In search of specific and highly selective sugar clusters for cell receptors, such as membrane lectins, various disaccharides were coupled to small peptide cores through an amide bond. In a first step, the reducing disaccharides, i.e. lactose and three different dimannoses, were converted into glycosyl-pyroglutamyl-β-alanine derivatives. The free carboxylic group of these conjugates was then coupled to the ω and ε amino groups of the core peptide (Lysn-Ala-Cys-NH₂) with n = 1 to 5, with complete substitution leading to homogeneous glycoclusters. The thiol group of the cysteine residue was used to tag the glycosylated oligosaccharides upon reaction with fluorescein iodoacetamide. The affinity of these glycoclusters towards two plant lectins was assessed by surface plasmon resonance. The selectivity of their cell uptake was investigated by flow cytometry using two types of cells: a human hepatoma cell line (HepG2 cells) expressing the plasma membrane galactose-specific lectin, and monocyte-derived dendritic cells expressing the plasma membrane mannose-specific lectin. The glycoclusters containing four or five disaccharides were shown to bind plant lectins and cell surface membrane lectins with a narrow selectivity and with a high affinity.

Key words: endocytosis, glycosynthons, flow cytometry, lectins, surface plasmon resonance.

INTRODUCTION

High affinity ligands for cell specific receptors, such as membrane lectins, belong amongst either complex oligosaccharides of natural origin, or synthetic clusters made of sugar units attached either to a protein (neoglycoprotein) or to a small peptide core. Knowing that sugar–lectin interactions depend on the density of the partners, several teams have synthesized multivalent glycopeptides by coupling, for instance, monosaccharide derivatives on to oligosaccharines [1–3]. Small multivalent synthetic glycopeptides with two to four mannose residues covalently linked through a spacer arm to the ω- and ε-amino groups of lysine, di-, and tri-lysines, were shown to be competitive inhibitors of rat alveolar macrophage uptake of a neoglycoprotein (mannosylated BSA) with 50% inhibition in the micromolar concentration range, while the galactosyl-peptide analogue did not inhibit the uptake of mannosylated BSA [4], in agreement with the fact that macrophages did not recognize galactose residues.

In search of synthetic high affinity ligands for a human mannose receptor isolated from placenta, Biessen and co-workers [5] synthesized a series of homologous lysine-based monosaccharides containing two to six terminal ω-d-mannose groups linked to the ω- and ε-amino groups of a linear oligolysine through a phenylthiocarbamyl arm. The synthesized ω-mannose clusters were able to displace the binding of ribonuclease B and tissue-type plasminogen activator from the isolated human mannose receptors. In order to improve both the specificity and the selectivity of sugar clusters, we decided to use disaccharides instead of monosaccharides. With the aim of avoiding the use of a phenylthiocarbamyl bond [5] which may lead to cytotoxic derivatives, we synthesized N-glycosyl-pyroglutamyl derivatives [6] bearing a C-terminal carboxylic group [7] able to be coupled to an amino group. In this approach, it is possible to obtain clusters in which the core ends with one reacting group which allows the cluster to be linked on to a molecule, such as a gene vector, an oligonucleotide, or a matrix. In a previous study [7], we showed that both natural oligomannosides and oligosaccharide partially substituted with a dimannose derivative were taken up by dendritic cells.

In this paper, we describe the synthesis of diglycosylpyroglutamyl-β-alanine derivatives (glycosynthons) and an improved synthesis of glycoclusters by coupling glycosynthons either to lysine or to an oligosaccharine containing up to five lysyl residues (Lysn-Ala-Cys-NH₂ peptide), leading to fully substituted biodegradable conjugates. In order to test the specificity and the selectivity, we synthesized four types of clusters: one containing lactose and three containing α2, α3 or α6 dimannose. With the aim of designing a high affinity cluster at the lowest possible cost, we synthesized a series of clusters containing two to six sugar moieties. The peptide core ended with a cysteine residue, the thiol group of which was either substituted with a pyridylthio group or with an acetylamidofluorescein group.

Two main properties of the clusters were investigated: (i) their affinity towards two immobilized plant lectins, a galactose-specific lectin Ricinus communis agglutinin (Rca) and a mannose-specific lectin Canavalia ensiformis agglutinin (ConA), assessed by surface plasmon resonance (SPR); and (ii) their selective uptake by HepG2 cells (a human hepatoma cell line) expressing a cell surface galactose-specific lectin [8] and blood
monocyte-derived dendritic cells expressing cell surface mannoseselective lectins [9,10], assessed by flow cytometry.

**EXPERIMENTAL PROCEDURES**

**Materials**

Lactose and N,N-di-isopropylethylamine (DIEA) were purchased from Janssen Chimica (Beerse, Belgium); dimannoses (Manz 2Man, Manz3Man, Manz6Man) (Figure 1) were from Dextra Laboratories (Reading, U.K.). Imidazole, DMSO, n-butanol, acetic acid, trifluoroacetic acid, ethanedithiol, molecular sieves (4 Å; 1 Å = 0.1 nm) and silica gel 60 were from Merck (Darmstadt, Germany). 2,2-Dithiodipyridine and monensin were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Tris-(2-carboxyethyl)phosphine (TCEP) and 2-Dithiodipyridine and monensin were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Tris-(2-carboxyethyl)phosphine (TCEP) and SDS (Darmstadt, Germany). Acetonitrile was purchased from SDS (Peypin, France). Acetonitrile was purchased from SDS (Peypin, France). Tris-(2-carboxyethyl)phosphine (TCEP) and SDS (Darmstadt, Germany). Acetonitrile was purchased from SDS (Peypin, France). Tris-(2-carboxyethyl)phosphine (TCEP) and SDS (Darmstadt, Germany). Acetonitrile was purchased from SDS (Peypin, France). Tris-(2-carboxyethyl)phosphine (TCEP) and SDS (Darmstadt, Germany).

**Cells**

HepG2 human hepatoma cells were obtained from American Type Culture Collection (8055 HB, Rockville, MD, U.S.A.). HepG2 cells were cultured in complete DMEM medium complemented with 5% (v/v) heat-inactivated FCS containing 2 mM l-glutamine and 1% antibiotics (50 units/ml penicillin, 50 μg/ml streptomycin). Dendritic cells named dendritophages DΦ (IDM, Paris, France) were obtained by apheresis from healthy volunteer peripheral blood mononuclear cells (PBMC) as follows: 1 × 10⁷ PBMC were washed 3 times in PBS and cultured for 7 days in non-adherent hydrophobic bags (Stedim, Aubagne, France) supplemented with 500 units/ml granulocyte macrophage cell stimulating factor (GM-CSF) and 50 ng/ml interleukin-13, at a density of 5 × 10⁶ cells/ml. Interleukin-13 was added on day 4 of culture. On day 7, DΦ were isolated by elutriation. The purity of DΦ ranged from 75 to 95% and the cell viability was higher than 95%.

**Chromatography**

Conjugates were purified by gel filtration on either a Trisacryl GF05 column (2 cm × 45 cm; Biosepra, Villeneuve-la-Garenne, France) stabilized and eluted with distilled water containing 5% n-butanol, or a BioGel column (P2, P4 or P6; Bio-Rad, Oxfordshire, U.K.) supplemented with 500 units/ml granulocyte macrophage cell stimulating factor (GM-CSF) and 50 ng/ml interleukin-13, at a density of 5 × 10⁶ cells/ml. Interleukin-13 was added on day 4 of culture. On day 7, DΦ were isolated by elutriation. The purity of DΦ ranged from 75 to 95% and the cell viability was higher than 95%.

**Electrospray ionization mass spectrometry (ESI-MS)**

The positive ion electrospray mass spectra were obtained using a platform quadrupole mass spectrometer (Quattro II, Micromass, Manchester, U.K.) equipped with a nebulizer-assisted electrospray source. A voltage difference of 3.11 kV was applied between the capillary and the counter electrode. The sample cone voltage was 20–25 V. The ion source was kept at 80 °C. Instrument control and data analysis were accomplished using the Masslynx application software (version 3.4; Micromass). Calibration of
the mass spectrometer was performed using horse myoglobin (Sigma). Samples were dissolved in a 4:1 (v/v) acetonitrile/water mixture containing 0.1 \% (v/v) formic acid. ESI mass spectra were run in both positive and negative ion modes. Clusters were also characterized by matrix-assisted laser-desorption ionization time-of-flight ("MALDI-TOF") with a Billips III spectrometer (Brucker, Wissembourg, France), in positive mode, using azo-cyan-4-hydroxycinnamic acid as a matrix.

**1H NMR spectroscopy**

For **1H NMR** analysis, compounds were dissolved at 25 °C in deuterated water, once with D$_2$O containing 99.9 \% D and, after freeze-drying, with D$_2$O containing 99.96 \% D (Sigma). **1H NMR** spectroscopy was performed at 300 K with a Varian Unity 500 MHz NMR spectrometer.

**TLC**

TLC was performed using silica gel F$_{254}$ preformed layers on a plastic sheet (Merck, Darmstadt, Germany), and the chloroform/methanol/water (13:8:2, by vol.) mixture was used as an eluent. Carbohydrates were visualized upon spraying with ammonium formamidobutyrate/H$_2$SO$_4$ and subsequent heating. Compounds containing free amino groups were visualized by ninhydrin spray and subsequent heating at 80 °C.

**Flow cytometry**

Adherent cells (4 × 10$^5$ HepG2 on day 1 after plating, or DPH on day 3 after cultivation) were incubated for 2 h at 37 °C in complete culture medium in the presence of the fluorescent labelled conjugates (0.05–20 \( \mu g/ml \)). After washing in PBS, the cell-associated fluorescence was determined by flow cytometry after a final incubation for 30 min at 4 °C in the presence or absence of 50 \( \mu M \) monensin [13,14]. The cell-associated fluorescence was analysed with a Becton Dickinson-LSR flow cytometer and data were analysed using the Cell Quest Software (Becton Dickinson, Le-Pont-De-Clair, France).

### Table 1 Characteristics of the lysine-based oligosaccharide clusters

<table>
<thead>
<tr>
<th>Oligosacetyl clusters*</th>
<th>Molecular mass</th>
<th>Calculated</th>
<th>Measured†</th>
<th>tR (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lac-Glp-( \alpha )Lys(Lac-Glp-( \beta )Ala)AlaCys(R)-NH$_2$</td>
<td>R = -Spy</td>
<td>3343.3</td>
<td>3342.9 (+2878.4)</td>
<td>12.8 (+14.3)</td>
</tr>
<tr>
<td></td>
<td>R = -Flu</td>
<td>3621.4</td>
<td>3622.8</td>
<td>19.1</td>
</tr>
<tr>
<td>Lac-Glp-( \alpha )Lys(Lac-Glp-( \beta )Ala)AlaCys(R)-NH$_2$</td>
<td>R = -Spy</td>
<td>3621.4</td>
<td>3622.7 (+2158.4)</td>
<td>17.6</td>
</tr>
<tr>
<td></td>
<td>R = -Flu</td>
<td>3621.4</td>
<td>3622.7</td>
<td>17.6</td>
</tr>
<tr>
<td>Lac-Glp-( \beta )Lys(Lac-Glp-( \alpha )Ala)AlaCys(R)-NH$_2$</td>
<td>R = -Spy</td>
<td>3343.3</td>
<td>3346.5</td>
<td>10.6</td>
</tr>
<tr>
<td></td>
<td>R = -Flu</td>
<td>3621.4</td>
<td>3622.7</td>
<td>17.6</td>
</tr>
<tr>
<td>Lac-Glp-( \beta )Lys(Lac-Glp-( \alpha )Ala)AlaCys(R)-NH$_2$</td>
<td>R = -Spy</td>
<td>3343.3</td>
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<td>10.6</td>
</tr>
<tr>
<td></td>
<td>R = -Flu</td>
<td>3621.4</td>
<td>3622.7</td>
<td>17.6</td>
</tr>
<tr>
<td>Man$_x$6Man-Glp-( \beta )Lys(Man$_x$6Man-Glp-( \beta )Ala)AlaCys(R)-NH$_2$</td>
<td>R = -Spy</td>
<td>3343.3</td>
<td>3346.5</td>
<td>10.6</td>
</tr>
<tr>
<td></td>
<td>R = -Flu</td>
<td>3621.4</td>
<td>3622.7</td>
<td>17.6</td>
</tr>
</tbody>
</table>

* The sugar linked to the Glp nitrogen is a \( \beta \) anomer. \( ^{\dagger} \) Masses in italics were measured by MALDI-TOF; \( t_R \) is retention time. \( ^{\ddagger} \) HPLC elution was performed using a C4 column with a gradient of 2% solvent B (95% CH$_3$CN/5% H$_2$O/0.1% TFA) in solvent A (95% H$_2$O/5% CH$_3$CN/0.1% TFA) for 5 min, followed by a linear gradient of 2–15% solvent B in solvent A over 15 min; elution was monitored by UV absorbance at 220 nm, 280 nm or 450 nm according to the cysteine side-chain substituent, using a photodiode array detector. Values in parentheses are the masses of minor components. Spy, 2-thiopyridyl; Flu, fluoresceinyl.

**SPR**

BIACore 2000 (BIACore, Uppsala, Sweden) was used to quantify interactions between lysine-based oligosaccharide clusters and lectins. Lectins were immobilized at pH 4.5 (10 mM sodium acetate buffer) on a CM5 sensor chip (BIACore). The chip carboxymethylated dextran matrix was activated and derivatized by using the amine coupling method [mixture of 100 mM N-hydroxysuccinimide (Sigma-Aldrich) and 400 mM N-ethyl-N'-[dimethylaminopropyl]-carbodi-imide (Acros, Noisy-le-Grand, France)]; the remaining activated sites were then blocked with 1 M ethanolamine, pH 8.5 (Sigma-Aldrich). The binding of any material on the chip induced a refraction index increase, expressed as resonance units (RU). All measurements, using the sensor surfaces, were carried out in 10 mM Hepes Balanced Saline buffer [10 mM Hepes (pH 7.4), 1 mM CaCl$_2$, 1 mM MgCl$_2$, 150 mM NaCl, 17 mM Na$_2$PO$_4$, 0.5 \% (v/v) surfactant P20 (BIACore)]. Analysis was performed at 25.0 °C and at a flow rate of 20 \( \mu l/min \). In the BIACore instrument, the sensor chip is divided into four flow cells which can be used to individually measure interactions. Flow cell number one was used as a control flow cell and treated in an analogous manner except that the lectin addition step was omitted. Analyte samples were prepared and diluted in the running buffer, pH 7.4. Surfaces were regenerated with 30 \( \mu l \) of 0.3 M lactose for Rca and 0.3 M \( \alpha \)-methylmannoside (Sigma) for ConA. Glycoclusters which have a low molecular mass do not give a significant signal. Also, we favoured a procedure based on the inhibition of the binding of a neoglycoprotein on an immobilized lectin. Inhibition constants were derived from experiments using analytes in a 0.25–100 \( \mu M \) concentration range, using a serial 3-fold dilution in running buffer containing 10 \( \mu g/ml \) (approx. 0.125 \( \mu M \)) neoglycoprotein Lac-BSA or Man-BSA, for Rca and ConA respectively; this
concentration is the half-saturation concentration which gives the half-maximal value, $RU_{\text{max}}/2$.

In the case of a classical association, the Langmuir equation [15] can be written, according to Scatchard [16], as

$$K \cdot (n-r) = r \cdot g^{-1} \quad \text{or} \quad r \cdot (n-r)^{-1} = K \cdot g$$  \hspace{1cm} (1)

where $K$ is the affinity constant between a lectin and a neoglyco-protein expressed as litre $\cdot$ mol$^{-1}$, $n$ is the total number of sites on the sensor chip, $r$ is the number of sites occupied by a neoglyco-protein and $g$ the concentration of the neoglyco-protein used as an analyte, expressed as mol $\cdot$ litre$^{-1}$.

It is well known from the law of mass action that a linear relationship between free ($f$) and bound ($b$) hapten concentrations allows the determination of the equilibrium constant $K$ when the combination of a hapten and an antibody is the same for all the combining sites, i.e. that there is no heterogeneity. More often, the plot $1/b$ versus $1/f$ exhibits a downward curvature attributed to combining sites heterogeneity [17]. The same behaviour is relevant in the case of neoglycoproteins and lectins. In this case, the heterogeneity comes from the neoglyco-protein which contains 25 sugars on average, and from a non-homogeneous density of immobilized lectins on the chip. The number of sugars present in a neoglyco-protein varies from one molecule to another, because about 25 lysines $\pm 3$ are glycosylated and more than 25 are not. Therefore the heterogeneity comes from a quantitative aspect, but also from a qualitative one: the position of the sugar moieties on the neoglyco-protein. Moreover, the interaction between such a neoglycoprotein and a lectin is complex because of an avidity effect [18,19].

Nisonoff and Pressman [17] used the distribution function of Sips [20], in which the data may be fitted to a straight line. The equation proposed by Sips is derived from the adsorption of a gas on a solid surface, but it may be transformed into an equation applicable to equilibria in solution, as is the case in SPR experiments where $RU$ is measured at equilibrium.

According to eqn. 1,

$$\log[r \cdot (n-r)^{-1}] = \alpha \cdot \log(K \cdot g)$$  \hspace{1cm} (2)

or

$$r \cdot (n-r)^{-1} = (K \cdot g)^\alpha$$  \hspace{1cm} (3)

Since $RU_{\text{max}}$ is related to $n$ and $RU$ is related to $r$, it follows that

$$RU_{\text{max}} \cdot (RU)^{-1} = n \cdot r^{-1},$$

so eqn. 3 gives

$$[RU_{\text{max}} \cdot (RU)^{-1}]^{1/\alpha} = (K \cdot g)^{-1}$$  \hspace{1cm} (4)

The slope $\alpha$ is called either the heterogeneity coefficient or the Sips coefficient.

For a homogeneous group of sites, $\alpha$ is equal to $+1$: decreasing values of $\alpha$ correspond to increasing degrees of heterogeneity, showing an avidity effect or reflecting a heterogeneity either related to the ligands or to the receptors. The lowest value of $\alpha$ corresponds to the highest heterogeneity.

The association constant of the inhibitor $K_i$ was calculated from the apparent affinity $K$ of a neoglycoprotein relevant for the immobilized lectin, measured in the presence of glycosylated clusters. In the presence of an inhibitor $i$, with an association constant $K_i$, the saturation equations giving $K$ and $K_i$ become

$$K \cdot (n-r-q) = r \cdot g^{-1}$$ \hspace{1cm} (5)

and

$$K_i \cdot (n-r-q) = q \cdot i^{-1}$$ \hspace{1cm} (6)

where $q$ is the number of sites occupied by a glycocluster and is equal to

$$q = r \cdot i \cdot K_i \cdot (K \cdot g)^{-1}$$ \hspace{1cm} (7)

and

$$K \cdot [n-r-r \cdot i \cdot K_i \cdot (K \cdot g)^{-1}] = r \cdot g^{-1}$$ \hspace{1cm} (8)

therefore

$$(n-r) \cdot r^{-1} = (K \cdot g)^{-1} + i \cdot K_i \cdot (K \cdot g)^{-1}$$ \hspace{1cm} (9)

When the binding is heterogeneous, eqn. 9 becomes

$$[(n-r) \cdot r^{-1}]^{1/\alpha} = (1 + i \cdot K_i) \cdot (K \cdot g)^{-1}$$ \hspace{1cm} (10)

$K_i$ is deduced from

$$(RU_{\text{max}} \cdot RU^{-1} - 1)^{1/\alpha} = (1 + i \cdot K_i) \cdot (K \cdot g)^{-1}$$ \hspace{1cm} (11)

Synthesis, purification and characterization of peptide cores

A series of peptide cores (Lys)$_n$-Ala-Cys with $n = 1$ to 5, and (Lys-Ala)$_2$-Cys with a C-terminal amide, were prepared by solid phase synthesis on an Applied 433-A peptide synthesizer (Foster City, CA, U.S.A.) equipped with a conductimetric monitor, using a fluorenly-9-methoxycarbonyl (Fmoc) strategy, and starting from an Fmoc-amide resin (0.64 mmol/g) (Applied Biosystems). Fmoc-protected amino acids [Lys(Boc) and Cys(trityl)] were from Senn Chemicals (Dielstorf, Switzerland). A cysteine residue was appended at the C-terminal end of the peptide to enable labelling of the conjugate with a fluorescent tag.

The peptide was deprotected and released from the resin by adding 5 ml of a TFA/ethanediol (EDT)/water mixture (190/5/5) for 3 h at 25 °C under stirring. The solid support was filtered off and washed with a TFA/EDT/water mixture. Peptides were precipitated from the filtrate by adding TBME. The precipitate was collected by centrifugation and washed thoroughly with TBME. The cysteine side chain was further protected with a 2-thiopyridyl (2-Spy) group which will be easily reduced with TCEP. The unprotected peptide was dissolved in water/methanol (1:1, v/v) under nitrogen. A degassed solution of 2,2-dithiodipiridyline (10 equivalents) in water/methanol (4:1, v/v) was added and the pH of the reaction mixture was increased to 8.2 with diluted NH$_4$OH. The mixture was stirred under nitrogen for 3 h. The excess of dithiodipiridyline and the pyridine-2-thione formed were extracted with ethyl acetate, whereas the aqueous phase containing the protected peptide was concentrated and freeze-dried. Peptides were analysed by HPLC and MS.

Synthesis of glutamyl-$\beta$-alanyl-benzyl ester (H-Glu-$\beta$-Ala-OBzL) (1)

The title compound was obtained by coupling Boc-Glu(OtBu)-OH with H-$\beta$-Ala-OBzL.Tos. Briefly, Boc-Glu(OtBu)-OH (3.03 g, 10 mmol) was dissolved in 20 ml of ethyl acetate and coupled with H-$\beta$-Ala-OBzL.Tos (3.51 g, 10 mmol) in the presence of N,N-dicyclohexylcarbodi-imide (2.2 g, 11 mmol) and 1,74 ml (10 mmol) of DIEA. After stirring overnight, the coupling efficiency was assessed by TLC in chloroform/methanol (9:1, v/v). The $R_p$ of the dipeptide was 0.86 and the reaction was completed within 18 h. The insoluble by-products were eliminated by filtration. The filtrate was washed twice with 40 mM KH$_2$SO$_4$ (25 ml), twice with distilled water (25 ml), twice with 60 mM NaHCO$_3$ (25 ml) and twice again with distilled water (25 ml). The organic layer was dried on sodium sulphate, filtered and evaporated under reduced pressure. The dipeptide Boc-Glu(OtBu)-$\beta$-Ala-OBzL was then dissolved in 30 ml of water/trifluoroacetic acid (5:95, v/v) and kept for 1 h at 25 °C leading to H-Glu-$\beta$-Ala-OBzL, TFA. The progress of the reaction was assessed by TLC in chloroform/methanol/water (13:8:2, by vol.); finally, a single spot was seen both under UV and upon staining with ninhydrin ($R_p$ = 0.56). The solvent was evaporated under reduced pressure, the solid material was dissolved in 3 ml...
was purified by gel filtration on a Trisacryl GF 05 column and was complete within 1 h. The glycoconjugate was precipitated from its NMPO solution by pouring it in 10 vol. of TBME and recovered at −20 °C upon adding TBME. The expected product was obtained in 90% yield.

Synthesis of oligosaccharidyl-pyroglutamate (Glp)/β-Ala-OBzl (2) and (3)

In a typical experiment (see Figure 2) according to Quétard et al. [21], the disaccharide (50 mg, 146 μmol), H-Glu/β-Ala-OBzl (90 mg, 292 μmol) and imidazole (40 mg, 584 μmol) were dissolved in 400 μl of NMPO. The solution was kept at 50 °C for 6 h. Without any purification, the glycosylamine formed (2) was then intramolecularly N-acylated by the γ-carboxyl group of glutamic acid, at 25 °C, by adding 146 mg (330 μmol) of BOP and 23 mg (330 μmol) of imidazole. The reaction was monitored by HPAEC and was complete within 1 h. The glycoconjugate was precipitated from its NMPO solution by pouring it in 10 vol. of TMBE; under such conditions, imidazole and hydroxybenozotriazole remained in the supernatant. Disaccharidyl-Glp/β-Ala-OBzl (3) was purified by gel filtration on a Trisacryl GF 05 column (2 cm × 45 cm; Biosepra, Villenueve-la-Garenne, France), stabilized and eluted with distilled water containing 5% n-butanol. The yield was 80–90%, depending on the disaccharide used. By using ESI-MS, for the lactosyl-Glp/β-Ala-OBzl, the observed mass of the molecular ion was 615.3 (615.2 calculated for C_{18}H_{25}N_{1}O_{7} [M + H]+). 1H NMR (500 MHz, 2H O), δ (p.p.m.): 7.44 (5 H, s, H aromatic), 5.18 (2 H, s, -OCH$_2$ Bzl), 5.10 (1 H, d, H$_{1}$-β-D-Glc), 4.65 (1 H, dd, J$_{1,2}$ = 7.8 Hz, αCH Glp), 2.83 (1 H, m, γCH$_{2}$ Glp), 2.56 (2 H, m, γCH$_{2}$ Glp and βCH$_{2}$ Glp), 2.28 (1 H, m, βCH$_{2}$ Glp).

Reduction of oligosaccharidyl-Glp/β-Ala-OBzl (4)

Compound (3) was dissolved in 10 ml of a water/methanol mixture (1:1, v/v). The solution was purged under nitrogen for 30 min. Then, 25 mg (10%) of palladium charcoal (Merck) was added to this solution. The suspension was stirred for 3 h under hydrogen at 25 °C. The reaction was monitored by TLC in chloroform/methanol/water (13:8:2, by vol.). The glycosynthon had an R$_f$ of 0.30. The palladium charcoal mixture was removed by filtration, and the methanol was removed under reduced pressure and the methanol-free solution containing the product (4) was freeze-dried. The yield was 95%. The mass of the molecular ion determined by ESI-MS [calculated for C_{18}H_{25}N_{1}O$_{7}$ (M + H)$^+$, 525.2] was 525.3; the deprotection of the C-terminal carboxylic group was shown to be complete by 1H NMR (500 MHz, 2H O).

Coupling of the oligosaccharidyl-Glp/β-Ala-OH on to an oligosaccharide leading to the cluster (5)

The synthesis of these oligosaccharide clusters is shown in Figure 2. The oligosaccharides used were Lys$_n$/Ala-Cys(Spy)-NH$_2$ with n = 1 to 5 and (Lys-Ala)$_n$/Cys(Spy)-NH$_2$. A solution of oligosaccharide (2 μmol) in NMPO (200 μl) with one equivalent of DIEA per amino group was added to disaccharidyl-Glp/β-Ala-OH (1.5 equivalents per -NH$_2$) dissolved in NMPO (200 μl) in the presence of 1.5 equivalent of DIEA, 1.5 equivalent of HOBT/HTBU, and 70 mg of 4 A molecular sieves. The mixture was gently stirred at 25 °C for 7 h. The reaction was monitored by TLC in chloroform/methanol/water (13:8:2, by vol.). The cluster was then precipitated by pouring the solution into 10 vol. of TBME and recovered upon centrifugation. The product was purified by gel filtration on a BioGel column (2 cm × 45 cm; Bio-Rad, Oxfordshire, England), stabilized and eluted with 50 mM acetic acid. The product collected was freeze-dried and analysed by reverse-phase HPLC and MS. Table 1 summarizes the physico-chemical characteristics of the synthesized clusters.

Labelling of oligosaccharidyl-Glp/β-Ala-[Lys(oligosaccharidyl-Glp/β-Ala)]$_n$/Cys(Spy)-NH$_2$

The lysine-based oligosaccharide cluster (0.6 mmol) was dissolved in 400 μl of phosphate buffer (50 mM, pH 7.2), then 50 μl of dimethylformamide (DMF) was added. The solution was gassed with nitrogen for 30 min; meanwhile a solution of TCEP (1.2 mmol, 0.34 mg) in 100 μl of phosphate buffer (50 mM, pH 7.2) was also gassed with nitrogen. This solution was added to the lysine-based oligosaccharide cluster solution in order to reduce the disulphide bridge. The mixture was stirred under nitrogen for 2 h. A solution of IAF (3 mmol, 1.5 mg) in 200 μl of DMF, gassed with nitrogen, was added on the reduced lysine-based oligosaccharide cluster. The mixture was stirred at 25 °C overnight. The crude product was purified on a BioGel P2 column (2 cm × 45 cm), stabilized and eluted with acetic acid (50 mM). The collected product was finally freeze-dried. The same procedure was used for labelling the cluster Lacβ-Glp/β-Ala-[Lys(Lacβ-Glp/β-Ala)-Ala]$_n$/Cys(Spy)-NH$_2$. The characteristics of the syn-
the synthesized fluoresceinylated clusters are reported in Table 1. The concentration of fluorescein-labelled clusters was determined from their UV absorbance at 490 nm at pH 7.4 (fluorescein $\epsilon_{490} = 80000$). The affinity of these compounds for the lectins was analysed by SPR and their cell uptake was investigated by flow cytometry.

**RESULTS**

**Structure of oligolysine-clusters**

$^1$H NMR showed that, in glycosynthons, the sugar linked to Glp is in a $\beta$ configuration. The yield of glycosynthons was almost quantitative, except for Man$\times$2Man-Glp-$\beta$/Ala-OH, for which the yield was 60 $\%_\circ$, and both $\alpha$ and $\beta$ configurations were present. This may be due to the compact shape of Man$\times$2Man which is less extended than the other disaccharides [22] (see Figure 1). The oligolysine-based saccharide clusters were synthesized starting from a series of peptide cores, Lys$_n$-Ala-Cys-NH$_2$ with $n = 1$ to 5, and (Lys-Ala)$_n$-Cys-NH$_2$, with the aim of investigating the influence of the number and spatial distribution of the oligosaccharides on their cell uptake. They were characterized by HPLC, $^1$H NMR and ESI-MS and matrix-assisted laser-desorption ionization-time-of-flight (‘MALDI-TOF’) MS (see Table 1). When the synthesis of oligolysine clusters was conducted without a dehydrating agent, the substitution of the amino groups of proteins was controlled by passing different concentrations of Na$_2$SO$_4$ in order to avoid any mass transfer problems. The duration of both the binding and the dissociation steps was set at 360 s to reach a plateau value. The regeneration of the chip was achieved by using 0.3 M lactose and 0.3 M $\alpha$-methylmannopyranoside for the Rca and ConA respectively. This large concentration of regeneration solution ($\approx 10\%$ from their UV absorbance at 490 nm at pH 7.4 (fluorescein $\epsilon_{490}$) was obtained from the sensor-chip, starting with a lectin concentration of 100 $\mu$g/ml for the Rca and 10 $\mu$g/ml for the ConA.

**SPR**

SPR allows the determination of both the time course of the binding and the affinity constants. ConA and Rca were linked to the sensor chip, starting with a lectin concentration of 100 $\mu$g/ml and leading to a large increase in the refractive index: $\Delta$RU = 3500. The neoglycoprotein in the absence or presence of inhibitors was injected at a flow rate of 20 $\mu$l/min in order to avoid any mass transfer problems. The duration of both the binding and the dissociation steps was set at 360 s to reach a plateau value. The regeneration of the chip was achieved by using 0.3 M lactose and 0.3 M $\alpha$-methylmannopyranoside for the Rca and ConA flow cells respectively. This large concentration of regeneration agent completely washes out any ligand from the flow cell without the loss of any immobilized lectin, owing to the neutral nature of such agents. The binding specificity of the neoglycoproteins was controlled by passing different concentrations of Man-BSA on immobilized Rca and Lac-BSA on ConA: in both cases, there was no detectable increase in refractive index. The number of resonance units (RU) was obtained from the sensorgrams and $RU_{\text{max}}$ deduced from the intercept of the linear transformation: $(1/\text{RU})$ versus $(1/g)^2$. The heterogeneity coefficient was $\alpha = 0.55$ for the Lac-BSA binding on to immobilized Rca and $\alpha = 0.7$ for the Man-BSA binding on to immobilized ConA. The neoglycoprotein concentration ($10 \mu$g/ml) corresponding to $RU_{\text{max}}/2$ was chosen to perform inhibition experiments with glycosylated clusters (Figure 3).

It appears that the lactose-based clusters containing either four or five lysines have the highest affinity towards Rca, which is $2 \times 10^5$ times larger than that of free lactose (Table 2). The binding constant of dimannosyl clusters onto ConA was deduced from their inhibitory effect on the binding of mannosylated bovine serum albumin on immobilized ConA. Man$\times$2Man-$\beta$-Glp-$\beta$/Ala-[Lys (Man$\times$2Man-$\beta$-Glp-$\beta$/Ala)$_n$-Ala-Cys(SPy)-NH$_2$

**Figure 3 Analysis of Lac-BSA binding on Rca with the glyocluster inhibitor Lac-Glp-$\beta$/Ala-[Lys(Lac-Glp-$\beta$/Ala)$_n$]-Ala-Cys(SPy)-NH$_2$**

Binding analysis of a sensorgram corresponding to the inhibition of the binding of Lac-BSA (10 $\mu$g/ml) on Rca with the glyocluster inhibitor Lac-Glp-$\beta$/Ala-[Lys(Lac-Glp-$\beta$/Ala)$_n$]-Ala-Cys(SPy)-NH$_2$ at concentrations of 0, 0.25, 0.5, 1.0, 2.5, 5.0 and 10.0 M. Resp. Diff. stands for response difference.

<table>
<thead>
<tr>
<th>Lysine-based lactose clusters</th>
<th>$K_i$ (litre $\cdot$ mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>$1.0 \times 10^3$</td>
</tr>
<tr>
<td>Lac$<em>\beta$/Glp-$\beta$/Ala-[Lys(Lac$</em>\beta$/Glp-$\beta$/Ala)$_n$]-Ala-Cys(SPy)-NH$_2$</td>
<td>$4.5 \times 10^5$</td>
</tr>
<tr>
<td>Lac$<em>\beta$/Glp-$\beta$/Ala-[Lys(Lac$</em>\beta$/Glp-$\beta$/Ala)$_n$]-Ala-Cys(SPy)-NH$_2$</td>
<td>$4.0 \times 10^5$</td>
</tr>
<tr>
<td>Lac$<em>\beta$/Glp-$\beta$/Ala-[Lys(Lac$</em>\beta$/Glp-$\beta$/Ala)$_n$]-Ala-Cys(SPy)-NH$_2$</td>
<td>$1.0 \times 10^5$</td>
</tr>
<tr>
<td>Lac$<em>\beta$/Glp-$\beta$/Ala-[Lys(Lac$</em>\beta$/Glp-$\beta$/Ala)$_n$]-Ala-Cys(SPy)-NH$_2$</td>
<td>$3.0 \times 10^5$</td>
</tr>
</tbody>
</table>

**Table 2 Inhibition of the interaction of immobilized Rca with Lac-BSA, by lysine-based lactose clusters**

The association constants ($K_i$) of the inhibitors were determined by surface plasmon resonance. The enhancement factors were quite similar for a given glycocluster with a variation within $\pm 10\%$ between independent experiments. The compound in bold is the most effective inhibitor, owing to its high affinity and small size.

<table>
<thead>
<tr>
<th>Lysine-based dimannose clusters</th>
<th>$K_i$ (litre $\cdot$ mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Man$<em>\times$2Man-$\beta$-Glp-$\beta$/Ala-[Lys(Man$</em>\times$2Man-$\beta$-Glp-$\beta$/Ala)$_n$]-Ala-Cys(SPy)-NH$_2$</td>
<td>$5.5 \times 10^6$</td>
</tr>
<tr>
<td>Man$<em>\times$2Man-$\beta$-Glp-$\beta$/Ala-[Lys(Man$</em>\times$2Man-$\beta$-Glp-$\beta$/Ala)$_n$]-Ala-Cys(SPy)-NH$_2$</td>
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<td>$3.0 \times 10^6$</td>
</tr>
</tbody>
</table>

**Table 3 Inhibition of the interaction of immobilized ConA with Man-BSA, by lysine-based dimannose clusters**

The association constants ($K_i$) of the inhibitors were determined by surface plasmon resonance. The enhancement factors were quite similar for a given glycocluster with a variation within $\pm 10\%$ between independent experiments.
had a higher affinity than ConA than the two other clusters synthesized from Man₃Manβ-Glp-βAla and Man₆Manβ-Glp-βAla, which have a low affinity (Table 3).

**Endocytosis of oligosaccharide clusters**

The endocytosis efficiency of two series of lysine-based disaccharide clusters, containing up to five lysine residues, was assessed by flow cytometry using two types of cells, HepG2 cells and DΦ dendritic cells; three or more independent experiments were performed and one typical experiment is presented. The endocytosis of lactose clusters by HepG2 cells (Figure 4A) was poor with bis- and tris-lactose clusters, K₁Lac₂, K₂Lac₃, K₄Lac₅, respectively, and large with tetrakis- and pentakis-lactose clusters, K₅Lac₆. The endocytosis was maximal with both hexakis-lactose clusters based on either a pentalsylamine derivative (K₆Lac₆) or a pentalsylaniline derivative (Lac[(Lac)₅]₆), showing that the presence of an intercalating amino acid between two ε-glycosylated lysines did not change the recognition efficiency.

Endocytosis of the dimannose (Man₃Man = Os) cluster by DΦ dendritic cells (Figure 4B) was very poor with bis- and tris-dimannose clusters, K₁Os₂, K₂Os₃, respectively, suboptimal with tetrakis-dimannose cluster (K₄Os₄) and maximal with both pentakis- and hexakis-dimannose clusters (K₅Os₅ and K₆Os₆, respectively).

The endocytosis specificity with regards to the cell type was clearly demonstrated by showing that optimal lactose clusters were efficiently taken up by HepG2 cells and not at all by DΦ dendritic cells, and conversely that optimal dimannose clusters were efficiently taken up by DΦ dendritic cells and quite poorly by HepG2 cells (Figures 4A and 4B).

Upon endocytosis, disaccharide clusters were localized in acidic compartments, as shown by a cell-associated fluorescence increase upon incubation at 4°C in the presence of 50 μM monensin (Figures 4A and 4B). Indeed, monensin is a proton/sodium ionophore which transports univalent cations (Na⁺ and H⁺) across membranes and, thereby, equilibrates the external and internal pH of organelles in intact cells [14]. Thus, the enhancement of the cell fluorescence intensity upon monensin treatment is due to the neutralization of endosomes and lysosomes. It is worthy of note that the monensin-dependent enhancement was much larger in the case of HepG2 cells than in the case of DΦ dendritic cells, reflecting the fact that the fluorescent clusters were delivered into more acidic compartments in the hepatoma cells than in dendritic cells.

Dimannose clusters were taken up with an efficiency which partially depended on the linkage between the two mannoses (Figure 5): Man₃Manβ-Glp-βAla[Lys(Man₃Manβ-Glp-βAla)]₅Ala-Cys(Flu)-NH₂.

**DISCUSSION**

Cells express on their surface a variety of sugar receptors called lectins (reviewed in [23,24]). These cell surface lectins, as well as their ligands, sugar moieties of specific glycoconjugates, are involved in several important phenomena, including cell-cell recognition, glycoconjugate-cell recognition and the uptake of glycoconjugates. This last property is the basis of glycotargeting (reviewed in [25–27]). Dendritic cells, which are key cells for antigen presentation and cellular vaccination (reviewed in [28]), express mannose-specific lectins [9,10,29] and are therefore suitable targets for drug delivery based on glycoconjugates containing mannose.

The recognition of a simple sugar by a lectin is usually in the very low affinity range (approx. 10⁻⁴ litre · mol⁻¹). Conversely,
complex oligosaccharides, neoglycoproteins, as well as saccharide clusters, bind lectins in the high affinity range (up to \(10^2\)–\(10^8\) litre \cdot mol\(^{-1}\); reviewed in [18,19]). For clinical purposes, animal glycoproteins including neoglycoproteins should be avoided because they may be contaminated by virus or prions etc., while synthetic carbohydrate clusters are suitable. Indeed, several authors have developed different glyoclusters (reviewed in [19,30,31], including glycodendrimers, glucostarburst, glucostarfish and glycosylated peptides. Glyoclusters may be synthesized using classical chemical approaches starting with fully protected sugars (reviewed in [32]).

With the aim of creating therapeutic tools for targeting oligonucleotides and genes, we designed glyoclusters that are made of natural metabolites, namely amino acids and sugars. Glyoclusters are made of glycosynthons [6] which are prepared according to a one-pot two-step reaction, starting with reducing sugars and a glutamate derivative. In the present paper, we used Glu-\(\beta\)-Ala-benzyl ester instead of Glu-\(\beta\)-nitroanilide [21] in order to get rid of the anilide moiety. Such glycosynthons, after benzyl group removal, were activated and linked to the amino groups of an oligosaclyse, in the presence of molecular sieves. Molecular sieves allow an increase in the reaction yield from approx. 60 % amino group substitution [7] up to completion. The use of oligosaclyse to prepare glyoclusters was pioneered by Ponpipom [1] and Robbins [4] and used by others including Toyokuni and Hakomori [33], as well as Biessen and co-workers [5,34], and Grandjean and co-workers [3,35]. The preparation of the glyoclusters presented in the present paper did not require any protection of the hydroxyl groups of the sugar moiety, in contrast with Ponpipom [1], Kragol [36] or Kichler [2], who used peracetylmannosylthioipropionate, peracetylmannosylserine or peracetylgalactosylthioethyloxyethyl derivatives respectively. The yield of glyoclusters using the preparation described in the present paper was quite high, up to 90 % for the tetrakis dimannosyl trislyxse or pentakis lactose tetralysine, in contrast with yields of 34 % for the tetrakis mannose trislyse [5] using monosaccharyl phenylisothiocyanate. The method used in the present paper allows the preparation of glyoclusters containing disaccharide as well as oligosaccharides (results not shown); the unique requirement is to use an oligosaccharide which terminates with a reducing sugar as the starting material. The possibility of using disaccharides or higher oligosaccharides was thought to enhance the selectivity. Indeed, plant and animal lectins recognize disaccharides and oligosaccharides more selectively than simple sugars. For instance, the macrophage mannose-specific lectin recognizes an oligomannoside containing a Man\(\alpha\)\(2\)Man\(\alpha\)\(6\)Man motif more efficiently than a monosaccharide [37]. Knowing that natural glycans contain Man\(\alpha\)2Man, Man\(\alpha\)3Man and Man\(\alpha\)6Man, we prepared glyoclusters containing these disaccharides, as well as glyoclusters containing lactose as a control.

The association constants for a series of lactose clusters and a series of dimannose clusters were deduced from SPR experiments. In most of the published data, the interactions are measured by running the lectin on immobilized oligosaccharides. We have chosen immobilization of the lectin in order to compare easily the affinities of a series of different glycoconjugates, and also because Shinohara and co-workers [38] showed that when the lectin was immobilized, the calculated affinity constant was in good agreement with values obtained using classical methods with lectins in solution. The analysis of the cluster binding to plant lectins indicated that Rca, a galactose-specific lectin, recognized lactose clusters, and amongst them, the pentakis lactose tetralysine with a high affinity, while it did not recognize any dimannose clusters. Similarly, ConA did not recognize the lactose clusters but it did bind the dimannose clusters, the best ligand being the cluster made of Man\(\alpha\)2Man, in agreement with published data (reviewed in [39,40]).

The glyoclusters as fluorescence-labelled derivatives were shown to be specifically recognized and taken up by cells expressing membrane lectins. HepG2 cells, which express a galactose-specific lectin [8,41] but not a mannose-specific lectin, optimally took up clusters containing at least four lactose groups, but failed to take up any dimannose clusters. Similarly, DF dendritic cells, which express mannose-specific lectins, a macrophage mannose-specific receptor (reviewed in [42]), as well as dendritic cell-specific ICAM-grabbing non-integrin (DC-SIGN, [29]), optimally recognized and took up glyoclusters containing five dimannoses, whereas they failed to recognize lactose clusters. In addition, the dimannose clusters containing Man\(\alpha\)6Man were more efficiently taken up by DF dendritic cells than those containing either Man\(\alpha\)3Man or Man\(\alpha\)2Man.

The preferential uptake of Man\(\alpha\)6Man clusters by dendritic cells is in agreement with the fact that oligomannosides containing Man\(\alpha\)6Man are the best ligands for the macrophage-like mannose receptor [37]. This selective uptake may mean either that DC-SIGN prefers the Man\(\alpha\)6Man based clusters, or that it is not predominantly involved in the uptake of any dimannoside clusters. This last hypothesis could be in accord with the recent results published [43], which showed, on the basis of three-dimensional studies, that the binding site of DC-SIGN accommodates high-mannose oligomannosides containing branched structures such as Man\(\alpha\)6(Man\(\alpha\)3)Man.

CONCLUDING REMARKS

The conditions for the preparation of well-defined sugar clusters based on disaccharides and an oligosaclyse core have been optimized; their binding capacity is optimal when five sugar moieties are present. The use of such sugar clusters as recognition signals for antigen and/or gene targeting in a vaccine approach, as well as the preparation and the use of glyoclusters containing glycosynthons made with three and more sugar units, are currently being investigated in our laboratory.

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