Metabolic Glycoengineering-Driven 'Clickable' Nanoemulsions for Biomarker-Agnostic Tumour Targeting

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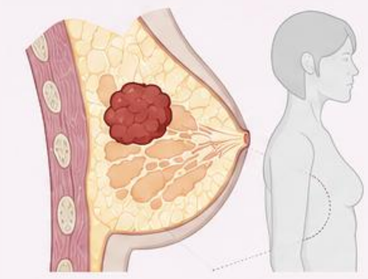
THE CHALLENGE

Triple-Negative Breast Cancer (TNBC)



Highly heterogenous and resistant to treatment

Lack of specific biomarkers

Limited treatment options: surgery, radio/chemotherapy

Limitations Targeted Therapies

Microenvironmental influences

Dynamic biomarker expression

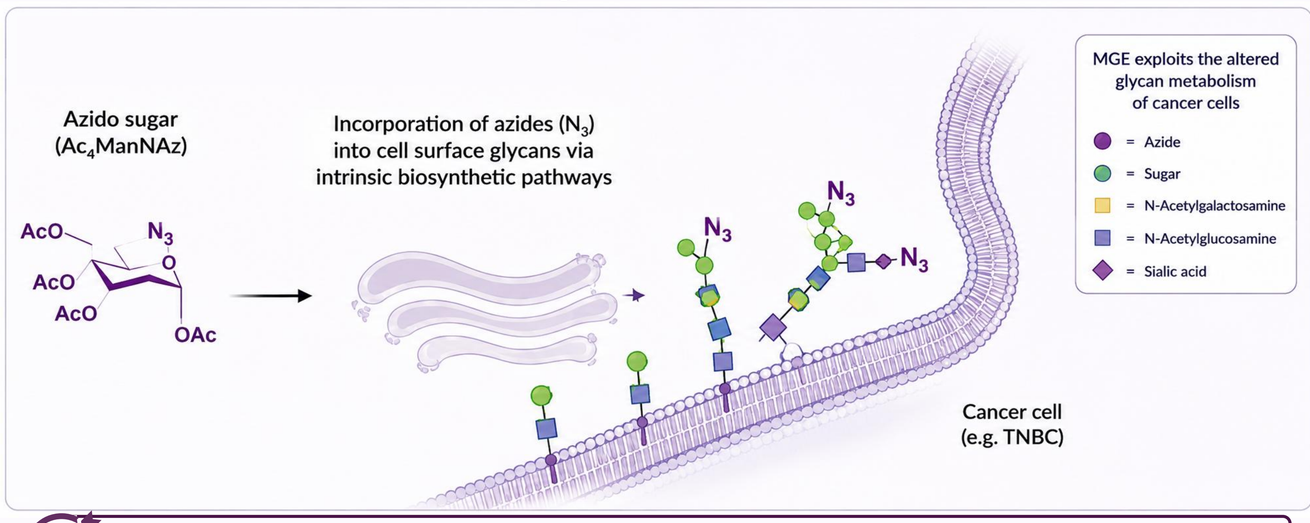
Technological limitations

Challenge in discriminating cancer Vs normal cells

INTRODUCTION

METABOLIC GLYCOENGINEERING (MGE)

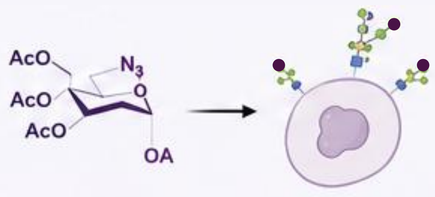
MGE enables the selective installation of bioorthogonal azides on tumour cell glycans, exploiting altered glycan metabolism of cancer cells and generating artificial biomarkers for 'click' conjugation with functionalized nanocarriers



Selectively labelling of cancer cells as alternative for targeted therapies to agnostic-biomarker tumors

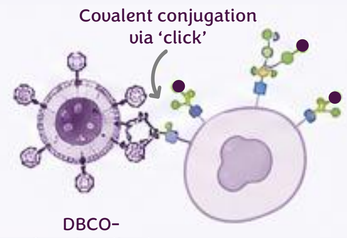
OUR STRATEGY

1. Azide labelling of cancer cells by MGE



Ac_4ManNAz — Cancer cell labelled with azide

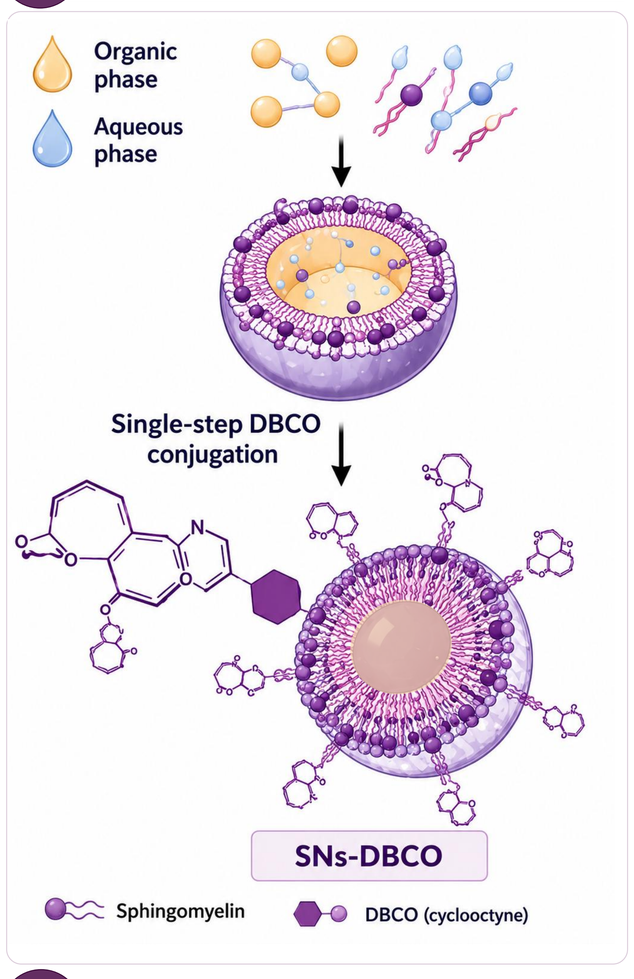
2. Click conjugation via SPAAC with DBCO-functionalized Nanoemulsions



DBCO-functionalized nanoemulsions (SNs-DBCO)

METHODS & RESULTS

1 Nanoemulsion Formulation



2 Physicochemical characterization

Table 1. Physicochemical characterization of SNs-DBCO at different DBCO concentrations in terms of hydrodynamic volume (size, nm), Polydispersity Index (PDI), and surface charge (Z-Potential, mV), measured by Dynamic Light Scattering (DLS) and Laser Doppler Anemometry (LDA).

SN	Molar ratio	[DBCO] (μM)	Size (nm)	PDI	Z-Potential (mV)
Vitamin E: Sphingomyelin: PEG2k-DBCO	1:0.1:0.01	16	86.9 ± 1.6	0.20 ± 0.10	-20.6 ± 3.3
	1:0.1:0.02	32	81.0 ± 1.7	0.26 ± 0.02	-23.0 ± 0.9
	1:0.1:0.04	64	71.1 ± 1.1	0.30 ± 0.02	-18.9 ± 1.9
	1:0.1:0.08	128	73.7 ± 1.3	0.30 ± 0.03	-21.2 ± 2.9
	1:0.1:0.1	160	96.7 ± 1.3	0.31 ± 0.12	-27.3 ± 2.6

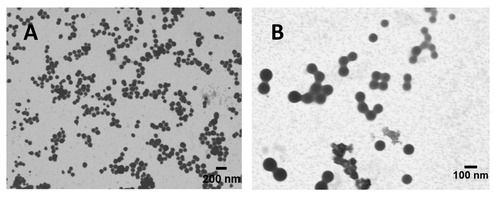


Fig 1. Transmission Electron Microscopy (TEM) images show the spherical morphology of SNs composed of vitamin E, sphingomyelin, and lipid DSPE-PEG2k-DBCO, with scale bars of **A.** 200 nm and **B.** 100 nm.

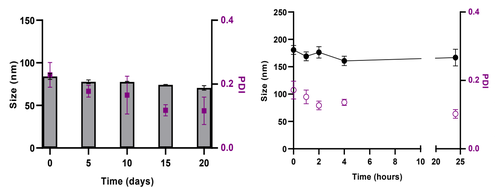
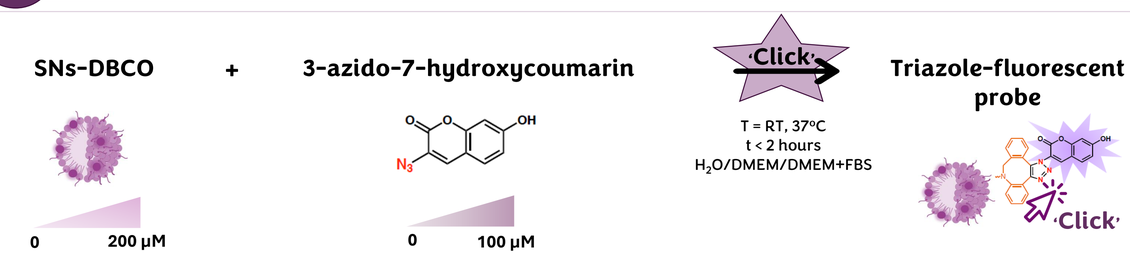


Fig 2. A. Colloidal stability of SNs-DBCO (1:0.1:0.01) over time during storage at 4°C. **B.** Stability of SNs-DBCO after incubation in supplemented cell culture medium at 37°C by monitoring size and PDI over time. Data is shown as mean ± SD (n = 9).

- **Small size** (<100 nm), narrow distribution (PDI~0.2) and negatively charged formulations at all molar ratios (increasing DBCO concentration).
- SNs-DBCO exhibit a **spherical morphology** with sizes ranging from 70 - 100 nm, consistent with Dynamic Light Scattering (DLS) characterization.
- **Colloidal stability** in water under storage conditions (4°C) up to 1 month, and 24 hours in physiological simulating conditions (cell culture medium, 37°C)

3 'Click' reactivity in suspension



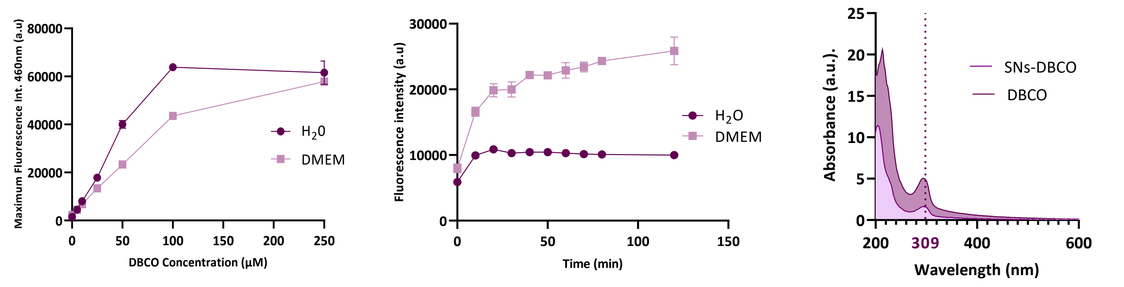


Fig. 3. Fluorogenic 'click' reaction between SNs-DBCO (0-100 μM DBCO) and 3-azido-7-hydroxycoumarin (50 μM N_3) incubated at 37°C for 1 h in H_2O and cell culture medium (DMEM).

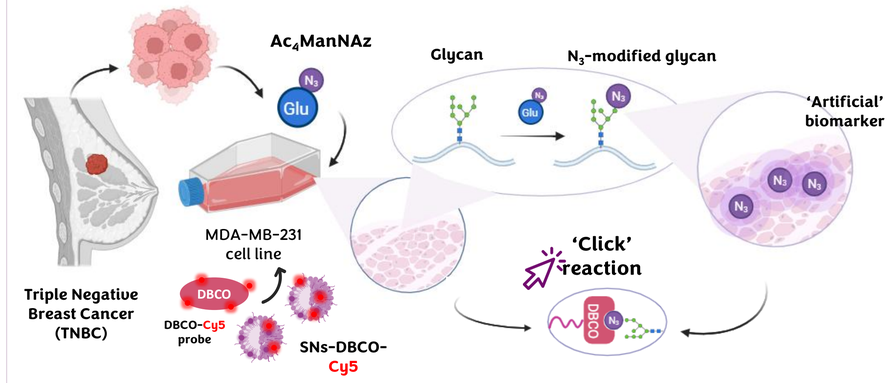
Fig. 4 Fluorogenic 'click' reaction between SNs-DBCO (100 μM DBCO) and 3-azido-7-hydroxycoumarin (20 μM N_3) incubated at 37 °C over time in H_2O and cell culture medium (DMEM).

Fig. 5. UV–Vis absorbance profiles of SNs-DBCO highlighting the decrease/increase in the characteristic DBCO peak at 309 nm. Variations in peak intensity were used as a reference to quantify DBCO conjugation efficiency and click reactivity.

- ✓ Reaction conditions evaluated and optimized (ratio, temperature, medium, reaction time).
- ✓ 'Click' reactivity assessment completed with SNs-DBCO to further translation in vitro.

Optimal ratio DBCO:N3	DBCO Conjugation Efficiency	'Click' Reactivity Efficiency
5:1	88%	~70%

4 'Click' reactivity in vitro



Viability assays

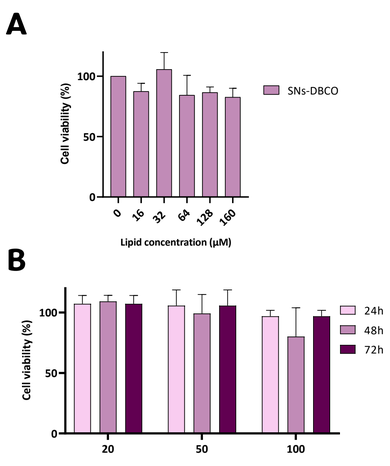


Fig. 6. Viability assays (alamarBlue reagent) to evaluate the potential cytotoxicity of: **A.** SN-DBCO at varying concentrations (0-160 μM) after 24 h of incubation in MDA-MB-231 cell model; **B.** the sugar precursor (Ac_4ManNAz) at varying concentrations (0-100 μM) after 24, 48 and 72 h of incubation in MDA-MB-231 cells. Results are expressed as the mean ± standard deviation from four independent experiments.

No cytotoxicity detected

Internalization assays

Flow Cytometry analysis

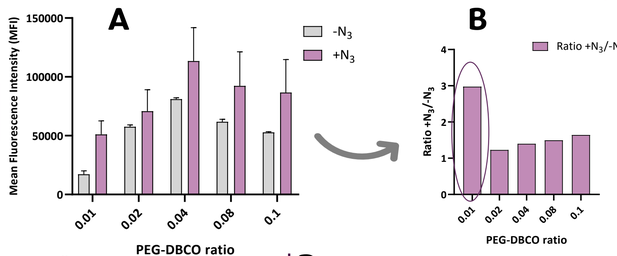


Fig. 7. Influence of PEG-DBCO density on azide-mediated nanoemulsion uptake. A. Mean Fluorescence Intensity (MFI) after pre-treating MDA-MB-231 cells with Ac_4ManNAz (100 μM, 48h; $+N_3$) following incubation SNs-DBCO formulations containing increasing PEG-DBCO surface densities. **B.** Ratio of mean fluorescence intensity between Ac_4ManNAz-treated ($+N_3$) and untreated ($-N_3$) cells. Higher $+N_3/-N_3$ ratios indicate greater azide-dependent nanoemulsion uptake.

↓ PEG-DBCO density → ↑ $+N_3/-N_3$ ratio

Fluorescence Microscope images

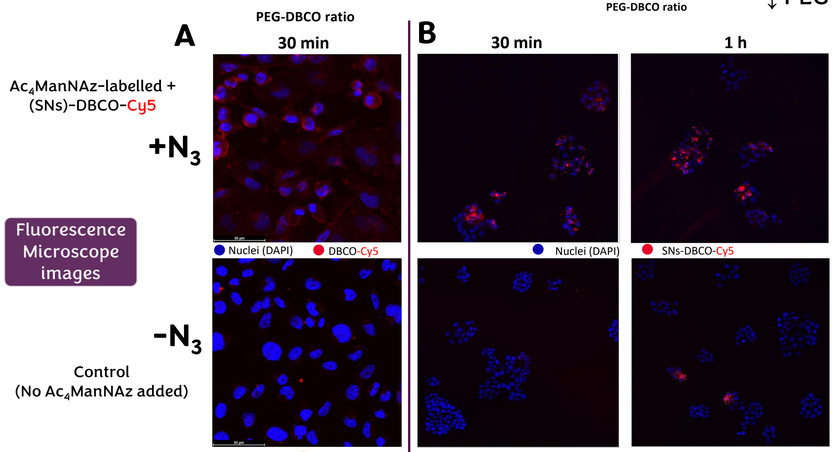


Fig. 8. Fluorescence microscopy images of the 'click' reaction in MDA-MB-231 cells pre-treated with Ac_4ManAz ($+N_3$) 100 μM for 48 h or without ($-N_3$), after incubation with **A.** DBCO-Cy5 probe (red) at 1 μM for 30 minutes; **B.** SNs-DBCO-Cy5 (red) at 20 μM DBCO concentration for 30 minutes and 1 hour.

1:0.1:0.01 → best SNs-DBCO molar ratio for click-mediated targeting

Steric crowding of PEG chains may limit the accessibility of DBCO groups to cell-surface azides

5 Highlights

- ✓ **Development of DBCO-sphingomyelin nanoemulsions** – SNs-DBCO with optimal physicochemical properties (<200 nm, PDI <0.2) successfully developed, highly biocompatible and stable for biomedical applications.
- ✓ **Click reactivity assessment in suspension** – SNs-DBCO exhibited high click reactivity (~70%) in suspension, with optimized formulation parameters (molar ratio, reaction time, media).
- ✓ **Efficient cellular glycoengineering** – MGE with Ac4ManNAz achieved efficient labelling of the tumour cell surface without compromising cell viability. The DBCO-N3 click interaction was validated in vitro as a precise and selective targeting strategy, supporting its potential for safer and more effective tumour-directed therapies.

6 Next steps

- ❑ Translation of the MGE strategy to other tumor cell lines (glioma model) to demonstrate the versatility of the approach.
- ❑ Validation of click reactivity in vitro with 3D tumor models.
- ❑ In vivo evaluation of the MGE labelling strategy in TNBC/glioma models.
- ❑ Therapeutic assessment of drug-loaded SNs-DBCO in TNBC.

ACKNOWLEDGEMENTS

GPC (IN607B2024_14)

Grant RED2022-134560-T funded by

PI24/00884

Grant CPP2023-010787 funded by MICIU/AEI/10.13039/501100011033/ERDF/EU

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