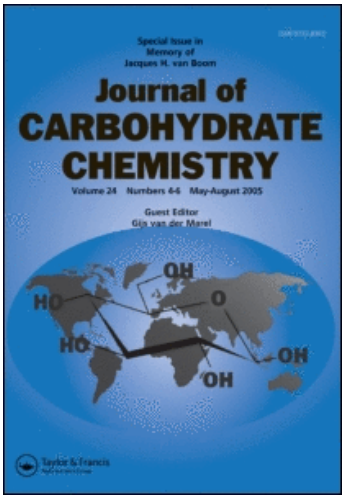

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NANOFILTRATION FOR PURIFICATION OF NUCLEOTIDE SUGARS

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ABSTRACT

Nanofiltration was used in downstream processing for the desalination and concentration of the nucleotide sugars, cytidine-5'-monophospho-β-*N*-acetylneuraminic acid (CMP-Neu5Ac) **1** and guanosine-5'-diphospho-α-D-mannose (GDP-Man) **2**. With appropriate membranes, the retention rate for CMP-Neu5Ac was 0.995 and for GDP-Man 0.983. Both nucleotide sugars were purified on a gram scale with a purity ≥95%.

INTRODUCTION

Reverse osmosis and nanofiltration are used for water desalination to produce drinking water and for treatment of waste water.[2,3] They have been employed for the separation of mixtures of mono- and divalent anions,[4] amino acids and peptides.[5] Applications for downstream processing after industrial fermentation processes have also been reported.[6] For cofactor dependent reactions, NAD(H) can be retained in continuously operated reactors, thereby increasing its total turnover number.[7] But until now, no application using this fast and mild method for purification of carbohydrates has been described. Here we present results showing how nanofiltration can be used to simplify and speed up purification of carbohydrates, with CMP-Neu5Ac **1** and GDP-Man **2** as examples.

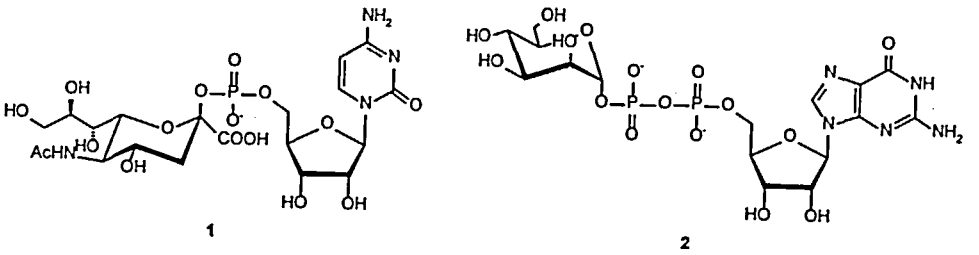

Figure 1. Structures of CMP-Neu5Ac **1** and GDP-Man **2**

Various examples of the importance of oligosaccharides and glycoconjugates have been reported. They serve as recognition sites for a variety of inter- and intracellular communication events, regulate growth and differentiation of cells and are associated with various pathological conditions including malignant transformation.[8,9] Nucleotide sugars are substrates for the Leloir glycosyltransferases widely used for *in vitro* synthesis of oligosaccharides.[10,11] These natural compounds and their synthetic derivatives are gaining increasing interest as possible pharmaceuticals.[12,13]

RESULTS AND DISCUSSION

Enzymatic synthesis of CMP-Neu5Ac **1**[14-17] and GDP-Man **2**[18-20] has been described up to multigram scale.[21] The standard procedure for product isolation is anion exchange chromatography[22] followed by gel filtration for desalting. The latter step is time-consuming and difficult to scale up. Thus, gel filtration was replaced by diafiltration, where the nucleotide sugar is retained by a filtration membrane and the salt is washed out by a continuous replacement of the solvent.

Several commercially available nanofiltration membranes were characterized in a pressurized cross flow filtration unit, which was modified to allow continuous feed of water with a piston pump. A constant volume corresponding to a constant filling level in the vessel was achieved by an adjustable electrode for conductivity measurement. Based on the conductivity, the pump was switched on or off by a controller. The flow scheme is given in Fig. 2.

The membranes were characterized by determination of the retention rate R (1) and the flux.

$$R = 1 - \frac{c_{\text{permeate}}}{c_{\text{feed}}} \qquad (1)$$

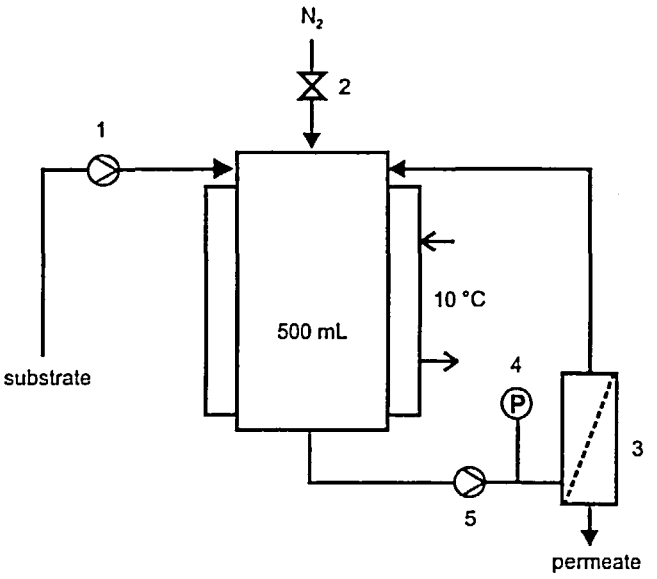

Figure 2. Flow scheme of cross flow filtration unit. 1 piston pump; 2 valve; 3 flat membrane module; 4 manometer; 5 gear pump

The course of diafiltration is described by an exponential function (2). The quotient V_D/V_0 describes the number of volumes exchanged.

$$c = c_0 \cdot e^{-(1-R)\cdot\frac{V_D}{V_0}} \qquad (2)$$

To minimize loss of material during the diafiltration step, retention for nucleotide sugars should be as high as possible and for salt as low as possible. Fig. 3 shows the concentration of the retained sugar as a function of the retention rate for two selected V_D/V_0 values. It becomes obvious that a retention of 0.95 is not sufficient when high recovery rates or complete desalination are required. On the other hand, retention up to 0.5 for the salt will cause no problems.

The volumes that have to be exchanged to give the desired product purity depends on the difference of the retention rates and the initial concentrations. A system consisting of two components can be described by equation (3). As the retention rate of ions increases with decreasing concentration, the calculated volumes have to be considered as minimum amounts.

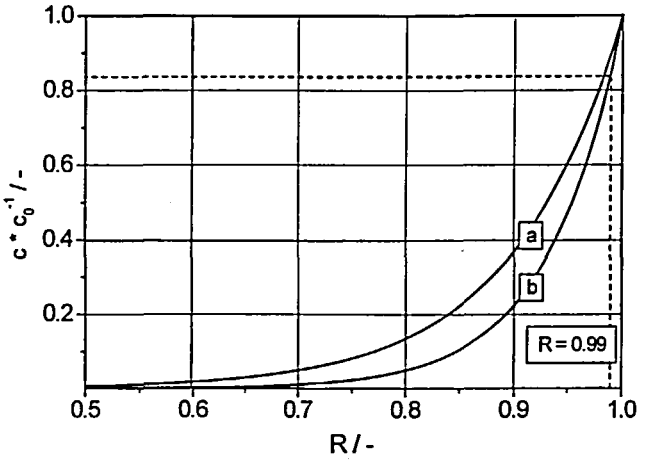

Figure 3. Concentration of the retained sugar as a function of the retention rate for two selected V_D/V_0 values. a 10; b 15.

$$\frac{V_D}{V_0} = \frac{\ln \frac{c_A \times c_{B0}}{c_B \times c_{A0}}}{R_A - R_B} \tag{3}$$

where:

V_D	volume of water used for diafiltration
V_0	initial volume of solution
c_A, c_B	concentrations in the retentate
c_{A0}, c_{B0}	initial concentrations
$R_{A,B}$	retention rates

Table 1 summarizes the results of the characterization of different membranes. Concentrations were chosen to resemble elution conditions from ion exchange columns during purification. The most suitable membranes were found to be ROM 375 and MX 07, showing the necessary retention rates together with a sufficiently high flux ensuring a fast process.

For treatment of smaller samples, commercially available stirred filtration cells can be used.[23] When desalting of CMP-Neu5AC 1 was performed in a 10 mL cell (Amicon type 8010 equipped with a ROM 375 membrane) the same values for retention and flux were obtained using the cross flow filtration unit described previously. In this case the water was added batchwise. The disadvantage of these cells is their limited pressure stability which reduces the number of possible membranes. Furthermore, only dilute solutions can be filtered as the pressure applied has to be higher than the osmotic pressure of the solution.

Table 1. Characterized membranes. Data of membranes finally used are printed bold face. Conditions: cross flow 2.9 m/s; 20 °C; pH 7.

membrane[a]	manufacturer	retention / -				p / bar	flux[b]
		CMP-Neu5Ac[c]	GDP-Man[c]	Na-For[d]	LiCl[e]		
BQ 06	Osmonics		0.905		0.040	8	0.14
CMS-KX-021	Celfa	0.995	0.939	0.484	0.036	8	0.04
CMS-KX-036	Celfa		0.553		0.028	8	0.07
MPF 44	Celfa	0.992	0.955	0.730	0.557	20	0.04
MX 07	Osmonics		**0.974**[g]		**0.035**	8	0.06
NF-PES 10	Hoechst		0.281		0	8	0.1
NF-TFC 50	Hoechst		0.710		0.020	8	0.03
ROM 350	Toray		0.876	0.213	0.024	15	0.04
ROM 362	Nitto		0.800		0.163	8	0.12
ROM 365	Toray	0.921	0.838		0.109	8	0.12
ROM 375	Toray	**0.992**[f]	0.936	**0.484**	0.152	8	0.12
ROM 380	Celfa	0.985	0.968	0.860	0.400	40	0.06
ROM 395	Nitto	0.974		0.173		30	0.25

a. ROM membranes are distributed by Amafilter, Langenhagen, Germany; b. mL/(cm^2×min);
c. 1 mmol/L; d. 100 mmol/L; e. 1000 mmol/L; f. 0.995 at 4 °C; g. 0.983 at 4 °C.

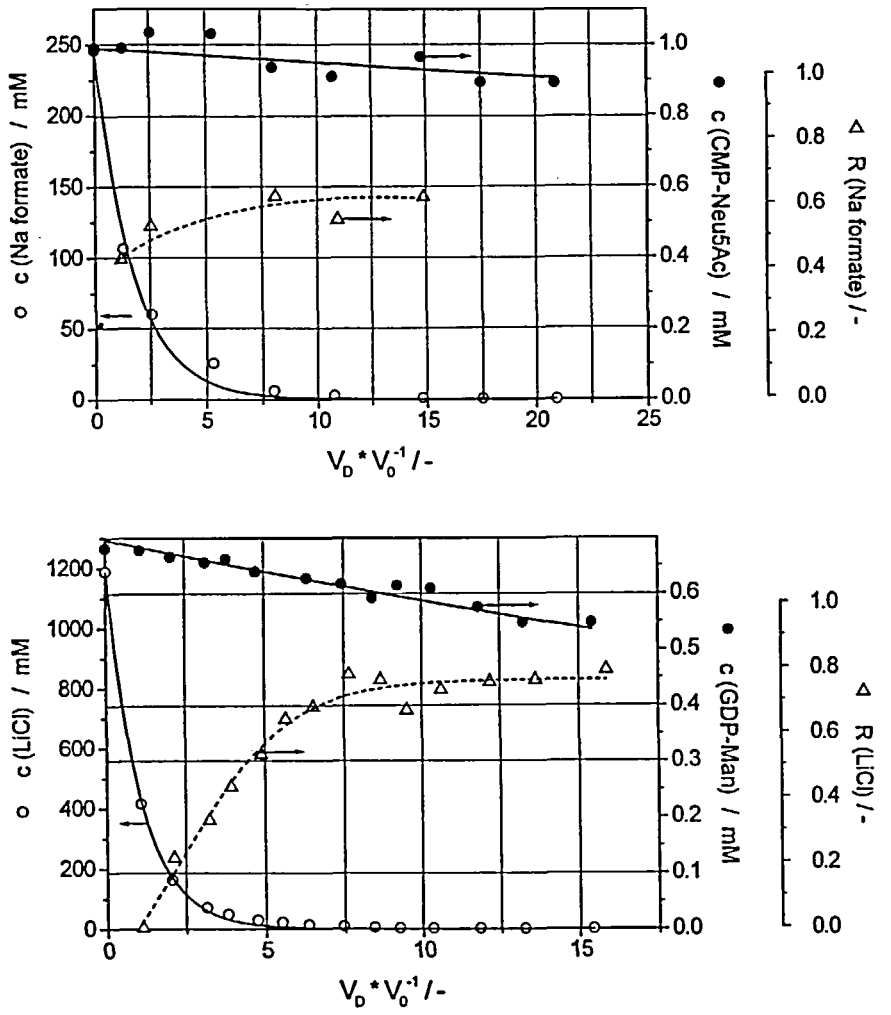

Figure 4. Course of concentrations during diafiltration. Conditions: 8 bar, 50 mL (CMP-Neu5Ac), 14 bar, 150 mL (GDP-Man), 50 mL, 4 °C, pH 7

Diafiltration runs were performed at 4 °C for optimal product stability, which generally leads to an increase in retention rates. Furthermore, retention rates of LiCl and Na formate were seen to rise during diafiltration, due to the decrease in concentration. After diafiltration, the products were concentrated 10-fold and lyophilized.

Purification of CMP-Neu5Ac **1** was carried out until the detection limit of sodium formate was reached (0.25 mM). Results from a typical diafiltration experiment

are shown in Fig. 4. A total of 5 g of the product could be obtained with a purity of >95% and a yield of >90% for the diafiltration step.

In the case of GDP-Man **2**, the course of a diafiltration on an analytical scale is shown in Fig. 4. The process was stopped after the exchange of 16 volumes, at which point the product purity was 92% and the yield was 81%. A second experiment with a more favorable GDP-Man/LiCl ratio (15.4 mM/1M) was performed on preparative scale. 1 g of the product was obtained in 95% purity with a yield of 88% after exchange of 11.5 volumes (data not shown).

CONCLUSION

By integrating nanofiltration in the downstream processing of CMP-Neu5Ac and GDP-Man, we demonstrated the efficiency of this method for the desalination of nucleotide sugars. Further applications of nanofiltration may include the separation of carbohydrates with limited stability from reagents after cleaving of protective groups used for synthesis, e.g., acetate.

EXPERIMENTAL

General methods. Membranes were obtained from Amafilter (Langenhagen, Germany), Celfa (Seewen, Switzerland), Hoechst (Frankfurt, Germany) and Osmonics (Minnetonka, USA). CMP-Neu5Ac[14-17] and GDP-Man[18-20] were synthesized as previously described. All other chemicals were obtained from Merck (Darmstadt, Germany).

Nanofiltration. For the characterization of the nanofiltration membranes as well as for preparative desalination, a cross flow filtration unit P 28 from Celfa (Seewen, Switzerland) was used. Operating volume was up to 500 mL. A cross flow of 2.9 m/s was provided by a gear pump. Flat membranes with a diameter of 75 mm and an area of 44 cm^2 were used. The effective membrane area was 28 cm^2. All were thin-film composite membranes which allow higher fluxes at reduced pressure. The unit was pressurized up to 40 bar with nitrogen. Temperature was controlled by a jacket and a water bath. The unit was modified to allow a continuous feed of water by a piston pump (P 500, Pharmacia, Uppsala, Sweden). To maintain a constant volume, the filling level was controlled by an adjustable electrode measuring the conductivity and a piston pump.

Analytical HPLC systems. Formate was measured at 195 nm using a HPX-87 cation exchange column (300 mm; Biorad, München, Germany), which was operated with 6 mM H_2SO_4 and a flux of 0.6 mL/min.

CMP-Neu5Ac and GDP-Man were detected using a method according to a previously reported procedure.[24] A reversed phase Hypersil ODS-5μm column (CS, Langerwehe, Germany) was used at a temperature of 40 °C with a methanol gradient (eluent A: 100 mM KH_2PO_4, 8 mM tetrabutylammonium hydrogensulfate (Serva, Heidelberg, Germany), pH 5.3; eluent B: 70 % eluent A, 30 % methanol gradient grade (Merck, Darmstadt, Germany), pH 5.95; 2.5 min 0 % B, 14 min 0-40 % B; 1 min 40-100 % B; 11.5 min 100 % B; 1 min 100-0 % B, 3.5 min 0 % B). Flow: 1.5 mL/min, detection by a UV spectrometer at 253 nm.

LiCl was detected by conductivity measurement using a DIGI-550 conductivity unit (WTW, Weilheim, Germany).

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17. Conditions of a representative fed batch synthesis of CMP-Neu5Ac: 4×5 mM CTP, 20 mM Neu5Ac, 80 mM $MgCl_2$, 1.75 U/mL inorganic pyrophosphatase, 0.2 U/mL CMP-Neu5Ac synthetase, pH 8, 30 °C, V = 450 mL, 60% yield.
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    GDP-Man: Dowex 1×2 resin, column: 125 mm × 20 mm, 4 °C, pH 7, flow rates: 32 mL/h (sample), 108 mL/h (150 mM LiCl), 108 mL/h (1 M LiCl, elution of GDP-Man).
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