture of human lung cells with different doubling time in a multiwell chamber for probing the competitive binding/uptake of Nps: A549 adenocarcinomic alveolar basal epithelial cells, MeT5A non-cancerous mesothelial cells, and MRC5 non-cancerous lung fibroblasts (scale bar = 20 μm).

The expected findings were anticipated to enrich the roadmap of glyco-engineering strategies for enabling cell-specific precision nanotherapeutic delivery.

**Materials and methods**

**Synthesis and spectroscopy analysis of DBCO-sugar (Ac₄ManNDBCO)**

N-DBCO, tetra-O-acetylated, D-mannosamine (Ac₄ManNDBCO) was synthesised in-house as follows:¹⁹ D-mannosamine hydrochloride (0.5 mmol, purity ≥ 98%, Sigma-Aldrich, MO USA) and triethylamine (1.0 mmol, purity ≥ 98%, Sigma-Aldrich, MO USA) were dissolved in methanol (20 mL, Merck, VIC Australia), followed by mixing with dibenzocyclooctyne-N-
hydroxysuccinimidyld ester (DBCO-NHS, 0.6 mmol, purity ≥97%, Lumiprobe, MD USA) at room temperature for overnight. The solvent was evaporated under reduced pressure atmosphere.

The crude product (ManNDBCO) obtained was purified by flash column chromatography under elution with methanol/chloroform (1:9 v/v, Merck, VIC Australia) to produce a light yellowish powder (45% yield). The nuclear magnetic

resonance (NMR) spectra of ManNDBCO were analysed using a Bruker 400 MHz spectrometer at 298 K. Chemical shifts were reported as δ in parts per million (ppm), i.e. 1H NMR (CDCl3, 400 MHz, δ ppm): 1.27-1.28 (m, 2H), 1.76-1.81 (m, 1H), 1.95-2.09 (m, 3H), 2.17-2.21 (m, 2H), 3.60-3.66 (m, 2H), 3.78-3.83 (m, 3H), 4.05-4.07 (m, 0.65H), 4.26-4.34 (m, 1.35H), 4.88-4.98 (m, 0.7H), 5.03-5.11 (m, 1.3H), 7.09-7.23 (m, 3H), 7.34-7.37 (m, 4H), 7.64-7.66 (m, 1H).

The purified ManNDBCO (0.2 mmol) was redissolved in pyridine (4 mL, purity ≥99%, Sigma-Aldrich, MO USA), followed by the addition of acetic anhydride (5 mL, Sigma-Aldrich, MO USA) and mixed at room temperature for overnight. The solvent was evaporated under reduced pressure atmosphere. The crude product was purified by flash column chromatography, eluted with chloroform to produce the final product (Ac4ManNDBCO) in the form of light yellowish powder (95% yield). The NMR spectra of Ac4ManNDBCO were analysed as described above. 1H NMR (CDCl3, 400 MHz, δ ppm): 1.33-1.53 (m, 4H), 1.92-2.21 (m, 16H), 3.80-3.83 (m, 1H), 3.80-3.83 (m, 1H), 4.06-4.18 (m, 1.65H), 4.31-4.41 (m, 1H), 4.52-4.60 (m, 0.65H), 4.72-4.73 (m, 0.35H), 5.05-5.06 (m, 0.35H), 5.14-5.35 (m, 2H), 5.57-5.62 (m, 0.2H), 5.83-5.90 (m, 0.4H), 6.02-6.10 (m, 1H), 7.02-7.46 (m, 7H), 7.17-7.77 (m, 1H).

Synthesis and physicochemical characterisation of gold nanoparticles (488/N3-AuNps)

Synthesis of 2-(bdecylichthiocarbonothioylthio)-2-methyl-propionic acid 3-azido-1-propanol ester (N3-DMP). DMP was synthesised according to the procedure described by Phillips et al.26 and characterised as follows. 1H NMR (CDCl3, 400 MHz, δ ppm): 3.22 (2H, t), 1.66 (6H, s), 1.60 (2H, p), 1.31 (2H, p), 1.24-1.19 (16H, m), 0.81 (3H, t) (Fig. S4, ESI†). IR cm⁻¹: 2955 (alkyl-H stretch); 1712 (C=O stretch); 1069 (S-(C=S)-S stretch). N3-DMP was synthesised according to the procedure described as follows. 1H NMR (CDCl3, 400 MHz, δ ppm): 4.18 (t, 2H, CH2O), 3.36 (t, 2H, CH2N3), 3.27 (t, 2H, SCH2), 1.90 (quint, 2H, CH2CH2N3), 1.69 (s, 6H, C(CH3)3), 1.66 (m, 2H, SCH2CH2), 1.38 (m, 2H, SCH2CH2CH2), 1.34-1.21 (br m, 16H, SCH2CH2CH2CH2), 0.88 (t, 3H, S(CH2)11CH3) (Fig. S5, ESI†). IR cm⁻¹: 2923 (alkyl-H stretch); 2096 (N3 stretch); 1734 (C=O stretch); 1254, 1154, 1126, 1064 (S-(C=S)-S stretch).

Synthesis of poly(N-hydroxyl ethyl acrylamide-co-hostosol methacrylate), p(HEA98-co-HMA2).

HEA (0.5 g, 4.34 mmol), hostosol methacrylate (39.7 mg, 86 µmol, kindly provided by Haddleton Group, University of Warwick),29 N3-DMP (19.8 mg, 0.044 mmol), 4,4′-Azobis(4-cyanovaleric acid) (ACVA) (2.5 mg, 0.009 mmol) were dissolved in 50:50 toluene:methanol (4 mL). Mesitylene (150 µL) was added as an internal reference. An aliquot was taken for NMR analysis in CDCl3. The solution was degassed under N2 for 30 mins. The reaction was stirred at 70 °C for 3 hours. An aliquot was taken for NMR analysis in MeOD. The reaction was rapidly cooled in liquid nitrogen and precipitated into diethyl ether. The polymer was reprecipitated into diethyl ether from methanol twice to yield an orange polymer product that was dried under vacuum. 98% conversion by NMR, Mw (Theoretical) = 12050 g·mol⁻¹, Mw (SEC) = 9800 g·mol⁻¹, Mw/Mn (SEC) = 1.33 (Scheme S1, Fig. S6–S7, ESI†).

Synthesis of gold nanoparticles (AuNps).

AuNps were synthesised by method developed by Bastús et al.30 A solution of 2.2 mM sodium citrate in Milli-Q water (150 mL) was heated under reflux for 15 min under vigorous stirring. After boiling had commenced, 1 mL of HAuCl4 (25 mM) was injected. The colour of the solution changed from yellow to bluish grey and then to pale pink in 10 min. Immediately after the synthesis of the Au seeds and in the same reaction vessel, the reaction was cooled until the temperature of the solution reached 90 °C. Then, 1 mL of a HAuCl4 solution (25 mM) was injected. After 30 min, the reaction was finished. This process was repeated twice. The sample was diluted by extracting 55 mL of sample and adding 53 mL of MilliQ water and 2 mL of 60 mM sodium citrate. This solution was then used as a seed solution, and the process was repeated a further seven times. AuNps of 30 nm was further functionalised in this study (Fig. S8–S9, Table S1, ESI†).

Functionalisation of AuNps with p(HEA98-co-HMA2) (488/N3-AuNps). 100 µL of polymer solution (10 mg·mL⁻¹) was added to 1 mL of AuNps and left for 30 minutes at room temperature on a tube roller. After 30 mins, particles were centrifuged at 7000 rpm the supernatant was removed and resuspended in 1 mL of Milli-Q H2O. This was repeated a further two times to ensure complete removal of any unattached polymer as determined by measuring fluorescence of the supernatant. Stability was confirmed by incubating in PBS for 30 minutes (Scheme S2, Table S2, Fig. S10–12, ESI†).

Metabolic-labeling screening in lung-derived cell lines

Cell culture. Three types of cell lines derived from human lungs were sourced from the American Type Culture Collection (ATCC, Manassas, VA USA): A549 adipocarcinomic alveolar basal epithelial cells (passage 5–8, ATCC® CCL-185™), MeTSA mesothelial cells (passage 21–24, ATCC® CRL-9444™), and MRC5 fibroblasts (passage 24-27, ATCC® CCL-171™). Each cell line was cultured using a T75 or T25 flask in a bio- incubator (37 °C, 5% CO2) and replenished every 3 days with the respective culture medium (Table S3, ESI†).

DBCO-sugar treatment. For DBCO-metabolic labelling screening, each cell line was mono-cultured in two sets of triplicate wells on a 24-well plate (BD Biosciences, Switzerland) to reach 50-60% confluence. The DBCO-sugar was pre-dissolved in DMSO (stock concentration = 10 mM Ac4ManNDBCO) prior to dilution with the corresponding culture medium. The cells were
then incubated (37 °C, 5% CO₂) with their corresponding medium containing the DBCO-sugar at the following doses: (i) 0 µM (containing 0.5% DMSO as the control solvent), (ii) ‘low’ dose at 25 µM (containing 0.25% DMSO), and (iii) ‘high’ dose at 50 µM (containing 0.5% DMSO). Following DBCO-sugar treatment for 6, 24 and 48 h, the cells were rinsed in triplicates with phosphate buffer saline (PBS 1X, Gibco, MD USA) prior to trypsinisation with 0.05% trypsin-EDTA solution (Gibco, MD USA). The suspended cells were collected by centrifugation (at 200 g for 3 min) and immediately fixed with 4% paraformaldehyde (for 15 min) to preserve the cell glycan properties.

**Flow cytometry.** The expression of cellular DBCO-glycan was determined by click-labelling with the complementary dye, cyanine5 azide (Cy5/N3) at 2 µg.ml⁻¹, LumiProbe C3030, MD USA) for 1 h prior to triplicate washes with PBS and centrifugal removal of the excess Cy5/N3 dye in the supernatant (at 10,000 g for 7 min). All cell samples were resuspended in 400 µL of PBS. The percentage of positive expression and median intensity of Cy5 signals (λex/em = 650/660 nm) were determined using the flow cytometric analysis on a LSRFortessa Instrument (BD Biosciences, Switzerland). The measurements were recorded at 10,000 events each. The control group treated with Cy5/N3 dye (i.e., ‘Control + Cy5/N3’) was used as the reference baseline to eliminate false positive signals originated from non-specific binding of the dye to the fixed cells (Fig. S13, ES†). Data were processed using the FlowJo™ software (for Windows, version 10.6.2, Becton, Dickinson and Company), and reported as mean ± standard deviation (S.D.).

**Viability assay.** The morphology of cells was observed via a bright field microscope for both control and DBCO-sugar treated cells, and the medium collected from each well and subjected to lactate dehydrogenase (LDH) cytotoxicity assay (Cat. No. 11644793001 manufactured by Roche, Sigma-Aldrich, Switzerland). The release of cytosolic enzyme (LDH), which is indicative of damage to the cell plasma membrane, was evaluated according to the product protocol: an aliquot of culture medium was mixed with the detection kit reagent at room temperature for 30 min, where the presence of LDH catalyses the conversion of lactate to pyruvate via NAD⁺ reduction to NADH; the oxidation of NADH by diaphorase then reduced a tetrazolium salt (INT) to a red formazan product that was measurable spectrophotometrically at 490 nm (Benchmark Plus Microplate Reader, Bio-Rad, USA). The level of formazan is directly proportional to the amount of LDH released. Cell viability was estimated from the absorbance values relative to that of the negative control cells.

**IncuCyte® cell proliferation assay.** Real-time quantification of cell proliferation was analysed using the IncuCyte® ZOOM live cell imaging system (Essen BioScience, MI USA). The cells were seeded at 1 x 10⁴ cells per well in a 96-well culture plate for 12 h, and cultured with the supplemented media containing either 0.5% DMSO as the negative media control, or 25–50µM DBCO-sugar for the treatment groups (37 °C, 5% CO₂). The system automatically monitored 9 regions per well (in triplicates for each sample), and calculated the cell density based on the occupied area of individual wells to represent the % confluence of cell images at each time point (i.e., every 4 h for 48 h).

**‘Feed-and-Click’ nanoparticle-targeting study**

**Multicellular culture and DBCO-sugar treatment.** Three types of human lung-derived cell lines A549, MeTSA and MRC5 were seeded separately at a density of approximately 1.5 x 10⁴ cells per cm² on a multwell co-culture dish (ibidi® µ-Slide 2 Well Co-Culture ibiTreat Dishes, Cat. No. 81806, Germany). Each cell line was left for incubation (37 °C, 5% CO₂) overnight to adhere to the dish, followed by replenishment and co-exposure to the same mixed media containing supplemented RPMI-1640, Media 199 and DMEM at a ratio of 1:1:1 (v/v/v). The cells were allowed to grow for an additional day to reach 50% confluence prior to use. On the third day after seeding, the A549/MeTSA/MRC5 culture was fed with the mixed media containing DBCO-sugar at the following doses: (i) 0 µM (containing 0.5% DMSO as the control solvent), (ii) ‘low’ dose at 25 µM (containing 0.25% DMSO), and (iii) ‘high’ dose at 50 µM (containing 0.5% DMSO). Following DBCO-sugar treatment for 6 h, the cells were rinsed in triplicates with blank mixed media prior to the subsequent nanoparticle exposure study.

**Co-exposure to gold nanoparticles (488/N3-AuNps).** 488/N3-AuNps were diluted and vortex-mixed with the mixed media to reach 100 µM (equivalent to 20 µg.mL⁻¹) in terms of Au concentration. An aliquot of 600 µL of the freshly prepared 488/N3-AuNps-containing media was added to each group of control and DBCO-sugar treated A549/MeTSA/MRC5 culture dishes. Following a 6 h of co-exposure to 488/N3-AuNps (at 37 °C, 5% CO₂), the cells were rinsed in triplicates with blank media prior to fixation with 4% paraformaldehyde (for 15 min).

**Immunostaining and confocal laser scanning microscopy (CLSM) imaging**

The fixed cells were blocked for non-specific binding with bovine serum albumin, BSA 1% (Sigma-Aldrich, Switzerland) for 30 min at room temperature. Phalloidin rhodamine (Invitrogen R415, Molecular Probes by Life Technologies, Thermo Fisher Scientific, Switzerland) was then added at a dilution of 1:50 to stain the F-actin cytoskeleton for 30 min. After duplicate washes with BSA 1%, the cell nuclei were stained with 4',6-diamidino-2-phenylindole, DAPI (Sigma-Aldrich, Switzerland) at 1 µg.ml⁻¹ for the cell nuclei. The cell membranes were not permeabilised prior to immunostaining in order to minimise possible perturbation to the cell membrane lipids.³⁻¹⁻³⁻¹ The fluorescently labelled samples were imaged using an inverted Zeiss LSM 710 confocal laser scanning microscope (Axio Observer.Z1, Carl Zeiss, Germany) equipped with Argon 488 nm, HeNe 543 nm and HeNe 633 nm lasers. Image acquisition was performed on both 63x and 40x oil immersion objective lenses for Z-stacks of 25 x 0.5 µm (with a pixel dwell of 6.3 µsec, scan time 15.5 sec) using the Zen 2010 software.
CLSM images of cell-nanoparticle association was analysed using Fiji (ImageJ, National Institutes of Health). The split-channel Z-stack images were reconstructed into a Maximum Intensity Z-projection, and the grayscale projection adjusted with a fixed threshold function using the control cell samples as the zero reference. The mean grey values were obtained and normalised by the number of cells in each image. The data were compared using a one-way ANOVA test with the level of significance set at $p < 0.05$. Numerical data are reported as mean ± standard deviation (S.D.) from three independent measurements of each experimental condition.

Enhanced dark-field/hyperspectral optical microscopy imaging

Samples of A549 cells with and without exposure to the DBCO-sugar and 488/N3-AuNps were imaged using a Cytoviva enhanced dark-field/hyperspectral optical microscopy (eDF-HSI Cytoviva®, Cytoviva Inc., Auburn, US) as a complementary analytical method to determine cell-Np association independent of fluorescent labels. The samples were scanned using a 100 × objective lens, numerical aperture 0.8 for enhanced-darkfield (frame size: 21.58 μm × 16.12 μm) and hyperspectral imaging setup for obtaining scattering feature of the samples (frame size: 87.77 μm × 87.77 μm). For quantification of number of particle agglomerates, image processing using FIJI was performed. Briefly, individual enhanced darkfield images (19 images for each condition) was subjected to background subtraction using background subtraction tool, and then object binarization in FIJI before particle counting was performed. Number of agglomerates per frame and their corresponding size (i.e., particle diameter and area) for each condition were obtained, and then a one-way ANOVA test with the level of significance set at $p < 0.05$ was performed.

Results and discussion

Characterisation of Ac4ManNDBCO and 488/N3-AuNps

$\text{N}$-DBCO, tetra-$\text{O}$-acetylated, $\text{D}$-mannosamine ($\text{Ac4ManNDBCO}$) was synthesised by reacting $\text{D}$-mannosamine with dibenzocyclooctyne-$\text{N}$-hydroxysuccinimidyl ester (DBCO-NHS), followed by peracylation of the hydroxyl groups. The less polar acetyl groups provide the sugar analogue with better cell permeability to enable effective glycan-labelling. This approach also enabled utility at micromolar units compared to non-acetylated sugars that are typically required in millimolar concentrations.

For the assessment of SPAAC-guided cell-nanoparticle targeting, AuNps with a core diameter of 30 nm were functionalised with $\text{N}_2$-terminated poly($\text{N}$-hydroxyethyl acrylamide-co-hostasol methacrylate, p(HEA$_{58}$-co-HMA$_2$)), prepared by reversible addition-fragmentation chain transfer (RAFT) polymerisation which introduces a terminal thiol group, for conjugation to the AuNp surface (Fig. 2). pHEA was chosen as a water-soluble, inert polymer that has been shown previously as a good stabilising polymer for AuNps. As a result of polymer grafting, the $\text{N}_2$-bearing gold nanoparticles

<table>
<thead>
<tr>
<th>Au surface functionalisation</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>Diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before (AuNps)</td>
<td>524</td>
<td>32.0 ± 0</td>
</tr>
<tr>
<td>After (488/N3-AuNps)</td>
<td>529</td>
<td>56.5 ± 9</td>
</tr>
</tbody>
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Fig. 2. Molecular structure, UV-vis absorbance and dynamic light scattering (DLS)-derived particle sizes of the gold nanoparticles before (i.e., AuNps) and after (i.e., 488/N3-AuNps) surface functionalisation with azide-terminated poly($\text{N}$-hydroxyethyl acrylamide-co-hostasol methacrylate, $\text{N}_2$-p(HEA$_{58}$-co-HMA$_2$)).

Cell-dependent uptake of DBCO-sugar and output of metabolic labelling

The use of azido- and DBCO-sugars in metabolic labelling has been reported to span across a wide range of concentration ($5$–$150$ μM) and incubation time (1–7 days) for different cell lines. Here, a systematic screening of the dose- and time-dependent cell tolerability as well as metabolic labelling output was performed on the three selected cancerous and non-malignant cell lines (A549, MeT5A, and MRC5) treated with the DBCO-sugar (Ac4ManNDBCO). The cell culture media for both negative control and sugar-treatment groups were consistently doped with 0.25–0.5% (v/v) DMSO as the solvent required to pre-dissolve the DBCO-sugar.

An initial screening of cell morphology under a bright-field microscope indicated negligible effects of the DBCO-sugar on A549 and MeT5A cells at doses up to 50 μM; however, MRC5 cells exhibited cell detachment and lysis when exposed to the DBCO-sugar at 50 μM for 24 h (Fig. 3a, ES1†). Through a lactate dehydrogenase (LDH) cytotoxicity assay, it was confirmed that doses at 25 μM and 50 μM of DBCO-sugar incubated over a shorter duration within 6 h were generally well-tolerated by all three types of cell lines without noticeable disruption to the cell membranes (Fig. 3b, ES1†).

Furthermore, a preliminary live-cell proliferation assay also revealed negligible effects of the DBCO-
sugar on the cell growth curve up to 12 h, but significant growth retardation was identified for prolonged incubation (Fig. S1c, ESI†). Therefore, a short-term dosing (6 h) using 25 µM (as a ‘low’ dose) and 50 µM (a ‘high’ dose) of DBCO-sugar was considered biocompatible and was uniformly adopted to compare the metabolic labelling efficiency across these cell lines.

The expression of DBCO-labelled glycans was detected using confocal laser scanning microscopy (CLSM) via click-labelling with an N3-conjugated Cy5-fluorescence probe (N3/Cy5). The N3/Cy5 dye was confirmed to produce minimal non-specific staining of the media-fed control cells; on the other hand, all three types of cell lines fed on the DBCO-sugar successfully expressed positive DBCO-N3/Cy5 signals predominantly within the nuclei and the cytoplasmic regions (Fig. 3a). This is different from several observations formerly reported for both azido- and DBCO-sugars, where the labelling intensity was mostly concentrated along the cell outer membranes with minimal distribution within the inner organelles.3,4,6,7,13,19 According to the general sialic acid biosynthesis route, the ManNAc analogues typically undergo several enzymatic conversions within the cytosol, followed by processing via the nucleus and Golgi apparatus before projecting through the cell surface glycans; sialoglycans on the cell surface or intracellular compartments may also be further recycled back into the sialic acid biosynthesis pathway (Fig. 1a).12 While the timeline of these intracellular processing steps has not been clearly defined, it is not surprising that the DBCO tags were expressed intracellularly given the current shorter incubation in hours as compared to those reported elsewhere for days. Importantly, such internalisation of the labelling molecules, which is consistent with some other studies,3,17,23 also verified cellular uptake of the DBCO-sugar rather than merely interfacial adsorption of the sugar.

To gain further insight into the percentage and mean fluorescence intensity (MFI) of cells expressing the DBCO-N3/Cy5 signals, the control and DBCO-tagged cells were further examined using flow cytometry (FC) analysis. With a threshold deliberately set at 80% for ‘complete positive labelling’, it can be seen that both A549 and MeT5A cells were successfully labelled within 6 h at both ‘low’ (25 µM) and ‘high’ (50 µM) doses of DBCO-sugar (Fig. 3b). In contrast, MRC5 cells displayed relatively lower expression of the DBCO-tags with only <10% and <65% positive labelling achieved, respectively at the ‘low’ and ‘high’ sugar doses in 6 h. The trend of metabolic labelling output (i.e., the % of cells but not the MFI) appears to go in line with the corresponding doubling time of the individual cells, i.e., A549 ≈ MeT5A > MRC5. It has been previously shown that smaller cells tend to undergo shorter doubling times.24 Therefore, it can be deduced that MRC5 cells presented a lower metabolic labelling efficiency in comparison with A549 and MeT5A cells, possibly due to their lower metabolic rate as well as slower process in cell division and elongation prior to forming a confluent colony achieving equal sialoglycan labelling of their extensively stretched membranes. The tolerability and metabolic labelling output collectively support the suitability of the short-term sugar incubation protocol in these cell lines to simultaneously probe the competition of AuNp click-targeting.

Fig. 3 DBCO-sugar uptake and glycan-labelling expression based on click-labelling with an N3-conjugated Cy5-fluorescence dye: (a) confocal laser scanning microscopy (CLSM) images of media-fed control cells and DBCO-sugar treated cells. The cell nuclei were stained with DAPI (blue), the F-actin filaments with rhodamine phalloidin (grey) and the DBCO expression indicated by the N3/Cy5 signals (yellow). Scale bar = 20 µm; (b) flow cytometry (FC) analysis quantifying the percentage (%) and mean fluorescence intensity (MFI) of cells expressing positive DBCO-N3/Cy5 signals (mean ± S.D., n = 2). 'NA' denoted non-availability of data due to cytotoxicity in MRC5 cells.
Selectivity of click-chemistry mediated nanoparticle targeting in multicellular environments

The applicability of metabolic glycoengineering coupled with SPAAC-guided targeting has been reported for a myriad of drug nanocarriers, including liposomes,13-15 chitosan nanoparticles,6 silica nanoconjugates,19 and polymers.4,16 The methodologies were mostly relied on cell monoculture or whole body imaging in tumour xenograft models. To enable simultaneous and direct comparison of the binding/uptake of N₃-AuNPs in a mixed cell environment, a multicellular culture of lung-derived cells (A549/MeT5A/MRC5) was employed. The cells were treated with DBCO-sugar for 6 h, followed by co-exposure to the Nps for 20 h. This Np exposure timeframe was referred from previous

Fig. 4 Comparative binding or uptake of 488/N₃-AuNPs in A549/MeT5A/MRC5 multicellular culture: (a) CLSM images of media-fed control cells and DBCO-sugar treated cells at a ‘low dose’ (25 µM) and ‘high dose’ (50 µM) for 6 h, followed by co-exposure to 488/N₃-AuNPs (at 100 µM in Au concentration) for 20 h at 37 °C, 5% CO₂. The cell nuclei were stained with DAPI (blue), the F-actin filaments with rhodamine phallolidin (grey) and the N₃-AuNPs indicated by the 488 fluorescence signals (yellow). Scale bar = 20 µm; (b) quantitative image analysis of the CLSM images comparing the percentage (%) and mean fluorescence intensity (MFI) of cell-associated 488/N₃-AuNPs in the absence (control) and presence (‘high sugar’) of DBCO-sugar treatment (mean ± S.D., n = 3, *p < 0.05); (c) CLSM cross-section view showing the distribution of 488/N₃-AuNPs (magenta/ arrows); and (d) correlation between positive DBCO-labelling and click-targeting of 488/N₃-AuNPs.
studies that have shown only minimal detectable cellular uptake of AuNps in shorter exposure time.\textsuperscript{39,40} It was also confirmed in the present work that the cell-Np association at 2h exposure time was too low for reliable quantification (Fig. S2, ESI†).

As illustrated in Fig. 4(a), the control A549, MeTSA and MRC5 cells did not show noticeable differences in their non-specific binding/uptake behaviours in the absence of glycan-modification. In the presence of glycan-labelling, A549 and MeTSA cells exhibited a clear trend of higher degree of cell-Np association with increasing doses of the DBCO-sugar. Notably, the effect of DBCO-sugar on the binding/uptake behaviours was less prominent in MRC5 cells. Quantitative image analysis in terms of cell percentage indicates substantial enhancement of Np selective targeting to the DBCO-modified A549 and MeTSA cells by 1.8-fold and 1.7-fold, respectively in comparison with the original control cells (Fig. 4b). A549 cells appeared to be better targeted through SPAAC than MRC5 cells in a co-exposed environment, whilst there were marginal differences between MeTSA and MRC5 modified cells. Interestingly, comparison of the MFI values showed a constant trend of DBCO-modified cells > control cells across all three cell lines. To further confirm that the cellular association of AuNps was reliably tracked using the fluorescent label without compromised molecular integrity or fluorescence quenching, we performed a supplementary enhanced dark-field hyperspectral imaging (eDF-HSI Cytoviva\textsuperscript{40}) of A549 cells exposed to the 488/N\textsubscript{2}AuNps under similar conditions and time duration.\textsuperscript{34} Cell-associated particles can be observed by hyperspectral imaging and their presence is confirmed by their high scattering intensity centered at 645 nm, which is attributed to the localised surface plasmon (Fig. S3, ESI†). Promisingly, image processing of the enhanced dark-field images (n = 19) shows consistent results with the fluorescence-based detections, where DBCO-modified cells similarly exhibited 1.8-fold higher cell-Np association than that of the control cells (which are mostly seen as particle agglomerates).

When examining the CLSM cross-section view, it could be seen that the N\textsubscript{2}AuNps were mostly internalised into the control cells (Fig. 4c). This is different from the DBCO-modified cells, where the distribution of N\textsubscript{2}AuNps broadly spanned throughout the cell interface as well as the cell interior. Likewise, it is also inferred from the complementary dark-field images that the AuNps were distributed in closer proximity to the nuclei for control cells, but more widely or homogeneously spread across the DBCO-treated cells (Fig. S3a, ESI†).

Taken together, the simultaneous competitive binding/uptake study reveals a close correlation between the efficiency of metabolic labelling and the SPAAC-promoted targeting of AuNps (Fig. 4d). A549 cells, although displaying a slightly higher percentage of cell-Np association in the multicellular setting, did not present an exclusively convincing cancer-selective labelling and SPAAC-guided AuNp targeting when competition exists in the presence of other fast-growing but non-malignant tissue cells (such as MeTSA cells in this case). Alternative techniques that adopt distinctive activation by cancer cells, such as modifying the synthetic sugar with chemical groups specifically cleavable by enzymes overexpressed in cancer cells,\textsuperscript{7} are considered necessary to minimise uptake in off-target neighbouring cells. This work provides further motivation to gain clearer insights into the metabolic competition between localised/ metastatic tumour cells and circulating/ resident phagocytic immune cells that are typically upregulated in the diseased states. In particular, the use of 3D multicell co-culture models would present a more complex microenvironment recapitulating the true physiology and local metabolic events to probe the trajectory of such clickable drug nanocarriers.\textsuperscript{41}

**Conclusions**

We have performed a competitive ‘Feed-and-Click’ study using an unconventional multicellular culture of cancer (A549 cells) and non-malignant cells (MeTSA and MRC5 cells) to evaluate the selectivity of metabolic glycan labelling in promoting SPAAC click-chemistry guided targeting of nanocarriers. Two major research questions were addressed through a dose- and time-dependent assessment using a DBCO-tagged synthetic sugar (Ac\textsubscript{4}ManNDBCO) as the metabolic labelling agent. Firstly, the metabolic labelling efficiency is dependent on the cell proliferation rate (i.e., A549 > MeTSA > MRC5) rather than the tumorigenic characteristics. Therefore, there is no clear cancer-selectivity in terms of sugar consumption and thus, the DBCO-glycan expression under a co-exposure environment. Secondly, a higher efficiency of metabolic labelling successfully led to higher degrees of click-targeting of the complementary N\textsubscript{2}-bearing gold nanoparticles towards the DBCO-modified cells in a sugar dose-dependent manner, especially among the fastest proliferating A549 and MeTSA cells. Such findings highlight the paramount importance of gaining more in-depth understandings of the correlations between cell behaviours and the output of metabolic glycoengineering in order to attain precise nanotherapeutic targeting to diseased cells with minimal perturbation to the adjacent healthy domains.

**Conflicts of interest**

There are no conflicts to declare.

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