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Identification of Lectins from Metastatic Cancer Cells through Magnetic Glyconanoparticles

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Abstract

Cancer cells can have characteristic carbohydrate binding properties. Previously, it was shown that a highly metastatic melanoma cell line B16F10 bound to galacto-side-functionalized nanoparticles much stronger than the corresponding less metastatic B16F1 cells. To better understand the carbohydrate binding properties of cancer cells, herein, we report the isolation and characterization of endogenous galactose binding proteins from B16F10 cells using magnetic glyconanoparticles. The galactose-coated magnetic glyconanoparticles could bind with lectins present in the cells and be isolated through magnet-mediated separation. Through Western blot and mass spectrometry, the arginine/serine rich splicing factor Sfrs1 was identified as a galactose-selective endogenous lectin, overexpressed in B16F10 cells, compared with B16F1 cells. In addition, galactin-3 was found in higher amounts in B16F10 cells. Finally, the glyconanoparticles exhibited a superior efficiency in lectin isolation, from both protein mixtures and live cells, than the corresponding more traditional microparticles functionalized with carbohydrates. Thus, the magnetic glyconanoparticles present a useful tool for discovery of endogenous lectins, as well as binding partners of lectins, without prior knowledge of protein identities.

Keywords

cancer; carbohydrates; lectin; magnetic nanoparticles

1. Introduction

Lectins are carbohydrate binding proteins that are neither enzymes nor antibodies, and they can recognize various carbohydrates attached to proteins and lipids with high specificity.^[1] The interactions between endogenous lectins and carbohydrates mediate a variety of biological processes, including cell signaling,^[2,3] cell adhesion,^[4] immune responses,^[5,6] and pathogen-host recognition.^[7,8] On cancer cells, some lectins have been found to be

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overexpressed, and they contribute to neoplastic transformation, angiogenesis, tumor invasion and metastasis, anti-apoptosis, and escape immune surveillance.^[9–14] As a result, cancer cells can have very different carbohydrate binding properties, compared with normal cells. Thus, methods that can aid in the detection, isolation, and purification of endogenous lectins from cancer cells, preferably from their native environments, are highly desired.^[15]

A new tool which has been developed in the last decade for studying carbohydrate protein interaction is functionalized magnetic nanoparticles.^[16–24] Compared with micron-sized beads or resins,^[22,25] nanoparticles (NPs) have high surface area-to-volume ratios. As a result, high-density glycans can be immobilized onto the NPs, leading to increased avidity, due to the polyvalency effect.^[25] The magnetic properties of the NPs can enable not only the detection of binding via magnetic resonance imaging,^[24] but also the separation of target proteins from bulk media, aided by a magnet.^[18,20,21]

Previously, using a panel of magnetic glyconanoparticles (MGNPs), we discovered that a range of cells, including malignant versus normal cells and closely related tumor cells, can be readily distinguished, based on their characteristic signatures in carbohydrate binding.^[24] For example, even though the B16F10 and B16F1 melanoma cells are derived from the same parent cell line (isogenic cells), the highly metastatic B16F10 binds much stronger to galactose (Gal) functionalized magnetic nanoparticles (Gal-NPs) than the less metastatic B16F1 cells. However, it was unclear which lectins were responsible for the enhanced galactoside binding by B16F10 cells. In this work, we aim to identify the galactoside binding lectins endogenous to B16F10 cells using MGNPs.

Although glyconanoparticles have been extensively utilized to analyze carbohydrate-lectin interactions,^[26,27] the majority of studies were performed using pure lectins, with only a few reports on lectin purification and characterization from complex mixtures.^[20,28,29] We demonstrate that MGNPs can be useful in the isolation and identification of lectins from the whole cell environment, and are much more effective than traditional microparticles.

2. Experimental Section

2.1 Materials and Instrumentation

All chemicals were reagent grade and were used as received from the manufacturer, unless otherwise indicated. Iron (III) chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) was purchased from Honeywell Riedel-de Haen. Iron (II) chloride tetrahydrate ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$), polyvinylpyrrolidone (PVP), fetal bovine serum (FBS), phosphate buffered saline (PBS), high-glucose Dulbecco's modified Eagle medium (DMEM), Fluorescein isothiocyanate labeled Concanavalin A (FITC, Con A) from *Canavalia ensiformis*, tetramethyl-rhodamine-isothiocyanate labeled lectin from *Bandeiraea simplicifolia* (TRITC-BS-I), HEPES, phenylmethanesulfonyl fluoride (PMSF), protease inhibitor cocktail, sodium dodecyl sulfate (SDS), N,N,N',N'-tetramethylethylenediamine (TEMED), Tween-20, Triton X-100, reduced glutathione, and sodium azide (NaN_3) were purchased from Sigma Aldrich. Fluorescein isothiocyanate labeled annexin V was purchased from Southern-Biotech Aposcreen. Chemiluminescent HRP antibody detection reagent was purchased from Denville Scientific Inc. Ammonium hydroxide was purchased from Fisher Scientific, tetraethoxysilane (TEOS),

aminopropyl triethoxysilane (APTES), dithiothreitol (DTT), iodoacetamide were purchased from Acros. Benotriazole-1-yl-oxytris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) was purchased from Peptides International, while hydroxybenzotriazole (HOBt) was purchased from Chem Impex International. Sodium chloride (NaCl), potassium chloride (KCl), sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$), calcium chloride (CaCl_2) were purchased from CCI. Sodium carbonate (Na_2CO_3), ammonium bicarbonate (NH_4HCO_3) were purchased from Jade Scientific. Piperazine di-hydrochloride monohydrate was purchased from Alfa Aesar. Immobilized glutathione beads were purchased from Thermo Scientific. Carboxylic acid-functionalized magnetic microparticles, with a mean diameter of 3.13 μm , were purchased from Bangs Laboratories, Inc.

B16F10 and B16F1 melanoma cancer cell lines were purchased from American Type Culture Collection (ATCC). L-glutamine, streptomycin, and penicillin were purchased from Gibco. UltraPure Tris, ethylenediamine-tetracetic acid (EDTA), and UltraPure glycine were purchased from Invitrogen. 30 % Acrylamide/0.8 % bisacrylamide solution and ammonium persulfate were purchased from BioRad. Sequencing grade modified porcine trypsin was purchased from Promega. Isopropyl- β -D-thiogalactopyranoside (IPTG) was bought from Research Organic, ampicillin from Roche, and chloramphenicol from United States Biochemical Corporation.

The purification of soybean agglutinin (SBA) and the generation and characterization of anti-SBA antibodies have been previously reported.^[30] The constructs for the expression of proteins from pGEX vectors (Pharmacia), including glutathione S-transferases (GST), glutathione S-transferases fused galectin-3 (GST-Gal3), and rabbit polyclonal antibody reagents against GST and Gal3 have also been described.^[31] The pGEX vector for the expression of Sfrs1 was a kind gift from Dr. Benoit Chabot (Universite de Sherbrooke, Sherbrooke, Quebec, Canada).^[32] Polyclonal antibodies directed against Sfrs1 (rabbit) and Sfrs5 (mouse) were purchased from Novus Biologicals. Mouse monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was purchased from Biodesign. *E. coli* BL-21 codon plus (DE3) cells were from Stratagene.

Zeta potential was performed on a Zetasizer Nano zs apparatus (Malvern, UK) ; Fourier Transform Infrared Spectroscopy (FT-IR) was carried out on a Mattson spectrometer, Galaxy series, FTIR 3000; Thermogravimetric Analysis (TGA) was carried out on a Thermal advantage (TA-Instruments-Waters LLC) TGA-Q500 series ; and Transmission Electron Microscope (TEM) was carried on a JEM-2200FS. Inductively coupled plasma (ICP) analysis was carried out on a Varian 710-ES Axial ICP-OES.

2.2 Synthesis and Characterization of Iron Oxide Magnetic Glyconanoparticles

Gal-NPs, Man-NPs, and TEOS-NPs were prepared following previously reported procedures.^[24] Briefly, a solution of iron (III) chloride hexahydrate, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (20.0 mmol, 5.41 g, 5 mL), iron (II) chloride tetrahydrate, $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (10.01 mmol, 2.00 g, 2.5 mL), 28 % ammonium hydroxide (20 mL), and polyvinylpyrrolidone (PVP) (0.0256 g mL^{-1} , 0.65 mL) were stirred for one hour at 80 °C. The resulting black precipitate (nanoparticles) was isolated by an external magnet, and washed with deionized water (DI) and ethanol. The nanoparticles were coated with silica by reacting with TEOS (4.50 mmol, 1.0 mL) in 2-

propanol : water (4 :1 v/v, 150 mL) and ammonium hydroxide (0.2 % v/v, 0.3 mL) for 1 hour, to yield silica-coated magnetic nanoparticles (TEOS-NP). To prepare amine-functionalized nanoparticles, TEOS-NP was suspended in ethanol (150 mL), sonicated for 30 minutes, and reacted with aminopropyl triethoxysilane (APTES) (2 % w/v, 12.8 mmol, 3.0 mL) at 60 °C for 18 hours. The resulting amine-functionalized nanoparticles (APTES-NP) were isolated by an external magnet, washed with deionized water and ethanol, and dried under a vacuum pump.

Mannose and galactose monosaccharide, with carboxylic acid linkers, were synthesized following reported procedures.^[23] They were conjugated to the APTES-NP by amide bond formation using BOP and HOBt coupling chemistry to generate Gal-NPs and Man-NPs, respectively. The resulting carbohydrate-functionalized NPs were characterized by Zeta potential, FT-IR, TGA, TEM, and fluorescent labeled plant lectin binding studies, as described previously.^[23,24] FITC labeled Con A (a mannose-selective lectin) and TRITC labeled BSI (a galactose-selective lectin) were used. Plant lectin binding studies verified that the carbohydrate immobilized on the NP surface retained their biological properties.^[24]

2.3 Lysate Preparation

Lysate was prepared following reported procedures, with slight modifications.^[33] Briefly, B16F10 melanoma cell line was cultured in 100 mm cell culture plates at 37 °C and 5% carbon dioxide in a high-glucose DMEM growth medium, supplemented with 10 % FBS, 1 % L-glutamine, and 1% streptomycin penicillin. At 80 % confluence (approximately six million cells), the growth medium was removed and the cells were washed with PBS. 300 µL of low-salt homogenization buffer (20 mM Hepes-NaOH pH 7.4, 0.2 mM EDTA pH 8.0, 1 mM PMSF, protease inhibitor cocktail) was added. Cells were scraped and collected in a 1.5 mL microcentrifuge tube. Another 300 µL of low-salt homogenization buffer was added to the dish, which was scraped again and pooled with the first fraction in the microcentrifuge tube. The cells were incubated on ice for 5 minutes, and then subjected to 10 strokes in a tight-fitting Dounce homogenizer. An equal volume of high-salt homogenization buffer (300 mM NaCl, 200 mM Hepes-NaOH pH 7.4, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, protease inhibitor cocktail) was added to the homogenizer, followed by 10 additional strokes. The cell homogenate was centrifuged at 1000 *g* for 10 minutes at 4 °C and the supernatant was collected.

2.4 Silver staining, Coomassie Blue Staining, and Western Blot

2.4.1 Silver Staining—Silver staining protocols^[34] were followed. Briefly, the proteins were resolved on a nonreducing 12 % SDS-PAGE. The gel was fixed overnight at room temperature in 50 % methanol, 12 % acetic acid, and 0.05 % formaldehyde in deionized water. The gel was washed with 50 % ethanol (3 times, 20 minutes each) then pretreated in freshly made Na₂S₂O₃ · 5H₂O (0.2 g L⁻¹) for 1 minute. After washing thoroughly with deionized water, the gel was impregnated in freshly prepared silver nitrate-formaldehyde solution (0.2 g AgNO₃+75 µL of 37% formaldehyde in 100 mL of water) for 20 minutes, washed with deionized water, and developed in a freshly prepared solution of Na₂CO₃ (60 g), Na₂S₂O₃ · 5H₂O (25 mg), and 37 % formaldehyde (500 µL) in 1 liter. Once the bands were visible, the staining was stopped by immersing the gel in a solution consisting 50 %

methanol and 12 % acetic acid for 10 minutes, followed by washing in 50 % methanol for 30 minutes.

2.4.2 Coomassie Blue Staining—The proteins were resolved on a 12% SDS-PAGE, after which the gel was fixed overnight at room temperature in 40 % methanol and 7% acetic acid in deionized water. The fixing solution was discarded, and 1X working solution of brilliant blue G-colloidal concentrate (16 % concentrate, 20% methanol, and 64 % deionized water) added, and rocked for at least two hours. The staining chamber was tilted to check if protein bands of interest were visible. If not, the staining was allowed to proceed for a longer time until the bands were visible. The staining solution was recycled, and wash solution #1 (10 % acetic acid, 25 % methanol, and 65 % deionized water) was added and washed for 30 seconds. Wash solution #2 (25 % acetic acid, 75 % deionized water) was added, and washed for 5 minutes. This procedure was repeated until the gel background was clear and the protein bands were clearly visible.

2.4.3 Western Blot—After gel electrophoresis, the proteins were electrophoretically transferred onto Immun-Blot PVDF membrane (Bio-RAD).^[35] The PVDF membrane and the gel were briefly equilibrated in the transfer buffer (25 mM Tris, 193 mM glycine, and 20 % methanol) and put into the transfer cassette. After the transfer, the membrane was blocked overnight in 10 % Spartan dry milk in Tris-buffered saline containing Tween 20, pH 7.6 (50 mM Tris, 0.5 M NaCl, 0.02 % Tween 20, (T-TBS)), and rocked overnight at room temperature. Finally, the membrane was incubated with primary antibody, followed by HRP-conjugated secondary antibody, and the proteins were visualized using chemiluminescence HRP detection reagent in the dark room. Antibodies for immunoblotting were diluted in T-TBS containing 1% Spartan dry milk. Primary antibodies, rabbit anti-Gal-3, rabbit anti-Sfrs1, rabbit anti-Sfrs5, and rabbit anti-SBA, were used at a 1 : 1000 dilution, while secondary antibody, horseradish peroxidase conjugated goat anti-rabbit IgG was used at 1 : 10,000 dilution.

2.5 Isolation of Endogenous and Exogenous Lectins

For endogenous lectin, 300 μ L of lysate was transferred into a 1.5 mL microcentrifuge tube and the volume was brought to 450 μ L with buffer D+ (Buffer D: 10 mM HEPES, pH 7.9, 20 % glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT; D+: 60% Buffer D and 40 % H₂O). 50 μ L was saved, and the rest was incubated with 500 μ g of Gal-NPs, corresponding to 19 mg, 1.06×10^{-7} mol of immobilized galactose for 2h at 4 °C. After 2 h, magnetic batch separation of the NPs was carried out using a magnetic separator (Dexter Magnetic LifeSep 50SX). The field gradient at full field was approximately 23.3 T m^{-1} . The separation process involved the placement of the tube containing the magnetic sample in the magnetic separator. After 15 minutes, the initially homogeneous solution became heterogeneous, and a black deposit formed on the back wall where the gradient field was the highest. The supernatant, which contained the unbound fraction, was saved. NPs were washed 3 times with buffer D+, followed by sequential elution with 250 μ L of 0.5 M mannose solution and 0.5 M galactose solution. The saved fraction (5 μ L), supernatant (5 μ L), wash fraction (10 μ L), and the eluted fraction (10 μ L each) were resolved on 12 %

cross-linked SDS-PAGE, followed by blotting for Gal-3. As a control, the same experiment, as above, was repeated using Man-NPs and TEOS-NPs.

For exogenous lectin, 500 μL of lysate was spiked with 10 μL ($5 \text{ mg } \mu\text{L}^{-1}$); soybean agglutinin (SBA), a galactose binding plant lectin, was placed in a 1.5 mL microcentrifuge tube, and the volume was brought to 1000 μL with buffer D+. 50 μL was saved, and the rest was incubated with 0.5 mg Gal-NPs for 2 h at 4 $^{\circ}\text{C}$. After 2 h, magnetic separation was done and the supernatant, which contained the unbound fraction, was saved. NPs were washed 3 times with buffer D+, followed by elution with 500 μL 0.5 M galactose solution. The saved fraction (10 μL), supernatant (10 μL), wash fraction (5 μL), and the elution fraction (5 μL) were resolved on 12 % cross-linked SDS-PAGE, followed by blotting for SBA and Gal3.

2.6 Isolation of Lectins after Gal-NP Binding to Live Cells

Cells were cultured, and at 80 % confluence (approximately six million cells), the growth media was removed, and the cells were washed with PBS. 10 mL of $100 \mu\text{g mL}^{-1}$ of Gal-NP in serum-free DMEM medium was added, and the cells were incubated with the NP for 12 h at 37 $^{\circ}\text{C}$ in a 5 % CO_2 incubator. The cell lysate was prepared, either by Triton X-100 based lysis buffer or by homogenization in a Dounce homogenizer. For Triton X-100 based lysis buffer, the media was aspirated out, and cells were washed 3 times with PBS (0.5 mL) to remove any unbound NPs. 500 μL lysis buffer (0.1 % Triton X-100, 75 mM Tris, pH 7.2, 55 mM CaCl_2 , 10 mM NaN_3 , PMSF, and protease inhibitor) was added and rocked at 4 $^{\circ}\text{C}$ for 20 minutes. The lysate was transferred into a 1.5 mL microcentrifuge tube and subjected to magnetic separation to separate the supernatant from the NPs. The supernatant contained the proteins that did not bind to the NPs. In the homogenization protocol, the procedures described in the lysate preparation section were followed. The lysate was transferred into a 1.5 mL microcentrifuge tube and subjected to magnetic separation. The NPs were washed 3 times with PBS, followed by sequential elution with 0.5 M mannose solution and 0.5 M galactose solution. The supernatant, PBS wash fraction, mannose and galactose-eluted fractions were resolved by SDS-PAGE, and protein bands were visualized through silver and Coomassie staining.

2.7 LC/MS/MS

After SDS-PAGE, the gel was stained with Coomassie Brilliant Blue G (Brilliant Blue G-colloidal concentrate). Protein bands of interest were excised from the gel, and subjected to tryptic digestion, according to reported procedures, with slight modifications.^[36] Briefly, gel slices were dehydrated using 100 % acetonitrile and incubated with 10 mM dithiothreitol in 100 mM ammonium bicarbonate, pH ~8, at 56 $^{\circ}\text{C}$ for 45 minutes, dehydrated again and incubated in the dark with 50 mM iodoacetamide in 100 mM ammonium bicarbonate for 20 minutes. Gel slices were then washed with ammonium bicarbonate and dehydrated again. Sequencing grade modified trypsin was prepared to $0.01 \mu\text{g } \mu\text{L}^{-1}$ in 50 mM ammonium bicarbonate, and ~50 μL of this was added to each gel band, so that the gel was completely submerged. Bands were then incubated at 37 $^{\circ}\text{C}$ overnight. Peptides were extracted from the gel by water bath sonication in a solution of 60 % acetonitrile and 1% trifluoroacetic acid, and vacuum dried to ~2 μL . Peptides were then resuspended in 2% acetonitrile and 0.1 % trifluoroacetic acid to 20 μL , and submitted for proteomics. From this, 10 μL was injected by

a Waters nanoAcquity Sample Manager (www.waters.com), and loaded for 5 minutes onto a Waters Symmetry C18 peptide trap (5 μm , 180 $\mu\text{m} \times 20 \text{ mm}$) at 4 $\mu\text{L min}^{-1}$ in 5% acetonitrile and 0.1 % formic acid. The bound peptides were then eluted into a Waters BEH C18 nanoAcquity column (1.7 μm , 100 $\mu\text{m} \times 100 \text{ mm}$) over 16 minutes, with a gradient of 5% B to 30 % B in 9 minutes, using a Waters nanoAcquity ultra performance liquid chromatography (UPLC) (Buffer A =99.9 % water/0.1 % formic acid, Buffer B = 99.9 % acetonitrile/0.1 % formic acid), with an initial flow rate of 1 $\mu\text{L min}^{-1}$.

Eluted peptides were sprayed into a ThermoFisher LTQ Linear Ion trap mass spectrometer outfitted with a MICHROM Bioresources advance nano-spray source. The top five ions in each survey scan were then subjected to data-dependent zoom scans, followed by low-energy collision-induced dissociation (CID), and the resulting MS/MS spectra were converted to peak lists, using BioWorks Browser v 3.3.1 (ThermoFisher), with the default LTQ instrument parameters. Peak lists were searched against the SwissProt mouse protein sequence database, using the Mascot searching algorithm, v2.4 (www.matrixscience.com). The Mascot output was then analyzed using Scaffold, v3.6.5 (www.proteomesoftware.com) to probabilistically validate protein identifications using the ProteinProphet computer algorithm. Assignments validated above the Scaffold 95 % confidence filter were considered true. Mascot parameters for all databases were as follows : up to 2 missed tryptic sites were allowed ; fixed modification of carbamidomethyl cysteine ; variable modification of oxidation of methionine ; MS/MS tolerance of 0.6 Da; and peptide charge state limited to +2/+3. The list of major proteins identified is presented in Table S5.

2.8 Binding of GST and GST Fused Proteins to Gal-NPs

GST-Sfrs1 was expressed and purified as a fusion protein, with glutathione S-transferase (GST), in the same fashion as the method reported previously for the preparation of GST-Gal3,^[31] with modifications. Briefly, the E-coli strain BL21(DE3) carrying GST-Sfrs1 plasmid was grown in LB (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 5 g L^{-1} NaCl), containing 100 $\mu\text{g mL}^{-1}$ ampicillin and 34 $\mu\text{g mL}^{-1}$ chloramphenicol, followed by induction with 0.1 mM isopropyl- β -D-thiogalactopyranoside for 3h at 37 °C. Cells were then centrifuged, and the pellet was resuspended in lysis buffer (20 mM piperazine-HCl, pH 9.5, 0.5 M NaCl, 1 mM DTT, 1 mM PMSF, and protease inhibitor cocktail). Lysozyme was added to a final concentration of 100 mg L^{-1} , followed by incubation on ice for 30 min. The cells were lysed using a probe sonicator, carefully avoiding foaming the sample. Triton X-100 was added to a final concentration of 1% and the sample was rocked for 30 minutes at 4 °C. The lysate was centrifuged at 10,000 rpm for 10 min at 4 °C and the supernatant was purified, on the basis of GST binding to glutathione agarose beads (Thermo Scientific). First, the supernatant was incubated with glutathione agarose beads at 4 °C for 2 h, centrifuged at 500 g for 2 minutes to remove the unbound protein, washed 4 times with lysis buffer, and thereafter transferred into a column. The bound protein was eluted from the glutathione column with the lysis buffer containing 20 mM reduced glutathione.

In the experiment to test the binding of GST-Sfrs1 to Gal-NPs, 300 μg of Gal-NPs, corresponding to 11.4 μg , 6.36×10^{-8} mol of immobilized galactose, was incubated with 380 ng of GST-Sfrs1 for 30 minutes at 4 °C in the presence of 60 % buffer D+. Magnetic

separation was performed to isolate the supernatant, and it was saved as the unbound fraction (UF). The NPs were washed 3 times with 100 μ L buffer D, and the wash fraction (WF) was saved. The NPs were then solubilized in 40 μ L of sample loading buffer (contains 1% SDS) to dissociate the bound protein, followed by magnetic separation to isolate the bound fraction (BF) from the NPs. The same experiment was repeated with GST (negative control) and GST-Gal3 (positive control). The UF, WF, and BF of GST, GST-Gal3, and GST-Sfrs1 were subjected to SDS-PAGE analysis, and blotted for GST, Gal3, and Sfrs1, respectively.

To test for the binding specificity of Sfrs1 to Gal-NPs, 12 μ L (10 μ g of GST-Sfrs1) was added to 490 μ L of buffer D+ (500 μ L total volume). 20 μ L was saved, and the remaining (480 μ L) was incubated with 500 mg of Gal-NP, corresponding to 19 μ g, 1.05×10^{-7} mol of immobilized galactose, at 4 $^{\circ}$ C for 1 h. Magnetic separation was performed to isolate the supernatant, and it was saved as the unbound fraction. The NPs were washed 3 times with 100 μ L buffer D, and the wash fraction was saved. The bound protein was eluted with 0.5 M mannose solution, followed by 0.5 M galactose solution, and was saved as eluted fraction (EF). The saved fraction, UF, WF, and eluted fractions were subjected to SDS-PAGE analysis, and blotted for Sfrs1. As a negative control, the same experiment was repeated using TEOS-NPs.

2.9 Comparison of Sfrs1 from F10 and F1 using Gal-NPs

Using Gal-NP, the amount of Sfrs1 in B16F10 was compared with that in B16F1. Each cell line, seeded at a density of 2.5×10^5 cells cm^{-2} in 60 $\times$ 15 mm dishes was cultured at 37 $^{\circ}$ C, 5% CO_2 overnight. The growth media was removed, and the cells were washed 3 times with PBS (2 mL). 4 mL of 100 $\mu\text{g mL}^{-1}$ of Gal-NPs (400 $\mu\text{g NP}$, corresponding to 15 mg, 8.48×10^{-8} mol of immobilized galactose) in serum-free DMEM medium was added, and the cells were incubated with the NP for 12 h at 37 $^{\circ}$ C, in a 5 % CO_2 incubator. The medium was aspirated out, and the cells were washed 3 times with PBS (2 mL) to remove any unbound NPs. Cells were lysed using Triton X-100 lysis buffer as described above. The lysate was transferred into a 1.5 mL microcentrifuge tube and subjected to magnetic separation. The NPs were washed 3 times with PBS, followed by sequential elution with 0.5 M mannose solution and 0.5 M galactose solution. Only galactose-eluted fractions were subjected to SDS-PAGE analysis and blotted for Sfrs1, as we were interested in Sfrs1 that was specifically bound to Gal-NPs.

The amounts of Sfrs1 in B16F10 and B16F1 were compared in a quantitative experiment. Cell lysate was prepared from an equal amount of B16F10 or B16F1 cells (5×10^5 cells were used). Equal volumes of the two lysates were resolved by SDS-PAGE and blotted for Sfrs1, and the amount of Sfrs1 represented by the band intensity was quantified using ImageJ software.

2.10 Quantification of Western Blot Protein Gel Band using ImageJ Software

The quantification process follows the Beer-Lambert law, i.e., the band intensity is proportional to the protein concentration. First, the protein band intensities were measured as areas and converted to a percentage of the total area of the measured peaks, following

procedures described in the ImageJ user guide.^[37] This area was taken to represent arbitrary units of protein contained in the volume of the sample that was loaded onto the SDS-PAGE. The total amount of protein was obtained by taking the total volume for each fraction into account. The fraction “before Gal-NP” was taken to represent the total protein present before depletion with Gal-NP, and therefore, the percentage protein was calculated using this value as the base value.

3. Results and Discussion

3.1 Synthesis and Lectin Binding Specificity of MGNPs

Fe₃O₄ magnetite NPs were synthesized via the coprecipitation method from a mixed solution of ferric chloride and ferrous chloride, which were subsequently coated with silica to protect the magnetic core (Scheme 1a). The NP **1** was then functionalized with amine, and derivatized with galactose and mannose carboxylic acids **3** and **4**, leading to Gal-NPs and Man-NPs, respectively (Scheme 1b).

We found that zeta potential was a convenient method to monitor the progress of NP functionalization. The zeta potential of TEOS-NP **1** was −23.1 mV. Upon successful amine functionalization, the zeta potential NP-**2** became +17.4 mV, presumably due to the protonation of amines at neutral pH. Amide formation with the galactosyl and mannosyl acids reduced the amount of ammonium ions on the surface, rendering more negative zeta potential values of −11.7 mV and −6.04 mV, respectively. The NPs were characterized by TEM, DLS, and TGA, which yielded core diameters of 10 nm, and weight percentages of 3.8 % galactose and 5.5 % mannose on Gal-NP and Man-NP, respectively (Figure S1).

The carbohydrates immobilized on NPs retain recognition specificities, as demonstrated by lectin binding experiments. Gal-NP (4 mg of NP corresponding to 0.15 mg of Gal) was incubated with a fluorescently labeled galactose-selective lectin, TRITC labeled *Bandeiraea simplicifolia* lectin (BSI) in buffer. Upon applying a desktop magnet, NPs were removed from the mixture. The amount of protein in the supernatant was quantified, showing a reduction of 78 % in fluorescence intensity (Figure S3a). Incubation of the isolated Gal-NPs, with a large quantity (1 M, 375 mg) of free galactose, eluted the bound BSI from the NPs (~65 % of originally bound BSI). A similar experiment was carried out by incubating FITC-Con A, a mannose-selective lectin, with Man-NP. Man-NP showed a reduction of 80 % in fluorescence intensity, and the addition of 375 mg of free mannose led to the recovery of about 50 % of the originally bound Con A (Figure S3b). The eluted fractions of BSI and Con A were resolved on SDS-PAGE to confirm that the lectins were indeed recovered from the NPs (Figure S4).

3.2 Isolation of Exogenous Lectins from Cell Lysate

Our goal is to identify lectins from cancer cells. As cellular proteins can potentially nonspecifically bind with the glyconanoparticles and block the glycans from biological interactions, the ability of the MGNPs to separate lectins from other cancer cell contents was first tested by spiking galactose binding lectin soybean agglutinin (SBA) into the lysate of B16F10 melanoma cells. After incubating the lysate with Gal-NP (500 µg of NP

corresponding to 19 mg, 1.06×10^{-7} mol of immobilized galactose), a magnet was applied to the mixture to collect the NPs, followed by thorough washing with PBS buffer. The galactose binding proteins on the NPs were then released by incubation with a concentrated solution of free galactose (18 mg of Gal). The presence of SBA in the supernatant, PBS wash, and galactose-eluted solution was detected and quantified by Western blot using an anti-SBA antibody. When SBA was blotted, two bands were observed: (a) a prominent band at ~30 kD, corresponding to the molecular weight of the SBA polypeptide; and (b) a band at ~60 kD, corresponding to the SBA dimer (Figure 1, Panel A, lane 1), consistent with previous reports of this lectin.^[30] The unbound supernatant fraction yielded very little SBA (Figure 1, Panel A, lane 2 ~9% of SBA added). The majority of SBA (~78 % of SBA added) was found in the galactose-eluted fraction (Figure 1, Panel A, lane 4 and Table 1), suggesting that most of the exogenously added lectin was bound to the Gal-NP and could be released through galactose elution. This demonstrated that cellular proteins did not significantly impact the lectin binding abilities of Gal-NPs.

3.3 Isolation of Endogenous Lectin from Cell Lysate

The isolation of lectins endogenous to B16F10 cells was explored next. The lysate of B16F10 cells was incubated with Gal-NP (500 µg of NP, corresponding to 19 mg, 1.06×10^{-7} mol of immobilized galactose), and the NPs were isolated through magnet-induced precipitation. To establish the lectin binding specificity to the NPs, the NPs were first washed with a solution of mannose, followed by galactose elution. The various fractions were collected and analyzed by SDS-PAGE.

B16F10 is known to express a galactoside binding lectin, galectin-3 (Gal3), which is a member of the galectin family.^[38] Gal3 has been associated with cell migration and invasion in melanoma and the induction of metastasis.^[38,39] The presence of Gal3 in B16F10 is confirmed by a ~30 kD band on SDS-PAGE of lysates, with immuno-blotting by an anti-Gal3 antibody (Figure 1, Panel B, lane 1). The unbound supernatant fraction yielded a faint Gal3 band (Figure 1, Panel B, lane 2), suggesting that most of the Gal3 in the lysate was bound to the Gal-NPs. No Gal3 could be detected in the PBS wash fraction (Figure 1, Panel B, lane 3), while a minute amount of the protein was found in the mannose-eluted fraction (Figure 1, Panel B, lane 4). The bulk of Gal3, accounting for 68 % of the Gal-3 in the original lysate, could be recovered in the galactose-eluted fraction (Figure 1, Panel B, lane 5 and Table 2). The near depletion of Gal3 in the unbound supernatant fraction of the lysate incubated with Gal-NP (Figure 1, Panel B, lane 2) suggested that the MGNPs had a high capacity for lectins.

Gal3 is selective towards galactose, with no mannose affinity.^[40] To confirm the binding selectivity, TEOS-NP **1** with no carbohydrates (Figure 1, Panel C) and Man-NP (Figure 1, Panel D) were incubated with B16F10 cell lysate followed by magnet-induced separation and PBS wash, as well as mannose and galactose elutions. Western blot showed that most of the Gal3 were found in the unbound fractions (Figure 1, Panel C, lane 2 and Panel D, lane 2). No Gal3 was detected in the mannose or galactose-eluted fractions of NP **1** (Figure 1, Panel C, lanes 4 and 5) or Man-NP (Figure 1, Panel D, lanes 4 and 5), indicating the depletion of Gal3 from the lysate requires a Gal3 binding carbohydrate on the NP.

To demonstrate the advantages of using NPs vs the more traditional resins and beads, commercially available magnetic microparticles (average diameter 3.13 μm) were derivatized with galactose. The efficiency of Gal3 isolation by galactose microparticles was compared with that of Gal-NP at equal amounts of galactose. The percentage of Gal3 that was isolated by Gal-NP out of the total pool of Gal3 was three times higher than that by the microparticles, presumably due to the smaller sizes and larger surface-to-volume ratio of Gal-NP, resulting in enhanced rate and capacity for lectin binding (Figure S10a and Tables S2 and S4).

3.4 Proteomic Analysis of Proteins Bound to Gal-NP

Using cell lysate to study endogenous lectin can have some drawbacks, as the cell lysis procedures utilize detergents to disrupt cellular membranes, which may denature some candidate proteins. It is desirable to use live cells, since the lectins are in their native environments, allowing binding studies under physiological conditions. NPs are compatible with whole cell analysis, as Gal-NPs have been found to enter B16F10 cells and do not cause cytotoxicity,^[24] thus providing a platform for lectin discovery in live cells.

To establish the possibility of identifying endogenous lectins from live cells, B16F10 cells were incubated with Gal-NPs overnight at 37 °C. Overnight incubation was established as the optimal incubation time, with large quantities of Gal-NP endocytosed into the cells (Figures S5 and S6). The unbound particles were washed off and the cells were lysed. NPs were then recovered by magnetic separation, which was followed by PBS, mannose, and galactose washes. The various fractions were resolved on an SDS-PAGE gel and analyzed via silver staining. While many bands were present in (a) the supernatant after magnetic separation, representing the unbound fraction; and (b) the PBS wash fraction (Figure 2, Panel A, lanes 1 and 2), the galactose-eluted fraction yielded two prominent sets of bands at 15–20 kD and 30–35 kD, respectively (Figure 2, Panel A, lane 4). To confirm the abilities of proteins in the galactose-eluted fraction to bind with galactose, this fraction was subjected to gel filtration to remove bound galactose, and incubated with a fresh batch of Gal-NP. The bound proteins were eluted with free galactose again and analyzed via SDS-PAGE. Similar patterns of bands were observed to those from the first affinity selection (Figure 2, Panel B, lane 4), thus confirming that the bindings of those proteins with Gal-NPs were galactose dependent.

To determine the identities of the proteins bound to Gal-NPs, proteomic analysis was performed. The gel slice containing bands at ~37 kD (area I highlighted in Figure 2, Panel B, lane 5) was cut out and digested with trypsin. LC-MS-MS analysis of the tryptic digest fragments revealed two distinct polypeptides: (a) there were 12 matches, representing 10 distinct tryptic peptides (each with a carboxyl terminal lysine or arginine), with the amino acid sequence of the murine homolog of splicing factor 2 (SF2)/alternative splicing factor (ASF), hereafter designated as Sfrs1; and (b) there were 37 matches, representing 25 tryptic peptides, with the amino acid sequence of murine annexin V. A similar proteomic analysis was carried out on the gel slice containing the bands at ~15 kD (area II highlighted for Figure 2, Panel B, lane 5). Three histone proteins (H4, H2B, H2A) were identified as the polypeptides accounting for the bands in area II.

As a comparison, proteomic analysis was also performed on the corresponding regions of the SDS-PAGE from the mannose-eluted fraction of Gal-NP isolation, as well as the galactose-eluted fraction of TEOS-NP 1 isolation. From these samples, all derived from B16F10 cells, no proteins corresponding to Sfrs1, annexin V, or histone proteins were identified by LC-MS, suggesting that isolation of these proteins required galactose on the NPs.

3.5 Confirmation of Sfrs1 in the Galactose-eluted Fraction of Gal-NP by Western Blot

To confirm the presence of Sfrs1, the various fractions from Gal-NP binding with B16F10 cells were developed on an SDS-PAGE gel and immunoblotted with anti-Sfrs1. There was little Sfrs1 in the unbound, PBS wash, and mannose elution fractions (Figure 3, Panel A, lanes 1–4). However, a prominent positive band was observed in the material bound to Gal-NP and eluted with galactose (Figure 3, Panel A, lane 5). In contrast, antibodies against an irrelevant protein, Sfrs5, failed to yield a positive immunoblot in the galactose-eluted fraction. Therefore, the identification of Sfrs1 in the galactose-eluted fraction by LC-MS was confirmed by Western blot analysis.

Besides the detection of Sfrs1, the gel was also immunoblotted with anti-Gal3 mAb. In this case, the supernatant fraction after magnetic separation, representing the unbound fraction, yielded a prominent Gal3 band (Figure 3, Panel B, lane 1). As Gal-NP was able to completely deplete Gal3 from the lysate of B16F10 cells, the presence of a large amount of Gal3 in the unbound fraction suggests that some Gal3 molecules were sequestered in the cells in locations not accessible by Gal-NPs. A significant amount of Gal3 was found in the galactose-eluted fraction (Figure 3, Panel B, lane 5), consistent with the results of Figure 1. The reason that Gal3 was not identified in the proteomics studies was most likely because Gal3 (~30 kD) migrated to just below the ~37 kD region on the gel excised for analysis (area I in Figure 2, panel B).

It should be pointed out that the method for cell lysis after NP binding was important. When cells were lysed using a detergent-based lysing buffer (0.1 % Triton X-100, 75 mM Tris, pH 7.2, 55 mM CaCl₂, 10 mM NaN₃, PMSF and protease inhibitor), low molecular weight proteins that blotted with anti-Gal3 or anti-Sfrs1 were observed, suggesting possible protein degradation under this condition (Figure S7). These degradation products were not observed when cells were lysed through homogenization (Figure 3).

The performance of Gal microparticles in isolation of lectins from live cells was also compared with Gal-NP. Equal amounts of Gal microparticles and NPs were incubated with B16F10 cells. After removing the free particles in the extracellular space, the amounts of particles endocytosed by the cells were quantified by inductively coupled plasma optical emission spectrometry (ICP-OES). With Gal-NP, more than a two-fold increase in the amount of intracellular iron was observed, indicating more facile uptake of the NPs vs the microparticles (Table S4). In addition, the lectin isolation experiments were performed with the whole cells, using both Gal NPs and microparticles. Consistent with the experiment using lysate (Figure S10A), Gal-NP showed superior efficiency in purifying Gal3 from the whole cell, compared with the Gal microparticles (Figure S10B and Table S3).

3.6 Binding Properties of Sfrs1 Expressed as a Fusion Protein with GST

In the above experiments, Sfrs1 was observed in the galactose-eluted fraction after the whole cells were incubated with Gal-NP. It was possible that the observed binding of Sfrs1 occurred via its association with a galactose binding protein for which we had neither an antibody reagent nor mass spectrometry information. A major challenge in specific isolation and purification of proteins is the copurification of highly abundant proteins with low affinity for the immobilized compound, leading to false positive results.^[41] To test for direct binding of Sfrs1 with Gal-NP, Sfrs1 was expressed and purified as a fusion protein with glutathione S-transferase (GST), following a similar procedure to the preparation of GST-Gal3.^[31]

When GST alone was incubated with Gal-NP, almost all of the protein was recovered in the unbound fraction (Figure 4, Panel A, lanes 1–3). Therefore, GST (M_r ~27 kD) by itself does not interact with the Gal-NP, serving as a negative control. The positive control was GST-Gal3 (M_r ~57 kD). Although small amounts of GST-Gal3 were present in both the unbound and wash fractions, the majority of the fusion protein was found in the galactose elution fraction (Figure 4, Panel B, lane 3). When the binding experiments were performed using GST-Sfrs1, all the GST-Sfrs1 incubated with Gal-NP was recovered in the bound fraction, with no GST-Sfrs1 in the unbound or the wash fraction (Figure 4, Panel C, lanes 1–3).

To test for the specificity of GST-Sfrs1 binding to Gal-NP, the binding experiment between GST-Sfrs1 and Gal-NP was repeated, but this time the bound GST-Sfrs1 was subjected to sequential elution, with mannose solution followed by galactose solution after the PBS wash. The decrease in the GST-Sfrs1 band intensity in the unbound fraction, as compared with before the addition of Gal-NP, is an indicator for binding, but not for specificity (Figure 5, Panel A, lanes 1 and 2). After eluting with mannose and galactose solution, the blotting results revealed the presence of a GST-Sfrs1 protein band in the galactose-eluted fraction, but very minimal in the mannose-eluted fraction or the PBS wash fraction, supporting binding dependence on galactose (Figure 5, Panel A, lanes 3–5).

To further substantiate the binding specificity of GST-Sfrs1 to Gal-NP, the binding experiment was repeated with TEOS-NP **1** as a negative control, to demonstrate the binding was not due to nonspecific absorption by NPs (Figure S8). As indicated by the blotting results, the majority of GST-Sfrs1 failed to bind to the NPs (Figure S8, lanes 1 and 2). In addition, there were no GST-Sfrs1 protein bands in the mannose or galactose-eluted fractions (Figure S8, lanes 4 and 5).

Glycan microarray screening is a powerful high throughput technique to decipher the carbohydrate binding activities of proteins.^[42] Recently, Fukuda and coworkers performed glycan microarray screening and ELISA assay of Sfrs1.^[43] Sfrs1 has been shown to bind with a wide range of glycans, including LacNAc and sialyl Lewis X, which contain galactose at the nonreducing terminal. Our results on Gal-NP binding by Sfrs1 are consistent with the findings from the Fukuda group.

As presented above, besides Sfrs1, annexin V and histone proteins have been identified via proteomics from the galactose-eluted fractions of Gal-NP bound proteins. To test the glycan

binding properties of these proteins, recombinant annexin V and histones (a mixture of H3, H4, H2B, H2A from chicken) were submitted to the Consortium for Functional Glycomics (CFG) and screened on a 610 member glycan microarray, covering a wide range of mammalian glycans at multiple concentrations. Interestingly, no significant binding signals were observed on the microarray, suggesting that these proteins exhibited little affinity to any of the glycan components up to 0.1 mM. In a separate experiment, histone proteins were incubated with Gal-NPs, which was followed by magnetic separation and PBS wash. Subsequent elution with a concentrated solution of galactose eluted few histone proteins. These results confirmed the low affinity of histones for galactoside.

3.7 Comparison of the Amount of Sfrs1 Isolated on Gal-NP from B16F10 versus B16F1 Cells

Our previous studies had documented that there was a higher binding of Gal-NP to B16F10, a more metastatic melanoma cancer cell line, compared with its less metastatic isogenic counterpart, B16F1.^[24] It is possible that the quantitative difference in Gal-NP binding observed between B16F10 and B16F1 cell lines could be explained, in part, by the difference in the expression levels of Sfrs1. To determine this, equal amounts of lysates from these two cell lines were resolved on an SDS-PAGE gel and blotted for Sfrs-1. As shown in Figure 6, B16F10 was found to express more Sfrs1, as compared with its isogenic counterpart B16F1 (Figure 6, Panel B). In separate experiments, B16F10 and B16F1 cells were incubated with Gal-NPs and subjected to magnetic separation and galactose elution. The galactose-eluted fractions were blotted for Sfrs1, which showed an elevated level of Sfrs1 in B16F10, as compared with B16F1 (Figure 6, Panel A), demonstrating higher amounts of Sfrs1 accessible to Gal-NP in B16F10 cells.

3.8 Discussion

Splicing factors are normally present in the nucleus mediating RNA splicing. The Fukuda group recently reported the novel finding that Sfrs1 exists on the cell surface of endothelial cells, functioning as a cell adhesion molecule to facilitate cancer cell metastasis.^[43] As discussed above, recombinant Sfrs1 can bind to a variety of glycans, including those terminated with galactose at the nonreducing end. It is important to note that the splicing factor binding to carbohydrate was calcium dependent, a characteristic exhibited by C-type lectins.^[43] Besides cell surface presence and their well-known site of action, i.e., in the nucleus, Sfrs proteins can be present in the cytoplasm. Cytoplasmic accumulation of Sfrs1 was reported to be controlled by arginine methylation. Mutations that block methylation and remove positive charges result in the cytoplasmic accumulation of Sfrs1.^[44] The carboxy-terminal arginine/serine-rich domain and the presence of active RNA-recognition motifs is required for Sfrs1 shuttling to the cytoplasm.^[45] Once in the cytoplasm, Sfrs1 was found to stimulate translation and the cytoplasmic mRNA targets for shuttling Sfrs1 have been identified.^[16,46] These properties of Sfrs1 proteins are consistent with those observed for Gal1 and Gal3. These two galectins have been reported to be present in the nucleus and cytoplasm, and on the cell surface, modulate pre-mRNA splicing activities, are overexpressed in metastatic cancer cells, play a role in tumor growth and development, and are able to bind carbohydrates.^[31,40,47–51] Taken together, Sfrs1 has strong similarities to

Gal3 in function, and joins a growing list of carbohydrate binding proteins that are multifunctional and exist in multiple cellular locations.

From our studies, higher levels of Sfrs1 have been isolated from the highly metastatic B16F10 cells, compared with those from the less metastatic counterpart B16F1 cells. The role of Sfrs1 in promoting cancer growth and development through alternative splicing has been reported.^[52,53] Sfrs1 was found to be upregulated in various human tumors, relative to their respective normal controls, including tumors of the colon, thyroid, small intestine, kidney, and lung.^[53,54] Its overexpression was found to be sufficient to transform immortalized cell lines. Moreover, Sfrs1 was shown to control the alternative splicing of the tumor suppressor BIN1, and the resulting BIN1 isoform lacked tumor suppressor activity. The oncogenic activity of Sfrs1 may also be due to its implication in multiple cellular programs, as it regulates the alternative splicing of genes implicated in proliferation, apoptosis, and cell motility, an activity important for tumor metastasis.^[53,55] These findings imply Sfrs1 has a significant role in cancer growth and development, and is therefore a potential target for the development of cancer therapies and diagnostics.

The glycan microarray screening results of annexin V and histone proteins suggest that they have weak affinities with all components, including galactosides, on the array. It should be pointed out that some members of the annexin family, including annexin V, have been documented to bind with carbohydrates. For example, annexins IV, V, and VI can bind with glycosaminoglycan affinity columns to facilitate purification, although the binding constants are not known.^[56,57] Annexin V has been reported to bind to sugar chains containing bisecting N-acetyl-glucosamine, with the relatively low affinity of 0.2 mM.^[58] Highly sulfated heparan sulfate/heparin were identified as preferred ligands for annexin A1.^[59] The CFG microarray does not contain glycosaminoglycans, and binding with K_d values higher than 0.1 mM could not be well detected. Thus, the lack of binding signals from annexin V and histones most likely reflects the weak affinities of these proteins with the components on the current glycan microarray under the experimental conditions.

The isolation of annexin and histone proteins by Gal-NP can be potentially explained by the interactions of these proteins with galactoside binding proteins, forming protein-protein complexes on the Gal-NPs. Related to our results, it has been reported that splicing factors, including Sfrs1, colocalize and interact with histones, contributing to the regulation of alternative splicing.^[60,61] Annexins have been shown to directly interact with Gal3.^[62,63] Thus, it is possible that the isolation of histones and annexin may be as a result of their complexations with Sfrs1 or Gal3 on the Gal-NP, resulting in their coisolation by magnet-mediated separation. The exact identity of the binding partners and the physiological importance of these interactions will require further investigation.

4. Conclusion

In this study, we report the utilization of MGNPs to isolate endogenous lectins from the B16F10 melanoma cancer cell line, using both the whole cell and the cell lysate. Gal-NP facilitated the purification of endogenous Gal3 through a magnet-induced purification protocol. The identity of Gal3 was confirmed by Western blot and the specificity of Gal-NP

binding to Gal3 was verified using carbohydrate elution protocols. The successful isolation of a well-known galactose binding lectin, Gal3, using Gal-NP implies the possibility of finding other lectins (either known or unknown) in the B16F10 cell line that can interact with galactose. Indeed, Sfrs1 has been isolated by Gal-NP, which was confirmed by mass spectrometry, Western blot, carbohydrate elution protocol, and direct binding of GST fused Sfrs1. Caution needs to be taken that not all proteins isolated by MGNPs are lectins, as some proteins may be co-isolated through their complexations with the lectins bound to the MGNPs. MGNPs have also been shown to be superior to the corresponding glyco microparticles in lectin isolation from both live cells and cell lysates. Therefore, MGNPs can be a useful tool for the field of glycoproteomics by providing exciting opportunities to discover novel lectins endogenous to cells, as well as the identification of the binding partners of the lectins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Binding of galactose-specific lectins to Gal-NP, Man-NP, and TEOS-NP 1, as detected by Western blot, using anti-SBA or anti-Gal3 antibodies, respectively. Panel A: Isolation of exogenous lectin (SBA) spiked in B16F10 lysate using Gal-NP; lane 1: before addition of glyconanoparticles ; lane 2: unbound fraction (supernatant); lane 3: PBS wash ; lane 4: galactose elution. Panel B: Blotting for Gal3 isolated from B16F10 cell lysate using Gal-NP; lane 1: B16F10 lysate; lane 2: unbound fraction (supernatant); lane 3: PBS wash; lane 4: mannose elution; lane 5: galactose-eluted fraction. Panel C: Isolation of Gal3 using TEOS-NP 1; lane 1: B16F10 lysate ; lane 2: unbound fraction (supernatant); lane 3: PBS wash ; lane 4: mannose elution; lane 5: galactose elution. Panel D: Isolation of Gal3 using Man-NPs; lane 1: B16F10 lysate; lane 2: unbound fraction (supernatant); lane 3: PBS wash ; lane 4: galactose elution; lane 5: mannose elution. The numbers on the left side of each panel indicate the positions of the corresponding molecular weight markers.

Figure 2.

Identification of endogenous proteins bound to Gal-NPs. Panel A: B16F10 cells were incubated with Gal-NPs overnight, lysed, subjected to magnetic separation, followed by elution of bound proteins ; lane 1: supernatant after magnetic separation ; lane 2: PBS wash fraction; lane 3: mannose-eluted fraction; lane 4: galactose-eluted fraction. Panel B: Galactose-eluted fraction passed through a desalting column to remove free galactose and subjected to fresh Gal-NP binding followed by sequential elutions ; lane 1: before addition of Gal-NPs ; lane 2: PBS wash; lane 3: mannoseeluted fraction; lane 4: galactose-eluted fraction. The bands around 37 kD and 15 kD were subjected to trypsinic digestion, followed by LC/MS/MS analysis. The numbers on the left side of each panel indicate the positions of the corresponding molecular weight markers.

Figure 3.

Identification of endogenous lectins from whole cells using Gal-NP. B16F10 cells were incubated with Gal-NPs overnight. After incubation, cells were lysed through homogenization, followed by the elution protocols described above. Panel A: Blotting for Sfrs1; lane 1: residual lysis buffer after magnetic separation (supernatant); lanes 2 and 3: PBS wash fraction; lane 4: mannose-eluted fraction; lane 5: galactose-eluted fraction. Panel B: Blotting for Gal3; lane 1: residual lysis buffer after magnetic separation (supernatant); lanes 2 and 3: PBS wash fraction; lane 4: mannose-eluted fraction; lane 5: galactose-eluted fraction. The numbers on the left side of each panel indicate the positions of the corresponding molecular weight markers.

Figure 4.

Binding of GST, GST-Gal3, and GST-SFRS1 to Gal-NPs. Panel A: Binding of GST to Gal-NPs; lane 1: unbound fraction (supernatant); lane 2: wash fraction; lane 3: bound fraction eluted with galactose. Panel B: Binding of GST-Gal3 to Gal-NPs ; lane 1: unbound fraction (supernatant); lane 2: wash fraction; lane 3: bound fraction eluted with galactose. Panel C: Binding of GST-Sfrs1 to GalNPs; lane 1: unbound fraction (supernatant); lane 2: wash fraction; lane 3: bound fraction eluted with galactose. All of the blotting was carried out with the same anti-GST antibodies. The numbers on the left side of each panel indicate the positions of the corresponding molecular weight markers.

Figure 5.

Further confirmation of the binding specificity of GSTSfrs1 to Gal-NPs. Western blot results with anti-Sfrs1, and histogram representation of band intensities, as determined by ImageJ. Lane 1: before addition of Gal-NP ; lane 2: unbound fraction (supernatant); lanes 3 and 4: PBS wash fraction; lane 5: mannose-eluted fraction; lane 6: galactose-eluted fractions. The numbers on the left side of the Western blot results indicate the positions of the corresponding molecular weight markers.

Figure 6.

Comparison of Sfrs1 in B16F10 and B16F1 cells. Panel A: 2.5×10^5 cells of either B16F10 or B16F1 were cultured overnight, Gal-NP was added and cultured for an additional 12 h. Cells were lysed, subjected to magnetic separation, washed with PBS and the bound fraction eluted sequentially with mannose and galactose solutions. The galactose-eluted fraction was resolved on SDS-PAGE and blotted for Sfrs-1. Western blot results and histogram representation of the band intensities, as analyzed by ImageJ software, are shown. Lane 1: B16F10; lane 2: B16F1. Panel B: Lysate was prepared from 5×10^5 cells of each cell line, and equal volumes of the lysate were resolved on SDS-PAGE and blotted for Sfrs1 and GAPDH. Western blot results and histogram representation of the band intensities, as analyzed by ImageJ, are shown. Lane 1: B16F10 ; lane 2: B16F1. Blotting for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the loading control. The numbers on the left side of the Western blot results indicate the positions of the corresponding molecular weight markers.

Scheme 1.
 Synthesis of glycolnanoparticles.

Table 1

Quantification of SBA in fraction after incubation with Gal-NP.

Fractions	Band intensity (From Figure 1 Panel A)	Volume (μL)	Amounts of SBA (Arbitrary units corrected for volume)	% of SBA in fraction
Before Gal-NP	53	1000	5301	
Unbound	5	1000	482	9
Wash	1	500	91	2
Galactose elution	41	500	4126	78

Table 2

Quantification of Gal3 in fraction after incubation with Gal-NP.

Fractions	Band intensity (from Figure 1, Panel B)	Volume (μL)	Amount of Gal3 (arbitrary units corrected for volume)	% of Gal3 in fraction
Before Gal-NP	26	450	2329	
Unbound	5	450	451	19
Wash	1	250	25	1
Mannose elution	5	250	118	5
Galactose elution	63	250	1586	68