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Specific and non specific interactions involving Le^X determinant quantified by lipid vesicle micromanipulation

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Carbohydate-carbohydrate recognition is emerging today as an important type of interaction in cell adhesion. One Ca^{2+} mediated homotypic interaction between two $Lewis^X$ determinants (Le^X) has been proposed to drive cell adhesion in murine embryogenesis. Here, the adhesion energies of lipid vesicles functionalised with glycolipids bearing monomeric or dimeric Le^X determinants were measured in NaCl or $CaCl_2$ media with the micropipette aspiration technique. These experiments on Le^X with an environment akin to that provided by biological membrane confirmed the existence of this specific calcium dependant interaction of monomeric Le^X. In contrast, dimeric Le^X produced a repulsive contribution. By using a simple model involving the various contributions to the adhesion free energy, specific and non specific interactions could be separated and quantified. The involvement of calcium ions has been discussed in the monomeric and dimeric Le^X lipids.
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Introduction

Many different glycoproteins and glycolipids are present at the surface of cells. Mother nature takes advantage of the enormous variety of structures which can be found in sugars to make the various weak biochemical bonds and recognition processes which take place in biology. In the past decades, the protein-carbohydrate interaction has been extensively studied and is now well documented [1–3]. At the same time, a direct interaction between carbohydrates was suggested. Hakomori pioneered a research on carbohydrate interactions in embryonal and tumor cells [4], and Misevic and Burger [5] reported carbohydrate interactions in marine sponge. This basic and novel recognition mechanism between two carbohydrates is now accepted by the scientific community. It however remains insufficiently documented because of the weakness of such an interaction which is difficult to probe by classical techniques. Nevertheless, in the last past years, the use of recent methods like atomic force microscopy [6], nuclear magnetic resonance [7–9], surface plasmon resonance [10–12] and micromanipulation experiments [13] on a few model systems especially designed to test carbohydrate-carbohydrate interactions have produced new data on this interaction [14–18]. Hakomori has identified different types of glycosphingolipids expressed at the surface of cells [19,20]. He has studied their implication in carbohydrate-carbohydrate interactions during the cell adhesion processes involved in murine embryogenesis and metastasis of melanoma and lymphoma mice cells [4]. One of these carbohydrates, the $Lewis^X$ determinant (Le^X) has been shown to be involved in murine embryogenesis. It is not expressed at the cell surface until the eight-cell stage, shows maximal expression at the 8–16 *morula* cells (*i.e.* compaction stage) and declines rapidly after compaction [20,21]. This compaction stage could be inhibited either by anti-Le^X antibodies, Le^X itself, or by inactivating the calcium with EDTA [22,23]. These results [4,22,23] led to the hypothesis of a calcium mediated Le^X-Le^X specific homotypic interaction as a basis for cell recognition. Experiments on model systems derived from biological products allowed to qualitatively test this hypothesis [24]. Nevertheless, such biological materials involve too many non-controlled parameters which hinder a detailed study of this weak Le^X-Le^X interaction. To avoid this difficulty, well-defined model systems

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involving Le^X determinants have been used in the techniques listed above. Each approach has contributed to the establishment of a calcium mediated homotypic recognition [7,24], providing information on its geometry, its structural requirements [8,9,25] and its energetics [12,13,18].

The aim of this study was to confirm the existence of a calcium dependant homotypic Le^X-Le^X recognition and to obtain quantitative physico-chemical information on this interaction. In natural glycosphingolipids, the Le^X determinant is attached to the aliphatic tails through a lactose group. Moreover, in nature, cells bear molecules wherein oligosaccharides are composed of one Le^X determinant but also several Le^X in series. To mimic nature, lipids bearing lactose connected to monomeric and dimeric Le^X were both synthesized. These Le^X bearing lipids were then inserted into vesicles (lipid bilayers) which provide a soft environment for the Le^X with mechanical features akin cell membranes. Two such vesicles in tight contact mimic two adherent cells during the compaction stage. Adhesion energies were measured by bringing two vesicles into contact using micropipette manipulation. An accurate quantitative analysis of this adhesion yielded detailed information on the energetics of the Le^X-Le^X interaction, and insights on the role of the calcium ions.

Materials and methods

Glycosphingolipids

Three glycolipids were synthesized. All of them were neutral, and had the same aliphatic tails but different oligosaccharide headgroups (Figure 1).

To ensure low solubility of the glycolipids in water, good bilayer cohesion and good translational mobility thought necessary for the interaction between Le^X groups, the hydrophobic moieties were composed of three branched hydrophobic chains. The aliphatic tail was connected to the oligosaccharide headgroup through a flexible PEO chain. This spacer provided the Le^X determinant with a high orientational mobility. The first glycolipid headgroup was composed of a disaccharide (lactose) (Figure 1a). The second one was a pentasaccharide made of lactose connected to a Le^X determinant (Figure 1b) [28]. The last was an octasaccharide with a lactose group and a Le^X dimer (two Le^X groups in series) (Figure 1c). The names of the glycolipids reflect the composition of their headgroups: Lac, Le^XLac, Le^XLe^XLac.

Vesicle adhesion energy measurements

Giant vesicles were formed from a 2:1 ethanol/chloroform solution of a 1:9 glycolipid/stearoyl-oleoylphosphatidylcholine (SOPC) mixture by lipid hydration, after evaporation of the solvant, in 320 mOsm sucrose solution. For the micromanipulation experiments, the vesicle suspension was added to an aqueous glucose solution chamber of the same osmolarity (320 mOsm). Two vesicles of cellular dimensions (10–50 μm) were then transferred to another chamber filled with salt solution (either NaCl or $CaCl_2$). In order to slightly deflate the vesicles and make them micromanipulable, the osmolarity in this chamber (360 mOsml) was slightly higher than that of the vesicles. Such an osmolarity was obtained by dissolving 0.11 mole of $CaCl_2$ salt (resp. 0.2 mole of NaCl) in 1 liter of ultrapure water (pH $\approx$ 5.5). The $CaCl_2$concentration was chosen in order to have one Ca^{2+} ion available per glycolipid in the aqueous film which separates the two vesicles in contact. In high calcium concentration, the vesicles were slightly more fragile than in glucose solution. They broke more easily upon being seized with a micropipette but once a vesicle was

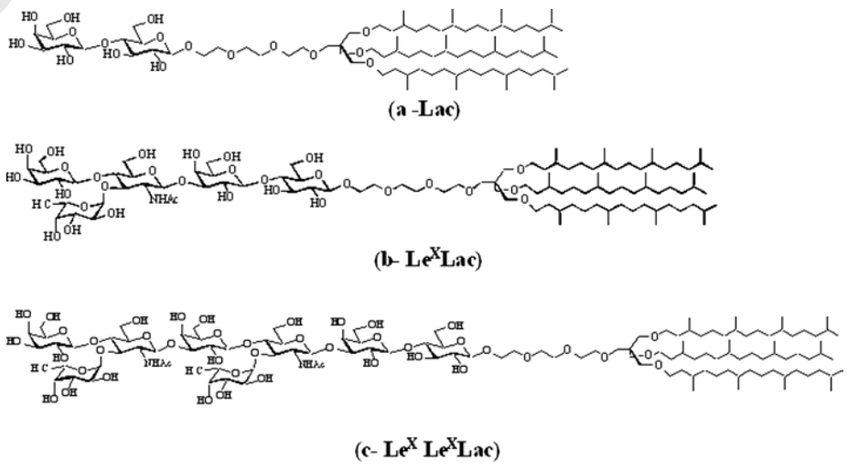

Figure 1. Glycolipids were composed of an oligosaccharide headgroup connected to a hydrophobic moiety through a flexible spacer. The hydrophobic part was composed of three branched aliphatic tails to ensure good bilayer cohesion and translational mobility. The flexible spacer was a small PEO chain which provided orientational mobility to the sugar headgroup. The oligosaccharide headgroup was (a) lactose group in the Lac molecule, (b) a Le^X determinant connected to a lactose group in the Le^XLac molecule, (c) a Le^X dimer connected to a lactose group in the Le^XLe^XLac molecule.

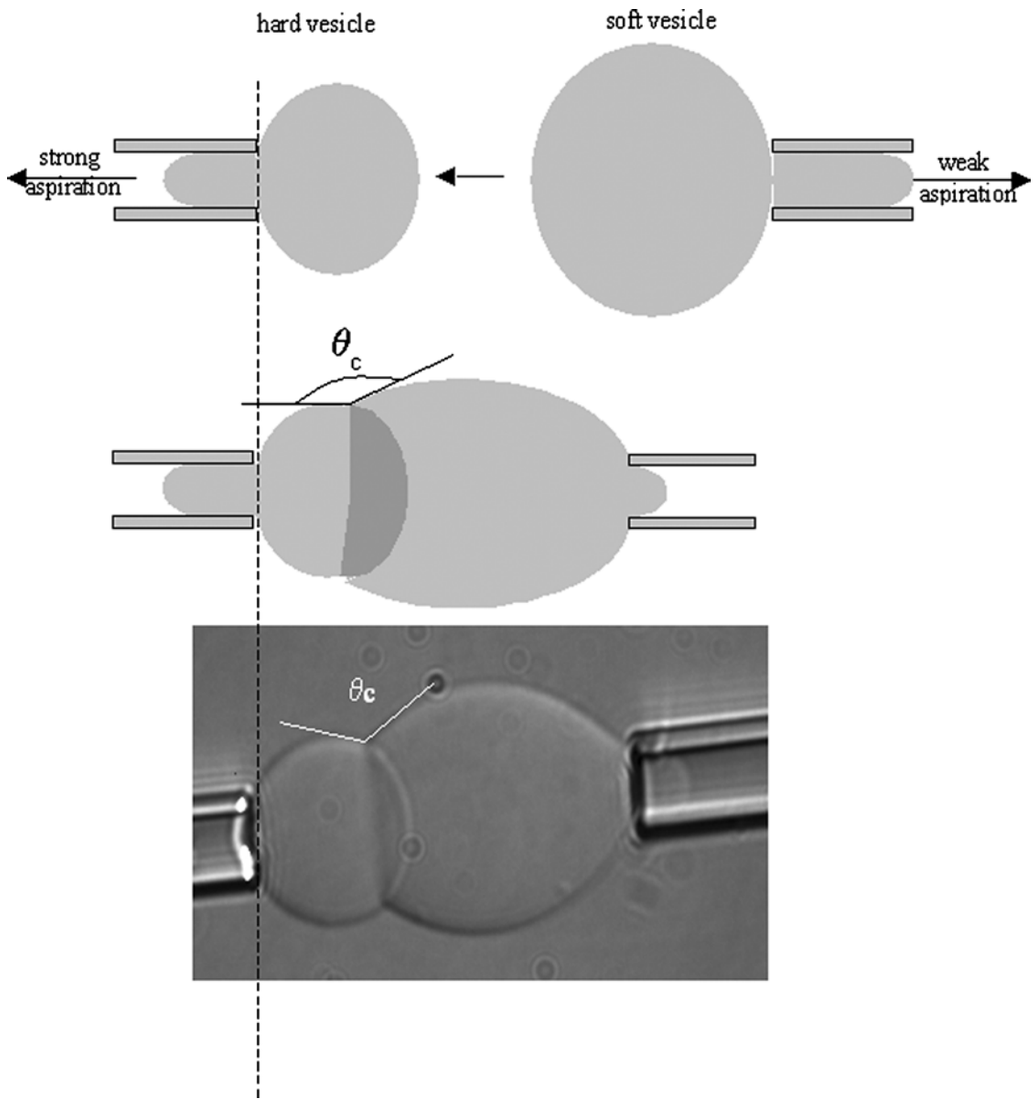

Figure 2. The two osmotically controlled vesicles held in micropipets by aspiration were observed by interference contrast microscopy. The suction pressure applied to the micropipets allowed the tension of the vesicle bilayers to be controlled. One of them (left) was pressurised into a tight-rigid sphere with large bilayer tension, whereas the adherent vesicle (right) was held with low pressure and remained deformable. The adhesion energy W_{adh} was obtained by determining the contact angle θ_c of the two vesicles and the tension τ_m of the membrane (30): $W_{adh} = \tau_m(1\text{-}\cos\theta_c)$.

maintained aspirated, it could be used for the whole duration of the measurements, *i.e.* from a few minutes up to two hours.

The two vesicles aspirated in micropipettes were then brought into contact (Figure 2).

The (negative) pressure ΔP in each pipette controlled the (positive) hydrostatic pressure in the vesicles and thus their membrane mechanical tension τ_m:

$$\tau_m = \frac{\Delta P}{2\left(\frac{1}{r_p} - \frac{1}{r_v}\right)} \tag{1}$$

where r_p and r_v are respectively the radius of the micropipette and of the vesicle. Both osmotically controlled vesicles were observed in interference contrast microscopy. One of them was pressurized into a rigid sphere by applying a large bilayer tension, whereas the adherent vesicle was held with low pressure and remained deformable. The adhesion energy W_{adh} was obtained by determining the contact angle θ_c of the two vesicles (Figure 2) and the tension τ_m of the flaccid vesicle membrane [27]:

$$W_{adh} = \tau_m\,(1 - \cos\theta_c) \tag{2}$$

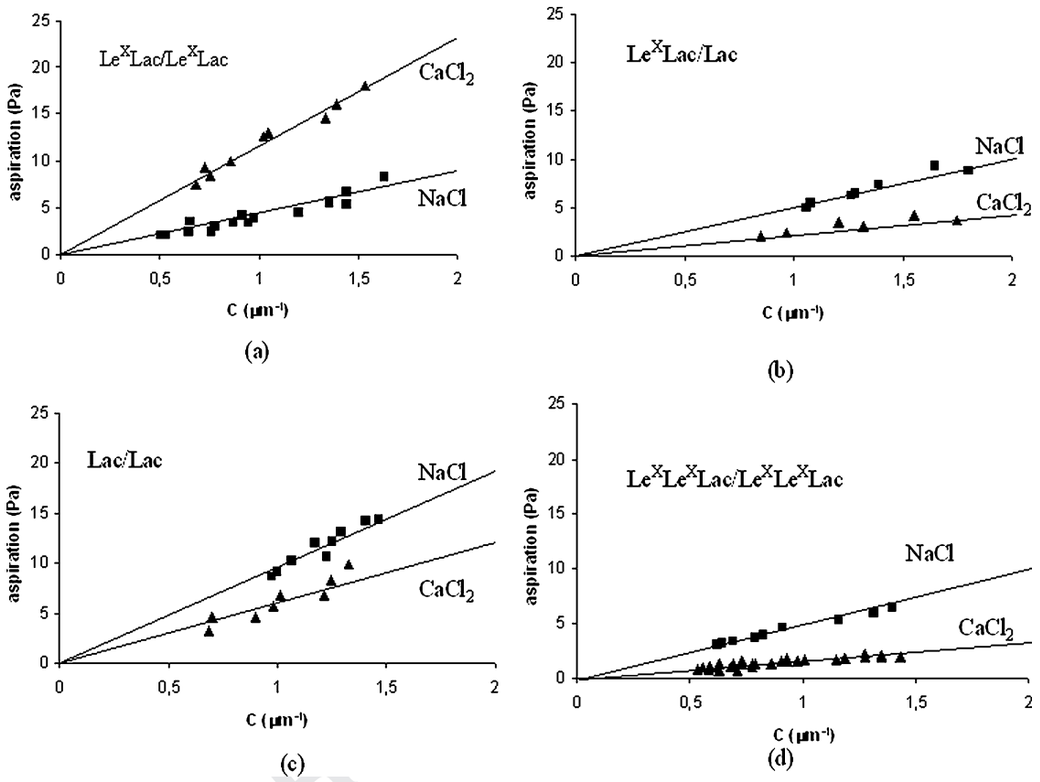

Figure 3. Aspiration pressure as a function of parameter C given in Eq. (1.3 and 1.4): (a) Le^XLac/Le^XLac pair, (b) Le^XLac/Lac pair, (c) Lac/Lac pair, (d) Le^XLe^XLac/Le^XLe^XLac pair. All the vesicles were composed of a 9 :1 mixture of SOPC and glycolipid. The triangles are for 0.11 M $CaCl_2$ solution and squares for 0.2 M NaCl. The straight lines are least square fits.

Combining Eqs. (1) and (2) yields a relationship between ΔP and W_{adh}:

$$\Delta P = C \cdot W_{\text{adh}} \tag{3}$$

where C depends only on the geometry of the system:

$$C = \frac{2\left(\frac{1}{r_p} - \frac{1}{r_v}\right)}{1 - \cos\theta_c} \tag{4}$$

θ_c was numerically deduced from geometrical parameters as indicated in ref. [27]. θ_c was determined for several tension values of the flaccid vesicle membrane by decreasing the aspiration and then increasing it in order to check the reversibility of the adhesion. Plots of ΔP versus C yield W_{adh}, the slope of the linear regression (Figure 3).

Results and discussion

Adhesion energy measurements were performed on five series of different vesicle pairs (see Table 1). At least three pairs of each series were tested in a 0.11 M $CaCl_2$ aqueous solution. The same was done in a 0.2M NaCl solution. Except for pure SOPC vesicles (named SOPC), the vesicles were all composed of a 1:9 mixture of glycolipid and SOPC, and are simply referred to by the name of their constituent glycolipid. Le^XLac/Le^XLac and Le^XLe^XLac/Le^XLe^XLac vesicle pairs were used to test Le^X-Le^X recognition and the influence (if any) of the number of Le^X determinants on adhesion. The adhesion energy of Le^XLac/Le^XLac pairs was compared to that of the Le^XLac/Lac control experiments in which the Le^X groups were absent on one of the vesicles. As calcium is known to produce sometimes peculiar effects on bilayer interactions [28] two additional controls were performed : Lac/Lac experiments in which the glycolipids

Table 1. Adhesion energy of vesicle pairs measured in $CaCl_2$ or NaCl aqueous media

	W_{adh} ($\mu J/m^2$)	
left vesicle/right vesicle	*in $CaCl_2$ (0.11 M)*	*in NaCl (0.2 M)*
pure SOPC vesicles	15 ± 2	14 ± 2
Lac/ Lac	6.0 ± 1.0	9.5 ± 0.5
Le^XLac/ Lac	2.5 ± 2.0	5.4 ± 1.0
Le^XLac/ Le^XLac	11.0 ± 2.0	4.5 ± 2.0
Le^XLe^XLac/Le^XLe^XLac	1 ± 1	4.5 ± 2.0

do not have the Le^X determinant and SOPC/SOPC experiment as SOPC is the main component of all the vesicles used in this study. Figure 3 shows the aspiration pressure as a function of the geometrical parameter C (Eq. (4)) for the four bearing glycolipid vesicle pairs. For each graph, the triangles correspond to the typical values obtained as a function of the aspiration in the right pipette (Figure 2) for vesicles in 0.11 M $CaCl_2$ solutions and the squares to 0.2 M NaCl. The lines are the associated fits. The adhesion free energies of the vesicles, given by the slopes are displayed in Table 1.

In a NaCl environment, one can see in Table 1 that the adhesion energies vary monotonically with the size of the headgroups: the larger the headgroups, the larger the steric repulsion and therefore the smaller the adhesion energies. With calcium ions, the adhesion energies are slightly smaller but the same variation with molecular sizes is observed, except for the Le^{X}Lac/Le^{X}Lac pairin which a dramatic increase in adhesion is found when calcium is present.

Non specific adhesion with NaCl

It is useful to understand and quantify the various contributions to the vesicle adhesion energy in order to distinguish the specific effects from the non specific ones. Because of their large headgroups (flexible PEO spacer plus di-, penta- or octa-saccharide), Lac, Le^{X}Lac and Le^{X}Le^{X}Lac vesicles can be schematically represented as shown in Figure 4: the SOPC plus the aliphatic tails

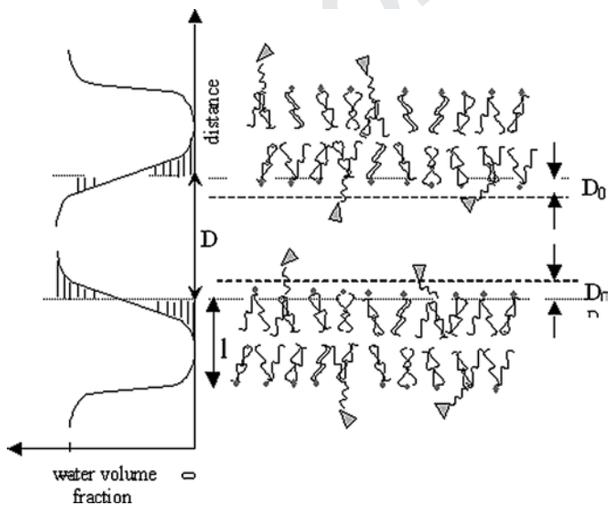

Figure 4. Representation of lipid bilayers in a vesicle adhesion experiment. Lipid chains connected to circles symbolize SOPC, whereas lipid chains connected to triangles (sugar headgroups) through small wiggles (PEO chains) symbolize glycolipids. The curve represents the water volume fraction (0 around the aliphatic chains and 1 in the gap between the layers). The distance D is taken between the water density weighted interface: the four striped zones have the same areas. D_0 is the distance between the surface of the vesicle and the plane of the charges.

of the glycolipids make a bilayers of thickness l, from which the large headgroups are protruding.

These extended sugar groups give rise to a steric repulsion equivalent to that produced by polymers [29]. The equilibrium distance between the vesicles and their adhesion free energy can be obtained by minimizing (relative to their separation distance) the free energy of interaction which involves several contributions: van der Waals attraction, undulation (or Helfrich) repulsion [30] which comes from spontaneous membrane undulations under thermal fluctuations, and steric repulsion due the the protuding headgroups. Note that hydration [31] and protrusion effects [32] of the glycolipids and/or SOPC also produce repulsive interactions. However, except for pure SOPC vesicle, the range of the repulsion they provide is shorter than the equilibrium distances of the vesicles. These interactions can therefore be neglected in this study.

In the case of two interacting vesicles, the van der Waals attraction has a power law distance dependence given by [33]:

$$W_{\mathrm{vdW}}(D) = \frac{H}{12\pi}\left[\frac{1}{D^2} - \frac{2}{(D+l)^2} + \frac{1}{(D+2l)^2}\right] \qquad (5)$$

where H is the Hamaker constant and l the bilayer thickness.

The Helfrich entropic repulsion also follows a power law distance dependence [30]. The expression derived in Ref. [34] is suitable for lipid bilayers and will be used here:

$$W_{\mathrm{Helfrich}}(D) = \frac{(k_B T)^2}{1.6\pi^2 k_c}\frac{1}{D^2} \qquad (6)$$

where k_c is the bilayer bending rigidity modulus.

The steric repulsion due the protruding glycolipid headgroups is given by [35,36]

$$W_P(D) = 36\Gamma k_B T e^{-\frac{D}{Rg}} \qquad (7)$$

where Γ is the surface density of glycolipids in the vesicle, and R_g is an effective radius of gyration independently obtained [29].

Assuming these contributions are additive (the approximation of additivity introduces an error smaller than the experimental one [37]), the theoretical interaction free energy can be calculated and compared to the adhesion energy experimentally obtained. For the vesicles composed of a 1:9 mixture of glycolipid and SOPC, the Hamaker constant H (in Eq. (5)) was taken as 9.5 10^{-21}J [29,37], the bilayer thickness l (Eq. (5)) is that of SOPC (Fig. 4) 4.06 nm, as measured in Ref. [31]. The bending rigidity modulus k_c (Eq. (6)) was approximated by that of SOPC 9.10^{-20}J [34]. In Eq. (7), Γgas obtained independently by isotherm measurements and is approximately 0.1 nm^{-2} for the three kinds of vesicles. The effective R_g value used in Eq. (7) was 0.52 nm for the Le^{X}Lac/Le^{X}Lac pair, 0.41 nm for Lac/Lac, 0.47 nm for Le^{X}Lac/Lac and 0.52 nm for Le^{X}Le^{X}Lac/Le^{X}Le^{X}Lac [29]. The adhesion energies calculated with these parameters are reported in Table 2 with the detail of all the contributions and the equilibrium distances.

Table 2. Vesicles adhesion free energy measured in NaCl and calculated. The detail of the van der Waals (W_{vdW}), Helfrich ($W_{Helfrich}$) and polymer (E_p) contributions and the equilibrium distance D_{eq} between the vesicles are also reported

in NaCl (0.2 M)	$W_{adh}(\mu J/m^2)$ *measured*	W_{adh} $(\mu J/m^2)$ *calculated*	D_{eq} *(nm)*	W_{vdW} $(\mu J/m^2)$	W_p $(\mu J/m^2)$	$W_{Helfrich}$ $(\mu J/m^2)$
Lac/Lac	9.5 ± 0.5	9.8	3.3	15.7	4.8	1.1
Le^XLac/Lac	5.4 ± 1	5.9	4.1	9.1	2.4	0.7
Le^XLac/Le^XLac	4.5 ± 2	4.0	4.7	6.4	1.8	0.6
Le^XLe^XLac/Le^XLe^XLac	4.5 ± 2	3.0	5.2	4.9	1.4	0.5

The calculated adhesion energies are in excellent agreement with the adhesion measured for all vesicle pairs. The main conclusion of this analysis is that the adhesion energy measured in NaCl environment results only from non specific interactions.

Le^X-Le^X recognition with calcium ions

Only for the Le^XLac/Le^XLac pair is the effect of adding calcium significant. The adhesion energy is 2.5 times that in NaCl (Table 1 and in Figure 3a) while the other pairs showed a small decrease of the adhesion energy in calcium. These results unambiguously show that one Le^X group is necessary on both vesicles for the calcium induced adhesion enhancement to occur. This is in agreement with the specific interaction scheme advocated by Hakomori [4].

The specific adhesion can be estimated as the difference between the adhesion energy measured with calcium ions and all non specific contributions. The specific adhesion (W_{spe}) is therefore given by:

$$W_{spe} = W_{adh}^{(Le^XLac/Le^XLac)_{CaCl_2}} - \left[W_{adh}^{(Le^XLac/Le^XLac)_{NaCl}} + \left(W_{adh}^{(Le^XLac/Lac)_{CaCl_2}} - W_{adh}^{(Le^XLac/Lac_{NaCl}}\right)\right] \quad (8)$$

Its value is about 10 ± 5 $\mu J/m^2$.

The calcium dependent Le^X-Le^X specific adhesion is therefore firmly established.

Glycolipids complex calcium ions

If two vesicles functionalized with Le^X show specific adhesion, one should expect a strengthening of adhesion with the di-Le^X system (Le^XLe^XLac/Le^XLe^XLac). Surprisingly, instead of enhancing adhesion, calcium lowered it significantly (by 80%) (Figure 3d). As previously mentioned, a decrease in adhesion was also measured with Le^XLac/Lac and Lac/Lac pairs (Table 1). Since $CaCl_2$ salt had no significant influence on the adhesion energy of pure SOPC vesicles (Table 1), one can assert that the decrease of adhesion is correlated to the glycolipids. It is very likely due to a charge effect resulting from the complexation of calcium ions with one or several functional groups of the glycolipid. In that case, an additional electrostatic double-layer repulsion would also contribute to the interaction energy. For a 1:2 electrolyte as $CaCl_2$, such a contribution is given by [38]:

$$W_{DL}(D) = 0.0833[CaCl_2]^{\frac{1}{2}} \tanh^2 \times \left(\frac{e}{4k_BT} \times 0.176 \times \frac{\sigma}{\varepsilon_0\varepsilon_r[CaCl_2]^{\frac{1}{2}}}\right) e^{-\frac{0.176(D-2D_0)}{[CaCl_2]^{\frac{1}{2}}}} \quad (9)$$

where σ is the density of charges and D_0 the distance between the vesicle surfaces defined as the density weight interface (because of the soft nature of membranes) and the plane of the charges as defined in Figure 4. The effect of charges on the adhesion energy of the vesicles was simulated allowing two parameters to vary: the surface density σ and the positions of the plane of the charges D_0 (Eq. (9)). With Le^XLac/Lac and Lac/Lac pairs, it has been shown that between 0.5 to 1 Ca^{2+} borne by the lactose groups would produce a repulsion equivalent to the observed adhesion energy decrease. In contrast, for Le^XLe^XLac/Le^XLe^XLac, such a complexation of Ca^{2+} and lactose group is not enough to account for the dramatic decrease of adhesion. The simulations suggest that dimeric Le^X determinant may also complex calcium ions which could make them unavailable for a Ca^{2+} mediated recognition and adhesion between the vesicles.

Conclusion

Monomeric Le^X determinants, when present on two vesicles, enhance their adhesion in a $CaCl_2$ environment. This Ca^{2+} mediated recognition between two Le^X, in a context similar to cells during embryogenesis validates the specific interaction scheme advocated by Hakomori.

With a micromanipulation technique we measured the adhesion energy of four pairs of vesicles bearing glycolipids. By using a simple model involving non specific interactions, we could separate and quantify non specific and specific contributions. With NaCl, the adhesion energy, resulting from van der Waals attraction, Helfrich repulsion and steric repulsion due to the protruding glycolipid headgroups for the four pairs, is non specific. In a $CaCl_2$ environment, our measurements indicate that lactose and calcium are able to associaate, producing an effective surface charge. If the resulting surface charge change is taken into account, the non-specific adhesion of Lac/Lac and Le^XLac/Lac pairs is completely accounted for. In contrast, the Le^XLac/Le^XLac adhesion involved a specific contribution due

to Le^X-Le^X recognition. An interesting observation of this study is the collapse of adhesion when di-Le^X determinants are used, which strongly suggests that di-Le^X can also complex calcium ions. Such a high affinity of di-Le^X determinants for calcium ions may play an important part in physiological situations.

Chemistry

The synthetic process for the preparation of these 3 glycolipids was described as follows.

The known lactosyl bromide **1** [40] was condensed with the readily prepared lipid **2** [26] in the presence of silver triflate to give the benzolated β-lactoside **3** in 80% yield which, after treatment with sodium methoxide at room temperature, provided the target lactosyl lipid in 90% yield (Figure 5).

The pentasaccharidic lipid **Le^{x}Lac** was synthesized using **Lac** as the starting material. Thus 3′,4′-*O*-isopropylidene of **Lac** was first formed by treatment of acetone dimethyl acetal in DMF containing camphorsulfonic acid in room temperature to afford **4** which, after benzylation, gave **5**. Treatment of **5** with trifluoroacetic acid provided diol **6**. Glycosylation of **6** with the previously described thioglycoside **7** [41] was performed in the presence of N-iodosuccinimide (NIS), trifluoromethanesulfonic acid (TfOH) [42] and molecular sieves (4 Å MS) to afford stereoselectively the pentasaccharidic block **8**. The stereochemistry of the newly introduced glycosidic linkage in **8** was determined to be β on the basis of the H-1c, H-2c coupling constant ($J_{1c,2c}$ = 8.4 Hz). Treatment of **8** with hydrazine in refluxing aqueous ethanol, followed by acetylation with acetic anhydride in pyridine, afforded the derivative **9** which was de-*O*-acetylated to give the compound **10**. Catalytic hydrogenolysis of **10** in methanol and ethyl acetate gave the target glycolipid **Le^{x}Lac** (Figure 6).

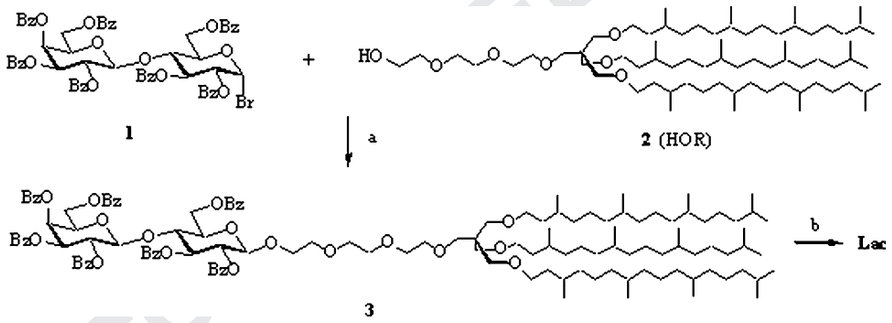

Figure 5. Preparation of lactosyl lipid: (a) AgOTf, CH_2Cl_2, -20°C; (b) NaOMe, MeOH, CH_2Cl_2, RT.

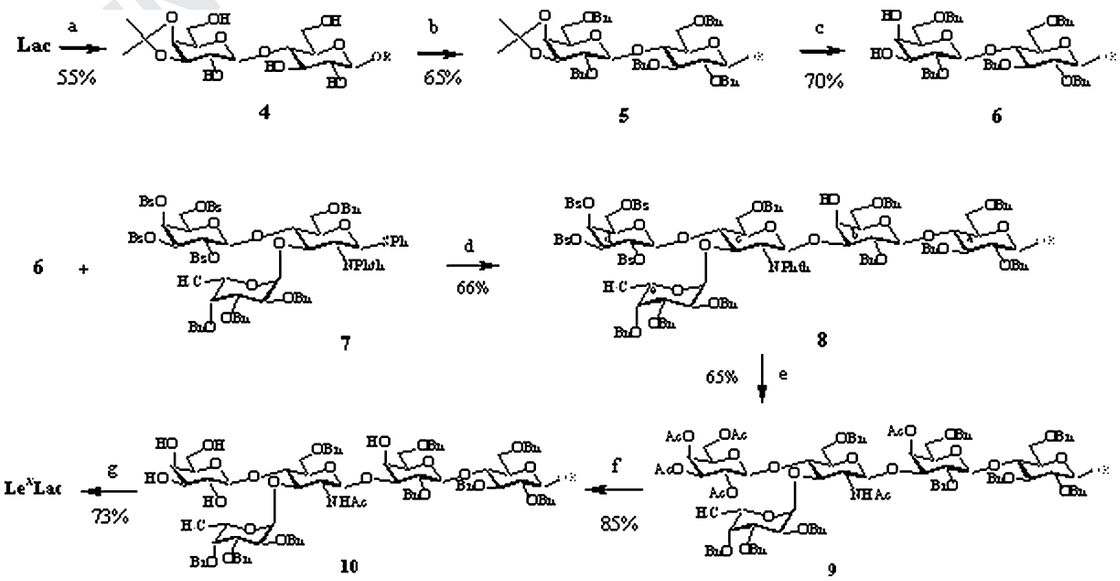

Figure 6. Synthesis of pentasaccharidic lipid: (a) $(CH_3)_2C(OCH_3)_2$, CSA, DMF, RT; (b) BnBr, NaH, DMF, 80°C; (c) CF_3COOH, CH_2Cl_2, RT; (d) NIS, TfOH, CH_2Cl_2, 4Å MS, -30°C; (e) NH_2NH_2, C_2H_5OH aq. reflux, then Ac_2O, Pyridine; (f) NaOMe, MeOH, CH_2Cl_2, RT; (g) H_2, Pd/C, MeOH, EtOAc.

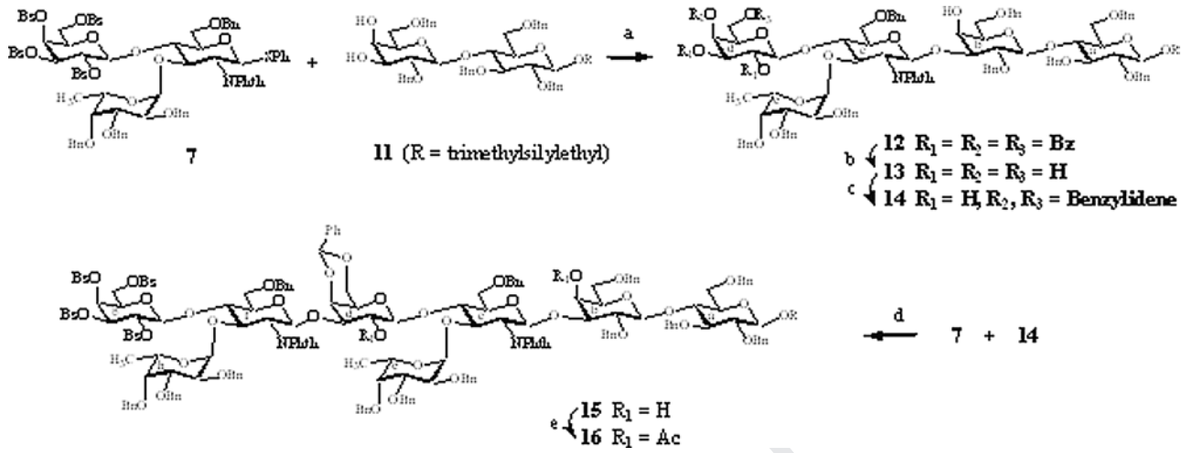

Figure 7. Synthesis of the protected octasaccharide**15**. (a) NIS-TfOH, CH_2Cl_2, 4Å MS, −20°C to RT; (b) NaOMe, MeOH-CH_2Cl_2, RT; (c) $PhCH(OMe)_2$, CSA, RT, 1h; (d) NIS-TfOH, CH_2Cl_2, 4Å MS, 0°C to RT; (e) Ac_2O, Pyridine, RT, quantitative.

For the synthesis of the octasaccharidic lipid **Le^{x}Le^{x}Lac**, a different strategy has been used. The oligosaccharide moiety was completely constructed before condensation with lipid part. Coupling of the previously used trisaccharide donor **7** with the known lactoside **11** [43] was performed in dichloromethane, promoted by NIS-TfOH, at −20°C for 1 h, then 12 h at room temperature. The desired pentasaccharide **12** was generated in 82% yield. The stereochemistry of the newly introduced glycosidic linkage in pentasaccharide **12** was determined to be β on the basis of the H-1c, H-2c coupling constant ($J_{1c,2c}$ = 8.4 Hz). Treatment of **12** with sodium methoxide in methanol—dichloromethane gave compound **13** in 89% yield which, after reaction with benzaldehyde dimethyl acetal in the presence of camphorsulfonic acid, gave the 4d,6d-*O*-benzylidene derivative **14** in 86% yield. Second coupling of trisaccharide donor **7** with the pentasaccharidic triol **14**, in the presence of NIS-TfOH, stereo- and regioselectively gave the protected octasaccharide **15** in 61% yield (Figure 7).

The stereochemistry of the newly introduced linkage in octasaccharide **15** was determined to be β on the basis of the H-1f, H-2f coupling constant ($J_{1f,2f}$ = 8.5 Hz). The regiochemistry of **15** was assigned from the ^{1}H NMR spectrum of **16**, obtained from **15** by acetylation, which revealed in $CDCl_3$ solvent, a deshielded signal for H-4b at 5.42 ppm (dd, $J_{4b,5b}$ < 1 Hz, $J_{3b,4b}$ = 3.3 Hz), and in $CDCl_3$–C_6D_6 (1:1) solution a deshielded signal for H-2d at 4.99 ppm (dd, $J_{1d,2d}$ = 8.3 Hz,

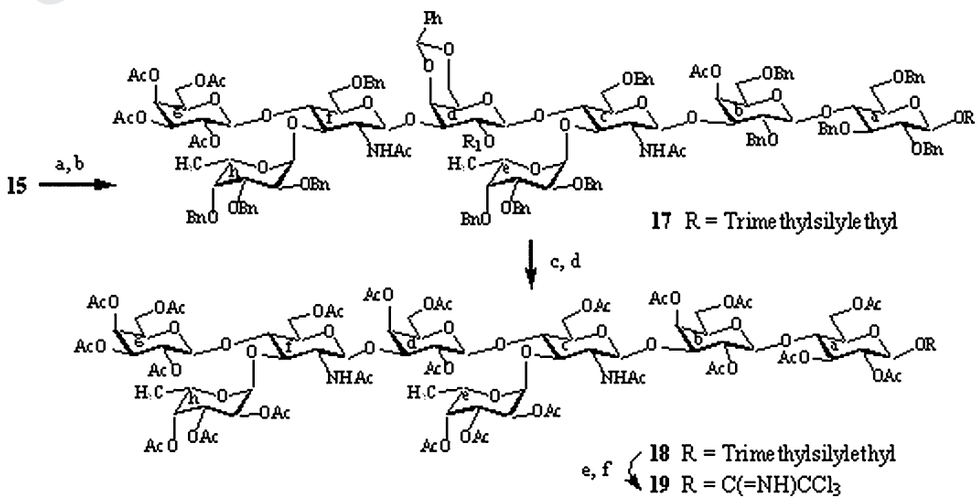

Figure 8. Synthesis of the fully acetylated octasaccharide **18** and transformation of **18** to donor **19**. (a) N_2H_4, C_2H_5OH, reflux, (b) Ac_2O, Pyridine, RT; (c) H_2, Pd/C, MeOH, EtOAc, RT; (d) Ac_2O, Pyridine, DMAP, RT to 40°C; (e) TFA, CH_2Cl_2, 0°C to RT; (f) Cl_3CCN, DBU, CH_2Cl_2, 0°C.

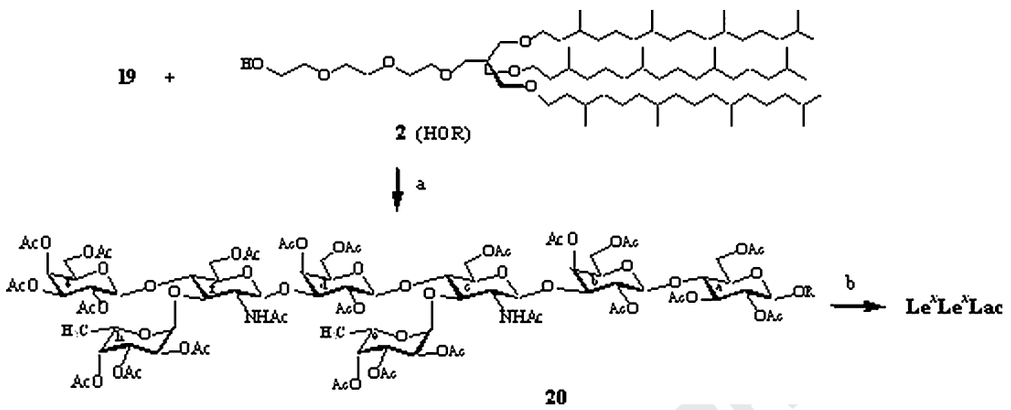

Figure 9. Synthesis of the glycolipid **Le^{x}Le^{x}Lac**. (a) TMSOTf, CH_2Cl_2, 4Å MS, 0°C; (b) NaOMe, MeOH, CH_2Cl_2, RT.

$J_{2d,3d} = 9.8$ Hz). We therefore confirmed that the position of the newly formed glycosidic linkages in **12** and **15** were OH-3b and OH-3d respectively.

Treatment of the octasaccharide **15** with hydrazine hydrate in refluxing ethanol, followed by acetylation with acetic anhydride in pyridine, gave the derivative **17** in 86% overall yield from **15**. Catalytic hydrogenolysis of **17** in methanol and ethyl acetate, followed by acetylation, gave the fully acetylated octasaccharide **18** in 73% overall yield. Acid catalysed cleavage of the 2-(trimethylsilyl)ethyl glycoside was performed in dichloromethane using trifluoroacetic acid [43] to give a hemiacetal which was directly treated with trichloroacetonitrile in the presence of DBU [44] to give the imidate **19** in 77% yield from **18** (Figure 8). According to ^{1}H-NMR, **19** exists essentially in α form.

Coupling of imidate **19** with the alcohol **2** promoted by trimethylsilyl trifluoromethanesulfonate (TMSOTf), gave the glycolipid **20** in 61% yield. The stereochemistry of the new glycosidic linkage was determined to be β, on the basis of H-1a, H-2a coupling constant from ^{1}H NMR ($J_{1a,2a} = 7.9$ Hz). De-*O*-acetylation of compound **20** in methanol-dichloromethane quantitatively provided the target product **Le^{x}Le^{x}Lac** (Figure 9) [45].

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