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Fluorophore-assisted carbohydrate electrophoresis: a sensitive and accurate method for the direct analysis of dolichol pyrophosphate-linked oligosaccharides in cell cultures and tissues

Ningguo Gao*

Department of Pharmacology, UT-Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9041, USA

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Abstract

Lipid-linked oligosaccharides (LLOs) such as $Glc_3Man_9GlcNAc_2$-P-P-dolichol are the precursors of asparagine (N)-linked glycans, which are essential information carriers in many biological systems, and defects in LLO synthesis cause Type I Congenital Disorders of Glycosylation. Due to the low abundance of LLOs and the limitations of the chemical and physical methods previously used to detect them, almost all studies of LLO synthesis have relied upon metabolic labeling of the oligosaccharides with radioactive sugar precursors such as [^{3}H]mannose or [^{14}C]glucosamine. In this article, a procedure is presented for a facile, accurate, and sensitive non-radioactive method for LLO analysis based on fluorophore-assisted carbohydrate electrophoresis (FACE). First, LLOs are extracted and partially purified. Next, oligosaccharides released from LLOs are labeled with negatively charged fluorophores: 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) or 7-amino-1,3-naphthalenedisulfonic acid (ANDS). A specialized form of polyacrylamide gel electrophoresis is then used to resolve and measure ANTS or ANDS labeled oligosaccharides. Finally, the resolved oligosaccharides are detected and quantified by fluorescence imagers using CCD cameras.

Keywords: Glycosylation; Lipid-linked oligosaccharide; Dolichol; Fluorophore-assisted carbohydrate electrophoresis

1. Introduction

In the lumenal space of the endoplasmic reticulum (ER), asparagine (N)-linked glycoproteins are formed by transfer of preformed oligosaccharide units from lipid-linked oligosaccharide (LLO) donors to nascent polypeptides with asparaginyl residues in the context Asn-X-Ser/Thr [1,2]. Completed LLOs have the structure $Glc_3Man_9GlcNAc_2$-P-P-dolichol, the preferred substrate for oligosaccharyl transferase compared with premature LLO intermediates [3]. LLO synthesis is highly conserved and requires a series of glycosyltransferase reactions. The first seven sugar transfer reactions take place on the cytoplasmic leaflet of the ER membrane and generate the lipid-linked intermediate $Man_5GlcNAc_2$-P-P-dolichol, which requires two equivalents of UDP-GlcNAc and five of GDP-mannose. By a mechanism still not fully understood, $Man_5GlcNAc_2$-P-P-dolichol flips to the lumenal leaflet, where it is the acceptor substrate for seven additional glycosyltransferase reactions using four equivalents of mannose-P-dolichol and three of glucose-P-dolichol as donors. This results in synthesis of $Glc_3Man_9GlcNAc_2$-P-P-dolichol, and this process is defective in the Type I congenital disorders of glycosylation (CDG), a family of at least 12 distinct genetic diseases [4–6]. After the oligosaccharide unit from $Glc_3Man_9GlcNAc_2$-P-P-dolichol is transferred to protein, the resultant N-linked oligosaccharide is processed in the ER by a series of glucosidases and

* Fax: +1 214 648 8626.
E-mail address: ningguo.gao@utsouthwestern.edu.

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mannosidases, in most cases ending in the structure $Man_8GlcNAc_2$. After exiting the ER, N-linked oligosaccharides of glycoproteins undergo additional processing reactions involving glycosidases and glycosyltransferases in the Golgi complex and *trans*-Golgi network. These N-linked oligosaccharides have specific roles in the folding, processing, and export of glycoproteins from the ER, as well as for functions that occur after exiting the secretory pathway [7].

A thorough knowledge of LLO synthesis is therefore essential to understand both ER function and the pathophysiology of diseases like CDG Type I. LLO analysis usually requires hydrolysis of the pyrophosphate bond, allowing the free water-soluble oligosaccharides to be fractionated and characterized, though recently an efficient fractionation of intact LLOs has been reported [8]. Since abundance of LLOs is low, typically of the order of 1 nmol/g tissue [9,10], detection of the oligosaccharides by physical and/or chemical means has been inefficient. It is common to study LLOs that have been made radioactive by incubations of cells, organelle preparations, or tissue slices with appropriate ^3H- or ^{14}C-labeled precursors. The incorporated isotopes then permit facile detection of the oligosaccharides.

Although isotopic approaches have been extremely useful for LLO analysis, they have a number of limitations: (1) Isotopic labeling in normal culture medium is often inefficient unless low glucose concentrations are used (5–10-fold below the physiological range), subjecting the cultures to potential glucose deprivation effects [11]. (2) The results obtained by metabolic labeling may not reflect the true steady-state LLO compositions since they are typically done for a brief incubation period (20–60 min). (3) Due to isotope dilution, it is difficult to determine the actual molar quantity of each LLO species from the amount of radioactivity incorporated. Intermediates with few sugars may be difficult to detect. (4) Metabolic labeling is difficult in living animals. Pool dilution and catabolism would require the use of very large quantities of radioactive compounds. Although LLO compositions from tissue of large animals, like bovine pancreas [9,10], have been studied by direct non-isotopic analysis of the oligosaccharides, these approaches were laborious, required large amounts of tissue, and might not be practical with small experimental animals such as the mouse or rat.

In this review, I describe the use of fluorophore-assisted carbohydrate electrophoresis (FACE) [12,13] to circumvent these problems. With the use of a commercial fluorescence scanner, multiple samples are easily processed. One to two picomoles of oligosaccharide released from each LLO species is detected, and detection is independent of the number of residues per oligosaccharide. In this article, protocols are provided for LLO extraction, partial purification, fluorophore labeling, and quantitation.

2. Methods

2.1. LLO extraction and partial purification

2.1.1. Reagents

CM: chloroform methanol mixed at a ratio of 2:1 (v/v).
CMW: chloroform, methanol, and water mixed at a ratio of 10:10:3 (v/v).
DEAE–cellulose (Bio-Rad, #731-1550), converted to acetate form by washing with 1 M acetic acid (in CMW).
A 3 M NH_4OAc stock solution prepared by dissolving 3 mol NH_4OAc into a total volume of 1000 ml methanol with 3% acetic acid.

2.1.2. Protocol

2.1.2.1. LLO extraction from cultured cells.

1. Wash the cells (90% confluence, $>10^7$ cells, typically four 15 cm dishes for dermal fibroblasts or one 10 cm dish for CHO-K1 cells) with ice cold PBS twice, add room temperature methanol, harvest the cells by scraping, and transfer to a 10 ml glass tube (Kimble, #73790-10).
2. Sonicate for 5–10 min in a waterbath sonicator, and dry under a stream of N_2 gas.
3. Add 10 ml CM to the tube, and sonicate for 5–10 min with occasional vortexing. Centrifuge (3000*g* for 10 min at room temperature) and discard the supernatant. Repeat once.
4. Resuspend pellet in 2 ml methanol by sonication, and dry under N_2 gas.
5. Add 10 ml water to the tube, sonicate for 5–10 min with occasional vortexing, centrifuge, and discard the supernatant. Repeat once.
6. Resuspend pellet in methanol by sonication. Dry under N_2 gas.
7. Add 10 ml CMW to the tube, sonicate 5–10 min with occasional vortexing. Centrifuge and collect the supernatant. LLOs are extracted in this fraction.

2.1.2.2. LLO extraction from animal tissue.

1. Harvested animal tissues should be used immediately or frozen in liquid nitrogen. Fresh or frozen tissues are disrupted into 10 volumes of methanol with a Bronson homogenizer ("polytron") for 30 s at a setting of 6.
2. The methanolic suspension is dried under N_2 gas.
3. The remaining steps are the same as for cultured cells beginning at step 3.

Note. When drying the samples in methanol under nitrogen gas in the first step of LLO extraction, the samples can easily form hardened pellet chucks. To avoid

this, carefully dry the samples until slightly damp with no liquid visible. Then, add 5–10 ml CM to resuspend the pellet by sonication. For large pellets, such as from animal tissues, sonication can be repeated 2 or 3 times. Samples dried in this way form a loose powder, and the LLO recovery is very high.

2.1.2.3. LLO partial purification.

1. Load above CMW extract onto a DEAE–cellulose column pre-equilibrated with CMW, using 1 ml bed volume per 5×10^7 cells or 500 mg wet tissue.
2. Wash with 10 bed volumes of CMW.
3. Wash with 10 bed volumes of 3 mM acetic acid in CMW.
4. Elute with 10 bed volumes of 300 mM NH_4OAc in CMW.
5. Add 4.3 bed volumes of chloroform and 1.2 bed volumes of water to the tube, and vortex. Centrifuge (3000*g*, 10 min). Remove the upper phase carefully without disturbing the middle layer (most of the salt is removed by this step). Dry the lower phase under N_2 gas.

Note. When loading the CMW extract onto a DEAE–cellulose column, or during the washing and eluting steps, recovery is optimal if the flowrate is kept below 0.5 ml/min. This can usually be achieved by restricting the flow with a 200 µl flat-end gel loading tip attached to the bottom of the column.

2.1.2.4. Release of oligosaccharide from the LLO.

1. To the dried residue of the previous step, add 2 ml 0.1 N HCl (in 50% isopropanol), vortex, incubate at 50 °C for 60 min, and dry under N_2 gas.
2. Add 1 ml butanol-saturated water to the tube, vortex, then add 1 ml water-saturated butanol. Vortex, centrifuge, and collect the lower phase containing the released oligosaccharide. Freeze dry.
3. Resuspend the dried oligosaccharide/salt mixture in 1 ml water, and add 200 µl (packed volume) of AG50W-X8 (hydrogen form) cation exchange resin (Bio-Rad). Mix for 5 min, centrifuge, and collect the supernatant.
4. Add 200 µl (packed volume) of AG1-X8 (formate form) anion exchange resin (Bio-Rad) to the tube, mix for 5 min, centrifuge, and collect the supernatant containing the oligosaccharide released from LLO.

2.2. Derivatization of released oligosaccharides with ANTS and ANDS

2.2.1. Reagents

Fluorophores: ANTS (8-aminonaphthalene-1,3,6-trisulfonate, catalog #A-350) is from Molecular Probes. ANDS (7-amino-1,3-naphthalenedisulfonic acid, #14644-7) and sodium cyanoborohydride (#15615-9) are from Aldrich.

Fluorescent oligosaccharide standards: a known amount of maltooligosaccharide mixture, such as that formerly offered by Phanstiehl (#M-138), is used as the oligosaccharide standard. In addition, a partial acid hydrolysate of glycogen can be used to create a ladder.

2.2.2. Protocol

1. Prepare a 0.15 M solution of ANTS or ANDS in 15% (v/v) acetic acid. These can be stored at −80 °C for at least 2 months. ANDS is more suitable for our imager filter (Bio-Rad filter #530DF60) and in our hands is 5-fold more sensitive than ANTS (Fig. 1).
2. Prepare a 1 M solution of sodium cyanoborohydride in dimethyl sulfoxide (DMSO), which can be stored at −80 °C for at least 2 months.
3. Dry the oligosaccharide samples released from LLOs in a centrifugal vacuum device in a 1.5 ml microcentrifuge tube (use a 0.5 ml tube if the sample is below 200 pmol).

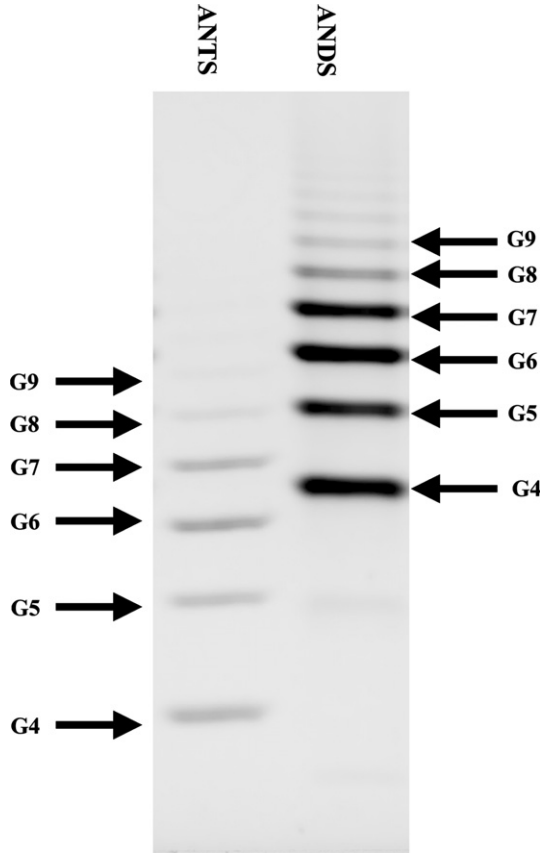

Fig. 1. ANTS- and ANDS-labeled glucose oligosaccharide standards. The same amounts of glucose oligomers were labeled with ANDS (net charge, −2) or ANTS (net charge, −3). The intensity of ANDS signal is about five times more than that obtained with ANTS, but ANTS-labeled oligosaccharides migrate faster. In this figure, the G6 oligomer is 20 pmol.

4. Add 5 μl (or 1 μl if the sample is below 200 pmol) of ANTS or ANDS solution. Vortex, centrifuge briefly.
5. Add 5 μl (or 1 μl if the sample is below 200 pmol) of sodium cyanoborohydride solution. Vortex, Centrifuge briefly, and react at 37 °C for 18 h.
6. Dry the reaction mixture under CEV. Dissolve in the desired volume of water.

2.3. Electrophoretic separation of ANTS- and ANDS-labeled oligosaccharides

2.3.1. Equipment and reagents

Gel electrophoresis apparatus: gels are sandwiched between 1.0 mm thick glass plates, and are 10 cm wide, 10 cm high, and formed with 0.5 mm thick spacers. Combs with eight 8 mm wide teeth (2 mm between teeth) are typically used to form loading wells, but 12-tooth combs can also be used. Pre-assembled FACE gels can be obtained from a commercial supplier (Prozyme, San Leandro, California, USA). Gel components can be recycled from used gels. The combs and spacer can also be prepared by any competent machine shop. The glass plates (EVR-type glass) must have very low ultraviolet absorbance; our laboratory has obtained them in bulk by special order from Erie Scientific Company, Portsmouth, New Hampshire (#SMC-2101 for front notched plate, #SMC-2102 for back plate). We have used two types of gel boxes for FACE. A specialized gel box is offered by Prozyme. This apparatus consisted of two chambers: a lower chamber cooled to −4 °C by circulating coolant and an upper chamber contacting the bottom chamber and containing electrolyte, in which gel sandwiches were mounted. The upper chamber reaches a running temperature of +4 °C during electrophoresis. This apparatus is convenient because gels can easily be removed and replaced for periodic inspection with ultraviolet light. A temperature controlled gel box with a matching gel-casting apparatus offered by Owl Separation Systems, Portsmouth, New Hampshire (#P8DS) may also be used, although periodic inspection of gels during the run with ultraviolet light is somewhat more cumbersome.

Imager: Many of the available fluorescence imagers using CCD cameras should be suitable for image acquisition and analysis. We have used the Bio-Rad Fluor-S Multi-imager with a 530DF60 filter and Quantity-One software supplied with the imager.

Reagents for preparing FACE gels:

Resolving gel stock solution: 38% (w/v) acrylamide and 2% (w/v) *N*,*N*′-methylenebisacrylamide.
Stock resolving gel buffer, 8×: 1.5 M Tris–Cl, pH 8.9.
Stacking gel stock solution: 10% (w/v) acrylamide and 2.5% (w/v) *N*,*N*′-methylenebisacrylamide.
Stock stacking gel buffer, 8×: 1.0 M Tris–Cl, pH 6.8.
Stock electrode buffer, 10×: 1.92 M glycine, 0.25 M Tris base, pH 8.3.
10% ammonium persulfate (prepared daily).
100% *N*,*N*,*N*′*N*′-tetramethylenediamine (TEMED).

2.3.2. Protocol

1. Assemble the gel-casting apparatus provided with the gel box and prepare the resolving gel solution by mixing: resolving gel stock solution (4 ml); stock resolving gel buffer, 8× (1 ml); water (3 ml); and 10% ammonium persulfate (30 μl).
2. Polymerization is initiated by addition of TEMED (10 μl), and the solution is poured into the casting apparatus to a height of 0.5 cm below the bottom of the teeth of the comb. Immediately overlay the gel solution with 1 cm of water. Polymerization occurs after 15 min.
3. Prepare the stacking gel solution by mixing: stacking gel stock solution (2 ml); stacking resolving gel buffer, 8× (0.5 ml); water (1.5 ml); and 10% ammonium persulfate (20 μl). Polymerization is initiated by adding TEMED (5 μl).
4. Pour off the water overlay, and fill the remaining space in the mold with stacking gel solution. Insert a comb to form sample wells. The stacker should polymerize within 15 min.
5. Dilute the stock electrode buffer 10-fold and cool to 4 °C. Pour into the electrophoresis apparatus (anode compartment), connected to a circulating cooler set to the appropriate temperature to maintain the gel at 4 °C.
6. Insert the gel sandwich into the apparatus. Add electrode buffer to the cathode compartment. Mix the sample (dissolved in water) with an equal volume of 2× loading buffer (0.01% thorin I (Aldrich #10456-6) in 20% glycerol). Apply 2–4 μl of mixture to the wells with flat end tips.
7. Connect to a power supply and set it to a constant current of 15 mA (a voltage in the range of 200–1200 V will result). Run until the thorin I marker dye exits the bottom of the gel, usually in 1 h. Turn off the current, remove the gel, and place in the gel imager with both glass plates still attached. A delay of more than 20 min may result in some diffusion of the fluorescent bands.

3. Conclusion

FACE is a simple, accurate, and quantitative method for the direct measurement of LLOs in cultured cells and animal tissues. For example, one 15 cm dish of CHO cells at 90% confluence gives a very good signal (Fig. 2) and is comparable with radiolabeling results (Fig. 3). LLOs from single mouse tissues can also easily be detected (Fig. 4). FACE circumvents pitfalls of radioisotope metabolic labeling, and in many cases permits

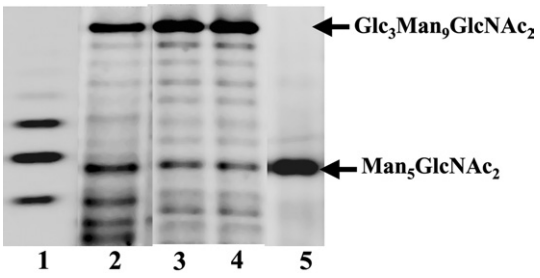

Fig. 2. Glycans from LLOs of cells characterized by FACE. Lane 1, glucose oligomer standards (Glc_{5-7}). Lane 2, LLOs from *Saccharomyces cerevisiae*. Lane 3, LLOs from CHO-K1 (culture used in this laboratory). Lane 4, LLOs from CHO-K1 (culture provided by Dr. Linda Hendershot). Lane 5, LLOs from Lec35 (a CHO-K1 mutant line that makes $Man_5GlcNAc_2$-P-P-dolichol instead of $Glc_3Man_9GlcNAc_2$-P-P-dolichol). In CHO-K1 and yeast cells, the major glycan is $Glc_3Man_9GlcNAc$. In Lec35 cells, the major glycan is $Man_5GlcNAc_2$. LLOs were labeled with ANDS.

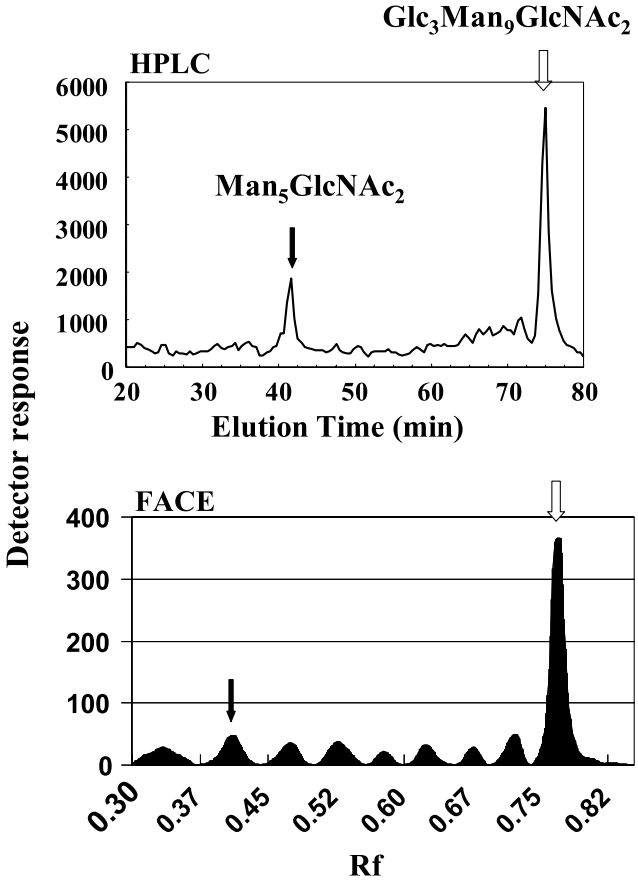

Fig. 3. Comparison of CHO-K1 LLO profiles detected by HPLC or FACE. Upper panel: LLOs were recovered from CHO-K1 cells labeled with [^{3}H]mannose in 0.5 mM glucose/Ham's F12 medium, and resolved by HPLC. Lower panel: LLOs were recovered from CHO-K1 cells grown in normal Ham's F12 medium (10 mM glucose) and analyzed by FACE (ANDS labeling). The FACE profile was generated with Quantity One software.

investigations not feasible by metabolic labeling. For example, we have used this method to investigate the cause of loss of metabolic labeling of LLOs during translation arrest [14], and have reported the first survey of LLO compositions in tissues of normal laboratory mice [15]. Currently, we are using FACE to investigate LLO abnormalities reported for the human disorders congenital disorder of glycosylation type I (CDG-I), and neuronal ceroid lipofuscinosis (NCL).

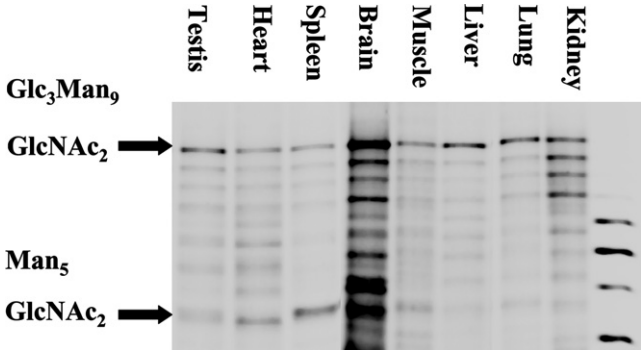

Fig. 4. LLO patterns from different mouse tissues detected by FACE. In all mouse tissues shown, the major LLO glycan is $Glc_3Man_9GlcNAc_2$ (30–60% of total LLO), although considerable amounts of intermediates are also present. In contrast, in cultured CHO-K1 cells $Glc_3Man_9GlcNAc_2$accounts for 70–90% of total LLOs, as in Figs. 2 and 3. LLOs were labeled with ANTS. The right-most lane contains Glc_{4-7} standards.

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References

[1] R. Kornfeld, S. Kornfeld, Annu. Rev. Biochem. 54 (1985) 631–664.
[2] A. Varki, R.D. Cummings, J.D. Esko, H.H. Freeze, G.W. Hart, J. Marth (Eds.), Essentials of Glycobiology, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1999.
[3] D. Karaoglu, D.J. Kelleher, R. Gilmore, Biochemistry 40 (2001) 12193–12206.
[4] T. Marquardt, H. Freeze, Biol. Chem. 382 (2001) 161–177.
[5] H. Schachter, J. Clin. Invest. 108 (2001) 1579–1582.
[6] C.E. Grubenmann, C.G. Frank, A.J. Hulsmeier, E. Schollen, G. Matthijs, E. Mayatepek, E.G. Berger, M. Aebi, T. Hennet, Hum. Mol. Genet. 13 (2004) 535–542.
[7] M.A. Lehrman, J. Biol. Chem. 276 (2001) 8623–8626.
[8] D.J. Kelleher, D. Karaoglu, R. Gilmore, Glycobiology 11 (2001) 321–333.
[9] J. Badet, R.W. Jeanloz, Carbohydr. Res. 178 (1988) 49–65.
[10] B.S. Gibbs, J.K. Coward, Bioorg. Med. Chem. 7 (1999) 441–447.
[11] A.E. Chapman, J.C. Calhoun, Arch. Biochem. Biophys. 260 (1988) 320–333.
[12] P. Jackson, Mol. Biotechnol. 5 (1996) 101–123.
[13] C.M. Starr, R.I. Masada, C. Hague, E. Skop, J.C. Klock, J. Chromatogr. A 720 (1996) 295–321.
[14] N. Gao, M.A. Lehrman, J. Biol. Chem. 277 (2002) 39425–39435.
[15] N. Gao, M.A. Lehrman, Glycobiology 12 (2002) 353–360.