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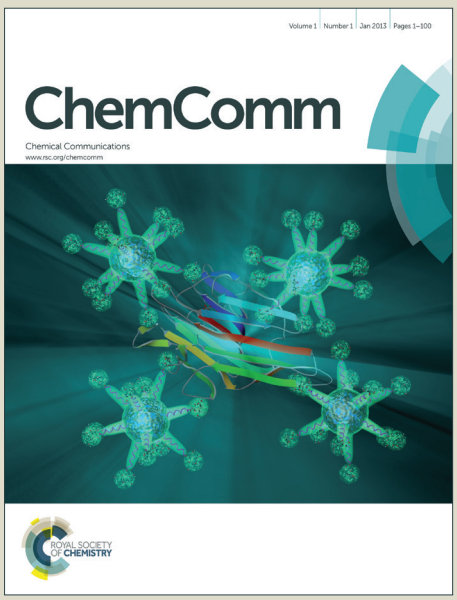

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Fructose-coated Nanoparticles: a Promising Drug Nanocarrier for Triple-negative Breast Cancer Therapy

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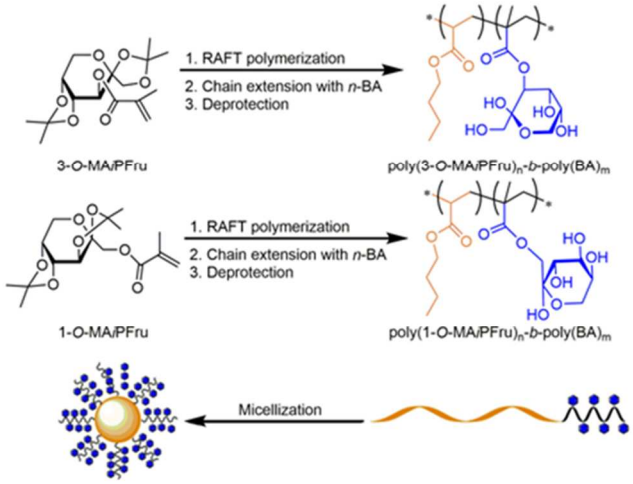
Fructose transporter GLUT5 is overexpressed in breast cancer cell lines, but not in healthy tissue. Micelles based on fructose, which were found to be low fouling, showed a high uptake by breast cancer cell breast cancer cells (MCF-7 and MDA-MB-231 cells), but only negligible uptake by macrophages.

Triple-negative breast cancer (TNBC), which was first mentioned in the literature[1] in 2005, is subject to intense investigations in oncology today. The lack of expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER-2/neu) makes TNBC therapeutic efficiency disappointing and frustrating. Therefore, a recognized target is in urgent need to achieve successful targeted therapies for TNBC. Recent research indicated that GLUT5, a specific fructose transporter, is overexpressed in breast cancer cells and tissues but not in healthy mammary tissues.[2] The strength of binding of different fructose derivatives toward GLUT5 is strongly influenced by the position of substitution.[3] The selective expression of GLUT5 in normal and cancerous tissues suggests that this fructose transporter could represent a potential therapeutic target. Successful application of this concept has been recently shown for small molecules with the selective uptake of fructose-substituted phosphorescent metal complexes.[4, 5]

Targeting tumors with long-circulating drug delivery systems such as polymeric micelles[6-9] is a promising strategy in systemic treatments for a variety of cancers. These nanocarriers accumulate in tumor tissue through the enhanced permeability and retention (EPR) effect[10] and / or binding to the receptors on the surface of cancer cells.[11] Compared with free drugs, nanomedicines have longer blood residence time and better drug distribution in the body, thus better therapeutic efficacy.[12] In addition, some features including size and shape can be designed to mediate cellular uptake,[13-15] body clearance[16] and biodistribution of nanoparticles.[17-19]



Scheme 1. Synthesis and Self-assembly of Fructose-based Amphiphilic Block Glycopolymers

We hypothesize that GLUT5 which is overexpressed by breast cancer cells can recognize fructose located on the surface of nanoparticles and induce receptor-mediated endocytosis. The specific affinity between GLUT5 and fructose moieties of nanoparticles can potentially induce the preferential binding of the nanocarriers to breast cancer cells rather than to normal cells, thus directing towards higher cellular uptake. Although glycopolymers have been extensively investigated due to their ability to bind to lectins, [20-23] no reports on fructose as a

building block are available despite its important biological role. Here, we describe novel fructose-coated nanoparticles that can be used for targeted drug delivery to triple-negative breast cancer cells (Scheme 1). We therefore prepared a library of fructose-coated micelles using RAFT polymerization[24] to study the effect of micelle size and the way fructose is conjugated to the polymer on the cellular uptake. The main focus was the evaluation of the selectivity of the fructose micelle towards breast cancer cell compared to healthy cell lines.

Fructose-coated micelles with varying sizes were prepared by self-assembly of amphiphilic glycopolymers (Scheme 1). The key features of this strategy are related to the preparation of two fructose-based glycomonomers and the synthesis of corresponding block glycopolymers. The glycomonomers were prepared according to the following procedure. Fructose was selectively protected to form isopropylidene derivatives,[25] followed by functionalization of the only remaining hydroxyl group with methacrylate. After purification, two glycomonomers 3-*O*-methacryloyl-1,2:4,5-di-*O*-isopropylidene-*β*-D-fructopyranose (3-*O*-MA*i*PrFru) and 1-*O*-methacryloyl-2,3:4,5-di-*O*-isopropylidene-*β*-D-fructopyranose (1-*O*-MA*i*PrFru) were obtained. The polymerization of glycomonomers was carried out under good control in the presence of 4-cyanopentanoic acid dithiobenzoate (CPADB) via reversible addition-fragmentation chain transfer (RAFT) polymerization. The investigations of the polymerizations (Figure S1) indicated pseudo-first order kinetics of the reaction for both monomers. The polymerization reaction rate of 1-*O*-MAiPrFru was much faster than that of 3-*O*-MAiPrFru due to the difference of steric hindrance between two monomers. The obtained homo-glycopolymers were then used as macro RAFT agents for the following chain extension with *n*-butyl acrylate (BA) and 1wt% fluorescein *O*-methacrylate. By controlling the ratio of BA to macro RAFT agent, a series of diblock copolymers with different hydrophobic chain lengths were synthesized, which was followed by the removal of the isopropylidene groups through acidic hydrolysis. To investigate the influence of substituted hydroxyl group position of fructose ring on cellular uptake, the polymerization degree of block copolymers prepared from both glycomonomers was kept similar. The block copolymers were subsequently self-assembled using DMF as a solvent, followed by the addition of water and dialysis against water. The sizes of micelles were measured by dynamic light scattering (DLS) (Table 1). The results revealed that the micelle sizes grow with the increasing hydrophobic chain length of the block copolymer. All the micelles prepared in this study had diameters between 20 and 50 nm with narrow polydispersity (PDI). The sizes and morphologies of micelles were also observed using transmission electron microscopy (TEM) (Figure 1). Micelle sizes measured by TEM are slightly larger than those obtained from DLS, due to the collapse of micelles during the specimen preparation. The butyl acrylate blocks that act as micellar cores have a relatively low glass transition temperature (T_g) and could not support the spherical structures when the specimens were dry.[26] As a result, some of the dried micelles took on shapes beyond the expected round shape.

Table 1. Hydrodynamic Diameter of Micelles Measured by DLS.

	Block Glycopolymer	Size (nm)	PDI
A	poly(3-*O*-MAFru)$_{33}$-*b*-poly(BA)$_{100}$	23	0.18
B	poly(3-*O*-MAFru)$_{33}$-*b*-poly(BA)$_{202}$	30	0.12
C	poly(3-*O*-MAFru)$_{33}$-*b*-poly(BA)$_{300}$	38	0.17
D	poly(1-*O*-MAFru)$_{35}$-*b*-poly(BA)$_{74}$	20	0.27
E	poly(1-*O*-MAFru)$_{35}$-*b*-poly(BA)$_{198}$	27	0.13
F	poly(1-*O*-MAFru)$_{35}$-*b*-poly(BA)$_{348}$	37	0.11

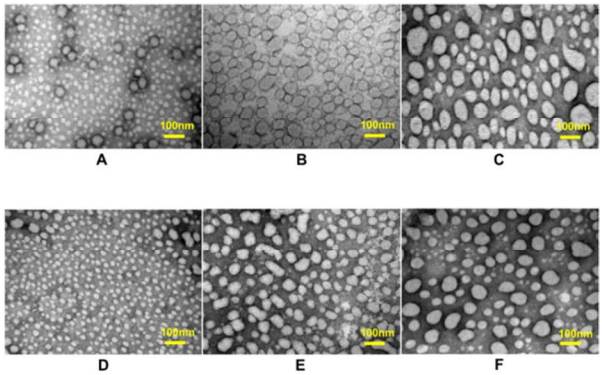


Figure 1. TEM Images of Micelles Prepared from Different Block Glycopolymers, Scale Bar: 100 nm, Uranyl Acetate Staining.

To compare cellular uptake of fructose-coated micelles, four different cell lines (Chinese hamster ovary CHO cells, RAW264.7 macrophages, MCF-7 and MDA-MB-231 breast cancer cells) were selected to evaluate their internalization behaviors. The breast cancer cell lines MCF-7, which is estrogen-receptor positive and MDA-MB-231, which is triple-negative, were used to mimic breast cancer tissues at early and late stages, respectively.

It needs to be considered that the cellular uptake is dependent on the stability of the micelle. Disassembly of the micelle can prevent the uptake by endocytosis[27] and will also affect the rate of exocytosis.[28] Therefore, the stability of the block copolymers were investigated using fluorescence spectroscopy revealing critical micelle concentrations (CMC) of around 1 μg/mL (Figure S5 in the Supporting Information).

Before the study of cellular uptake specificity, the cytotoxicity of micelles was assessed by SRB assay. The proliferation of breast cancer cell line MCF-7 in contact with the micelles, prepared from poly(3-*O*-MAFru)$_{33}$-*b*-poly(BA)$_{100}$ and poly(1-*O*-MAFru)$_{35}$-*b*-poly(BA)$_{74}$ respectively, was investigated at different concentrations over a period of 24 h (Figure S6 in the Supporting Information). Even at a high concentration of 250 μg/mL, none of the polymeric micelles were found to significantly inhibit cell proliferation, as compared to cells exposed to normal growth conditions. Therefore, the toxicity resulting from the synthetic procedure could be excluded.

Uptake specificity can often be hampered by the unspecific absorption of proteins before the drug carrier reaches its target. The fructose coated micelles were therefore incubated in a solution of 0.5 and 5wt% of BSA and the the hydrodynamic

diameter was determined after 1 and 10 h. Depicted in Figures S7-S9 in the Supporting Information, the hydrodynamic diameter did not change even after a long incubation time and at high BSA concentrations. The two population corresponding to micelle and BSA coexist without interfering with each other.

Internalization by cells was measured by flow cytometry after 24 h of exposure to micelles at a concentration of 50 µg/mL, which is well above the CMC (Figure 2). The amount of micelles taken up by cells was proportional to the measured fluorescence intensity since the fluorescent moieties were chemically linked to block glycopolymers. Prior to the flow cytometry analysis, the fluorescence intensity of the different micelles was determined to be able to adjust the output using the mean fluorescence intensity (MFI) of micelles, which was normalized after subtraction of the cellular autofluorescence (Table S2). As expected, the amount of micelles taken up by breast cancer cells (MCF-7 and MDA-MB-231) is much higher than that of normal cells (CHO and RAW264.7) in all six samples. This is likely due to the fact that MCF-7 and MDA-MB-231 cells overexpress fructose transporter GLUT5 on the cell membrane, while normal cells show limited expression.[2] The affinity between GLUT5 and fructose moieties on the micelle surface enhances the selective accumulation of micelles in MCF-7 and MDA-MB-231 cells over other normal cells. The limited amount of micelles internalized by RAW264.7 means that fructose-coated particles are 'invisible' to macrophages. The 'stealth effect' exhibited by these micelles will prolong their circulation time in the blood compartment and increase selective extravasation and accumulation in tumor sites when used as drug delivery systems.

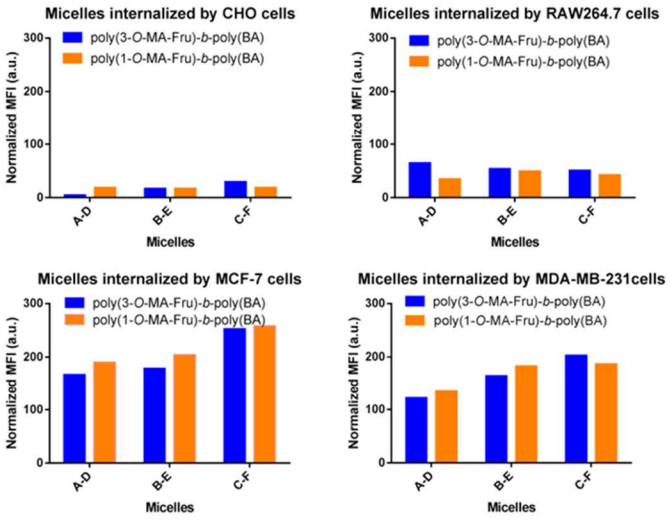


Figure 2. Flow Cytometry Analysis of Cellular Uptake of Different Micelles by CHO, RAW.264.7, MCF-7 and MDA-MB-231 Cells After 24 h of Incubation.

The internalization of micelles by MDA-MB-231 cells and CHO cells was also confirmed using confocal fluorescence microscopy (Figure 3). The higher fluorescence intensity indicates that more micelles were taken up by MDA-MB-231 cells than CHO cells. In addition, the merged image of MDA-MB-231 cells in Figure 3 (2) reveals that most micelles are accumulated in lysosomes after endocytosis. The extensive cellular uptake by both triple-positive and triple-negative breast cancer cells indicate the promising potential of fructose-coated micelles as a drug carrier for breast cancer therapy, especially for TNBC.

In addition, the quantitative uptake of micelles by MCF-7 and MDA-MB-231 cells was dependent on micelle size. All micelles prepared in this study are below 50 nm. In this scale range, the increase of micelle size significantly enhances the cellular uptake by breast cancer cells. This size effect is in agreement with a previous study by Chan's group.[29, 30] They reported that nanoparticles with a size around 50 nm exhibit the maximum uptake by cells via receptor-mediated endocytosis. Nanoparticles with a diameter less than 50 nm must be clustered together before internalization due to lack of free energy. It is also in agreement with the observation by ultrastructural immunocytochemistry that GLUT5 transporters are located in cell membranes 30 to 50 nm away from each other.[31] Therefore, the larger micelles should have a minimum size to enable multivalent binding of more than one fructose units at the same time.

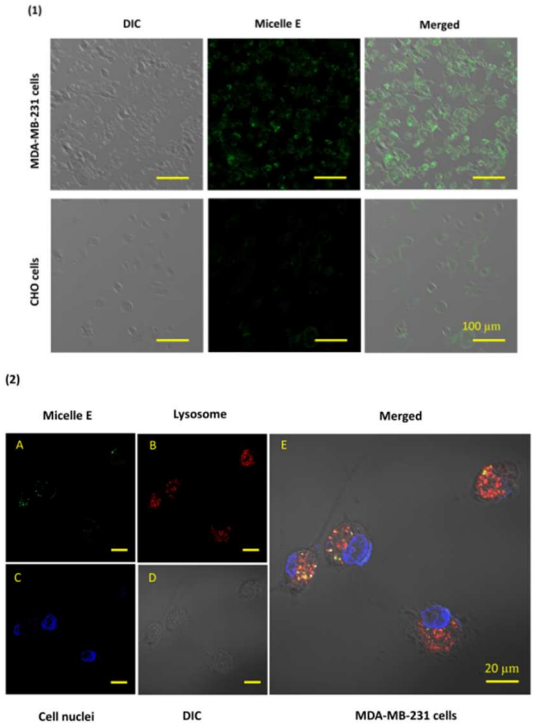


Figure 3. Cellular Uptake Measured by Confocal Fluorescence Microscopy. (1) Selective Uptake of Micelle E by MDA-MB-231 Cells and CHO Cells, Scale Bar: 100 µm; (2) Internalization of Micelle E by MDA-MB-231 Cells, Scale Bar: 20 µm. A. Green, Micelles; B. Red, Lysosome; C. Blue, Nuclei; D. Grey; E. Merged.

The micelles prepared from two different fructose-based monomers also show different cellular uptake selectivity. Both MCF-7 and MDA-MB-231 cells show higher uptake of micelles prepared from poly(1-*O*-MAFru)-*b*-poly(BA) than those from poly(3-*O*-MAFru)-*b*-poly(BA). On one hand this might be due to the fact that sugar rings are much closer to

backbone in the structure of poly(3-*O*-MAFru)$_n$-*b*-poly(BA)$_m$ than that of poly(1-*O*-MAFru)$_n$-*b*-poly(BA). On the other hand it has been reported that 3-*O*-substituted fructoses (3-*O*-allyl-substituents) are poorly tolerated (binding constant K_i = 90 mM) because the OH-group at 3-position is needed to closely interact with the GLUT5 pore. The more suitable conformation for binding to GLUT5 is the pyranose isomer of fructoses substituted at 1-position (K_i = 105 mM).[3]

Conclusions

To summarize, the development of smart nanoparticles that can achieve active targeting is a very valuable tactic to improve the therapeutic efficiency of triple-negative breast cancer. In this study, we reported two fructose-coated nanocarriers that have great potential to achieve targeted drug delivery to this type of cancer. These micelles exhibit breast cancer cell-specific uptake which is heavily dependent on micelle size and attachment site of fructose moieties. Since the internalization of micelles by triple-negative breast cancer cells is much higher than by normal cells, these non-cytotoxic, fructose-coated micelles can be used for targeted drug delivery to triple-negative breast cancer.

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Notes and references

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Electronic Supplementary Information (ESI) available: [Synthesis of monomers and block copolymers, preparation of micelles, NMR spectra, fluorescence spectra, CMC analysis, cytotoxicity assays, TEM, DLS in the presence of BSA, flow cytometry and confocal microscopy measurements can be found in the electronic supporting information.]. See DOI: 10.1039/b000000x/

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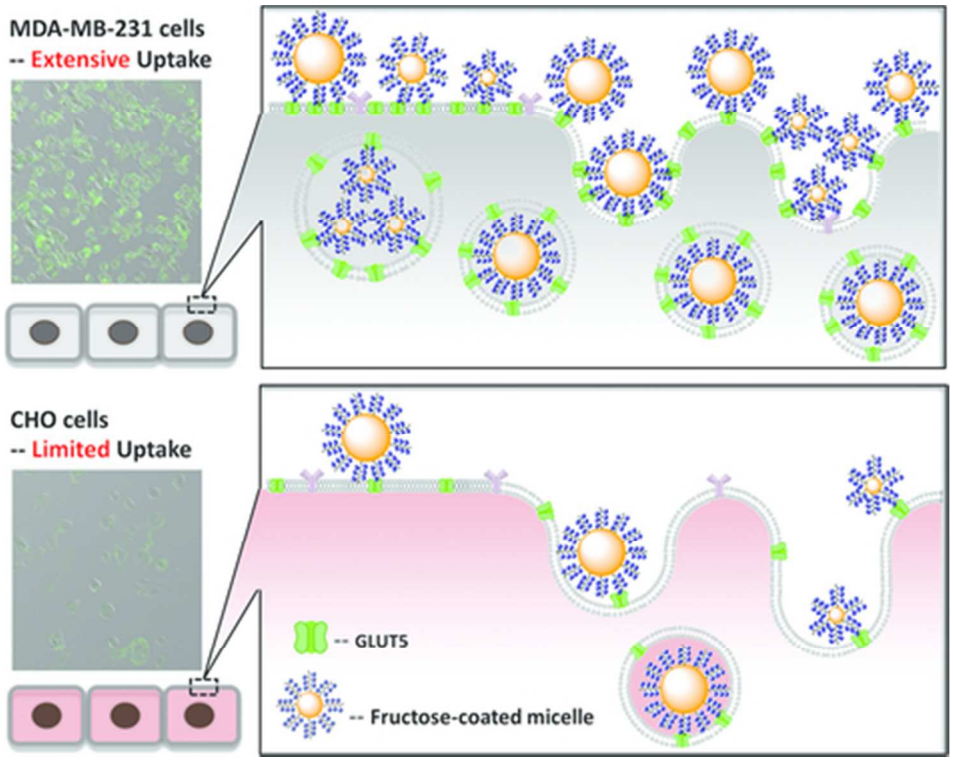

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